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AVALIAÇÃO DO MECANISMO ANTICÂNCER DO PEPTÍDEO BIOINSPIRADO CROTAMP14 CONTRA LINHAGEM DE CÂNCER DE MAMA TRIPLO NEGATIVO

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Tese apresentada ao Programa de Pós-Graduação Stricto Sensu em Ciências Biológicas (Biologia Molecular) da Universidade de Brasília, como requisito parcial para obtenção do Título de Doutor em Ciências Biológicas.

Orientador: Prof. Dr. Octávio Luiz Franco

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A presente tese está estruturada da seguinte forma:

Introdução;

Revisão da literatura;

Objetivos;

Artigo original;

Discussão;

Referências;

Publicações.

RESUMO

LEITE, Michel Lopes. **AVALIAÇÃO DO MECANISMO ANTICÂNCER DO PEPTÍDEO BIOINSPIRADO CROTAMP14 CONTRA LINHAGEM DE CÂNCER DE MAMA TRIPLO NEGATIVO.** 2024. 320 páginas. Doutorado em Ciências Biológicas (Biologia Molecular) – Universidade de Brasília, 2024.

O câncer de mama triplo negativo (CMTN) é a neoplasia com maior incidência em mulheres em todo mundo, representando entre 10% e 20% dos diagnósticos. Embora a quimioterapia seja a abordagem padrão para o tratamento de CMTN, ela gera efeitos colaterais deletérios. Desse modo, novas terapias com menor potencial de gerar efeitos colaterais precisam ser desenvolvidas. Atualmente, os peptídeos multifuncionais com atividade antitumoral (PACs) são moléculas promissoras para o desenvolvimento de terapias alternativas contra o CMTN. PACs são mais seletivos aos seus alvos, e com menor propensão a efeitos colaterais. No presente trabalho, o peptídeo crotAMP14 foi escolhido para ter o seu mecanismo anticâncer elucidado. Os dados demonstraram que o crotAMP14 atua sobre a membrana plasmática das células de CMTN, aumentando a sua rigidez sem causar um processo oxidativo. Esta é a primeira vez que tal mecanismo de ação é descrito para um PAC. A interação entre peptídeo e membrana ocorre por meio da região N-terminal do crotAMP14 com os oxigênios dos fosfolipídios da membrana plasmáticas. A elucidação desse possível novo mecanismo de ação permite gerar conhecimento para o desenvolvimento de novos fármacos a partir de peptídeos bionspirados.

Palavras-chave: neoplasia, câncer de mama, CMTN, crotalicidina, ACP.

ABSTRACT

LEITE, Michel Lopes. **EVALUATION OF THE ANTICANCER MECHANISM OF THE BIOINSPIRED PEPTIDE CROTAMP14 AGAINST TRIPLE-NEGATIVE BREAST CANCER LINE.** 2024.

Triple-negative breast cancer (TNBC) is the most prevalent neoplasm in women worldwide, accounting for 10% to 20% of diagnoses. Although chemotherapy is the standard approach for TNBC treatment, it induces harmful side effects. Thus, new therapies with fewer potential side effects need to be developed. Currently, multifunctional peptides with antitumor activity (PACs) are promising molecules for the development of alternative therapies against TNBC. PACs are more selective to their targets and have a lower likelihood of causing side effects. In this study, the peptide crotAMP14 was chosen to elucidate its anticancer mechanism. The data showed that crotAMP14 acts on the plasma membrane of TNBC cells, increasing its stiffness without causing oxidative stress. This is the first time such a mechanism of action has been described for a PAC. The interaction between the peptide and the membrane occurs through the N-terminal region of crotAMP14 with the oxygens of the plasma membrane phospholipids. The elucidation of this potential new mechanism of action provides valuable insights for developing new drugs based on bio-inspired peptides.

Keywords: Neoplasia, breast cancer, TNBC, crotalicidin, ACP.

Sumário

Dedico a todos os Lopes, Leites,
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“O homem nasceu para aprender tanto quanto a vida lhe permita”

João Guimarães Rosa

1 INTRODUÇÃO

Nas últimas décadas, apesar dos esforços voltados à criação de novas terapias que seriam ao mesmo tempo mais eficazes e que apresentassem efeitos colaterais mínimos, o câncer permanece como uma das principais causar de mortandade em todo o mundo, contabilizando quase uma em cada seis mortes (16,8%) e uma em cada quatro mortes (22,8%) por doenças crônicas não transmissíveis (BRAY et al., 2024). Além disso, neoplasias influenciam diretamente na redução da expectativa de vida em todos os países do mundo (SUNG et al., 2021). Com base nas estimativas da Agência Internacional de Pesquisa sobre Câncer (IARC), apenas no ano de 2022 foram cerca de 20 milhões de novos casos (BRAY et al., 2024). Os tipos de câncer mais comumente diagnosticados são o de pulmão, que representa 11,6% de todos os casos, o de mama feminino (11,6%) e o de colorretal (10,2%) (WHO, 2020). Além disso, em 2020, pessoas com 65 anos ou mais representaram aproximadamente metade de dos novos casos (PILLERON et al., 2022).

Na América Latina e Caribe (ALC), desconsiderando o câncer de pele não melanoma, foram diagnosticados mais de 1,4 milhão de novos casos, totalizando 600 mil óbitos, apenas no ano de 2020. Os tipos de neoplasias mais comuns foram o de mama (15% dos casos), de próstata (14%), de colorretal (9%), de pulmão (7%) e de estômago (5%) (BARRIOS et al., 2021). As projeções apontam para um aumento gradual do número de casos anualmente até atingir, em 2040, o patamar de 2,4 milhões de novos diagnósticos, representando um acréscimo de 70% para a América do Sul e 47% para o Caribe (WERUTSKY et al., 2022). Embora o câncer tenha impacto em todas as populações, as consequências devem ser analisadas considerando as importantes desigualdades observadas na saúde em todo o mundo (BARRIOS, 2022).

Entre os principais tipos de cânceres, o câncer de mama representa um desafio de saúde pública global significativo (WILKINSON; GATHANI, 2022), sendo o mais prevalente entre mulheres no mundo todo, representando mais de 2 milhões de diagnósticos anualmente (TARI, 2024). A taxa de sobrevida, em 5 anos, de pacientes com câncer de mama metastático tem sido inferior a 30%, mesmo com quimioterapia adjuvante (KASHYAP et al., 2022). Recentemente, a cada oito novos diagnósticos, um é de câncer de mama, totalizando 2,3 milhões de novos casos, em ambos os sexos combinados, de modo que sua incidência ultrapassou o número de novos casos de

câncer de pulmão (ARNOLD et al., 2022). Dentre os subtipos moleculares, o câncer de mama triplo negativo (CMTN) representa uma incidência entre 10% e 20% de todos os casos de câncer de mama (LU et al., 2023; YIN et al., 2020), apresentando, na maioria dos casos, comportamento agressivo, incluindo recorrência precoce e metástase (KEENAN; TOLANEY, 2020).

Nas últimas décadas, o desenvolvimento de novas tecnologias proporcionou um aumento na sobrevida, pois permitiu o rastreio de neoplasias mamárias, facilitando o diagnóstico precoce, potencializando a eficácia das terapias sistêmicas (PEDERSEN et al., 2022). As estratégias terapêuticas atuais, incluindo cirurgia, radioterapia, quimioterapia, ou a combinação destes tratamentos, são capazes de prolongar a expectativa de vida dos pacientes (JAFARI et al., 2022). Geralmente, a quimioterapia tem sido estratégia adotada como método padrão. No entanto, ela exibe efeitos colaterais graves, bem como pode gerar um fenótipo resistente a múltiplas drogas, levando a uma resposta insuficiente entre células neoplásicas e saudáveis (GUO et al., 2022).

Portanto, esforços voltados para o desenvolvimento de novos agentes anticancerígenos capazes de eliminar eficientemente células neoplásicas, sem ocasionar efeitos colaterais deletérios às células saudáveis do paciente, se fazem necessários (AGHAMIRI et al., 2021). Alguns dos candidatos promissores para o desenvolvimento de terapias alternativas contra o câncer são os peptídeos antimicrobianos (PAMs) (BASHI; MADANCHI; YOUSEFI, 2024; GUO, F. et al., 2022). Os PAMs são moléculas curtas, anfifílicas e, na maioria dos casos, catiônicas que possuem sequências altamente variáveis. Estes peptídeos apresentam múltiplas atividades antimicrobianas, sendo efetivos contra bactérias Gram-negativas e Gram-positivas, fungos e parasitas (LUO; SONG, 2021). No entanto, os PAMs também são capazes de erradicar células neoplásicas, os quais são denominados de peptídeos anticancerígenos (PACs) (KORDI et al., 2023).

Desse modo, os PACs podem ser utilizados no tratamento alternativo contra o câncer, sozinhos ou em combinação com os quimioterápicos convencionalmente administrados (AGHAMIRI et al., 2021). Além disso, compreender as diferenças genéticas, bem como as diversas respostas aos tratamentos de câncer entre as populações, certamente levará a melhores diagnósticos e terapias contra o câncer (CAMACHO; PANIS, 2022). Desse modo, o presente trabalho tem como principal

objetivo a elucidação do mecanismo de ação antitumoral do peptídeo crotAMP14, derivado da catelicidina ctn, contra células neoplásicas MDA-MB231.

2 REVISÃO DA LITERATURA

2.1 CÂNCER: DEFINIÇÃO E CARACTERÍSTICAS

Ao longo da vida, cada uma das células do corpo de um indivíduo acumula mutações, algumas vezes a combinação de várias delas desencadeia o surgimento e a progressão de um câncer (CHEEK; NAXEROVA, 2022). Na maioria dos casos, estas mutações podem ativar oncogenes e/ou silenciar genes supressores de tumor, resultando no crescimento celular descontrolado e na inativação de mecanismos apoptóticos, ou câncer (DAKAL et al., 2024). A origem etimológica da palavra câncer deriva dos termos gregos *καρκίνος* (*karkinos*) e *καρκίνωμα* (*karkinoma*), utilizados pelo médico Hipócrates (460-370 a.C.) para descrever tumores (INCHINGOLO et al., 2020). Atualmente, o termo câncer é utilizado para agrupar, em uma única categoria, mais de 270 tipos de doenças que acometem diferentes tecidos e tipos celulares (HASSANPOUR; DEHGHANI, 2017), mas que compartilham características que lhes permitem o crescimento celular desordenado e a aquisição de propriedades metastáticas (SARKAR et al., 2013).

No início dos anos 2000, Douglas Hanahan e Robert Weinberg propuseram um conjunto de capacidades funcionais que as células humanas saudáveis precisam adquirir, durante o processo de carcinogênese, para sustentar o desenvolvimento da massa tumoral (HANAHAN, 2022). Nesta revisão seminal, os pesquisadores elencaram as seis alterações fisiológicas essenciais que coordenam o surgimento da maioria, ou mesmo totalidade, das neoplasias (HANAHAN; WEINBERG, 2000). De acordo com as proposições aventadas por Hanahan e Weinberg, as células saudáveis se tornam neoplásicas apenas quando são capazes de produzir seus próprios sinais mitogênicos, de não responder aos sinais inibitórios de crescimento, de escapar à morte celular programada (apoptose), de possuir um potencial replicativo ilimitado, de sustentar a produção de novos vasos (angiogênese) e de invadir outros tecidos próximos ou distantes (metástase) (SENGA; GROSE, 2021).

O crescente acúmulo de evidências, associado a novas descobertas no campo da oncologia permitiram que, onze anos após a sua publicação seminal, Hanahan e Weinberg revisitassem o seu trabalho e acrescentassem duas outras alterações, às seis já existentes, responsáveis pela formação de um tumor (Figura 1) (HANAHAN; WEINBERG, 2011). A primeira alteração, que as células neoplásicas devem possuir, é a instabilidade genética, pois permite o acúmulo de mutações, herdadas ou induzidas, auxiliando no desenvolvimento tumoral (GUO et al., 2023). A segunda, é a capacidade de evadir o sistema imune do hospedeiro por meio de diversos mecanismos como restrição do reconhecimento de antígeno, inibição do sistema imunológico e indução da exaustão das células T (KIM; CHO, 2022).

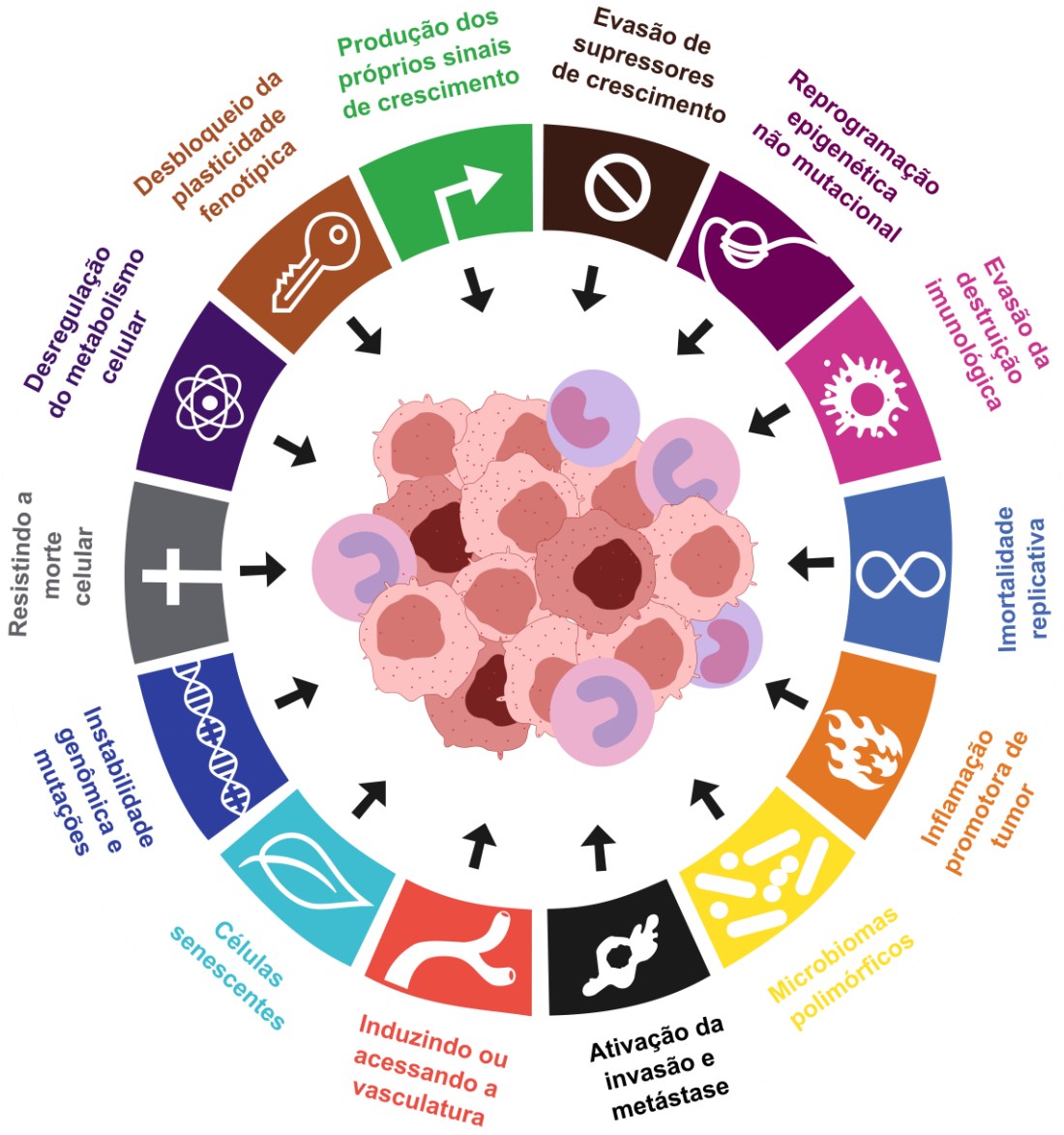

Figura 1. Alterações e características necessárias às células neoplásicas. As células neoplásicas necessitam adquirir todas estas etapas para serem capazes de se proliferar, migrar e invadir outros tecidos. Adaptado de (HANAHAN, 2022).

Recentemente, Hanahan propôs algumas características, as quais denominou-as de habilitantes (ou capacitadoras), que, associadas às oito alterações já descritas, são também responsáveis pela tumorogênese (HANAHAN, 2022) (Figura 1). A capacidade de desbloquear a plasticidade fenotípica, é a primeira característica capacitadora, a qual permite que as células sejam capazes de passar pelo processo de transdiferenciação. A segunda é a habilidade de reprogramação epigenética não mutacional, característica que resulta na instabilidade genética. A terceira é a existência de microbiomas polimórficos, pois a variedade polimórfica entre indivíduos de uma mesma população desempenha um importante papel nos fenótipos do câncer. Por último, a presença de células senescentes que, embora possa atuar como guardiões, em certos contextos podem estimular variadamente o desenvolvimento do tumor e a progressão maligna (HANAHAN, 2022).

O desenvolvimento do câncer pode ser um processo que ocorre em várias etapas, nas quais as células saudáveis aumentam em malignidade progressivamente, por meio da aquisição de alterações no padrão de marcações epigenéticas e mutações em genes essenciais de controle (Figura 1) (BUDER et al., 2019). Os genes supressores de tumor desempenham um papel central na manutenção do desenvolvimento celular normal, prevenindo a transformação maligna, mas quando sofrem variações genéticas, estes genes promovem a tumorogênese (YAO et al., 2022). O primeiro gene supressor de tumor molecularmente definido foi o gene de susceptibilidade ao retinoblastoma (*RB1*) (HUANG, M.-F. et al., 2024; KNUDSON, 1971; MANDIGO et al., 2022). O *RB1*, localizado no cromossomo 13q14.2, possui 27 éxons, 26 íntrons e uma sequência promotora que codifica a proteína retinoblastoma (pRb), composta por 928 resíduos de aminoácidos (YAO et al., 2022).

A pRb é uma proteína associada à cromatina que limita a transcrição de genes do ciclo celular, principalmente por meio da regulação do fator de transcrição E2F (CHANG et al., 2024). Sua via inclui os inibidores de quinases dependentes de ciclinas (CDK) da família das proteínas INK4, que regulam negativamente a proliferação celular, bem como ciclinas do tipo D de ação positiva, as quais formam complexos quinases ativos em associação com CDK4 e CDK6. As CDKs fosforilam a pRb, inativando sua atividade de modo que, a atividade tanto das CDKs quanto da pRb é regulada por fatores mitogênicos e anti-mitogênicos (MYERS et al., 2023). Desse modo, a superexpressão de pRb em diversos tipos de câncer resulta na perda da

função supressora de tumor. Embora a relação entre o aumento na expressão gênica e alterações no câncer não estejam completamente elucidada, várias possibilidades podem existir (USMAN et al., 2021).

Outro exemplo de gene supressor de tumor importante é o *TP53*, que codifica para a proteína p53 (HASSIN; OREN, 2023; TEMAJ et al., 2024). A p53 é um fator de transcrição conhecido como o “guardião do genoma” devido ao seu papel crucial na preservação da integridade genômica. Sua importância é tal que ele se encontra mutado em, aproximadamente, metade dos tumores humanos, incluindo o câncer de mama, cólon, pulmão, fígado, próstata, bexiga e pele (MAREI et al., 2021). Por meio tanto da transativação quanto da transrepressão de genes alvos, a p53 desempenha papeis críticos em diversos processos biológicos importantes, incluindo apoptose, parada do ciclo celular, senescência, reparo do ácido desoxirribonucleico (DNA), metabolismo celular e defesa antioxidante (ZHANG et al., 2020). A perda de função do alelo selvagem da p53, por meio de mutações e outros mecanismos como a superexpressão de antagonistas (MDM2, MDM4 e PPM1D), pode estar diretamente relacionada com o início e/ou progressão de diversos cânceres (ZHANG et al., 2020). Em pacientes de diferentes grupos étnicos com câncer de mama, especialmente o tipo triplo-negativo, foram relatadas diferentes mutações no gene *TP53*, as quais frequentemente ocorrem no éxon 4 e íntron 3 (KAUR et al., 2018). Devido a relevância desse gene, diversas terapias direcionadas a p53/*TP53* estão sendo desenvolvidas (KAUR et al., 2018).

Em um estudo recente, um nanosistema anticâncer foi desenvolvido, utilizando vesículas extracelulares (VE) derivadas de tumores de câncer de mama vazias, que atuam como nano-carreadores, realizando a entrega seletiva da p53 recombinante exógena, o que resulta na morte celular por apoptose (JIAO et al., 2022). Compostos tricíclicos-indol fundidos em 3,4 (TFI) podem ser utilizados como agentes seletivos da p53. O composto sintético TFI-6D demonstrou ser citotóxico contra diversas linhagens de cânceres humanos, induzindo à morte celular por meio da ativação da via apoptótica relacionada à mitocôndria a jusante p53, proporcionando o aumento de membros da família Bcl-2 pró-apoptóticos em relação aos anti-apoptóticos (INOUE et al., 2022).

Em relação a mudanças estruturais, a membrana plasmática das células neoplásicas podem passar por alterações em sua composição lipídica (Figura 2)

(LEITE; DA CUNHA; COSTA, 2018). As células eucariotas saudáveis apresentam uma membrana plasmática altamente assimétrica. No folheto externo encontram-se a maior parte de fosfatidilcolina (PC) e, provavelmente, todos os esfingolipídios, enquanto os demais tipos como fosfatidilserina (PS), fosfatidiletanolamina (PE) e fosfatidilinositol (PI), estão localizados no folheto interno (KUR; WEIGERT, 2024; SKOTLAND; KAVALIAUSKIENE; SANDVIG, 2020). Por outro lado, em células neoplásicas e apoptóticas, esta assimetria é perdida, parcial ou completamente, devido à exposição de PS no folheto externo da membrana. No entanto, esta característica é conhecida como um importante marcador, o qual aciona os macrófagos para a eliminação de células danificadas, apoptóticas e cancerígenas (LIRUSSI et al., 2023).

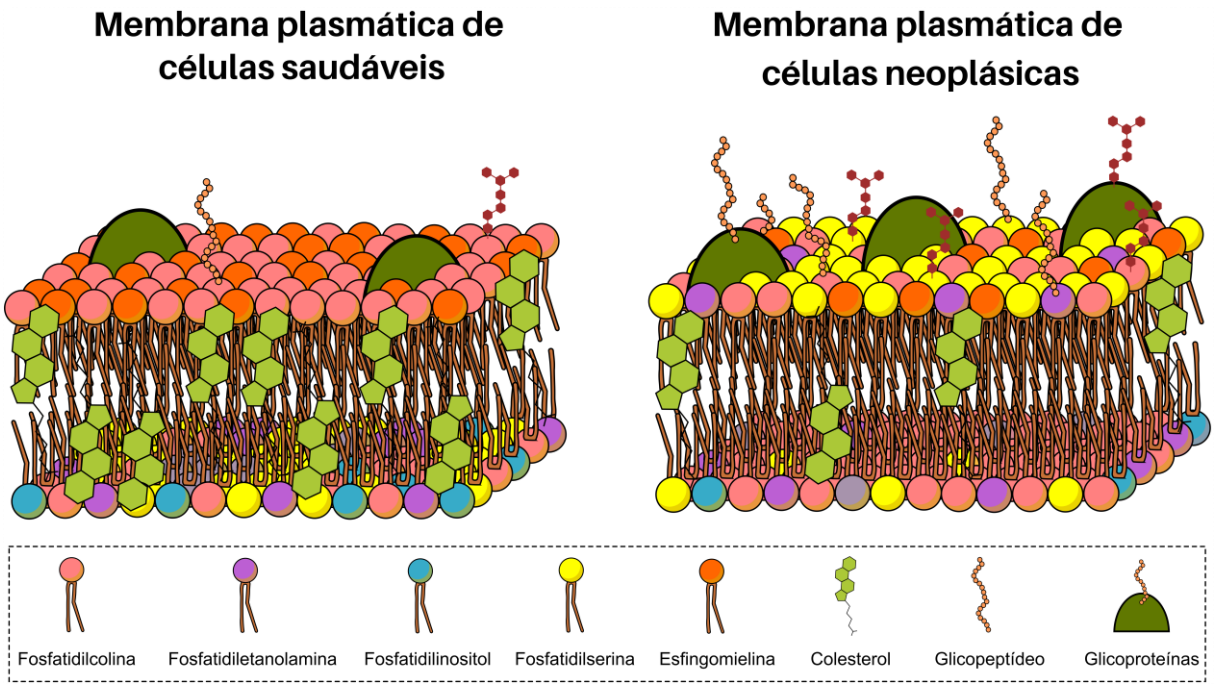

Figura 2. Representação esquemática da diferença na composição lipídica entre células saudáveis e neoplásicas. No lado esquerdo do painel está representada a membrana plasmática de uma célula considerada saudável. A membrana plasmática de células neoplásicas pode ser vista no lado direito do painel. É possível observar diferenças substanciais entre ambas como, por exemplo, na membrana plasmática de tipos celulares malignos há uma maior quantidade de moléculas aniônicas, tais como glicoproteínas e glicolipídios, bem como uma menor concentração de colesterol, quando comparado com a célula saudável. Outra característica importante é a presença do fosfolipídio fosfatidilserina na camada externa da bicamada. Todas essas características contribuem para a carga líquida negativa da membrana plasmática de células neoplásicas. Abaixo no painel estão as legendas dos tipos de lipídios e componentes da bicamada.

É importante salientar que a composição lipídica também pode ser variável entre os diferentes tipos de câncer (SZLASA et al., 2020). No entanto, uma maior

presença de PS no folheto externo das células neoplásica é uma característica que pode ser explorada como um alvo efetivo para o tratamento do câncer (PRETA, 2020), uma vez que a disposição destes fosfolipídios aniônicos, associada com a exposição de glicoproteínas, contribui para a carga global negativa da membrana plasmática destas células (BAXTER et al., 2017). Esse rearranjo lipídico ocorre, em parte, devido ao fenômeno conhecido como glicólise aeróbica, no qual células neoplásicas absorvem avidamente a glicose, convertendo-a em lactato por meio da via glicolítica, independente da presença de oxigênio. Estas alterações metabólicas fornecem às células além de energia, o carbono necessário para a síntese de blocos de construção celulares, incluindo nucleotídeos e lipídios (BUTLER et al., 2020).

O metabolismo do colesterol também pode ser desregulado em células neoplásicas, o que resulta em maior ou menor quantidade de colesterol na membrana plasmática dessas células, quando comparadas com as saudáveis, permitindo uma variação na fluidez da membrana. Esta é uma característica importante, pois possibilita a deformação da membrana plasmática, facilitando a entrada dessas células nos vasos sanguíneos, permitindo a ocorrência de metástase (ZALBA; TEN HAGEN, 2017). Além disso, as células neoplásicas possuem um grande número de resíduos de ácido siálico (SUN et al., 2024), proteoglicanas (LEE et al., 2015), mucinas *O*-glicosiladas (PEDRAM et al., 2024), microvilosidades (HUANG et al., 2012) e a glicosilação de proteínas e lipídios associados à membrana plasmática (LIU et al., 2015), bem como um aumento do nível de expressão de alguns receptores de membrana como, por exemplo, o receptor de ácido fólico (TANG et al., 2014).

Embora todas essas alterações possibilitem que células neoplásicas sejam capazes de contornar os mecanismos de inibição, permitindo que elas se proliferem indiscriminadamente, o aumento da massa tumoral induz à privação crônica de nutrientes e reduz as concentrações de oxigênio, pois apresenta uma vascularização ineficiente. Para sobreviver e se adaptar a esses estresses ambientais severos, as células neoplásicas podem modificar suas vias metabólicas visando à captura de metabólitos externos e maximizar a eficiência das atividades das enzimas metabólicas (PARK; PYUN; PARK, 2020).

Além disso, outra estratégia para contornar essa limitação consiste na produção de novos vasos sanguíneos, processo denominado de angiogênese (QI et al., 2022). Embora estes novos vasos apresentem uma arquitetura vascular anormal, eles

permitem que o tumor possa aumentar a sua permeabilidade, diminuindo as limitações nutricionais e aumentando as taxas de oxigenação e excreção de resíduos, bem como possibilita uma variação no pH e perfusão (BYRNE; BETANCOURT; BRANNON-PEPPAS, 2008; DANHIER; FERON; PRÉAT, 2010; DIGESU et al., 2016; MAEDA; BHARATE; DARUWALLA, 2009). Todas estas características fazem do câncer uma doença complexa e multifatorial, dificultando seu diagnóstico e tratamento.

2.1.1 Câncer de mama

O câncer de mama é bastante heterogêneo, podendo ser divido clinicamente em três subtipos baseados na imuno-histoquímica de acordo com a expressão dos receptores hormonais, estrogênio (ER) e progesterona (PR), e do receptor tipo 2 do fator de crescimento epidérmico humano (HER2) (DING et al., 2024). Desse modo, os subtipos podem ser classificados como luminal ER-positivo e PR-positivo (os quais são subdivididos em luminal A e B), HER2-positivo e CMTN (LOIBL et al., 2021). Os tumores luminais A possuem um prognóstico favorável, podendo ser tratado com opções terapêuticas que incluem a terapia endócrina isolada. Tumores luminais B exibem uma baixa assinatura de proliferação e marcadores, além de uma menor responsividade, devido aos baixos níveis de ER ou PR, altos níveis de Ki-67 e alta carga tumoral, tornando a escolha terapêutica uma combinação entre terapia endócrina e quimioterapia (ACOSTA-CASIQUE et al., 2023).

HER2 consiste em um receptor de tirosina quinase transmembrana em que o seu gene é amplificado, ou superexpresso, em aproximadamente 20% das neoplasias de mama, característica que confere a estes tumores um comportamento agressivo (NICOLÒ; TARANTINO; CURIGLIANO, 2023). Estratégias terapêuticas direcionadas contra esse receptor, em tumores HER2+, tem sido bem-sucedidas na clínica (ACOSTA-CASIQUE et al., 2023). Além disso, variantes patogênicas de *TP53* possuem uma consistente associação com o câncer de mama HER2+, como demonstrado por estudo, no qual dos 24 carcinomas, surgidos no contexto da neurofibromatose tipo 1, houve uma maior prevalência de HER2+, quando comparado com o controle da mesma idade (SOKOLOVA et al., 2023).

O câncer de mama é o tipo de câncer não cutâneo mais diagnosticado em pessoas do sexo feminino no mundo, contribuindo com, aproximadamente, 11,6% do

número total de casos (ROONEY; MILLER; PLICHTA, 2023; WU, Y.; ZHONG; MA, 2023). Em pessoas do sexo masculino, embora a incidências de novos casos tenha aumentado em todo mundo, este tipo de câncer é considerado raro, representando menos de 1% de todos os cânceres de mama (FOX; SPEIRS; SHAABAN, 2022). No Brasil, o câncer de mama contribui atualmente com 29,5% de todas as neoplasias entre mulheres (GOMES et al., 2022).

O sexo feminino constitui um dos principais fatores de risco associado ao aumento de neoplasias mamárias, principalmente devido aos constantes ciclos de aumento da estimulação hormonal (ŁUKASIEWICZ et al., 2021). Outro fator de risco é a obesidade, definida como um índice de massa corporal (IMC) ≥ 30 Kg/m^2, a qual está associada com o alto risco de adenocarcinoma mamário (PICON-RUIZ et al., 2017). A deposição de gordura ectópica, expansão patológica do tecido adiposo branco em áreas que não deveriam estar (como intra-hepática, intra-abdominal, intramiocelular), pode causar, por meio de múltiplas vias, alterações metabólicas, inflamatórias e imunológicas que afetam o reparo de DNA, as funções gênicas, a taxa de mutação celular, bem como alterações epigenéticas que permitem a transformação e progressão maligna (AVGERINOS et al., 2019).Indivíduos obesos correm um risco maior de desenvolver diabetes melitus do tipo 2 (DM2), pois frequentemente são acometidos por síndrome metabólica (KANG; LEROITH; GALLAGHER, 2018).

Diabetes é um fator de risco para diversos cânceres sólidos, incluindo o de mama (SHAHID et al., 2021). A resistência à insulina, a hiperinsulinemia e as alterações na sinalização dos hormônios de crescimento e esteroides, associadas à diabetes podem aumentar as chances de desenvolver neoplasia mamária (ABUDAWOOD, 2019). O estilo de vida também pode representar um fator de risco, uma vez que o consumo de bebidas alcóolicas aumenta a probabilidade do surgimento de câncer de mama (BROWN et al., 2024; SARHANGI et al., 2022). Isso ocorre devido ao metabolismo do etanol (EtOH), pois a álcool desidrogenase (ADH) metaboliza o EtOH em acetaldeído, principal metabólito carcinogênico em células de câncer de mama, que se acumula nos tecidos mamários após o consumo de álcool (SHIELD; SOERJOMATARAM; REHM, 2016).

A terapia de reposição hormonal (TRH), particularmente a combinação de estrogênio com progestagênio, durante a menopausa representa um risco (SANDVEI et al., 2019), pois a terapia hormonal da menopausa está associada a um aumento de

23% do risco de desenvolvimento de um câncer de mama (WANG, X. et al., 2022). A terapia hormonal é frequentemente prescrita para mulheres visando aliviar os sintomas climatérios associados à menopausa (FAIT; VRABLIK, 2024). Os históricos pessoal e familiar de casos da doença é outro fator importante, pois um estudo demonstrou que mulheres com câncer de mama unilateral apresentam risco de câncer de mama contralateral de, aproximadamente, 0,4% ao ano, persistindo ao longo do período de acompanhamento de 25 anos (GIANNAKEAS; LIM; NAROD, 2021). Outros fatores de risco, considerados não modificáveis, para pessoas do sexo feminino são a idade, a raça, a etnia, a densidade do tecido mamário (ŁUKASIEWICZ et al., 2021), menarca precoce e a idade do primeiro parto (ROONEY; MILLER; PLICHTA, 2023).

Até o momento, há uma escassez de literatura avaliando a relação entre certas comorbidades médicas e o risco do desenvolvimento de câncer em pessoas do sexo masculino (SOGUNRO et al., 2023). No entanto, assim como nos demais tipos de cânceres, a idade é um fator de risco para o desenvolvimento de neoplasias de mama neste grupo de indivíduos. Fatores como obesidade, síndrome de Klinefelter (47 XXY), administração de drogas e hormônios exógenos provocam um desequilíbrio hormonal, podendo resultar no desenvolvimento de câncer de mama. Outros fatores relacionados são lesões e tumores que prejudicam a função testicular, como caxumba, criptorquidia e malignidades testiculares (FOX; SPEIRS; SHAABAN, 2022). Além disso, elevados níveis de estrogênio também estão relacionados com neoplasias de mama em pessoas do sexo masculino (KHAN; TIRONA, 2021).

A genética também pode desempenhar um papel importante para o desenvolvimento de neoplasias mamárias. Entre 5% e 10% dos cânceres de mama são hereditários, com mutações germinativas nos genes supressor de tumor *breast cancer* 1 (*BRCA1*) e *breast cancer* 2 (*BRCA2*) respondendo pela maioria dos casos (ROONEY; MILLER; PLICHTA, 2023). No entanto, *PALB2*, *CHEK2*, *ATM*, *CDH1*, *PTEN*, *TP53* e *STK11* são outros genes de susceptibilidade ao câncer de mama, embora com menor grau de risco ao longo da vida, quando comparados ao *BRCA1* e *BRCA2* (WEN; COLLINS, 2023). BRCA1/2 são proteínas envolvidas no reparo de quebras do DNA de fita dupla por meio da recombinação homóloga (SOKOLOVA et al., 2023). As mutações germinativas mais comuns associadas ao câncer de mama estão nestes genes, com um risco cumulativo médio ao longo da vida de cerca de 70% (LOIBL et al., 2021).

Câncer de mama possuindo variantes deletérias do *BRCA1* tem uma maior probabilidade de ser negativo para o receptor de progesterona, além de potencializar o surgimento de CMTN a partir de progenitores luminais. *BRCA1* pode inibir a atividade transcricional do receptor de progesterona por meio da ubiquitinação, levando a sua degradação, bem como ao silenciamento epigenético de promotores-alvo (LI et al., 2022). Além das mutações nestes genes terem um risco aumentado (40% para *BRCA1* e 26% para *BRCA2*) para o câncer de mama contralateral, elas também estão associadas a um risco vitalício significativamente elevado para o desenvolvimento de outros tipos de câncer como o de ovário (SOKOLOVA et al., 2023).

Com relação a alterações nestes genes em pessoas do sexo masculino, há uma associação mais fraca entre o câncer de mama e mutações no *BRCA1*, quando comparado com os indivíduos portando mutações no *BRCA2* (KHAN; TIRONA, 2021). Além disso, mutações somáticas no *BRCA2*, incluindo a perda da heterozigosidade, representam 12% dos casos de câncer de mama masculino esporádicos (FOX; SPEIRS; SHAABAN, 2022). O risco vitalício de câncer de mama em indivíduos do sexo masculino com as variantes patogenéticas do *BRCA2* é de 8,9%, 80 a 100 vezes maior do que na população em geral (PENSABENE; VON ARX; DE LAURENTIIS, 2022). Outra consequência de mutações nesses genes é a susceptibilidade ao desenvolvimento de outras malignidades, particularmente o câncer de próstata (*BRCA1/2*) e o pancreático (*BRCA2*) (SOKOLOVA et al., 2023).

2.1.2 Câncer de mama triplo negativo

O câncer de mama triplo-negativo (TNBC), responsável por 15 a 20% dos carcinomas de mama, está associado a uma maior incidência de metástase visceral, bem como a um alto grau de recorrência precoce e mau prognóstico. (LI, R. Q. et al., 2024). Na América Latina, é alta a frequência de tumores de mama do tipo triplo negativo em pacientes com câncer, representando um importante desafio no tratamento para estas pessoas (AYALA et al., 2023). Outra característica clínica relevante é o fato de que, quando comparado com os demais subtipos, o CMTN apresenta um prognóstico desfavorável devido à falta de terapias direcionadas (PATEL; PRINCE; HARRIES, 2024). O CMTN é uma coleção heterogênea de

cânceres de mama caracterizado pela ausência, ou detecção em concentrações muito baixas, de ER, PR e HER2, também conhecido como ERBB2 (BARANOVA et al., 2022; LI et al., 2022).

O CMTN é o subtipo mais comum em mulheres na pré-menopausa com menos de 40 anos e, quando comparado com outros câncer de mama (ER+, PR+ e HER2+), possui um maior potencial metastático, bem como um desfecho clínico pior quando iniciado em pessoas com idade mais precoce (LU et al., 2023). Além disso, este tipo de neoplasia possui um elevado grau histológico patológico, bem como uma alta taxa de proliferação celular, podendo ser destacados os carcinomas ductal invasivo, metaplásico, apócrino e medular. Adicionalmente, pacientes com CMTN possuem uma prevalência de mutações germinativas nos genes *BRCA1*/2 (CIPRIANO; MESQUITA, 2021). Levando em consideração as diferentes características moleculares, o CMTN pode ser caracterizado em quatro subtipos, os quais são o receptor de andrógeno luminal (LAR), o imunossupressor tipo basal (BLIS), imunomodulador (IM) e subtipo mesenquimal (MES). Essa subdivisão é importante, pois auxilia na tomada de decisões clínicas, visando o tratamento mais promissor para cada um dos subtipos (HU et al., 2024; LEHMANN et al., 2016). Cada subtipo é enriquecido em ontologias gênicas distintas, bem como em diferenças no padrões de expressão gênica (CIPRIANO; MESQUITA, 2021). Novas tentativas de caracterização vêm sendo propostas como, por exemplo, a classificação molecular dos CMTN com base em características metabólicas com o intuito de complementar o estadiamento clínico atual (ZHOU et al., 2022). Um dos fatores que torna o CMTN tão agressivo é a sua capacidade de invadir outros tecidos. Recentemente, foi demonstrado (utilizando-se de um modelo de camundongo geneticamente modificado com xenoenxertos de CMTN humano) que a maior parte da população de células *in vitro* epiteliais-mesenquimais híbridas que levam à invasão *ex vivo*, expressam características epiteliais e mesenquimais. Além disso, os dados sugerem que a vimetina (Vim) é importante para o processo de metástase por meio da transição epitélio/mesênquima (GRASSET et al., 2022). Uma vez que as células tenham invadido outros tecidos, estabelecendo a metástase, a doença é incurável, sendo a quimioterapia a principal opção terapêutica com sobrevida variando entre 2 e 3 anos (SO et al., 2022).

Novas estratégias para diagnóstico e tratamento do CMTN estão sendo propostas. A ceritinibe, uma pequena molécula inibidora da tirosina quinase

administrada em pacientes com câncer de pulmão de células não pequenas, inibiu eficientemente o crescimento de células LAR (DONG, S. et al., 2022). Outra abordagem, em potencial, é a utilização de inibidores de MEK/ERK para evitar a migração celular, diminuindo a formação de metástase (ACOSTA-CASIQUE et al., 2023). No entanto, biomarcadores sensíveis e específicos ainda estão faltando para o CMTN, de modo que a busca por novos se faz urgente. Regiões diferencialmente metiladas no genoma de células de CMTN se configuram como um potencial biomarcador (MANOOCHEHRI et al., 2023). Outro alvo epigenético que pode ser explorado é a histona deacetilase, HDAC7, a qual inibe a proliferação celular por meio da modulação negativa da expressão de GGH e NudCD1 (ZHU et al., 2022). No entanto, compreender a complexidade genética, bem como a modulação da expressão gênica desse subtipo de câncer de mama após a exposição a potenciais fármacos é de extrema importância para o desenvolvimento de novos tratamentos. O estudo do transcriptoma tem sido empregado para investigar biomarcadores putativos e potenciais alvos terapêuticos para diversos câncer sólidos humanos. O uso de microarranjos (*microarray*) possibilitou identificar uma variedade de genes relacionados com o desenvolvimento do câncer de mama. A ampla utilização de tecnologias de sequenciamento de ácido ribonucleico (RNA), RNA-seq, ampliou drasticamente o conhecimento a respeito do câncer de mama, sendo possível a quantificação de genes expressos em níveis extremamente baixos (LI et al., 2022)

Embora avanços significativos possibilitem um diagnóstico mais confiável, sobretudo na classificação dos subtipos de câncer de mama, o desenvolvimento de novas estratégias (mais seletivas e menos nocivas às células saudáveis), bem como de novos alvos terapêuticos é urgente (LU et al., 2023). Atualmente, uma das classes de biomoléculas que apresenta um maior potencial para o desenvolvimento de terapias alternativas, sejam administradas sozinhas ou em combinação adjuvante, são os PAMs (GUO et al., 2022). Estes peptídeos possuem propriedades como alta especificidade, um baixo potencial de provocar efeitos colaterais e penetração tumoral aceitável, de modo que, podem ser utilizados na prevenção do câncer em diferentes estágios como iniciação, promoção e progressão (NOROUZI; MIRMOHAMMADI; HOUSHDAR TEHRANI, 2022).

2.2 PEPTÍDEOS MULTIFUNCIONAIS

Mais de oito década atrás, em 1939, o microbiologista René Dubos isolou, de uma cepa de *Bacillus* de solo, a gramicidina, molécula reconhecida como o primeiro PAM descoberto (DUBOS, 1939). Em estudos posteriores, ficou demonstrada a capacidade que a gramicidina tem em proteger camundongos contra infecções causadas por pneumococos, além de ser eficaz no tratamento tópico de feridas (DUBOS; HOTCHKISS, 1941). Atualmente, já foram descritos mais três mil PAMs, entre naturais e sintéticos, de acordo com o banco de dados de peptídeos antimicrobianos (APD3) (WANG; LI; WANG, 2016). Os PAMs são oligopeptídeos curtos (entre 15 e 45 resíduos de aminoácidos), com carga positiva, produzidos desde bactéria a mamíferos como mecanismo de defesa contra patógenos (AHMED, A. et al., 2019; CIUMAC et al., 2019; DONG, Z. et al., 2024; MAGANA et al., 2020).

Os PAMs apresentam amplo espectro antimicrobiano contra bactérias (Gram-positivas e Gram-negativas), fungos, vírus e diversos patógenos (LUONG; THANH; TRAN, 2020; MULUKUTLA et al., 2024). Embora sejam reconhecidos como fármacos alternativos promissores para o tratamento de doenças infecciosas, diversos PAMs estão sendo explorados como agentes antitumorais (NG; LEE, 2020), pois são considerados relativamente seguros, altamente seletivos, possuem boa tolerabilidade, relativamente fáceis de modificar e sintetizar, além de exibirem um perfil farmacológico promissor (SCHADUANGRAT et al., 2019). Desse modo, peptídeos que, além de possuírem atividade antimicrobiana, também são capazes de erradicar células neoplásicas são denominados de PACs (KORDI et al., 2023).

Com base na composição de aminoácidos e suas posições, os PACs adquirem diferentes estruturas secundárias (Figura 3) (JI et al., 2024; TRIPATHI; VISHWANATHA, 2022). No entanto, os dois principais tipos de estruturas, importantes para a atividade de PACs, são α-hélice e folha β-pregueada (folhas β) (NOROUZI; MIRMOHAMMADI; HOUSHDAR TEHRANI, 2022). Além disso, os PACs podem ser compostos por uma combinação de ambas α-hélice e folha β, denominados de mistos (LUONG; THANH; TRAN, 2020). Há ainda peptídeos lineares estendidos (SCHADUANGRAT et al., 2019), que, devido ao seu alto teor de resíduos de prolina e/ou glicina, carecem de estruturas secundárias clássicas (LUONG; THANH; TRAN, 2020).

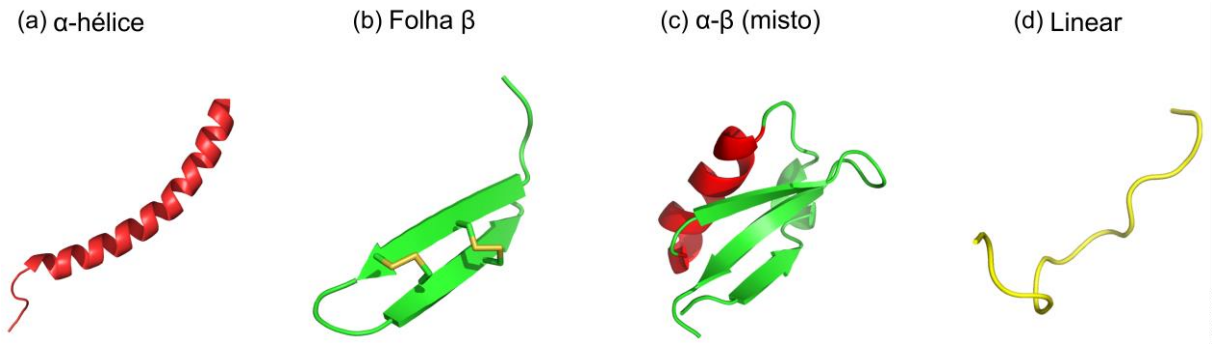

Figura 3. Representação esquemática das quatro classes estruturais dos PACs. Os peptídeos podem ser do tipo α-helicoidal, como a catelicidina humana LL-37 (PDB: 2K6O), visto em (a); folha β, como a potegrina-1 (PDB: 1PG1), visto em (b); de estrutura mista (α-β), como a defensina de planta (PDB: 1TI5), visto em (c); e linear estendido, como a indolicidina (PDB: 1G89), visto em (d). A visualização foi realizada no *software* PyMol v1.6.

Geralmente, a maioria dos PACs α-helicoidais é desestruturada em solução aquosa, formando estruturas anfifílicas apenas quando em contato com membranas biológicas (DRAYTON et al., 2021; JUNG et al., 2023), ou na presença de trifluoretanol, micelas de dodecil sulfato de sódio (SDS), vesículas fosfolipídicas e lipossomas (BLONDELLE; LOHNER; AGUILAR, 1999; BROGDEN, 2005; MWANGI et al., 2019). Por outro lado, devido às ligações dissulfeto, os PACs em folha β parecem ser mais estruturados em solução, levando a menores alterações conformacionais ao interagirem com as membranas lipídicas (DRAYTON et al., 2021; NGUYEN; HANEY; VOGEL, 2011; ZHANG, L.; GALLO, 2016). A transição entre desestruturados e estruturados ocorre devido às interações eletrostáticas com a superfície da células neoplásicas (CHIANGJONG; CHUTIPONGTANATE; HONGENG, 2020).

Este fenômeno também pode contribuir para a seletividade dos PACs, pois eles interagem, com maior afinidade, com a membrana aniônica das células neoplásicas do que com a das células de mamíferos saudáveis, a qual possui uma carga líquida neutra devido à maior presença de lipídios zwitteriônicos (HADIANAMREI et al., 2022a). Tal especificidade ocorre por causa do fato de que a maioria dos PACs é frequentemente carregada positivamente, com cargas variando entre +2 e +9 (em pH fisiológico), devido à presença de vários resíduos de lisina e arginina ao longo da estrutura primária (HANEY; STRAUS; HANCOCK, 2019; KUMAR; KIZHAKKEDATHU; STRAUS, 2018; MOOKHERJEE et al., 2009). Além disso, os PACs também possuem aproximadamente 50% de resíduos hidrofóbicos, favorecendo a formação de uma estrutura anfipática quando interagem com as membranas-alvo (MANSOUR; PENA; HANCOCK, 2014; WU, X. et al., 2024).

Outra vantagem é que, embora a carga líquida positiva dos peptídeos seja responsável por sua maior seletividade em relação a membranas específicas, seu caráter anfifílico permite sua interação com o núcleo hidrofóbico da membrana (KABELKA; VÁCHA, 2021). Esta é uma das características principais dos peptídeos de penetração celular (PPCs), os quais após aderirem à membrana, se translocam através da bicama lipídica para o interior da célula (LI, C. M. et al., 2021). Além disso, dados demonstram que as proteínas transmembranares que possuem regiões hidrofílicas podem aumentar a translocação de PACs anfifílicos, estabilizando-os na membrana. No entanto, há uma anfifilicidade aparentemente ótima, uma vez que o peptídeo pode ficar superestabilizado no estado transmembranar, no qual a dissociação peptídeo-proteína é dificultada, limitando a translocação do peptídeo (BARTOŠ; KABELKA; VÁCHA, 2021).

Tanto as características estruturais quanto as propriedades físico-químicas desses peptídeos influenciam diretamente nos seus mecanismos de ação (Figura 4) (WU, X. et al., 2024). A ruptura da membrana induzida por PACs ocorre, principalmente, por meio de três modos: tipo carpete, formação de barril e modelo de poro toroidal (DONG, Z. et al., 2024). No modelo de carpete (Figura 4), os PACs adsorvem paralelamente à bicamada lipídica, por meio de interações eletrostáticas, até atingirem uma concentração limiar, a qual permite cobrir a superfície da membrana (formando um “tapete”), levando à formação de micelas e à ruptura da bicama lipídica (KUMAR; KIZHAKKEDATHU; STRAUS, 2018). Neste mecanismo de ação, não há a formação de canais nem a inserção dos PACs no centro hidrofóbico da membrana (ZHANG, Q.-Y. et al., 2021).

No modelo de barril (Figura 4), os PACs inicialmente se inserem perpendicularmente (em orientação vertical) na porção hidrofóbica da membrana e, em seguida, interagem uns com ou outros para formar uma estrutura em forma de barril, semelhante a uma aduela. Os poros formados funcionam como canais iônicos, causando o vazamento de materiais citoplasmáticos, bem como diminuindo o potencial da membrana (NOROUZI; MIRMOHAMMADI; HOUSHDAR TEHRANI, 2022). No modelo de poro toroidal, os peptídeos também se inserem perpendicularmente na bicama lipídica, mas as interações peptídeo-peptídeo específicas não estão presentes. Em vez disso, os peptídeos, frouxamente associados com os grupos polares dos lipídios, induzem a uma curvatura local da

bicamada lipídica com os poros parcialmente formados por peptídeos interdigitados e as cabeças dos fosfolipídios (KUMAR; KIZHAKKEDATHU; STRAUS, 2018). Em contraste com o modelo de barril, os peptídeos permanecem associados aos grupos principais dos lipídios e não permeiam através das regiões hidrofóbicas da bicama (ALGHALAYINI et al., 2019).

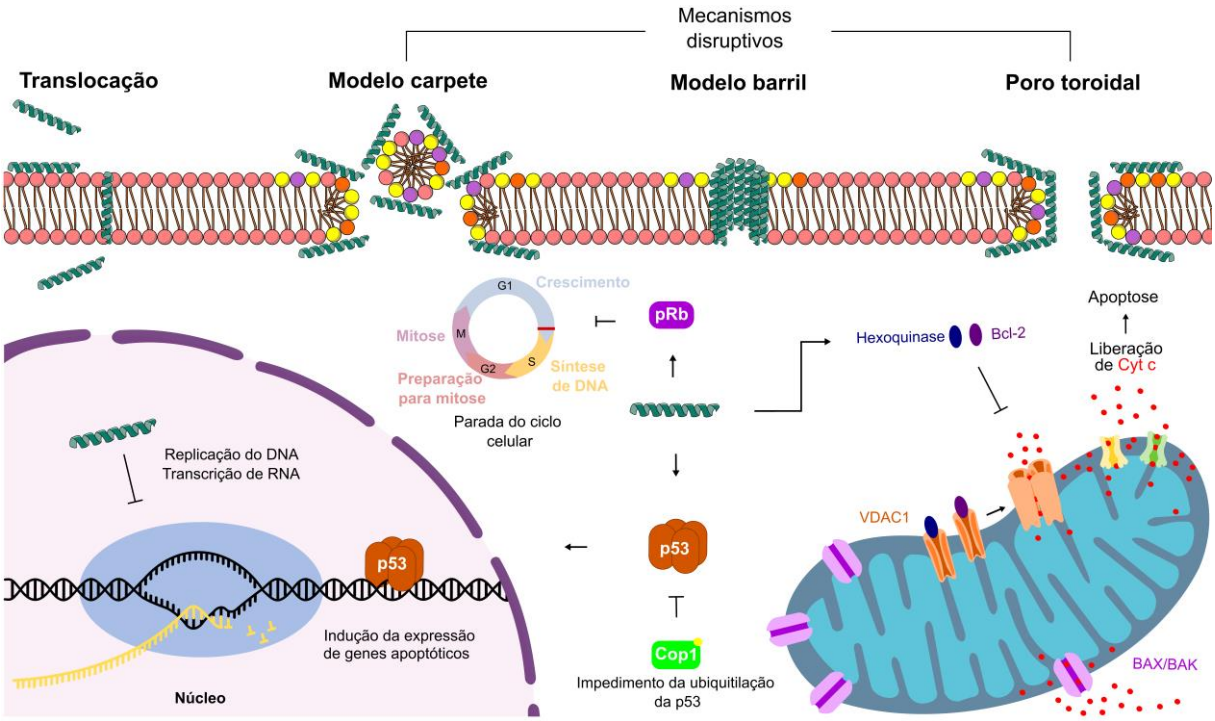

Figura 4. Mecanismos de ação dos PACs. Os peptídeos possuem atividade disruptiva e não disruptiva. No primeiro caso, os PACs interagem, por meio de interações eletrostáticas com a membrana plasmáticas das células alvos, produzindo poros (toroidais e em forma de barril) ou desestruturando a membrana com um efeito semelhante ao causado por moléculas surfactantes (modelo carpete). No mecanismo não disruptivo, os PACs atravessam a membrana, por meio do processo de translocação espontânea, e atuam em alvos intracelulares (mitocôndria, inibição de replicação do DNA, transcrição e tradução do RNA), induzindo à morte celular dependente de mitocôndria. Adaptado de (Soon et al., 2020).

No entanto, como já mencionado, alguns PACs são capazes de atravessar a membrana plasmática e atuarem em alvos intracelulares (Figura 4). Geralmente, uma vez que estes peptídeos entram no citoplasma, eles podem perturbar a integridade da membrana da mitocôndria, liberando, consequentemente, o citocromo c (Cyt c), o que induz à apoptose dependente da mitocôndria (DAI et al., 2016; HWANG et al., 2022). A liberação do Cyt c induz à oligomerização do fator de apoptose-protease 1 (apaf-1), à ativação da caspase 9 e à conversão da pró-caspase 3 em caspase 3, a qual é responsável por muitos eventos apoptóticos (NOROUZI; MIRMOHAMMADI;

HOUSHDAR TEHRANI, 2022). Em adição, os PACs também podem promover a morte celular programada por meio da inibição da síntese de DNA e da formação de poros na membrana da mitocôndria (CHIANGJONG; CHUTIPONGTANATE; HONGENG, 2020).

O peptídeo denominado poropeptídeo BAX [106-134], desenvolvido a partir de um segmento da proteína de membrana de BAX, pode induzir diretamente à liberação de fatores apoptogênicos residentes nas mitocôndrias, por meio da formação de poros na membrana dessa organela, levando as células neoplásicas à morte celular programada dependente de caspase, de forma imediata e irreversível (VALERO et al., 2011). Por meio de uma abordagem semelhante, Sohn e colaboradores desenvolveram o peptídeo NAF1$^{44-67}$ de um fragmento da proteína transmembrana NAF1, contendo um segmento *N*-terminal hidrofóbico, que está embutido na membrana mitocondrial, e uma parte *C*-terminal carregada que é encontrada em solução aquosa. O NAF1$^{44-67}$ demonstrou atividade contra a linhagem de mama MDA-MB-231, sem afetar as células saudáveis, perturbando a membrana da mitocôndria e do retículo endoplasmático, desencadeando a morte celular com características de apoptose, ferroptose e necroptose (SOHN et al., 2022).

Outro alvo é a hexoquinase, enzima glicolítica chave ligada à mitocôndria, envolvida na glicose aeróbica, que promove a tumorogênese, por meio da interação com a isoforma mais abundante do canal aniônico dependente de voltagem (VDAC), denominado de VDAC1. Células neoplásicas evitam a apoptose superexpressando a hexoquinase (SOON et al., 2020). Células B de leucemia linfocítica crônica tratadas com o PPC Antp-LP4, baseado em VDAC1, apresentaram a perda do potencial de membrana mitocondrial ($\Delta\psi$), levando à diminuição da produção de ATP, disfunção mitocondrial, oligomerização de VDAC1 e liberação de Cyt c, eventos que levam à morte celular por via apoptótica. Isso ocorre devido ao fato de o Antp-LP4 interage com a hexoquinase e a Bcl-2, dissociando-se de VDAC1 (PREZMA et al., 2013).

Os PACs também podem atuar em proteínas centrais no processo de tumorogênese, como a p53 (LI, H. et al., 2023; YU et al., 2023). Em testes *in vitro*, células A549 de adenocarcinoma pulmonar e células A431 de carcinoma epidermoide vulvar tiveram seus crescimentos suprimidos pela ação da β-defensina humana recombinante (rec-hBD2). Esta defensina ocasionou a parada do ciclo celular em

G1/S, em ambas as linhagens via desfosforilação de pRb, além de inibir a expressão de ciclina D1, sem alterar a expressão de p53 (ZHURAVEL et al., 2011). Outro exemplo é o peptídeo α-helicoidal de 28 resíduos de aminoácidos (p28), derivado da azurina (uma proteína redox contendo cobre de 128 aminoácidos secretada de *Pseudomonas aeruginosa*). O p28 se liga à p53, selvagem e mutante, inibindo sua ubiquitinação mediada pela proteína morfogênica constitucional 1 (Cop1), evitando à degradação da p53, parando o clico celular em G2/M e levando a uma eventual apoptose (YAGHOUBI et al., 2020).

Entre as diversas classes de peptídeos, as catelicidinas se destacam por serem um grupo que apresentam atividade antitumoral. A catelicidina humana LL-37 é capaz de ativar a apoptose independente de caspase em células de câncer de cólon (REN et al., 2012). Além disso, peptídeo derivados do LL-37 também demonstraram atividade contra neoplasias. O peptídeo FF/CAP18 provoca a perda do potencial de membrana mitocondrial no estágio inicial da apoptose, resultando em um efeito antiproliferativo de células de câncer de cólon HCT116. Outro peptídeo derivado do LL-37, o FK-16, além de induzir à apoptose independente de caspase, também induziu morte celular autofágica em células neoplásicas (REN et al., 2013). Desse modo, catelicidinas compõem uma classe de peptídeos promissores para o desenvolvimento, seja por meio do isolamento de novas moléculas ou desenho racional de peptídeos naturais, de novos fármacos para o tratamento do câncer.

2.2.1 Catelicidinas

As catelicidinas são uma das duas principais classes de peptídeos expressas endogenamente por células epiteliais e imunes de vários organismos e a expressão demonstrou surgir em caso de infecção e inflamação (JAVED et al., 2024). As catelicidinas são peptídeos pleiotrópicos, filogeneticamente antigos (sendo encontrados no peixe-bruxa sem mandíbula, o que indica uma origem de, ao menos, 400 milhões de anos), expressos em diversas formas de vida como um mecanismo de defesa da imunidade inata (FALCAO, C. B. et al., 2014). Assim como em outras famílias de PAMs, catelicidinas estruturalmente divergentes são encontradas, mesmo em uma única espécie de mamífero. Existem ao menos sete catelicidinas em bovinos, equinos, suínos, ovinos e caprinos. No entanto, há algumas exceções como humanos,

macacos rhesus, camundongos, ratos e porquinhos-da-índia que possuem apenas um único exemplar de catelicidina (WANG, Y. et al., 2008). Além de apresentam amplo espectro de atividade microbicida contra bactérias, vírus envelopados e fungos, as catelicidinas também podem desencadear respostas de defesa específicas no hospedeiro (KOŚCIUCZUK et al., 2012).

As catelicidinas são moléculas curtas, catiônicas e anfipáticas (BALS; WILSON, 2003; DE BARROS et al., 2019), liberadas de seus precursores inativos correspondentes por meio de clivagem proteolítica (WANG, Y. et al., 2008). Todos os membros da família catelicidina são caracterizados pela presença de um domínio catelina (um inibidor da cisteína proteinase catepsina L) aniônico altamente conservado (ZHAO et al., 2008). Nos precursores das catelicidinas, os domínios de catelina altamente conservados, compostos por cerca de 100 resíduos de aminoácidos, são flanqueados por um fragmento de peptídeo sinal (aproximadamente 30 resíduos de comprimento) em seu *N*-terminal e por uma região de peptídeo antimicrobiano catiônico estruturalmente variável em sua região *C*-terminal (WANG, Y. et al., 2008). Os domínios *C*-terminais, em algumas catelicidinas, podem apresentar conformações α-helicoidal ou folha-β e em alguns peptídeos podem ser ricos em prolina/arginina, com o peptídeo maduro variando em tamanho de 12 a 80, ou mais, resíduos de aminoácidos (ZANETTI, 2005).

As catelicidinas são produzidas na pele, tecidos epiteliais da mucosa, secreções como saliva e suor (ALFORD et al., 2020; MURAKAMI et al., 2002), bem como nas glândulas de veneno de serpentes (OGUIURA et al., 2023; PERUMAL SAMY et al., 2017). Na classe Reptilia, os PAMs relacionados à catelicidina (CRAMPs) do veneno natural de serpentes constituem uma importante família envolvida na defesa do sistema imunológico, apresentando propriedades não apenas antibacterianas, mas também imunomoduladoras (DE BARROS et al., 2019). Até o momento, 16 catelicidinas foram identificadas em serpentes pertencentes às famílias Elapidae, Viperidae e Boidae (WANG, A. et al., 2019). Entre elas podem ser citadas a catelicidina-BF (WANG, Y. et al., 2008), a OH-CATH (ZHAO et al., 2008), a NA-CATH (BLOWER; BARKSDALE; VAN HOEK, 2015) e a crotalicidina (Ctn) (FALCAO, C. B. et al., 2014). A Ctn (KRFKKFFKKVKKSVKKRLKKIFKKPMVIGVTIPF) foi identificada a partir de bibliotecas de cDNA de glândulas de peçonha da jararaca sul-americana *Crotalus durissus terrificus* (FALCAO, Claudio Borges; RADIS-BAPTISTA, 2020). Por

ser membro das catelicidinas, a Ctn possui o peptídeo sinal, seguido do domínio catelina, o qual encontra-se dobrado por duas pontes dissulfeto e a região *C*-terminal, composta por uma fração aniônica variável rica em Glu (entre 25 e 27 resíduos) e uma porção final catiônica, devido a presença de resíduos de Lys e Arg, responsável pela atividade antimicrobiana desse peptídeo (FALCAO, C. B. et al., 2014). A Ctn é um peptídeo catiônico (+16) α-helicoidal na sua porção *N*-terminal e apresenta-se não estruturado na região *C*-terminal (PERUMAL SAMY et al., 2017). Testes antimicrobianos demonstraram que a Ctn possui atividade contra bactérias (principalmente Gram-negativas), fungos e células neoplásicas (DE AGUIAR et al., 2020; PÉREZ-PEINADO et al., 2018).

A partir da dissecação *in silico* da Ctn, dois fragmentos (Ctn[1-14] e Ctn[15-34]) foram observados e realizados testes antimicrobianos (contra bactérias Gram-negativas e Gram-positivas), antitumorais (carcinoma epitelial do colo do útero (HeLa S3), leucemia Jurkat E6.1 (linhagem de células T), HL-60 (linhagem de promielócitos), U937, THP-1 e MM6 (linhagem de monócitos-macrófagos)) e testes de citotoxicidade contra células saudáveis (fibroblastos humanos 1BR3G) (FALCAO, Claudio Borges et al., 2015). O Ctn[15-34] foi tóxico paras as linhagens sensíveis a Ctn (IC50 igual a 1 μM, 6,25 μM e 25 μM para HeLa S3, U937 e THP-1, respectivamente). No entanto, o outro fragmento (Ctn[1-14]) apresentou atividade apenas contra a linhagem de HeLa S3, com valor acima de 10 μM, e praticamente não exerceu efeitos nas células leucêmicas (FALCAO, Claudio Borges et al., 2015). Portanto, o fragmento ativo Ctn[15-34] pode ser um modelo simples de como os pró-peptideos modulam as propriedades físicas e biológicas de peptídeos naturais mais longos (JÚNIOR et al., 2018).

2.2.1.1 CrotAMP14

Devido ao fato de a catelicidina Ctn ter demonstrado, em testes anteriores, atividade antibacteriana tanto contra cepas sensíveis como isolados clínicos multirresistentes, além de não apresentar efeitos hemolíticos ou citotóxicos para as células saudáveis (OLIVEIRA-JÚNIOR et al., 2018), ela foi escolhida para ter sua atividade potencializada, originando o peptídeo catiônico (+8) sintético crotAMP14. Como o nome sugere, seu tamanho foi reduzido a 14 resíduos de aminoácidos

(KRLKKIFKKMIKIF-NH_2), por meio da abordagem de desenho racional, na qual Oliveira e colaboradores (2020) utilizaram a estratégia de *design* guiada por propriedades físico-químicas (Figura 5), visando a redução do tamanho, bem como preservar (ou potencializar) a atividade antibacteriana e a baixa citotoxicidade do peptídeo parental (OLIVEIRA et al., 2020).

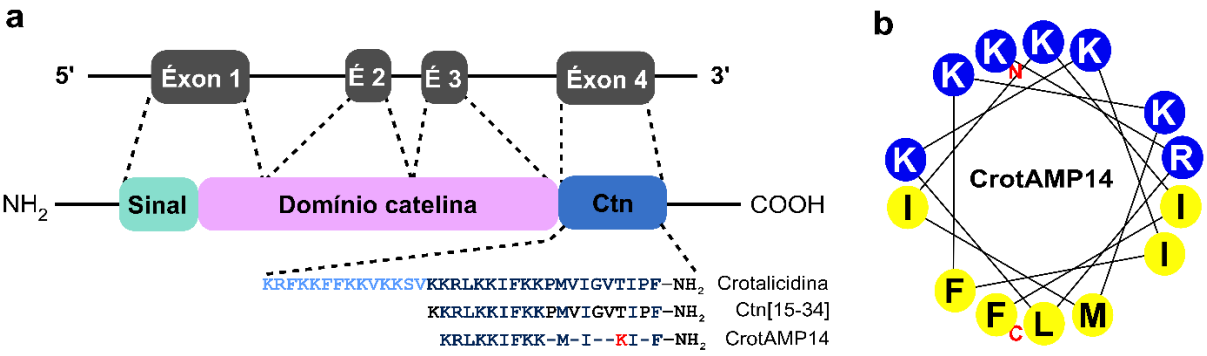

Figura 5. Desenho racional do crotAMP14. (a) A partir de modificações de alguns resíduos de aminoácidos da região antimicrobiana *C*-terminal da catelicidina crotalicidina (especificamente o fragmento Ctn[15-34]), foi obtido o peptídeo sintético crotAMP14. Os resíduos grafados em preto na estrutura do Ctn[15-34] foram removidos para originar o crotAMP14. O resíduo grafado em vermelho, representa a adição de um resíduo de lisina. (b) Análise de dicroísmo circular demonstrando o arranjo dos resíduos carregados positivamente (em azul) e os não polares e aromáticos (em amarelo). Fonte: Autor. Adaptado de (OLIVEIRA et al., 2020).

O crotAMP14 possui atividade contra ambos microrganismos suscetíveis e isolados clínicos, com concentrações inibitórias mínimas (MIC) variando entre 1,5 μM e 12,5 μM, além de ser pouco tóxico contra células Caco-2 (OLIVEIRA et al., 2020). Análises de dicroísmo circular e de dinâmica molecular indicaram que, em ambiente análogo a de uma membrana, o crotAMP14 assume uma conformação estrutural α-helicoidal a qual é capaz de interagir com fosfolipídios da bicamada lipídica, por meio de ligações de hidrogênio e interação eletrostática, resultando na desestabilização da membrana bacteriana, algo sugerido por microscopia (OLIVEIRA et al., 2020). Uma vez que o peptídeo parental, no qual o crotAMP14 foi obtido, apresenta atividade antitumoral (PÉREZ-PEINADO et al., 2018), o crotAMP14 foi escolhido para análise da modulação da expressão gênica em linhagem de câncer de mama triplo negativo, MDA-MB-231 após a triagem de vários PAMs.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Elucidar o mecanismo de ação antitumoral do peptídeo sintético crotAMP14 contra linhagem de câncer de mama triplo negativo, MDA-MB-231.

3.2 OBJETIVOS ESPECÍFICOS

- Selecionar um peptídeo candidato a antitumoral por meio de ensaio de viabilidade celular;
- Definir o menor período em que o peptídeo é ativo contra células neoplásicas.
- Realizar testes de citotoxicidade a fim de demonstrar que o crotAMP14 não é ativo contra células saudáveis;
- Avaliar se a morte celular das células expostas ao crotAMP14 ocorre por meio de apoptose, utilizando análise de expressão gênica por qPCR;
- Avaliar a ativação das caspases 3 e 7 nas células expostas ao crotAMP14;
- Elucidar a estrutura tridimensional do peptídeo candidato (crotAMP14) por meio de análises de Ressonância Magnética Nuclear (RMN);
- Analisar uma possível interação entre o crotAMP14 e a membrana plasmática das células de MDA-MB-231 por espectroscopia de EPR;
- Avaliar o grau de rigidez da membrana plasmática de células neoplásicas (MDA-MB-231) e células de cultura primária de fibroblastos humanos;
- Simular a interação do peptídeo crotAMP14 em membranas de células neoplásicas (MDA-MB-231) e saudáveis (hFib) por meio de ensaios de dinâmica molecular;
- Definir as interações eletrostáticas entre os resíduos de aminoácidos do crotAMP14 e os fosfolipídios carregados negativamente da membrana plasmática de células neoplásicas (MDA-MB-231).

4 ARTIGO ORIGINAL DO DOUTORADO

Universidade Católica de Brasília – UCB Centro
de Análises Proteômicas e Bioquímicas
Pós-graduação em Ciências Genômicas e Biotecnologia

Chief editor

Nature Communications

Dr. Lukman,

Please find enclosed the manuscript entitled “Unveiling the mechanism of action of a crotalicidin-derived peptide in combating triple-negative breast cancer cells” that we are submitting for consideration publication as an Original Article in **Nature Communications**. Neither the present manuscript nor any similar article has been submitted or published in any other scientific journal.

In this article, we screened 17 peptides from different sources (natural and synthetic) to evaluate their potential antitumor activity. Based on the selection criteria (number of peptide-sensitive lines and the lowest number of residues), we selected crotAMP14 for further analysis. The data demonstrated that the peptide is active against triple-negative breast cancer cells at concentrations up to 8 μM, generating caspase-independent killing. The mechanism of action of crotAMP14 occurs through interaction with the plasma membrane of the target cell, where, when in contact, it assumes an alpha-helix structural conformation, and inserts itself into the membrane, increasing its rigidity. This is the first time that the mechanism of action of crotAMP14 has been investigated against a breast cancer cell line, so the data generated are important for understanding the possible mechanisms of action of the peptide, in addition to aiding in the development of new ACPs from AMPs.

Based on this, we look forward to hearing from you regarding the suitability of this manuscript for publication in Nature Communications.

Best regards,

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Unveiling the mechanism of action of a crotalicidin-derived peptide in combating triple-negative breast cancer cells

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ABSTRACT

Triple-negative breast cancer (TNBC) is the most common female neoplasm type, accounting for between 10% and 20% of all diagnoses. As TNBC does not have estrogen, progesterone, and HER2 receptors, it presents a very aggressive behavior with an unfavorable prognosis. Therefore, new, more effective alternatives with reduced side effects are necessary to treat this neoplasia. Anticancer peptides (ACPs) seem promising for cancer treatment due to their greater affinity with their targets and low side effects. This study evaluated the antitumor potential of 17 peptides of various origins. After the screening, we chose crotAMP14 (14 aa), which is active against MDA-MB-231, PMC-42, and MCF-7 cell lines, to elucidate the mechanism of antitumor action. CrotAMP14 alters the gene expression profile of both anti-apoptotic and apoptotic genes but inhibits caspase activity. Gene expression analyses by qPCR demonstrated that crotAMP14 alters the expression profile of genes involved in the cell cycle and death by apoptosis. However, crotAMP14 inhibits caspase activity by 100%. EPR spectroscopy and molecular dynamics analyses revealed that the peptide acts specifically on the plasma membrane of MDA-MB-231 cells neoplastic cells, increasing its rigidity without increasing oxidation, demonstrating cell death by necrosis. The data generated here shed some light on the crotAMP14 antitumor mechanism of action and how this knowledge enables the development of new therapeutic strategies against breast cancer.

Keywords: TNBC, crotalicidin, anticancer peptides, breast cancer.

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INTRODUCTION

Breast cancer (BC) is the most commonly diagnosed cancer, with 2.3 million cases in 2021 alone[1], and the second leading cause of death among women worldwide[2–4]. BC is a pretty heterogeneous disease, being clinically divided into three subtypes based on immunohistochemistry according to the expression of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor type 2 receptor (ERBB2, also HER2)[5]. These subtypes can be classified as luminal ER+/PR+ (which are subdivided into luminal A and B), HER2+, and triple-negative breast cancer (TNBC)[6]. Among these subtypes, TNBC is a highly aggressive tumor characterized by remarkable intra-tumoral heterogeneity (ITH) and plasticity, representing the most lethal and treatment-resistant breast cancer with limited treatment options and effectiveness[7,8].

TNBC is defined by the absence of both ER and PR and does not have amplification of the HER2 gene or overexpression of the HER2 protein[9–11]. TNBC is the most common subtype in premenopausal women under 40 years of age and, when compared with other breast cancers (ER+, PR+, and HER2+), it has more significant metastatic potential, as well as a worse clinical outcome when initiated in people at an earlier age[12]. Currently, chemotherapy is considered the standard treatment against TNBC, which includes the administration of DNA intercalating agents (doxorubicin, a member of the anthracycline class), alkylating agents (cyclophosphamide), which prevent the formation of microtubules (taxane) and a cellular antimetabolite (fluorouracil, 5-FU)[13]. However, despite its efficiency, chemotherapy causes a series of side effects such as nausea, alopecia, vomiting, myelosuppression, and fatigue[14].

Given this scenario, it is urgent to search for new therapeutic molecules that have a mechanism of action that is more specific to their targets and a lower potential for generating resistance and side effects in patients with cancer. Currently, multifunctional peptides, antimicrobial peptides (AMPs) with different activities, such as antibacterial, antifungal, antiviral, immunomodulatory, and anticancer, are considered plausible alternatives to traditional medicines for cancer treatment[15]. AMPs are short oligopeptides (5–45 amino acid residues), usually presenting a net-positive charge, and produced by bacteria, fungi, plants, and animals as their first line of defense against pathogens[16–18]. Some AMPs have dual antibacterial and antitumor activity, being called anticancer peptides (ACPs)[19], which can be explored as an alternative treatment for breast cancer[20].

Cathelicidins stand out as a group that exhibits anticancer activity and can be classified, in some cases, as ACPs[21–24]. All cathelicidin family members are characterized by a highly conserved anionic cathelin domain (an inhibitor of the cysteine proteinase cathepsin L)[25], as well as highly conserved cathelin domains, flanked by a signal peptide fragment (~30 aa) at their N-terminus, and by a structurally variable cationic antimicrobial peptide at their C-terminal region[26]. Additionally, in some cathelicidins, the C-terminal domains may have α-helical or β-sheet conformations and, in some cases, the peptides may be rich in proline/arginine residues, with the mature peptide varying from 12 to 80 amino acids in length[27]. Cathelicidins isolated from snake venom have been pinpointed for their potential as novel drugs in the context of breast cancer[28,29].

Crotalicidin (ctn), a peptide previously identified from cDNA libraries of venom glands of the South American pit viper *Crotalus durissus terrificus*[30], has shown antitumor activity against numerous cancer types, including HeLa S3,

U937, and THP-1[31]. Ctn is a cationic peptide (+16) that adopts a hybrid structural scaffold comprised of an α-helical segment in its N-terminal portion and an unstructured one in the C-terminal region[32]. The antitumor activity of this peptide seems to be associated with the ctn[15-34] fragment, generated from the *in silico* dissection of ctn, which was toxic to ctn-sensitive cell lines (IC_{50} equal to 1 μM, 6.25 μM and 25 μM against HeLa S3, U937, and THP-1 cell lines, respectively)[31]. From the ctn[15-34] fragment, the synthetic peptide crotAMP14 used in the present study was obtained through physicochemical properties-guide design strategies, aiming at reducing the parental peptide's size, as well as preserving or enhancing the antibacterial activity and, finally, reducing possible cytotoxic effects toward healthy mammalian cells[33]. Circular dichroism and molecular dynamics analyses indicated that, in membrane-like environments, crotAMP14 assumes an α-helical structural conformation that is capable of interacting with phospholipids of the lipid bilayer through hydrogen bonds and electrostatic interaction, resulting in bacterial membrane destabilization[33].

Herein, after screening 17 peptides from different sources (natural and synthetic), we selected the synthetic peptide crotAMP14 to evaluate its antitumor potential against the triple-negative cell line MDA-MB-231. We performed several analyses to elucidate the mechanism of antitumor action of crotAMP14. The data demonstrate that the peptide interacts with the plasma membrane of neoplastic cells through electrostatic interactions. Once in contact with the cells, crotAMP14 causes a disruption in the membrane in the first 15 min of exposure, leading to cell death by mechanisms that are independent of caspase activation.

RESULTS AND DISCUSSION

Screening for peptides with antitumor activity

All 17 peptides (Table S1), either from natural sources or rationally designed, were chosen to screen for an anticancer candidate because they are effective against Gram-negative bacteria[34]. The plasma membrane of Gram-negative has a net negative charge due to the presence of phosphatidylglycerol (PG) and cardiolipin (CL)[35]. Similarly, the plasma membrane of neoplastic cells also presents a negative net charge due to phosphatidylserines (PS) in the membrane's outer layer[36]. Otherwise, the outer leaflet of the plasma membrane of healthy eukaryotic cells contains most of the phosphatidylcholine (PC) and probably all of the sphingolipids, resulting in a neutral net charge[37]. These characteristics contribute to ACP selectivity since most are often positively charged (+2 – +9) at physiological pH due to several lysine and arginine residues throughout the primary structure[38,39].

To perform peptide screening, a cell viability (MTT) assay, by quantifying the damage induced by an agent in cellular carbohydrate metabolism, by evaluating the activity of mitochondrial dehydrogenases, was carried out using different concentrations (64, 32, 16, 8, 4, 2, and 1 μM) of each of the peptides (Figure 1a). The cells were initially exposed to the peptides for 24 hours. Except for peptides LL-37 and IDR1021 (insufficient quantity for testing), all other peptides were tested against the three mammary adenocarcinoma lines MDA-MB-231 (ER-, PR- and HER2-), MCF-7 (ER+) and PMC-42 (ER+ and PR+).

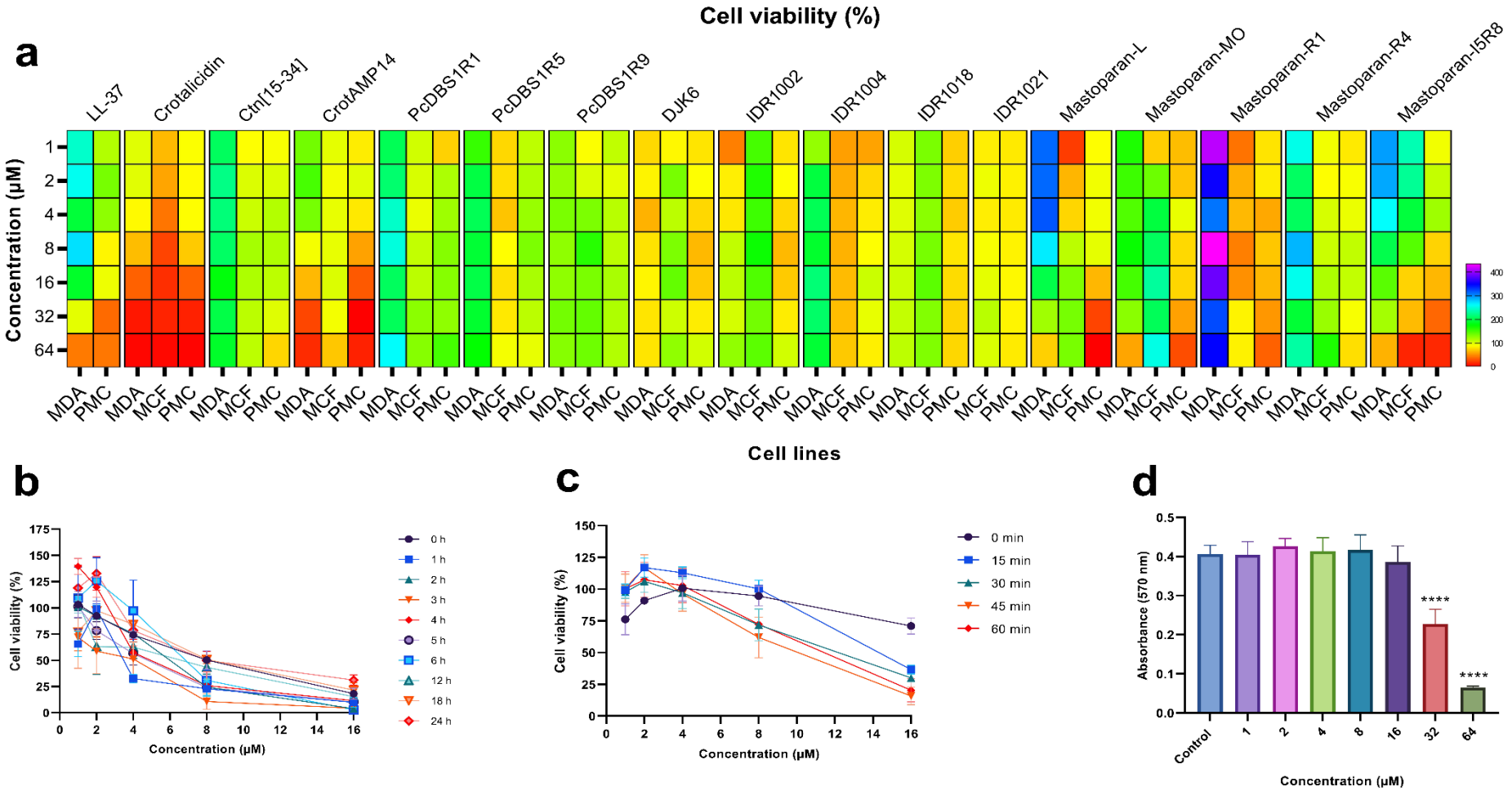

Figure 1. Screening of peptide with antitumor activity. a Seventeen peptides were evaluated at different concentrations (y-axis) against three breast cancer cell lines (x-axis). Except for LL-37 and IDR1021, all other peptides were tested against the three cell lines. **b** Cells (MDA-MB-231) were exposed to crotAMP14 during different periods (within a 24-hour interval) of treatment. The plate was then read at a wavelength of 570 nm. The graphs represent the mean and standard error of the absorbance of three triplicate biological replicates. **c** Cells (MDA-MB-231) were exposed to crotAMP14 during treatment periods (within 60 min). The plate was then read at a wavelength of 570 nm. The graphs represent the mean and standard error of the absorbance of three triplicate biological replicates. **d** Primary cultured human fibroblast cells were exposed to crotAMP14 during the 24-hour treatment period. The plate was then read at a wavelength of 570 nm. The graphs represent the mean and standard error of the absorbance of three triplicate biological replicates. The one-way ANOVA test and Bonferroni post-test verified statistical differences. ****$p < 0.0001$.

Although several peptides have been shown to have antitumor activity, even against a single cell line (mastoparan-L, mastoparan-MO, mastoparan-R1, mastoparan-I5R8, and LL-37), or all of them (ctn), the ctn-derived crotAMP14 peptide was chosen for future testing. The choice of crotAMP14 met two criteria: (i) the number of cell lines for which the peptide has activity and (ii) the size of the molecule. Compared to the other peptides, crotAMP14 was effective against the cell lines (even though for MCF-7, it was effective at 64 μM, Figure S1). Compared to ctn, crotAMP14 is smaller, reducing the production cost by chemical synthesis. Ctn is a member of the cathelicidins found in the venom gland of the South American pit viper[30]. Cathelicidins from different species have been shown to have antitumor activity[40,41]. Evidence demonstrates that motifs present in these peptides may be related to their activity[42], such as the FK-16 peptide, derived from LL-37, which induces colon cancer cells to caspase-independent apoptosis[43].

CrotAMP14 was effective against the triple-negative breast cancer cell line MDA-MB231 at concentrations of 64, 32, and 16 μM (Fig. 1a–c and Figure S1). Among all breast neoplasms, TNBC is the most diagnosed subtype, comprising between 10% and 20% of cases[44]. Due to its complexity, treatment for breast cancer is multidisciplinary, with conventional surgery associated with radiotherapy or mastectomy being recommended in women with early-stage disease[45]. Thus, the success of curative therapy may be linked to its administration within a defined period[46]. However, patients diagnosed with TNBC have an unfavorable prognosis due to the lack of targeted therapies[47]. Thus, crotAMP14 can be explored as an alternative for developing unconventional therapies, either alone or as an adjuvant.

Compared to ctn (34 aa), the parental peptide, crotAMP14, is smaller (14 aa), reducing the production cost through chemical synthesis. This way, strategies such as rational peptide design that reduce the peptide length while maintaining the parental activity can be explored to create novel antitumor peptides. Herein, we tested the croAMP14 peptide from the redesign of ctn[15-34] through the physicochemical-guided design strategy, in which unfavorable residues (K^{1}, P^{11}, V^{13}, G^{15}, V^{16}, T^{17}, and P^{19}) for the electrostatic surface were excluded, maintaining alternation between positively charged and hydrophobic residues. This strategy enhanced the crotAMP14 activity without showing an increase in cytotoxicity against healthy mammalian cells[33].

When the crotAMP14 was tested against the MCF-7 cell line, only 30% of cells were killed at the highest concentration (64 μM). However, 80% of cells from the triple-negative MDA-MB-231 cell line died when exposed to 32 μM of crotAMP14. At a concentration of 16 μM, although there was a decrease in cell death, the percentage of inhibition was 65% (similar was observed in PMC-42 at concentrations of 32 and 16 μM). Due to this survival rate, the concentration of 16 μM was chosen for further testing. Cell viability tests were then performed to assess the minimum interval necessary for crotAMP14 to induce cell death (Figure 1b).

Tests were carried out at different intervals to assess the shortest time required for crotAMP14 to be effective (0, 1, 2, 3, 4, 5, 6, 12, 18, and 24 h). Cells were exposed to 16 μM of the peptide at these different intervals. The data demonstrated that after one hour of exposure, it is possible to observe a death rate of 90.1% (16 μM), 77.1% (8 μM), and 67.4% (4 μM) (Fig. 1b). The data demonstrate that crotAMP14 could cause cell death in the first 60 min of exposure

at 16 and 8 μM. Over time (2 to 24 h), this result was maintained, indicating that the activity of the peptide begins one hour after encountering the cells and continues to be capable of eradicating part of the cells for 24 h, at least at the highest concentrations tested. Further, we tried to identify how long it would take for the crotAMP14 peptide to induce cell death. Therefore, another cell viability test was conducted at 0-, 15-, 30-, 45-, and 60-min intervals. The results suggest that cells begin cell death after 15 min of exposure to the peptide at 16 μM (Figure 1c). Over time, cell death is also visible at 8 μM at 30- and 45-minute intervals. However, only at 16 μM, the percentage of cell death is 63.5% (15 min), 69.9% (30 min), 84.2% (45 min), and 79.7% (60 min) (Fig. 1c).

The last stage of screening the crotAMP14 peptide consisted of carrying out a cytotoxicity test against the primary culture of human fibroblasts (hFib) (Figure 1d). The cells were exposed to 64, 32, 16, 8, 4, 2, and 1 μM of the crotAMP14 peptide during 24 h. The results demonstrated that the crotAMP14 peptide was not toxic against the human fibroblasts at concentrations between 16 and 1 μM, being toxic only at the highest concentrations (32 and 64 μM). Therefore, the concentration chosen for gene expression modulation analysis in triple-negative mammary adenocarcinoma is not harmful to the primary hFib culture and can still induce the cells to death, which can be explored in treatments aimed at controlling the proliferation of tumor cells.

Relative expression analysis (qPCR)

During the screening, we noticed that the cells were detached and reduced in size (Figure 2a, red arrows). Furthermore, flow cytometry analyses using

forward scatter (FSC) and side scatter (SSC) showed a decrease in cell size after exposure to crotAMP14 (data not shown). Cellular shrinkage is a morphological change associated with the process of early apoptosis[48], and, in some cases, the decrease in cell size can represent 38% of the average areas of cells that will die by apoptosis[49]. Thus, we selected 30 anti-apoptotic and apoptotic genes (Table S2) to evaluate the relative gene expression of MDA-MB-231 cells after exposure to 16 μM of the crotAMP14 peptide. Initially, cells were exposed to treatment with crotAMP14 at 16 μM for 0, 15, 30, 45, and 60 min (Fig. 2a).

Cells from the treatment, both from the supernatant and those remaining on the plate, were collected, and the total RNA was extracted (Figure 2b). Then, the RNA was treated with DNAse, and cDNA was generated. To assess the quality of the cDNA, a conventional PCR was performed using primers designed for RNA28S, one of the reference genes (Figure 2c and Table S2). Analysis of the relative expression of genes related to cell cycle and programmed cell death revealed that cells in the supernatant, compared to the control (Figure 2d-f and Tables S3-S5), showed an increase in apoptosis-related gene expression. Among them, caspase 3, 8, and 9 can be highlighted, which are upregulated at 0 min. An increase in the expression of the BID, BAK1, and BAX genes was also observed at different exposure times. At the same time, genes related to the cell division cycle (CDK4/6) were downregulated (15 min). Interestingly, genes related to cell proliferation (JNK1, PIK3CA) also increased expression levels.

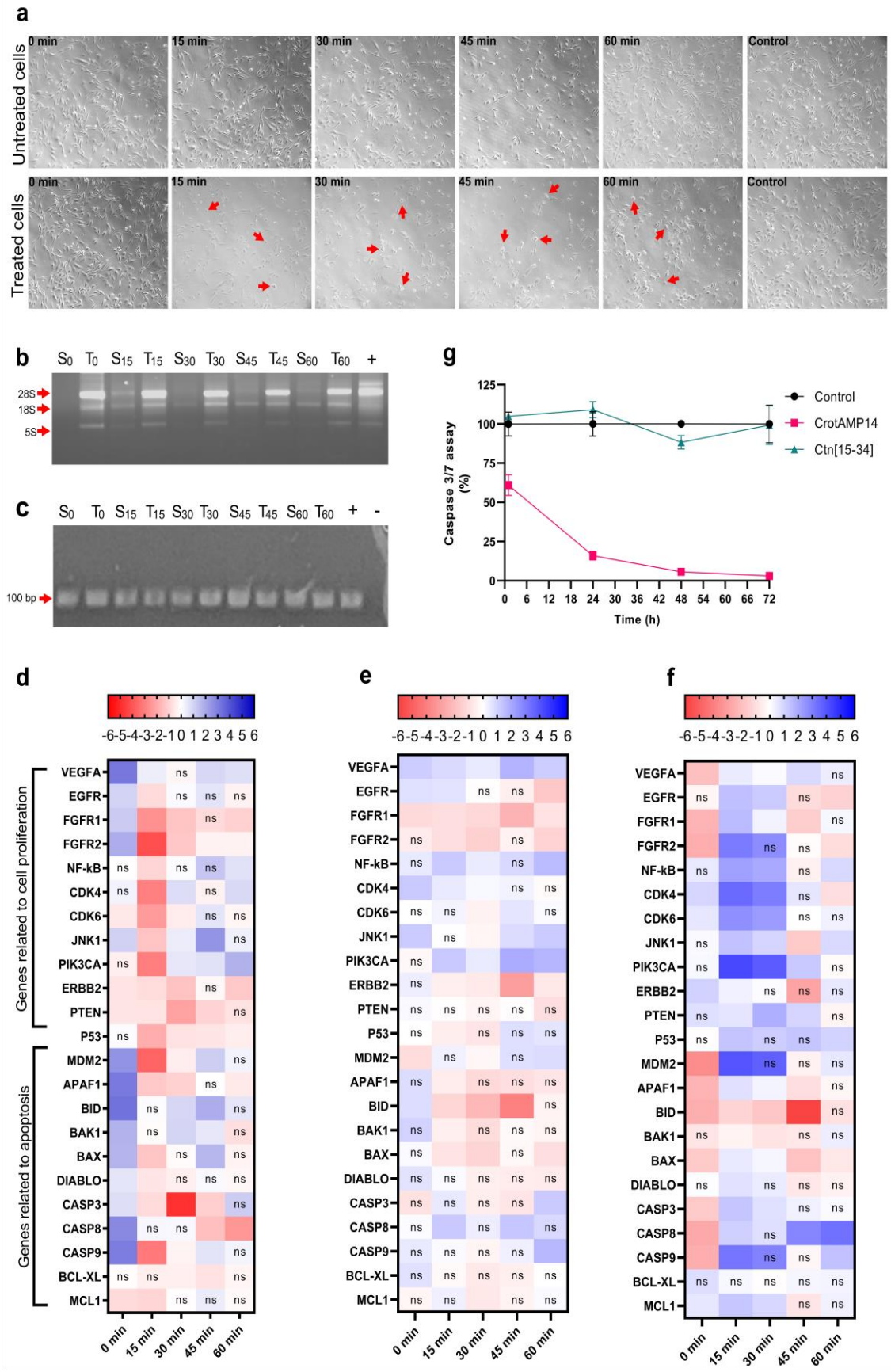

Figure 2. Total RNA extraction from cells treated with 16 µM crotAMP14 and heatmap of relative gene expression analysis by qPCR. a Cells photographed during the experiment showed untreated cells (top, untreated cells) and treated cells (bottom treated cells). Note that, as time passes, the cells become

detached from the bottom of the plate, remaining in suspension (globular cells pointed by red arrows). **b** 1% (w/v) agarose gel electrophoresis of total RNA extracted from treated cells. **c** 1% (w/v) agarose gel electrophoresis of the amplicons generated by conventional PCR to verify the quality of the cDNA. **d** Comparison between control (untreated cells) and dead cells collected in the supernatant. **e** Comparison between control and live cells (adhered). **f** Comparison between dead and living cells. **g** Caspase 3/7 activity assay. MDA-MB-231 cells were exposed to 16 µM at different intervals (1, 24, 48, and 72 h) to evaluate caspase 3/7 activity (red squares). Untreated cells were used as a control (black dots), and cells treated with the ctn[15-34] peptide were used as a negative control (green triangles) since this peptide was not active against the MDA-MB-231 strain. **ns** stands for non-statistical significance. **S** stands for supernatant cells (dead cells); **T** stands for attached cells (live cells); Subscript numbers mean the treatment time for each sample. **+** stands for positive control, **-** stands for negative control (H_2O), and **control** (untreated cells).

Regarding to the cells that remain attached, a reduction in the expression of genes related to programmed cell death was generally observed (Fig. 2e). However, most genes did not show statistically significant variations (ns). Caspase 8 showed increased expression at 15- and 45-minute intervals and caspases 3 and 9 showed increased expression at 60 min of peptide exposure. *BAX* and *BID* were downregulated. By contrast, genes involved in the cell cycle (CDK4/6) had their levels elevated in some intervals. Furthermore, *VEGFA* showed an increase at all exposure times. The most significant difference in the expression level of the evaluated genes was observed when comparing supernatant and adherent cells (Fig. 2f). Caspase 8 expression was expressed at intervals of 45- and 60-min. Caspase 3 and 9 showed an increase in expression after 15 min of peptide exposure. Furthermore, *P53* increased expression at 15, 45, and 60 min. By contrast, there was also an increase in the expression of *CDK4*/6, *PIK3CA*, *FGFR2*, and *EGFR*.

Although these data are relevant, the cell expression profile observed after exposure to the peptide does not correlate with that found in the literature. The opposite is generally true, as demonstrated in a study in which crotalicidin BMAP-27 reduces colon cancer cell proliferation by upregulating tumor suppressor genes (*CASPASE3*, *BAX*, *TP53*, *AXIN1*) while downregulating oncogene expression (*BCL-2*, *CDK-6*, *PCNA*, *WNT11*, and *CTNNB1*)[50]. On the other hand, the present study showed simultaneous upregulation of caspase genes and genes involved in the cell cycle (*CDK4/6*). Due to this discrepant gene expression profile, we evaluated the activation caspases-3/7 to identify whether cell death occurs through apoptosis mechanisms.

Caspase activity assay

The caspase activation tests (Figure 2g) data demonstrated that, although there is an increase in gene transcription, this does not reflect the activation of these proteins since crotAMP14 inhibits the activity of caspases 3 and 7 by 39% in the first hour of incubation. After 24h of exposure, the crotAMP14 peptide inhibited the activity of caspases 3 and 7 by 83.9%, increasing to more than 90% in 48h, remaining until the end of the experiment (72h). We used the parental peptide ctn[15-34] as a control since it has no activity against the triple-negative strain MDA-MB-231 (Figure 1a). Ctn[15-34] neither increased nor decreased caspase activation compared to the control (Fig. 2g).

Caspase-3/7 are cysteine-aspartic acid proteases responsible for directly triggering cell death by apoptosis followed by sequential activation of other caspases (8 and 9)[51]. Interestingly, crotAMP14 inhibited caspase-3/7 activity in MDA-MB-231 cells by approximately 100% after 48 h of exposure. Although previous data suggested a possible apoptotic cell death, caspase inhibition by crotAMP14 demonstrated that cell death can occur by another caspase-independent mechanism, such as necrosis. Thus, we began considering other targets on which the peptide could act directly. Since most of the peptides interact with the plasma membranes of cells[52], we focused on this as a possible target that could explain the observed response. Further evidence supporting this claim is that crotAMP14 showed similarities with the oncolytic peptide chemokinestatin-1 (CKS1)[53]. CKS1 is a 24-mer peptide derived from the chemokine CXCL1 that, when in contact with 4T1 cells, results in the detachment of these cells and rapid cell death (30 min). It has been shown that CKS1 causes severe damage to the plasma membrane, inducing cell death by necrosis[53]. To evaluate a possible

interaction between the crotAMP14 peptide and the plasma membrane of triple-negative breast cancer cells, we elucidated the three-dimensional structure of the peptide using nuclear magnetic resonance.

Elucidation of the 3D structure of crotamp14 by NMR

The NOE correlation data indicated medium and week intra-residues and sequential H_N-H_α connectivities across all residues, with specific H_N-H_B interactions observed between the final three residues 12Lys-13Ile-14Phe. Both, short distances (HN-HN, i,i+2; HN-Hα, i,i+2), and medium distances (H_N-Hα, i,i+3), and long-range connectivities (H_N-H_α, i,i+4) connectivity were identified. The crotAMP14 chemical shifts are present in Table S6. In the experimental conditions, the carbon alpha and carbon beta of the 1Lys, 3Leu, 6Ile, 7Phe, 11Ile, 13Ile, and 14Phe were not identified, but this did not impair the identification of the chemical shifts of other carbons and the not-mentioned residues. The three-dimensional structures were calculated using distance restraints derived from NOE intensities and dihedral angles calculated in the DANGLE software (Figure 3a and Figure S4).

As shown in Fig. S4, the residues are disposed of in the structure in an amphipathic configuration, except for 11Ile, which is disposed of in the hydrophilic region. The summary of the structural statistics of crotAMP14 in the presence of SDS-d_{25} micelles is presented in Table S7. Notably, the RMSD of the superimposed peptide backbone's secondary structures shows little deviation (0.14 +/- 0.5), indicating the stability of the crotAMP14 structure under the studied conditions. The Ramachandran plot analysis (Figure S5) indicates that most

residues (93.6%) are in the most favored region, and 6.44% are in the additionally allowed region.

The biological function of AMPs is influenced by key physicochemical properties such as charge, helicity, hydrophobicity, sequence length, amphipathicity, and solubility.[54] Understanding the structure-function relationship is crucial for drug development.[54] Our NMR findings demonstrate that crotAMP14 adopts an α-helical conformation in SDS, aligning with molecular dynamics simulations published by our group.[33] Cationic α-helical ACPs, including crotAMP14, are known for their anticancer properties, typically acting on neoplastic cells through electrostatic interactions with cell membranes, leading to lysis.[55–57] CrotAMP14, a short cationic α-helical peptide (+8), exhibits potential antitumor activity, as it interacts with bacterial cell membranes.[33] Spectroscopy data further demonstrate crotAMP14's interaction with the plasma membrane of triple-negative breast cancer cells.

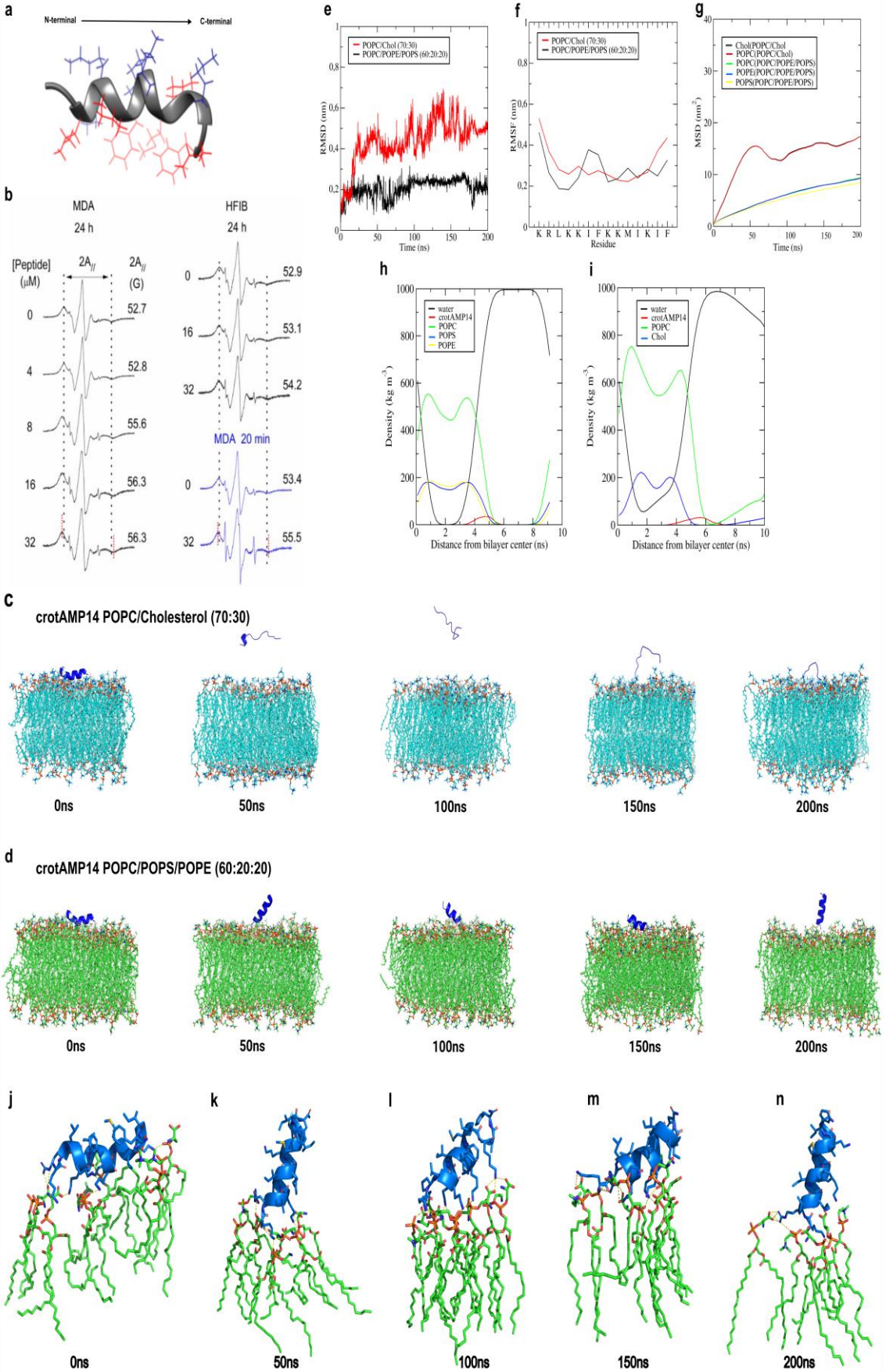

Figure 3. CrotAMP14 3D structure elucidation and membrane interaction analysis. a CrotAMP14 peptide NMR structure; the lowest energy structure in cartoon representation. **b** Representative EPR spectra of spin label 5-DSA incorporated into the plasma membranes of MDA-MB-231 and hFib for untreated

samples (control) and treated with the crotAMP14 peptide at indicated concentrations. The treatment was conducted with 24 h and 20 min incubation periods using 6 x 10^5 cells. The values of the EPR parameter $2A_{//}$ (outer hyperfine splitting) are indicated for each EPR spectrum. $2A_{//}$ is given by the magnetic field separation between the first peak and the last inverted peak (indicated by dotted vertical lines). The estimated experimental error of $2A_{//}$ is 0.5 G. The intensity of the spectra is in arbitrary units (Y-axis), and the total scan range of the magnetic field in each EPR spectrum was 100 G (X-axis). MDA = MDA-MB-231. **c, d** MD simulations of the crotAMP14 peptide in lipid bilayer systems, including POPC/Chol (70:30), mimicking the healthy membrane (**c**) and POPC/POPE/POPS (60:20:20), mimicking the cancer membrane (**d**). The simulations were performed with 75 $Å^2$ bilayers during 200 ns and represented at 50 ns intervals. **e–l** MD simulation analyzes crotAMP14 in lipid bilayer systems, including POPC/Chol (70:30) and POPC/POPS/POPE (60:20:20). The RMSD (**e**) and RMSF (**f**) of crotAMP14 were calculated for each simulated system. Moreover, the influence of crotAMP14 on the mean square lipid shift (MSD) was also evaluated for each lipid individually (**g**). Partial density maps are also shown for the crotAMP14-POPC/POPS/POPE (**h**) and crotAMP14-POPC/Chol (**i**) molecular complexes. **j–n** Mapped atomic interactions between the molecular complex crotAMP14-POPC/POPS/POPE. Structural snapshots at 0 ns (**j**), 50 ns (**k**), 100 ns (**l**), 150 ns (**m**) and 200 ns (**n**).

Electron paramagnetic resonance (EPR) spectroscopy of labeled MDA-MB-231 membranes

EPR spectra of 5-doxyl stearic acid (5-DSA) inserted into the plasma membranes of MDA-MB-231 and hFib cells for samples treated with different concentrations of crotAMP14 (Figure 3b). Treatment of the MDA-MB-231 cell with the crotAMP14 caused membrane rigidity from a concentration of 8 μM with a pronounced increase of ~2 G in the $2A_{//}$ parameter value. For concentrations of 16 and 32 μM, the rise in $2A_{//}$ was ~3 G.

On the other hand, treatment of the hFib cell with the peptide caused a reduction in fluidity only at the concentration of 32 μM, with a modest increase in $2A_{//}$ of 1.3 G. Therefore, as shown in Fig. 1d, cell death does not occur with 16 μM of crotAMP14 since this concentration is not sufficient to increase the rigidity of the plasma membrane of the hFib primary culture (Fig. 3b). Furthermore, even though at 32 μM there was cell death (Fig. 1d), the EPR data demonstrate that there was a minimal increase in stiffness.

EPR spectra indicated that the membranes of MDA-MB-231 cells have fluidity similar to that of the J774.A1 macrophage and the *Leishmania* parasite with $2A_{//}$ values of approximately 53 G[58,59]. Membrane rigidity associated with oxidative stress has been observed in the *Leishmania amazonensis* parasite. Several compounds that promote increased ROS formation, such as chalcone[58,59], β-carboline[60], and isoxazole derivatives[61], caused membrane rigidity after 24 h of treatment at concentrations of their respective IC_{50} values. However, unlike these compounds, the crotAMP14 showed increased rigidity in the MDA-MB-231 cell membrane for a short incubation period. This result

indicates that the crotAMP14 causes rigidity due to its physical presence in the membrane instead of triggering oxidative processes that require a longer period. Furthermore, it was found that the peptide can cause rigidity in isolated MDA-MB231 membranes shortly after treatment (EPR spectra not shown), indicating its interaction directly with the membrane.

In the cell membrane, it has been shown that the 5-DSA spin label behaves like annular or boundary lipids that preferentially surround the hydrophobic surface of membrane proteins and this effect restricts the mobility of the spin probe[62]. Thus, the greater the protein content of the membrane, the lower its fluidity. For instance, in the erythrocyte membrane that has a protein content of approximately 50%, the $2A_{//}$ value is greater than 56 G[63]. Based on these findings, it is expected that the presence of the crotAMP14 in a possible α-helix structure may restrict the mobility of cell membrane lipids. Furthermore, it is also likely that the peptide may hinder the packaging of lipid chains and thus increase the probability of pore formation in the cell membrane. The crotAMP14 also caused a reduction in membrane fluidity in fibroblast cells, but the effect was only observed at a concentration of 32 μM. The fact that there is a lesser effect on the fibroblast may be related to the lower entry of the peptide into the cell membrane.

CrotAMP14 effect on membrane bilayers

MD simulations were performed to investigate the interactions of crotAMP14/membranes at the atomic level using the lowest free energy crotAMP14 NMR structure as input for all simulations. Figures 3c and d show the peptide/bilayer structural snapshots during 200 ns, at 50 ns intervals. As depicted

386 in Figure 3c, the crotAMP14 peptide detaches from the membrane and loses its
387 a-helical secondary structure during the MD simulations. At 200 ns, the peptide
388 slightly re-attaches to the membrane through its termini regions. However, no
389 evident insertion in the membrane is observed. By contrast, when in contact with
390 a cancer lipid bilayer, the crotAMP14 peptide conserves its structure throughout
391 the simulations and preserves its interactions with this anionic membrane system
392 through the N-terminus positively charged region.

393 As observed in Figure 3e, the RMSD reveals that crotAMP14 presents a
394 lower deviation in its trajectory when in contact with cancer membranes than
395 zwitterionic membranes. By contrast, the crotAMP14 peptide displayed similar
396 RMSF profiles when evaluated in both membrane systems for 200 ns of MD
397 simulations (Figure 3f). Interestingly, the MSD (Fig. 3g) data show that the
398 crotAMP14 peptide may induce more significant lipid displacement in POPC/Chol
399 compared to the POPC/POPE/POPS membrane system. This data supports our
400 *in vitro* findings in which the crotAMP14 peptide triggers rigidity in cancer cell
401 membranes. Finally, the partial density maps in POPC/Chol (Fig. 3h) and
402 POPC/POPE/POPS (Fig. 3i) indicate that the peptide is more deeply inserted in
403 the cancer membrane than the zwitterionic membrane. In the latter, most of the
404 peptide is in the water interface in the POPC/Chol system (Fig. 3i). It is also worth
405 noting that the crotAMP14 peptide mainly interacts with the POPC/POPE/POPS
406 membrane through hydrogen and saline bonds involving positively charged
407 residues from the N-terminus (Fig. 3j-n; Table S8). As depicted in Table S8, from
408 50 ns to 200 ns of MD simulations, more than ten atomic interactions were
409 mapped for each point evaluated, responsible for the peptide/membrane complex
410 stabilization throughout the dynamics.

411 The MD simulations corroborated the data from other experiments. In the
412 cytotoxicity tests, the crotAMP14 peptide was not effective at concentrations 16,
413 8, 4, 2, and 1 μM (Fig. 1d). Furthermore, MD simulations (Fig. 3c and d)
414 corroborated the fact that the peptide assumes the 3D structure only when in
415 contact with plasma membranes, something demonstrated by NMR (Fig. 3a). As
416 mentioned previously, this phenomenon occurs due to the difference in the lipid
417 composition of healthy and neoplastic cells. In a study investigating the in situ
418 mass spectrometry profile of different breast cancer cell lines, it was
419 demonstrated that the levels of phosphatidylinositol (PI(36:1)), PC(36:1), and
420 PC(36:2) are increased in MDA-MB-231 cells when compared with other five cell
421 lines[64]. This variation in the profile with significantly elevated levels of
422 monounsaturated lipids may be associated with the degree of malignancy of
423 these neoplasms[64]. Furthermore, lipid profile changes in cancer may occur at
424 different stages of progression[65].

425 Although crotAMP14 has a relatively high net positive charge (+8),
426 experiments have shown that in silico production of ACPs with improved
427 anticancer activity does not necessarily require a maximal increase in charge and
428 hydrophobicity[66]. Rather, these properties constrain and influence each other
429 until a balance is reached that achieves improved anticancer activity[66].
430 Something similar was adopted in the redesign of crotAMP14[33]. The data
431 presented here demonstrate that crotAMP14 can be explored as a potential
432 antitumor agent since, being cationic and in α-helix, it acts specifically on the
433 plasma membrane of MDA-MB-231 triple-negative breast cancer cells.

434 In summary, although the crotAMP14 peptide altered the gene expression
435 profile of MDA-MB-231 cells, the expression did not result in apoptotic cell death,

as seen in caspase activation assays. CrotAMP14 acts on the plasma membrane, increasing its rigidity and leading to cell death within a few minutes of exposure. This interaction is likely due to electrostatic interactions between the positively charged peptide and the negatively charged plasma membrane of the target cell. This is the first time a study attempting to evaluate the mechanism of action has been performed for crotAMP14. The investigation of the potential antitumor activity of AMPs allows the development of new drugs, including peptide derivatives, to treat breast cancer. Such treatments may be more effective and have fewer side effects for patients with breast cancer. In addition, understanding the mechanism of action of ACPs is crucial because it sheds light on new pharmacological targets and allows the development of new molecules capable of eradicating cells through electrostatic interactions.

METHODS

Peptide synthesis and purity degree

The fluorenylmethoxycarbonyl protecting group (Fmoc) strategy synthesized all peptides used in this work by Peptide 2.0 (Purity >95%). The mass of the peptides was confirmed by Matrix Assisted Laser Desorption Ionization – Time of Flight (MALDI-ToF) on mass spectrometer Ultraflex MALDI-ToF III (Bruker Daltonics). The synthetic peptide concentrations were determined using measurements of UV-spectroscopy at 205, 215, and 225 wavelengths. Afterward, the peptides were resuspended in ultrapure water (milli-Q grade) at a stock concentration of between 1 and 4 $mg.mL^{-1}$ at a ratio of 1:1 (w:v), filtered with 0.22 µm pore filter membranes and stored at −80 °C.

460

461 **Cell culture**

462 Human adenocarcinoma cell lines (MDA-MB-231, MCF-7, and PMC-42)
463 and the primary fibroblast culture isolated from donor abdominal tissue were used
464 in the present study to determine the activity of the peptides against neoplastic
465 cells as well as cytotoxicity of these molecules against healthy cells. All human
466 adenocarcinoma cell lines were grown in Roswell Park Memorial Institute (RPMI)-
467 1640 medium supplemented with 10% fetal bovine serum (FBS). The human
468 fibroblast cells were grown in Dulbecco's Modified Eagle's Medium (DMEM)
469 medium supplemented with 10% FBS. The cells were incubated at 37 °C in 5%
470 CO_2 and humidity from 85 to 95%.[67]

471

472 **Cell cytotoxic assay**

473 The cells were placed in 96-well plates (Kasvi, Brazil) at 1 x 10^4 cells per
474 well and incubated for 24 h to allow the cells to adhere to the bottom of the wells.
475 Then, cell viability tests were carried out after a 24-h incubation period. All tests
476 were performed in technical replications (n=9) at different peptide concentrations
477 (64, 32, 16, 8, 4, 2, and 1 μM). Wells containing only culture medium and cells
478 were positive controls and represented 100% cell viability. Those containing cells
479 in culture medium and adding 50 μL of lysis solution (10 mM Tris, 1 mM EDTA,
480 and 0.1% Triton X-100, pH 7.4) correspond to the negative control and represent
481 0% cell viability. Triplicates containing only culture medium represented the blank
482 of the experiment. After 24 h of incubation, the medium from each well was
483 removed and 90 μL of fresh medium plus 10 μL of 3-(4, 5-dimethylthiazol-2-yl)-2,

484 5-diphenyltetrazolium bromide (MTT, 50 µg) per well and the plates were
485 incubated again at 37ºC, at a concentration of 5% CO_2 and 95% humidity for an
486 interval of 4h. After the incubation period, 100 µL of dimethyl sulfoxide (DMSO)
487 (Sigma-Aldrich, USA) was added to each of the wells, and homogenization was
488 carried out so that the crystals were completely solubilized. Finally, the
489 absorbance reading was performed on a microplate reader, Eon Microplate
490 (BioTek, USA), at a wavelength of 570 nm[68].

491

492 **RNA isolation and real-time polymerase chain reaction (qPCR)**

493 Total RNA was extracted using the phenol/hot acid method[69].
494 Complementary DNA (cDNA) was subsequently synthesized from 5 µg of total
495 RNA using GoScript™ Reverse Transcriptase (#A5000; Promega Corporation).
496 qPCR was performed with GoTaq qPCR Master Mix (#A6002; Promega
497 Corporation) using the 7300 Real-Time PCR System thermocycler (Applied
498 Biosystems). The reaction parameters were: 95ºC for 10 min for activation of the
499 Taq DNA polymerase enzyme (hot start), DNA amplification for 40 cycles
500 (comprising denaturation at 95ºC for 15 s, annealing of primers and extension of
501 the new strand at 60ºC for 60 s) and a final cycle of extension at 72ºC for 5 min.
502 The sense and antisense sequences of the primers used in this study are shown
503 in Table S2.

504

505 **Caspase activity assay**

506 Measurements of caspase activities in cells were performed using the
507 commercially available Caspase-Glo 3/7 Assay (Promega, Madison, WI). Briefly,

1 x 10^4 cells per well were treated with 16 µM of crotAMP14 or 16 µM ctn[15-34] for 1, 24, 48, and 72 h at 37ºC. After the peptide's incubation, 100 µl of the reagent Caspase-Glo 3/7 was added to each well, and after homogenization, the plate was incubated for 3 h at room temperature. Caspase 3/7 activity was determined by luminescence (Multimode Plate Reader, PerkinElmer, Waltham, MA, USA).

Nuclear magnetic resonance (NMR)

The structural analysis of crotAMP14 was performed using NMR spectroscopy, specifically [^{1}H-^{1}H] TOCSY, [^{1}H-^{1}H] NOESY, and [^{1}H-^{13}C] HSQC, in SDS-d_{25} micelles. The spin systems were assigned based on the TOCSY contour map and sequential assignment was carried out using the amide region of the NOESY contour map. Verification of the spin systems was achieved through a hydrogen-carbon chemical shift in HSQC. The spin system correlations were identified in the NOESY spectrum, and the dihedral angles were predicted from the chemical shifts of H_α, H_β, C_α, and C_β. (Figures S2 and S3). NMR spectra of 1.5 mM crotAMP14 (pH 6.5) in the presence of 75 mM of SDS-d_{25} micelles were acquired at 25°C on a Bruker Avance III 500 spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). The sample contained 0.5% sodium trimethylsilylpropionate (TMSP) and 10% D_2O for internal reference. [^{1}H-^{1}H] TOCSY experiments were acquired with 4096 × 1024 points, 48 scans, and a mixing time of 70 ms. [^{1}H-^{1}H] NOESY experiments were acquired with 4096 × 1024 points, 40 scans, and a mixing time of 200 ms. [^{1}H-^{13}C] HSQC spectra were recorded with 4 k × 512 points and 50 scans. All the spectra were processed using Bruker TopSpin 4.0.7 and analyzed using CcpNMR Analysis 2.4.2[70]. The

three-dimensional structures were calculated using distance and dihedral angle restraints. The NOE correlations were converted into distance restraint using 1.72 Å as the lower distance limit, 3.2 Å as the reference distance, and 8.0 Å as the upper distance limit. Dihedral angle restraints were determined from the chemical shifts of $^{1}H_{\alpha}$, $^{1}H_{\beta}$, $^{13}C_{\alpha}$, and $^{13}C_{\beta}$ by using the DANGLE software[71] from the CcpNMR platform. The ARIA 2.3 software[72] was used for structure calculation with the CCPN data as input. A protocol of 8 iterations, with 200 structures in each step, was performed, besides a final step of water refinement, which generated 20 structures. The 10 structures with the lowest energy were selected. ARIA software integrated package PROCHECK[73] was used for structural analysis. Structures were visualized using the UCSF Chimera program[74]. The Ramachandran plot was obtained using PyRAMA code (https://github.com/gerdos/PyRAMA). The structure analysis and structure calculation were performed in NMRBox, a resource filled with biomolecular software[75].

Spin labeling and EPR spectroscopy.

The triple-negative breast cancer line MDA-MB-231 was incubated for 24h with the crotAMP14 peptide at different concentrations 1, 2, 4, 8, 16, and 64 μM. After exposure, the cells were incubated for 5 min with 1X trypsin in PBS, collected and centrifuged at 25,000 x *g* for 5 min, and washed once more in 1X PBS. After removing the supernatant, cells were suspended in 50 μL of PBS, and spin labeling was performed. To incorporate the spin label into the plasma membranes, a 1 μL aliquot of ethanolic solution containing the spin label 5-DSA

(Sigma-Aldrich, St. Louis, MO, USA) diluted at a rate of 2 mg/ml was added to each sample. Alternatively, untreated cells washed in PBS were treated with the crotAMP14 peptide and then spin-labeled with 5-DSA to perform the EPR measurement for approximately 20 min after treatment. After spin labeling, the cells were transferred to a 1-mm-i.d. capillary tube, which was flame-sealed on one side. The capillary was centrifuged at 25,000 × *g* for 5 min, and the approximately 2 mm cell pellet was placed in the center of the resonance cavity of a Bruker EMXplus EPR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). The instrumental settings were microwave power, 10 mW; microwave frequency, 9.45 GHz; modulation amplitude, 1.0 G; magnetic field sweep, 100 G; scan time, 168 s; and sample temperature, 25 ± 1°C.

Molecular dynamics in lipid bilayers

The lowest free energy NMR structure for crotAMP14 was used for molecular dynamics in lipid bilayers. Membrane systems were constructed using the CHARMM-GUI server, including 60% 1- palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 20% 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), and 20% 1-palmitoyl-2-oleoyl-phosphatidylehtanolamine (POPE) for cancer cells; and POPC/cholesterol (Chol) (70:30) for healthy mammalian cells[76]. The CHARMM36 force field was used, and the crotAMP14 peptide was positioned approximately 0.27 nm from the lipid bilayers. Simulations were conducted using GROMACS version 5.0.4, under 0.15 M NaCl ionic strength. The geometry of the water molecules was maintained using the SETTLE algorithm[77], whereas the LINCS algorithm[78] was employed to preserve the atomic bond lengths. For electrostatic corrections, the Particle Mesh Ewald (PME)

method was applied with a cutoff radius of 1 nm, aiming at minimizing the computational simulation time. The exact cutoff value was used for van der Waals interactions. The neighbor list for each atom was updated every 10 simulation steps, each lasting 2 fs. Energy minimization was performed using the steepest descent algorithm for 50,000 steps. Subsequently, the systems underwent temperature and pressure normalization, stabilizing at 310 K and 1 bar through the velocity rescaling thermostat (NVT) (Bussi, 2007) and the Parrinello–Rahman barostat (NPT) for a duration of 100 ps. After reaching minimized energy and equilibrated temperature and pressure, molecular dynamics (MD) simulations were performed for 200 ns. The data were analyzed using root mean square deviation (RMSD), root mean square fluctuation (RMSF), mean square displacement (MSD), partial density maps, and a structural snapshot of the peptide/membrane molecular complexes at 50 ns intervals.

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Author contributions

This work was conceived and designed by M.L.L, K.B.S.O, and O.L.F. Material preparation, experiments, and data analysis were performed by M.L.L., M.M.R., D.A.B.M., N.L.C., B.P.O.S., M.T.Q.M., J.B.B., L.M.L., A.A., E.N.B., A.M.N., F.S.A., E.C.I.B., L.R.L., M.H.C., K.B.S.O. The final critical analysis of data was executed by O.L.F, K.B.S.O, M.H.C, and M.L.L. All authors read and approved the final manuscript.

Competing interests

The authors declare that there are no competing interests.

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Unveiling the mechanism of action of a crotalicidin-derived peptide in combating triple-negative breast cancer cells

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Figures

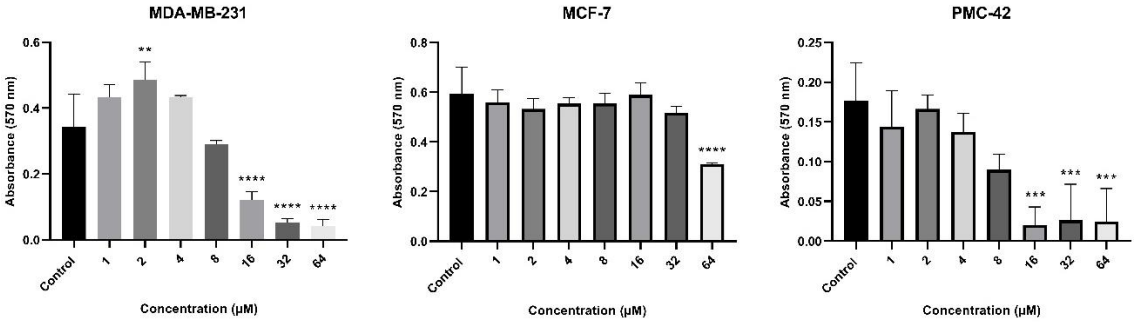

Figure S1. In vitro cell viability tests using crotAMP14. The strains (MDA-MB-231, MCF-7, and PMC-42) were exposed to the peptide during a 24-hour treatment period. The plate was then read at a wavelength of 570 nm. The graphs represent the mean and standard error of the absorbance of three triplicate biological replicates. The one-way ANOVA test and Bonferroni post-test verified statistical differences. *$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$.

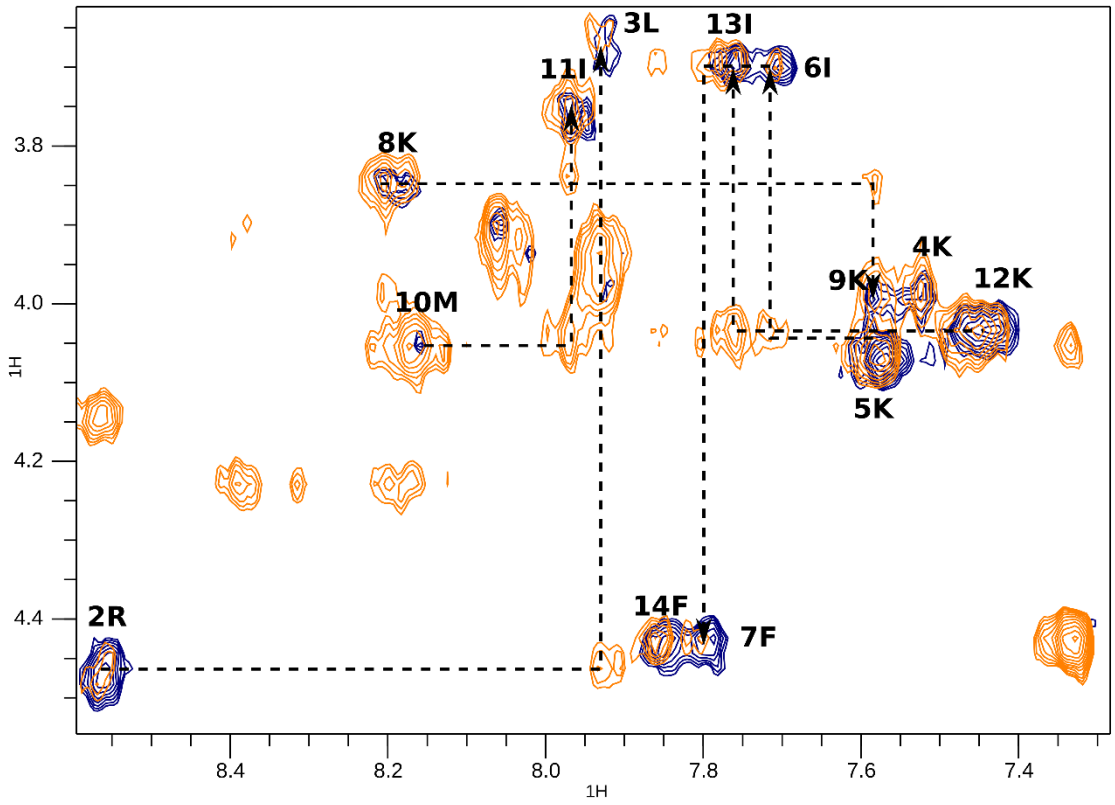

Figure S2. CrotAMP-14 H_N-H_α correlation peaks. The TOCSY experiment (navy blue) was superimposed with the NOESY experiment (orange) to highlight the identified spin systems and their correlations. Partial sequential assignment H_N-H_α (i,i+1) is demonstrated by dashed arrows.

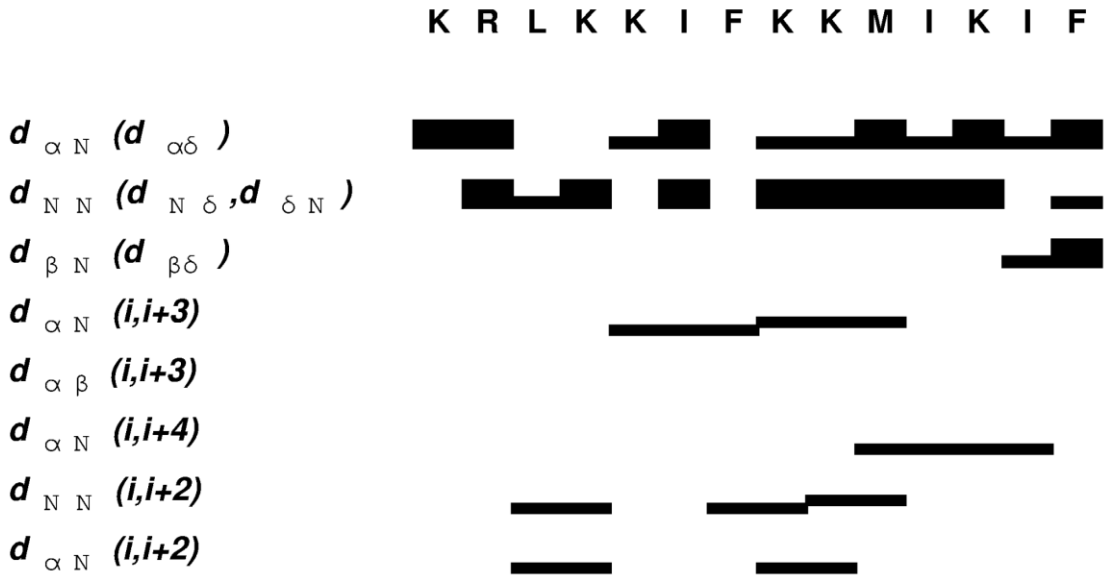

Figure S3. Summary of the sequential and medium range NOE connectivities for CrotAMP-14 in SDS-d_{25} micelles. The peptide sequence is described at the top with the one-letter code. The chart presents the NOE connectivity, the secondary chemical shifts, and the predicted three-dimensional structure.

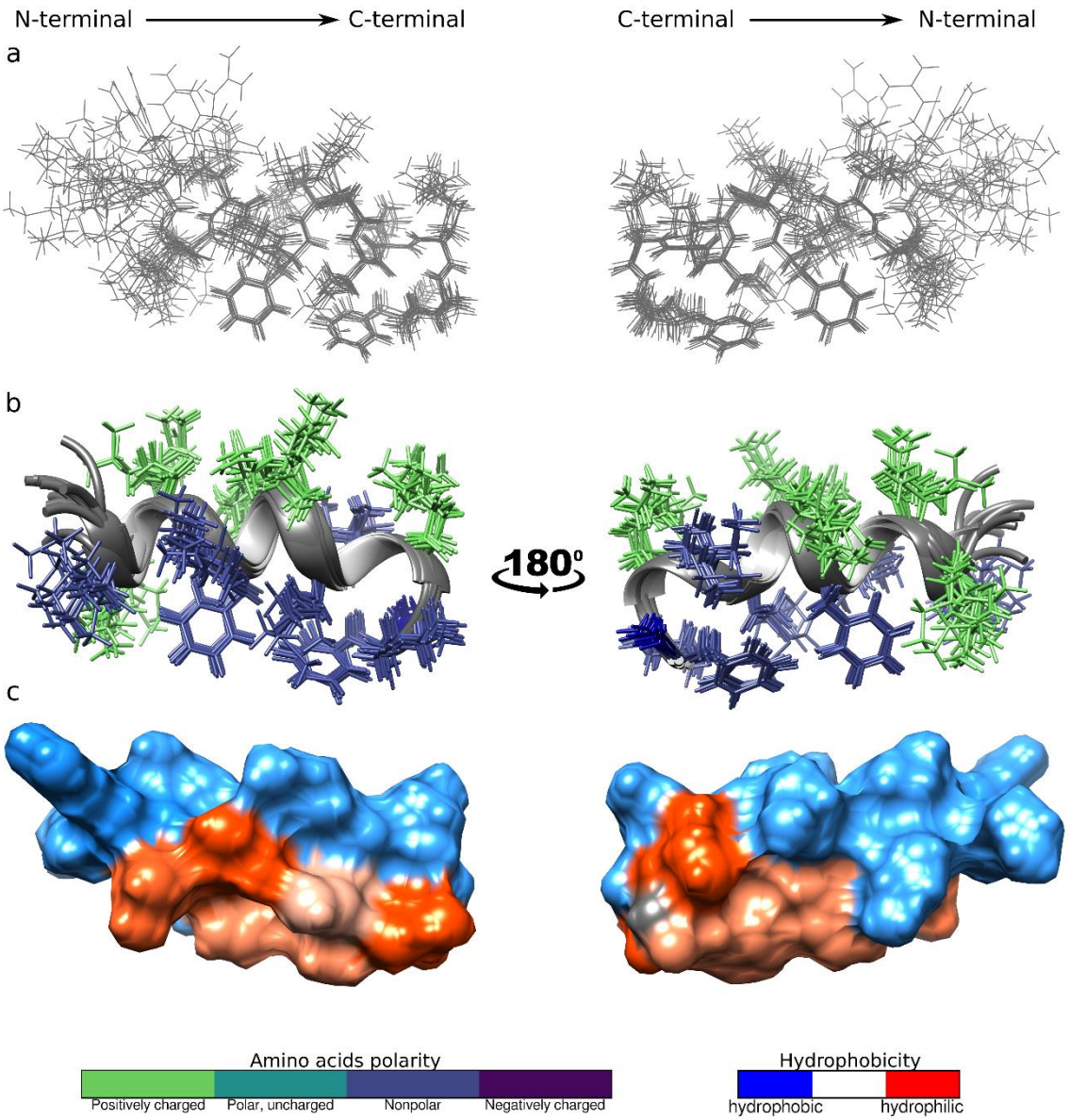

Figure S4. CrotAMP14 peptide NMR structures. Superposition of the 10 lowest energy structures of the peptide in the presence of SDS-d_{25} micelles as stick (A) and cartoon (B). Surface representation of the lowest energy structure (C). Side chains of cartoon structure are colored by polarity (viridis color scheme). Surface representation is colored by KyteDooLittle's hydrophobicity color scheme, from hydrophobic (blue) to hydrophilic (orange).

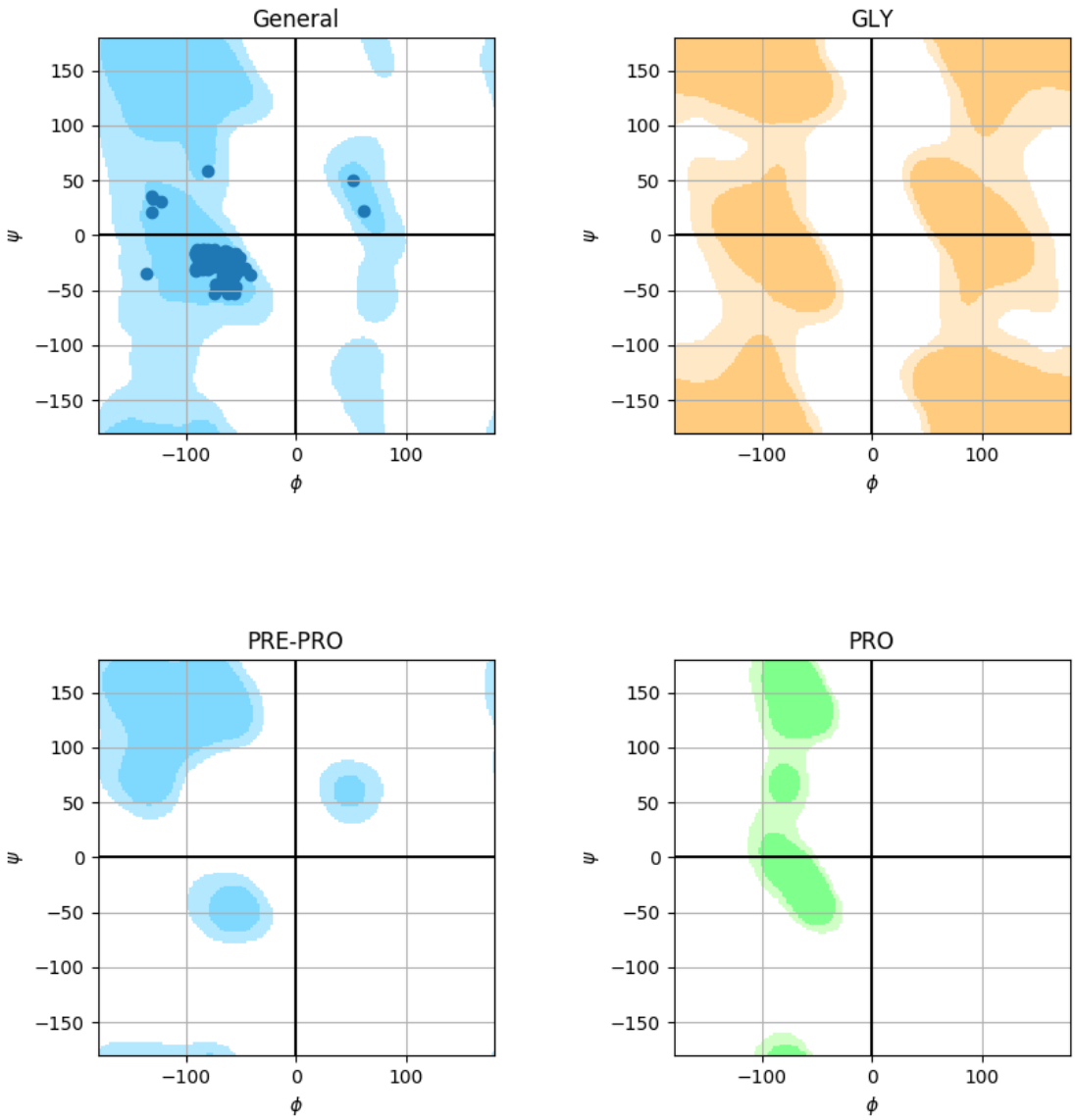

Figure S5. Ramachandran plot of the 10 lowest-energy solution NMR structures of CrotAMP-14 in 75mM of SDS-d_{25}.

Tables

Table S1. List of peptides used in the present study. The remaining peptides were developed through rational redesign except for LL-37 (isolated from humans).

Peptide name	aa sequence	Number of aa	Mass (Da)
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	37	4493,32
Crotalicidin	KRFKKFFKKVKKSVKKRLKKIFKKPMVIGVTIPF	34	4152.37
Ctn[15-34]	KKRLKKIFKKPMVIGVTIPF	20	2372,09
CrotAMP14	KRLKKIFKKMIKIF-NH2	14	1821,43
PcDBS1R1	PKLAIRITCKIHKKVAISV	19	2118,70
PcDBS1R5	PMNAIKLLCRVHKKIAISV	19	2134,72
PcDBS1R9	PMNAINFTCKLLKKVAIKI	19	2145,74
DJK6	VQWRRIRVWVIR (D-aa)	12	1667,04
IDR1002	VQRWLIVWRIRK-NH2	12	1653,05
IDR1004	RFWKVRVKYIRF	12	1698,09
IDR1018	VRLIVAVRIWRR-NH2	12	1536,93
IDR1021	VRLRIRVAV	9	1081,37
Mastoparan-L	INLKALAALAKKIL	14	1479,91
Mastoparan-MO	FLPIIINLKALAALAKKIL	19	2063,68
Mastoparan-R1	KILKRLAAKIKKIL	14	1636,19
Mastoparan-R4	INLKKLAARIKKKI	14	1637,13
Mastoparan-I5R8	INLKILARLAKKIL	14	1607,10

Table S2. General information on the primers used to evaluate gene expression of the triple-negative breast cancer line MDA-MB-231.

Access number (NCBI)	Primer names	Sequences	Bases	Tm (ºC)	Amplicon (bp)
NM_001188.4	BAK1_F	ACGACATCAACCGACGCTAT	20	60.5	101
	BAK1_R	GGTGGCAATCTTGGTGAAGT	20	60.0	
NM_138761.4	BAX_F	AACATGGAGCTGCAGAGGAT	20	59.8	104
	BAX_R	CAGTTGAAGTTGCCGTCAGA	20	60.0	
NM_001196.4	BID_F	AAGAAGGTGGCCAGTCACAC	20	60.2	117
	BID_R	GTCCATCCCATTTCTGGCTA	20	59.9	
XM_047437733.1	BCL2_F	GAGGATTGTGGCCTTCTTTG	20	59.7	116
	BCL2_R	GCCGGTTCAGGTACTCAGTC	20	59.7	
NM_138578.3:382-1083	Bcl-xL_F	GGAGCTGGTGGTTGACTTTC	20	59.7	115
	Bcl-xL_R	CTCCGATTCAGTCCCTTCTG	20	59.8	
NM_021960.5	Mcl1_F	TGGGTTTGTGGAGTTCTTCC	20	59.9	100
	Mcl1_R	TGCCAAACCAGCTCCTACTC	20	60.4	
NM_002392.6	MDM2_F	CTTCGTGAGAATTGGCTTCC	20	59.8	100
	MDM2_R	CAAAGCCCTCTTCAGCTTGT	20	59.6	
NM_001372051.1	CASP8_F	AAGCAAACCTCGGGGATACT	20	60.0	115
	CASP8_R	TGCATCCAAGTGTGTTCCAT	20	60.0	
NM_001229.5	CASP9_F	CTAGTTTGCCCACACCCAGT	20	60.0	112
	CASP9_R	ATGTCGTCCAGGGTCTCAAC	20	60.0	
NM_004346.4	CASP3_F	GGTTCATCCAGTCGCTTTGT	20	60.1	100

	CASP3_R	AATTCTGTTGCCACCTTTCG	20	60.1	
NM_001371333.1	DIABLO_F	TATCAAACTGGCGCAGATCA	20	60.4	112
	DIABLO_R	CCAGCTTGGTTTCTGCTTTC	20	60.0	
NM_005923.4	ASK1_F	CACATCACAACCCTCATTGC	20	60.0	103
	ASK1_R	CGAAGTCCAGCTCCAGTTTC	20	60.0	
NM_001323329.2	JNK1_F	ACTACAGAGCACCCGAGGTC	20	59.3	118
	JNK1_R	TCCCTTCCTGGAAAGAGGAT	20	60.0	
NM_181861.2	APAF1_F	GCCCTGCTCATCTGATTCAT	20	60.2	118
	APAF1_R	CGTCCAAGAAGGTGTCCATT	20	60.0	
NM_000546.6	p53_F	GTTCCGAGAGCTGAATGAGG	20	60.0	123
	p53_R	TTATGGCGGGAGGTAGACTG	20	60.1	
NM_000314.8	PTEM_F	AGTTCCCTCAGCCGTTACCT	20	60	135
	PTEM_R	AGGTTTCCTCTGGTCCTGGT	20	60.0	
NM_005228.5	EGFR_F	GGCTGGGTAGGGTAGAGATT	20	57.2	114
	EGFR_R	TGAACTGCTGGTGAGGATGA	20	60.4	
NM_023110.3	FGFR1_F	CACTTCATCCCCTCCCAGA	19	61.0	140
	FGFR1_R	CAAAACAGACCAAACCGACA	20	59.6	
NM_000141.5	FGFR2_F	GCTGGGGTCGTTTCATCTG	19	61.6	120
	FGFR2_R	GGTATTTGGTTGGTGGCTCT	20	58.9	
NM_003376.6	VEGFA_F	ACTCGCCCTCATCCTCTTC	19	59.3	127
	VEGFA_R	TCAACCACTCACACACACACA	21	59.6	
NM_006218.4	PIK3CA_F	GCAAGAAAATACCCCCTCCA	20	61.2	130

	PIK3CA_R	GAAAAAGCCGAAGGTCACAA	20	60.2	
NM_004448.4	ERBB2_F	CCCTCATCCACCATAACACC	20	60.0	121
	ERBB2_R	CCCACACACTCGTCCTCTG	19	60.3	
NM_000075.4	CDK4_F	CTGGACACTGAGAGGGCAAT	20	60.3	141
	CDK4_R	TGGGAAGGAGAAGGAGAAGC	20	60.8	
NM_001145306.2	CDK6_F	GTGAACCAGCCCAAGATGAC	20	60.5	133
	CDK6_R	GTGGAGGAAGATGGAGAGCA	20	60.3	
NM_003998.4	NFKB1_F	GCCTCTAGATATGGCCACCA	20	60.1	117
	NFKB1_R	TCAGCCAGCTGTTTCATGTC	20	60.0	
		Reference genes			
NM_002046.7	GAPDH_F	TCAAGAAGGTGGTGAAGCAG	20	59.1	113
	GAPDH_R	AAAGGTGGAGGAGTGGGTGT	20	59.1	
XR_007090869.1	RNA28S_F	TCATCAGACCCCAGAAAAGG	20	60.0	102
	RNA28S_R	GATTCGGCAGGTGAGTTGTT	20	60.1	
NM_001284240.2	CCSER2_F	GCCACCCTGACCACTATCAT	20	59.8	122
	CCSER2_R	CTTCTGAGCCTGGAAAAAGG	20	59.0	
NM_006196.4	PCBP1_F	GGTTACTATCACTGGCTCTGCT	22	57.7	125
	PCBP1_R	GAAAGGGGTTATTGAGGGAAC	21	58.8	

Table S3. Relative expression of selected genes between supernatant cells and control (untread cells). The relative gene expression and statistics analyses were performed using REST software, employing genes CCSER2 and PCBP1 as internal control. $p \leq 0.05$.

	Log_2																			
Gene	0 min				15 min				30 min				45 min				60 min			
	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result
VEGFA	3,192	0,362	0,414	UP	0,399	0,392	0,364	UP	-0,208	0,379	0,390	ns	0,904	0,390	0,422	UP	0,751	0,345	0,392	UP
EGRF	1,025	0,677	0,543	UP	-0,824	0,654	0,441	DOWN	0,092	0,716	0,525	ns	0,603	1,149	1,882	ns	-0,317	0,638	0,476	ns
FGFR1	1,236	0,333	0,268	UP	-2,608	2,148	1,329	DOWN	-1,373	0,266	0,299	DOWN	-0,829	1,177	1,673	ns	-1,086	0,356	0,298	DOWN
FGFR2	1,907	0,298	0,385	UP	-4,158	0,450	0,441	DOWN	-1,311	0,288	0,433	DOWN	-0,308	0,348	0,417	DOWN	-0,317	0,368	0,394	DOWN
NF-kB	0,001	0,830	0,729	ns	-0,991	0,804	0,785	DOWN	0,058	0,771	0,743	ns	1,324	2,033	2,093	ns	0,684	0,768	0,748	UP
CDK4	0,274	0,386	0,365	ns	-3,000	0,442	0,435	DOWN	0,795	0,346	0,369	UP	-0,308	0,664	0,624	ns	0,888	0,407	0,366	UP
CDK6	-0,554	0,680	0,660	DOWN	-2,358	0,774	0,696	DOWN	-0,448	0,644	0,720	DOWN	0,596	2,022	1,873	ns	-0,219	0,735	0,883	ns
JNK1	1,060	0,215	0,202	UP	-1,404	0,438	0,389	DOWN	0,439	0,302	0,267	UP	2,512	0,188	0,238	UP	0,346	0,473	0,561	ns
PIK3CA	-0,398	0,685	0,438	ns	-3,047	0,749	0,532	DOWN	0,545	0,748	0,497	UP	0,696	0,731	0,482	UP	1,831	0,738	0,575	UP
ERBB2	-0,642	0,466	0,354	DOWN	-0,806	0,333	0,285	DOWN	-1,423	0,208	0,215	DOWN	-0,173	1,106	1,321	ns	-1,283	0,199	0,215	DOWN
PTEN	-0,593	0,203	0,231	DOWN	-0,644	0,283	0,298	DOWN	-2,191	0,267	0,334	DOWN	-1,032	0,268	0,288	DOWN	-0,680	1,928	1,498	ns
P53	0,050	0,313	0,302	ns	-1,932	0,292	0,289	DOWN	-0,671	0,312	0,313	DOWN	-0,613	0,268	0,262	DOWN	-0,417	0,277	0,265	DOWN
MDM2	2,518	0,597	0,423	UP	-3,644	0,594	0,472	DOWN	-0,400	0,597	0,387	DOWN	1,145	0,581	0,451	UP	0,279	0,592	0,490	ns
APAF1	3,136	0,496	0,515	UP	-1,276	0,581	0,548	DOWN	-1,089	0,510	0,518	DOWN	-0,003	1,813	1,930	ns	-0,531	0,543	0,558	DOWN
BID	3,316	1,016	0,596	UP	0,064	0,839	0,848	ns	0,938	1,052	0,605	UP	1,916	0,932	0,654	UP	0,572	1,343	1,778	ns

BAK1	1,802	0,672	0,564	UP	-0,088	0,757	0,596	ns	1,010	0,604	0,449	UP	0,548	0,610	0,488	UP	-0,735	1,349	1,334	ns
BAX	1,736	0,507	0,476	UP	-1,355	0,594	0,431	DOWN	-0,103	0,505	0,427	ns	1,677	0,477	0,428	UP	-0,279	0,512	0,430	ns
DIABLO	0,611	0,549	0,728	UP	-0,599	0,632	0,714	DOWN	-0,381	0,557	0,790	ns	0,106	0,594	0,773	ns	-0,198	0,571	0,675	ns
CASP3	0,763	1,027	0,669	UP	-0,870	0,946	1,001	DOWN	-4,837	1,042	0,704	DOWN	-1,152	0,943	0,700	DOWN	1,159	2,209	3,137	ns
CASP8	2,776	0,887	0,572	UP	0,169	0,903	0,663	ns	0,145	0,890	0,581	ns	-1,515	1,497	1,299	DOWN	-2,458	0,864	0,611	DOWN
CASP9	3,056	0,374	0,400	UP	-3,133	0,458	0,498	DOWN	-0,297	0,404	0,419	DOWN	0,632	0,390	0,397	UP	0,236	0,392	0,423	ns
BCL-XL	-0,076	0,494	0,599	ns	-0,364	0,485	0,637	ns	-0,390	0,472	0,666	DOWN	-0,694	0,480	0,690	DOWN	-0,253	0,641	0,645	ns
MCL1	-0,837	1,079	0,727	DOWN	-0,980	1,134	0,766	DOWN	-0,035	1,120	0,745	ns	0,427	1,822	2,755	ns	-0,150	1,139	1,738	ns

Table S4. Relative expression of selected genes between adherent cells and control (untread cells). The relative gene expression and statistics analyses were performed using REST software, employing genes CCSER2 and PCBP1 as internal control. $p \leq 0.05$.

	Log_2																			
Gene	0 min				15 min				30 min				45 min				60 min			
	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result
VEGFA	1,160	0,373	0,419	UP	0,853	0,360	0,410	UP	0,493	-0,770	-0,371	UP	1,766	1,276	1,297	UP	1,166	0,784	0,623	UP
EGRF	0,726	0,729	0,608	UP	0,638	0,682	0,441	UP	-0,044	-1,123	-0,221	ns	-0,523	1,536	2,030	ns	-1,732	0,758	1,097	DOWN
FGFR1	-1,146	0,381	0,451	DOWN	-1,012	0,206	0,228	DOWN	-1,218	-0,434	-0,252	DOWN	-2,388	0,948	1,615	DOWN	-0,900	0,891	0,665	DOWN
FGFR2	-0,653	1,449	1,593	ns	-1,059	0,431	0,420	DOWN	-1,490	-0,851	-0,209	DOWN	-0,417	1,521	2,024	ns	-1,388	1,177	0,900	DOWN
NF-kB	0,487	1,412	1,284	ns	1,241	0,789	0,772	UP	0,452	-1,561	-0,262	UP	0,875	1,317	1,536	ns	1,578	1,184	1,151	UP
CDK4	1,236	0,474	0,469	UP	0,481	0,324	0,367	UP	0,157	-0,692	-0,200	DOWN	0,279	2,565	1,823	ns	-0,144	0,839	1,254	ns
CDK6	-0,003	0,639	0,836	ns	0,257	0,678	0,638	ns	-0,421	-1,315	-0,214	DOWN	0,594	0,554	0,745	UP	0,103	1,274	1,198	ns
JNK1	1,201	1,266	1,754	UP	0,014	0,324	0,288	ns	-0,309	-0,612	-0,189	DOWN	0,818	0,989	1,427	UP	1,206	1,155	0,729	UP
PIK3CA	-0,250	1,531	2,215	ns	1,261	0,807	0,567	UP	0,454	-1,374	-0,450	UP	1,950	1,765	1,484	UP	1,701	0,766	0,836	UP
ERBB2	0,397	1,397	1,729	ns	-0,486	0,222	0,198	DOWN	-0,708	-0,420	-0,126	DOWN	-3,059	0,930	1,490	DOWN	-0,747	1,169	0,685	DOWN
PTEN	0,138	1,325	0,791	ns	-0,051	0,337	0,317	ns	-0,318	0,993	0,968	ns	-0,050	1,444	1,618	ns	-1,020	2,302	1,311	ns
P53	-0,010	0,647	0,811	ns	-0,550	0,279	0,342	DOWN	-0,829	-0,621	-0,270	ns	0,750	1,438	1,474	ns	0,606	1,121	0,843	ns
MDM2	-1,071	1,340	1,646	DOWN	0,329	0,594	0,390	ns	-0,265	-0,984	-0,297	DOWN	0,736	1,831	1,391	ns	0,806	0,889	0,876	UP
APAF1	0,781	1,231	1,742	ns	-0,573	0,559	0,470	DOWN	-1,133	-1,030	-0,182	ns	-1,068	1,422	2,044	ns	-0,803	1,374	1,068	ns
BID	0,787	0,991	0,630	UP	-1,293	0,958	0,650	DOWN	-2,252	-1,608	-0,271	DOWN	-3,989	1,585	1,804	DOWN	-0,394	2,268	1,933	ns
BAK1	1,017	1,345	2,031	ns	-0,494	0,598	0,452	DOWN	-1,092	-1,050	-0,386	ns	-0,199	2,291	1,770	ns	-0,335	1,092	1,370	ns

BAX	0,098	1,398	1,718	ns	-0,860	0,517	0,398	DOWN	-1,377	-0,915	-0,101	DOWN	-0,241	2,349	2,091	ns	-1,032	0,974	0,882	DOWN
DIABLO	0,648	1,521	1,736	ns	0,076	0,560	0,794	ns	-0,484	-1,354	-0,332	ns	-0,624	1,038	1,246	ns	-0,635	0,930	0,980	ns
CASP3	-0,886	1,414	2,086	ns	0,483	1,040	0,663	ns	-0,556	-1,703	-0,296	ns	-0,826	1,540	1,899	ns	1,247	1,650	1,616	UP
CASP8	0,059	1,325	2,012	ns	1,265	0,876	0,598	UP	0,390	-1,474	-0,333	ns	1,320	0,986	1,432	UP	0,872	1,498	1,353	ns
CASP9	0,507	1,423	1,842	ns	0,107	0,424	0,430	ns	-0,317	-0,854	-0,257	ns	0,352	1,091	1,558	ns	1,654	0,454	0,509	UP
BCL-XL	0,682	1,523	1,697	ns	-0,196	0,501	0,670	ns	-0,697	-1,171	-0,166	ns	-0,486	1,246	1,404	ns	-0,016	1,172	0,896	ns
MCL1	-0,286	0,901	0,904	ns	0,336	1,082	0,779	ns	-0,747	-1,862	-0,425	DOWN	-0,381	1,492	1,774	ns	0,076	1,645	1,697	ns

Table S5. Relative expression of selected genes between supernatant cells and adherent cells. The analyses of relative gene expression and statistics were performed employing REST software, employing the CCSER2 and PCBP1 as internal control. $p \leq 0.05$.

Log_2

Gene	0 min				15 min				30 min				45 min				60 min			
	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result	Expression	Error positive	Error negative	Result
VEGFA	-2,029	0,337	0,385	DOWN	0,453	0,345	0,313	UP	0,108	-0,658	-0,316	UP	0,862	1,225	1,323	UP	0,416	0,727	0,548	ns
EGRF	-0,299	0,393	0,399	ns	1,461	0,242	0,196	UP	1,219	-0,438	-0,348	DOWN	-1,127	2,482	2,289	ns	-1,419	1,047	0,800	DOWN
FGFR1	-2,381	0,405	0,421	DOWN	1,599	1,322	2,206	UP	0,277	-3,528	-0,218	DOWN	-1,561	2,297	2,132	DOWN	0,187	0,909	0,673	ns
FGFR2	-2,556	1,432	1,659	DOWN	3,098	0,434	0,394	UP	2,665	-0,828	-0,182	ns	-0,108	1,440	1,942	ns	-1,074	1,110	0,826	DOWN
NF-kB	0,485	1,307	0,970	ns	2,232	0,221	0,226	UP	2,011	-0,447	-0,442	UP	-0,450	2,597	1,635	ns	0,893	1,229	0,778	UP
CDK4	0,962	0,426	0,467	UP	3,483	0,425	0,378	UP	3,058	-0,804	-0,199	DOWN	0,586	2,563	1,791	ns	-1,032	0,804	1,246	DOWN
CDK6	0,550	0,440	0,449	UP	2,618	0,308	0,277	UP	2,310	-0,584	-0,168	DOWN	-0,001	1,765	2,129	ns	0,323	1,524	1,245	ns
JNK1	0,140	1,286	1,782	ns	1,418	0,448	0,478	UP	0,971	-0,926	-0,350	DOWN	-1,694	0,986	1,401	DOWN	0,860	1,110	0,921	UP
PIK3CA	0,147	1,782	1,932	ns	4,305	0,457	0,429	UP	3,848	-0,886	-0,361	UP	1,255	2,110	1,297	UP	-0,130	0,709	0,614	ns
ERBB2	1,038	1,543	1,732	UP	0,321	0,253	0,275	UP	0,068	-0,528	-0,231	ns	-2,889	1,791	1,743	DOWN	0,536	1,237	0,687	ns
PTEN	0,732	1,336	0,797	ns	0,593	0,318	0,364	UP	1,873	1,095	0,921	UP	0,983	1,453	1,626	ns	-0,338	2,721	3,001	ns
P53	-0,059	0,652	0,758	ns	1,385	0,220	0,255	UP	1,165	-0,475	-0,249	ns	1,362	1,430	1,417	UP	1,023	1,135	0,760	UP
MDM2	-3,591	1,468	1,445	DOWN	3,977	0,281	0,231	UP	3,697	-0,512	-0,224	ns	-0,409	2,010	1,335	ns	0,527	1,088	0,683	ns
APAF1	-2,358	1,181	1,770	DOWN	0,703	0,455	0,384	UP	0,249	-0,838	-0,367	UP	-1,065	2,509	2,336	ns	-0,274	1,379	0,941	ns
BID	-2,531	0,265	0,216	DOWN	-1,358	0,418	0,407	DOWN	-1,776	-0,824	-0,247	DOWN	-5,878	1,087	1,527	DOWN	-0,966	2,328	2,247	ns

BAK1	-0,786	1,412	1,787	ns	-0,405	0,465	0,344	DOWN	-0,870	-0,808	-0,272	DOWN	-0,747	2,374	1,488	ns	0,399	1,627	1,113	ns
BAX	-1,639	1,545	1,573	DOWN	0,497	0,289	0,298	UP	0,208	-0,587	-0,496	DOWN	-1,916	2,406	1,952	DOWN	-0,751	1,101	0,733	DOWN
DIABLO	0,037	1,425	1,728	ns	0,675	0,234	0,228	UP	0,441	-0,462	-0,290	ns	-0,730	0,919	1,325	ns	-0,438	0,884	0,907	ns
CASP3	-1,648	1,352	1,705	DOWN	1,354	0,625	0,448	UP	0,729	-1,073	-0,182	UP	0,325	1,077	1,676	ns	0,088	1,769	2,499	ns
CASP8	-2,718	1,341	1,712	DOWN	1,097	0,366	0,310	UP	0,731	-0,676	-0,221	ns	2,834	1,912	1,664	UP	3,327	1,691	1,033	UP
CASP9	-2,548	1,330	1,926	DOWN	3,239	0,342	0,356	UP	2,898	-0,698	-0,222	ns	-0,281	0,933	1,658	ns	1,417	0,422	0,348	UP
BCL-XL	0,758	1,406	1,766	ns	0,169	0,220	0,248	ns	-0,051	-0,468	-0,242	ns	0,208	1,065	1,500	ns	0,236	1,096	0,916	ns
MCL1	0,550	0,566	0,455	UP	1,317	0,382	0,383	UP	0,935	-0,764	-0,313	DOWN	-0,806	2,820	2,096	ns	0,289	2,168	2,171	ns

Table S6. Chemical shifts (^{1}H and ^{13}C) in parts per million (ppm) of the synthetic peptide crotAMP14. A solution of 1.5 mM crotAMP14 was made in 75 mM SDS-d_{25} micelles and analyzed at 25°C.

Position	Residue	H	HA	HB	HG	HD	HE	HZ	CA	CB	CD	CE	CZ
1	Lys		4.14										
2	Arg	8.56	4.47	1.83,1.92	1.68,1.68	3.12,3.12			57.30	30.80	43.77		
3	Leu	7.93	3.64	1.90,1.90	1.71	0.79,1.04							
4	Lys	7.52	3.98	1.88,1.88	1.52,1.52	1.69,1.69	2.97,2.97		58.16	32.35	29.24	42.14	
5	Lys	7.58	4.04	1.87,1.87	1.47,1.47	1.68,1.68	3.12,3.12		58.87	32.25	29.07	42.12	
6	Ile	7.71	3.70	1.93	1.55,0.69	0.30					16.77		
7	Phe	7.80	4.43	2.82,3.24		7.33	7.18	7.06			131.77	131.14	129.21
8	Lys	8.20	3.84	1.90,1.90	1.47,1.47	1.69,1.69	3.12,3.12		59.70	32.35	29.57	42.15	
9	Lys	7.58	3.99	1.91,1.91	1.48,1.48	1.67,1.67	3.12,3.12		59.19	33.02	28.71	42.16	
10	Met	8.17	4.05	2.06,2.28	2.68,2.48				58.27	32.77			
11	Ile	7.97	3.75	1.89		0.74					13.5		
12	Lys	7.45	4.04	1.92,1.92	1.51,1.51	1.66,1.66	2.96,2.96		58.55	32.32	29.14	42.17	
13	Ile	7.77	3.70	1.92	0.70,1.54,1.54	0.29					16.66		
14	Phe	7.85	4.43	2.83,3.25		7.33	7.18	7.06			132.03	130.91	129.12
15	Nh2	7.38,6.92											

Table S7. Summary of structural statistics of CrotAMP14 in SDS-d_{25} micelles.

Distance and dihedral restraints				
NOEs				
Intra Residue		88		
Sequential		30		
Short range		9		
Medium range		1		
Long range		1		
Total unambiguous		129		
Ambiguous		73		
Total NOE-derived		202		
Dihedral angles (psi + phi)		38		
Structural statistics				
Ramachandran Plot (%)	**value**	**error**	**min**	**max**
Most favored	93.6	5.16	83.3	100
Additionally allowed	6.44	5.16	0	16.7
Generously allowed	0	0	0	0
Disallowed regions	0	0	0	0
Structural precision				
RMSD Backbone (secondary structure/all)	0.139599 (+/- 4.903223E-02) / 1.32994 (+/- 0.454047)			
RMSD Heavyatom (secondary structure/all)	0.824521 (+/- 0.142518) / 2.12025 (+/- 0.494964)			
NOE violations				
>0.1 A		0		

Table S8. Atomic interactions involving the lowest free energy solution NMR structures determined for crotAMP14 and POPC/POPE/POPS (60:20:20) membrane bilayer during 200 ns of MD simulations at 50 ns intervals.

crotAMP14 – 0 ns			**Distance (Å)**	**Membrane – 0ns**			**Interactions**
Residue	**Position**	**Atom**		**Residue**	**Position**	**Atom**	
Arg	2	NE	3.4	POPS	20	O13B	HB
Arg	2	NH2	2.6	POPS	20	O13B	HB
Lys	5	NZ	3.0	POPS	16	O13B	HB
Lys	8	NZ	2.8	POPS	15	O14	SB
Lys	9	NZ	2.7	POPS	19	O13	SB
Lys	12	NZ	2.6	POPS	18	O14	SB
Lys	12	NZ	2.7	POPC	57	O14	HB
crotAMP14 – 50 ns				**Membrane – 50ns**			
Lys	1	N	2.8	POPS	15	O13A	HB
Lys	1	N	2.9	POPS	15	O13B	HB
Lys	1	N	3.0	POPS	16	O13A	HB
Lys	1	NZ	2.5	POPS	17	O13A	HB
Lys	1	NZ	2.7	POPS	15	O13A	HB
Lys	1	NZ	2.7	POPS	15	O13A	HB
Lys	1	NZ	2.7	POPE	175	O14	SB
Arg	2	NH2	2.5	POPS	20	O13B	HB
Arg	2	NH1	2.6	POPS	20	O13A	HB

Arg	2	NH1	2.8	POPS	16	O13B	HB
Lys	4	NZ	2.7	POPS	17	O13	SB
Lys	4	NZ	2.8	POPS	21	O13B	HB
Lys	4	NZ	3.0	POPS	21	O13A	HB
	crotAMP14 – 100 ns			**Membrane – 100ns**			
Lys	1	N	2.7	POPS	15	O13B	HB
Lys	1	N	2.8	POPC	76	O13	HB
Lys	1	NZ	2.6	POPS	15	O13A	HB
Lys	1	NZ	2.9	POPS	17	O13A	HB
Arg	2	NH1	2.7	POPS	16	O13A	HB
Arg	2	NH1	2.8	POPS	20	O13A	HB
Arg	2	NH2	2.7	POPS	20	O13B	HB
Arg	2	NH2	3.1	POPS	20	O13A	HB
Lys	4	NZ	2.6	POPS	15	O14	SB
Lys	4	NZ	2.6	POPS	21	O14	SB
Lys	5	NZ	2.7	POPS	19	O14	SB
Lys	5	NZ	3.0	POPS	16	O13	SB
Lys	8	NZ	2.5	POPS	19	O13A	HB
Lys	8	NZ	2.8	POPS	21	O13	SB
Lys	12	NZ	2.7	POPS	31	O13A	HB
Lys	12	NZ	2.7	POPS	21	O13A	HB

Lys	12	NZ	2.8	POPS	21	O13B	HB
crotAMP14 – 150 ns				**Membrane – 150ns**			
Lys	1	O	3.3	POPS	16	O13	HB
Lys	1	N	2.7	POPC	76	O13	HB
Lys	1	NZ	2.7	POPS	16	O13B	HB
Lys	1	NZ	3.5	POPS	16	O13A	HB
Arg	2	NE	2.7	POPS	16	O14	HB
Arg	2	NH1	2.8	POPS	20	O13A	HB
Arg	2	NH1	3.2	POPS	20	O13B	HB
Arg	2	NH2	2.8	POPS	16	O14	SB
Lys	5	NZ	2.8	POPS	20	O13B	HB
Lys	9	NZ	2.7	POPS	19	O13	SB
Lys	9	NZ	2.7	POPS	20	O14	SB
Lys	9	NZ	3.0	POPC	61	O13	HB
crotAMP14 – 200 ns				**Membrane – 200ns**			
Lys	1	N	2.5	POPS	15	O13B	HB
Lys	1	N	3.0	POPC	60	O13	HB
Lys	1	N	3.2	POPS	15	O13A	HB
Lys	1	NZ	3.1	POPC	76	O32	HB
Arg	2	NH1	2.7	POPS	23	O13B	HB
Arg	2	NH1	3.5	POPS	23	O13A	HB

Arg	2	NH2	2.8	POPS	23	O13A	HB
Arg	2	NH2	3.4	POPS	23	O13B	HB
Arg	2	NH2	3.6	POPC	60	O14	HB
Lys	4	NZ	2.6	POPS	17	O13A	HB

HB: Hydrogen bond; SB: saline bonds (electrostatic interaction).

7 DISCUSSÃO

Embora a humanidade tenha testemunhado, nos últimos anos, notáveis avanços científicos no tratamento do câncer, esta doença continua sendo uma das maiores causas de morbidade e mortalidade em todo o mundo (TIAN et al., 2022). O câncer é uma doença caracterizada pela alteração na sinalização e no metabolismo celular que gera divisão descontrolada, sobrevivência de células transformadas e invasão de outros tecidos (UPADHYAY, 2021). Além disso, em tumores sólidos, células não neoplásicas como as estromais e as do sistema imune também desempenham papéis críticos na progressão tumoral, impactando a resposta terapêuticas dos pacientes com câncer (XIAO; YU, 2021).

Entre os indivíduos do sexo feminino, o câncer de mama é o tipo de neoplasia mais comumente diagnosticado, em que o CMTN corresponde por cerca de 10% a 20% de todos os casos (WOJTUKIEWICZ; POGORZELSKA; POLITYNSKA, 2022). Devido à sua complexidade, o tratamento para o câncer de mama é multidisciplinar, sendo aconselhada cirurgia convencional, associada a radioterapia ou mastectomia em mulheres com estágio inicial da doença (MOO et al., 2018). Desse modo, o sucesso da terapia curativa pode estar atrelado à sua administração dentro de um período definido (MUTEBI et al., 2020). No entanto, pacientes diagnosticados com CMTN apresentam um prognóstico desfavorável devido à falta de terapias direcionadas (KANG; LEROITH; GALLAGHER, 2018).

Atualmente, a quimioterapia é considerada o tratamento padrão contra o CMTN, a qual inclui a administração de agentes intercaladores de DNA (doxorrubicina, membro da classe das antraciclinas), alquilantes (ciclofosamida), que impedem a formação dos microtúbulos (taxano) e a inibição da timidilato sintase, enzima que catalisa a síntese de pirimidinas (fluorouracil, 5-FU) (WON; SPRUCK, 2020). Embora seja a abordagem mais utilizada, a quimioterapia causa uma série de efeitos colaterais nos pacientes oncológicos como náuseas, alopecia, vômitos, mielossupressão e fadiga. Além disso, a quimioterapia pode ocasionar mucosites, oral e/ou gastrointestinal, resultando em ulcerações, dor, anorexia, perda de peso, anemia e chances de formação de sepse (PRADHAN et al., 2023).

Diante de tais limitações, novas terapias precisam ser desenvolvidas de modo a tornar o tratamento mais específico e menos nocivo às células saudáveis que

apresentam a mesma taxa de replicação que as neoplásicas (LU et al., 2023). Uma dessas novas estratégias foi apresentada em um estudo clínico não randomizado de fase 1, no qual foi testada uma vacina de DNA plasmidial, que codifica para o domínio intracelular de ERBB2 (HER2), visando aumentar as células T auxiliares, podendo induzir à imunidade a ERBB2 na maioria dos pacientes (DISIS et al., 2023). Os resultados demonstraram que a imunização com a dose de 100 µg da vacina foi associada à geração de células T tipo 1 que reconhecem especificamente ERBB2 na maioria dos pacientes com câncer de mama que expressa ERBB2, podendo ser utilizada como um tratamento alternativo (DISIS et al., 2023).

No entanto, como citado anteriormente, as neoplasias de mama que não expressam os receptores ER, PR e ERBB2 apresentam um quadro bastante agressivo com um prognóstico desfavorável, de modo que terapias endócrinas ou anti-HER2 não são efetivas para estes pacientes (CHOI; KIM, 2023). Desse modo, alternativas que independam do reconhecimento de receptores transmembranares e que gerem pouco ou nenhum efeito colateral precisam ser exploradas. Atualmente, peptídeos com atividade antitumoral vêm sendo estudados como moléculas alternativas promissoras para o tratamento do câncer, incluindo o de mama (GUO, F. et al., 2022; LI, K.; LIU; ZHANG, 2020). No presente trabalho, após uma triagem com 17 peptídeos de diferentes fontes, naturais e sintéticos, selecionamos o crotAMP14 para avaliar o seu mecanismo de ação. Os critérios de seleção se basearam no número de linhagens em que o peptídeo é ativo e a menor quantidade de resíduos de aminoácidos.

O crotAMP14 é derivado da ctn, um membro das catelicidinas encontrada na glândula de peçonha da jararaca sul-americana (FALCAO, Claudio Borges; RADIS-BAPTISTA, 2020), que possui atividade antibacteriana, antifúngica e antitumoral (DE AGUIAR et al., 2020; PÉREZ-PEINADO et al., 2018). As catelicidinas além de proeminente família de PAMs, também desempenham papéis importantes no sistema imune inato de praticamente todas as espécies de vertebrados (KLUBTHAWEE et al., 2020). Em humanos, a LL-37 é constitutivamente expressa em diferentes células de defesa do hospedeiro, incluindo macrófagos, neutrófilos, células epiteliais e células endoteliais, desempenhando papéis importantes não apenas no combate a bactérias, fungos, vírus e parasitas, mas também na regulação de várias funções imunes, como reações inflamatórias, proliferação celular, apoptose, parada do ciclo celular, angiogênese e liberação de citocinas (CHEN, J. et al., 2020).

Catelicidinas de diversas espécies possuem atividade antitumoral como as de frango CATH-(1, 2 e 3), as quais foram efetivas contra linhagem de câncer de mama MCF-7 nas concentrações de 10, 20 e 40 $\mu g.ml^{-1}$ (MAHMOUD et al., 2022). Outro exemplo é a catelicidina humana LL-37, a qual também demonstrou atividade antitumoral contra células de carcinoma espinocelular oral por meio do mecanismo subjacente que pode incluir a indução de apoptose dependente de caspase-3 (via P53-Bcl) via de sinalização -2/BAX (CHEN, X. et al., 2020). Além disso, o estudo estrutural das catelicidinas pode indicar motivos capazes de apresentar atividade com uma redução de tamanho, algo observado no peptídeo FK-16, derivado do LL-37, o qual induz células de câncer de cólon à apoptose independente de caspase (REN et al., 2013). Desse modo, as catelicidinas se configuram como uma fonte de peptídeos que podem ser explorados para o tratamento de neoplasias.

O crotAMP14 foi obtido a partir do redesenho da ctn, mais especificamente do seu fragmento compreendendo do 15º aa ao 34º aa, o ctn[15-34]. Por meio de uma estratégia de *design* físico-quimicamente guiada, foram excluído resíduos considerados desfavoráveis para a superfície eletrostática do peptídeo crotAMP14, mantendo uma alternância entre resíduos carregados positivamente e hidrofóbicos (OLIVEIRA et al., 2020). Além disso, outra modificação foi a exclusão de vários resíduos (K^{1}, P^{11}, V^{13}, G15, V^{16}, T^{17} e P^{19}), bem como a adição de lisina (K) na posição 12 do crotAMP14. Tais modificações resultaram em um peptídeo menor (14 aa), quando comparado com a ctn (34 aa), seu parental, com a atividade antimicrobiana aumentada, no entanto, sem causa citotoxicidade contra células saudáveis (OLIVEIRA et al., 2020). Dessa forma, estratégias como o design racional de peptídeos, permitindo a redução do tamanho de peptídeo parental, associado ao aumento da atividade sem comprometer a citotoxicidade podem ser exploradas para o desenvolvimento de novos PACs. Por meio de um redesenho, Tzitzilis e colaboradores (2020) desenvolveram novos peptídeos análogos do segmento da catelicidina de 13 aminoácidos 17–29 ($F^{17}KRIV^{21}QR^{23}IK^{25}DF^{27}$LR-NH2). Entre estes peptídeos, o peptídeo 1 apresentou atividade contra a linhagem de câncer epitelial brônquico A549 (IC50 20,13 $\mu g.mL^{-1}$) sem ser tóxico para eritrócitos (TZITZILIS et al., 2020), demonstrando a eficiência da estratégia de redesenho funcional de peptídeos para aplicações terapêuticas contra o câncer.

O crotAMP14 foi efetivo contra linhagem triplo-negativa de câncer de mama (MDA-MB-231) nas concentrações de 64, 32, 16 e 8 μM. Após 24 horas de exposição aproximadamente 100% das células morreram em 64 e 32 μM e 45% em 16 μM. Devido ao fato de que para realizar a análise de expressão gênica das células após a exposição ao peptídeo, adotamos a concentração de 16 μM para os futures testes uma vez que não há 100% de morte celular, permitindo a coleta do RNAm. Além disso, foi demonstrado que o peptídeo é ativo contra a linhagem de adenocarcinoma nos primeiros 15 minutos de exposição. Durante os testes de citotoxicidade, foi observado que as células se desprendem diminuem de e ficam em suspensão no meio. Análises de citometria de fluxo (FSC e SSC) indicaram que as células diminuem em tamanho consideravelmente ao ponto de gerar duas populações nos histogramas (dados não mostrados).

A diminuição no tamanho das células é uma alteração morfológica fortemente associada ao processo de morte celular por apoptoses (ÖLANDER; HANDIN; ARTURSSON, 2019). Em alguns casos, a redução de tamanho representa 38% da área média das células que irão disparar os mecanismos de morte celular programada (CACHOUX et al., 2023). Desse modo, um possível mecanismo de ação do crotAMP14 por apoptose foi avaliado. Para tal, 30 genes pró-apoptóticos e antiapoptóticos foram selecionados e tiveram a sua expressão gênica avaliada por meio de qPCR. De modo geral, nas comparações entre os tratamentos (células do sobrenadante versus controle, células aderidas versus controle e células do sobrenadante versus células aderidas) houve um aumento na expressão tanto de genes apoptóticos (caspases 3, 8 e 9) quanto de genes envolvidos no ciclo celular (CDK4/6).

Embora os dados gerados pela análise da expressão relativa destes genes sejam relevantes, o perfil de expressão das células observado não corresponde com os descritos na literatura científica. É comumente observado um aumento na expressão de genes pró-apoptóticos concomitantemente com a diminuição da expressão de genes antiapoptóticos, induzindo a célula à morte programada. Recentemente, foi demonstrado que a crotalicidina BMAP-27 reduz a proliferação de células de câncer de cólon por meio da regulação positiva de genes supressores de tumor (CASPASE3, BAX, TP53, AXIN1) enquanto regula negativamente a expressão de oncogenes (BCL-2, CDK-6, PCNA, WNT11 e CTNNB1) (DAS et al., 2023). No

entanto, não foi possível observar essa correlação nas células expostas ao crotAMP14.

Embora as análises de citometria de fluxo utilizando anexina-V e iodeto de propídio indicaram morte celular por apoptose (dados não mostrados), os dados de expressão gênica não foram conclusivos para corroborar a hipótese de morte celular programada. Desse modo, foram feitos testes de ativação de caspases 3 e 7 para avaliar o aumento da expressão dos genes das caspases foi acompanhado da ativação dessas proteínas. Interessantemente, o crotAMP14 inibi a ativação das caspases em aproximadamente 80% nas primeiras 24 horas chegando a 100% após 48 horas.

Caspase-3/7 são proteases de ácido cisteína-aspártico responsáveis por desencadear diretamente a morte celular por apoptose seguida pela ativação sequencial de outras caspases (8 e 9) (SHIM et al., 2017). Curiosamente, o crotAMP14 inibiu a atividade da caspase-3/7 em células MDA-MB-231 em aproximadamente 100% após 48 h de exposição. Embora dados anteriores sugerissem uma possível morte celular apoptótica, a inibição da caspase pelo crotAMP14 demonstrou que a morte celular pode ocorrer por outro mecanismo independente da caspase, como a necrose. Desse modo, a hipótese de que o peptídeo atua formando poros na membrana foi levantada, pois os dados de citometria podem ter acusado um falso positivo, uma vez que ao formar poros na membrana plasmática, a anexina-V pode entrar na célula e se ligar às fosfatidilserinas presentes na camada interna da membrana plasmática.

Outra evidência que apoia essa hipótese é o fato de que o crotAMP14 mostrou semelhanças com o peptídeo oncolítico quimiocinestatina-1 (CKS1) (FURUKAWA et al., 2024). CKS1 é um peptídeo 24-mer derivado da quimiocina CXCL1 que, quando em contato com células 4T1, resulta no desprendimento dessas células do fundo do poço e morte celular rápida (30 min). Foi demonstrado que CKS1 causa danos severos à membrana plasmática, induzindo morte celular por meio de necrose (FURUKAWA et al., 2024). Além disso, a maioria dos interage com as membranas plasmáticas das células (XIE; LIU; YANG, 2020). Desse modo, foram feitos testes para avaliar uma possível interação entre o crotAMP14 e a membrana plasmática das células de MDA-MB-231. O primeiro deles consistiu na elucidação estrutural do peptídeo por meio de ressonância magnética nuclear.

A atividade biológica dos AMPs depende, em grande parte, de determinantes físico-químicos cruciais, incluindo carga, grau de helicidade, hidrofobicidade, comprimento de sequência, anfipaticidade e solubilidade (AHMED, T. A. E.; HAMMAMI, 2019). Portanto, entender a relação entre a estrutura e a função dessas biomoléculas é essencial para o desenvolvimento do novo fármaco (AHMED, T. A. E.; HAMMAMI, 2019). Os dados de RMN demonstraram que o crotAMP14 adota uma estrutura α-helicoidal, quando em contato com membranas miméticas, corroborando dados publicados anteriormente (OLIVEIRA et al., 2020).

Os ACPs α-helicoidais catiônicos são uma classe essencial de peptídeos antitumorais (HUANG, Y. et al., 2015), geralmente atuando em células neoplásicas por meio de interação eletrostática com a membrana dessas células (ABD EL-AAL et al., 2023), causando lise celular (GHALY et al., 2023). Conforme demonstrado recentemente, a atividade anticâncer pode estar relacionada ao seu conteúdo helicoidal e hidrofobicidade (HADIANAMREI et al., 2022b). O crotAMP14 é um peptídeo curto sintético α-helicoidal catiônico (+8), o que pode explicar sua atividade antitumoral demonstrada aqui. Além disso, como foi reportado anteriormente, o crotAMP14 interage com a membrana plasmática de células bacterianas (OLIVEIRA et al., 2020). Dados de espectroscopia de EPR gerados aqui demonstraram o que crotAMP14 atua na membrana plasmática das células neoplásica, aumento a sua rigidez sem, no entanto, aumento a oxidação da membrana plasmática o que indica uma provavelmente morte celular por necrose.

As análises de EPR também demonstraram que o peptídeo não causa redução na fluidez da membrana plasmática de cultura primária de fibroblastos humanos, corroborando dados observados nos testes de viabilidade celular, nos quais as células apenas nas concentrações mais elevadas (32 e 64 μM) houve morte celular. Um pequeno aumento em 2A// de 1,3 G foi observado na concentração de 32 μM. O crotAMP14 aumentou a rigidez na membrana celular de MDA-MB-231 em um curto período de incubação. Este resultado indica que o crotAMP14 causa rigidez devido à sua presença física na membrana em vez de desencadear processos oxidativos que exigem mais tempo. Além disso, foi descoberto que o peptídeo pode causar rigidez em membranas MDA-MB231 isoladas logo após o tratamento indicando sua interação diretamente com a membrana.

Na membrana celular, foi demonstrado que o marcador de spin 5-DSA se comporta como lipídios anulares ou de limite que circundam preferencialmente a superfície hidrofóbica das proteínas da membrana e que esse efeito restringe a mobilidade da sonda de spin (MARSH, 2010). Assim, quanto maior o conteúdo de proteína da membrana, menor sua fluidez. Por exemplo, na membrana do eritrócito que tem um conteúdo de proteína de aproximadamente 50%, o valor 2A// é maior que 56 G (ALONSO et al., 2019). Com base nessas descobertas, espera-se que a presença do crotAMP14 em uma estrutura de α-hélice possa restringir a mobilidade dos lipídios da membrana celular. Além disso, também é provável que o peptídeo possa dificultar o empacotamento de cadeias lipídicas e, assim, aumentar a probabilidade de formação de poros na membrana celular. O fato de haver menor efeito no fibroblasto pode estar relacionado à menor entrada do peptídeo na membrana celular.

Embora os dados de EPR são gerados por testes empíricos, foram feitas simulações *in silico* para avaliar as interações entre o peptídeo crotAMP14 e a membrana plasmática das células alvos. Os dados obtidos por meio de dinâmica molecular corroboraram os dados obtidos nos outros experimentos. O fato de praticamente não haver redução na fluidez da membrana de fibroblastos por parte do crotAMP14 está associado com a perda de sua estrutura α-helicoidal devido a menor entrada do peptídeo na membrana plasmática dessas células. Por outro lado, quando em contato com uma bicamada lipídica de células neoplásicas, o peptídeo crotAMP14 conserva sua estrutura durante as simulações e preserva suas interações com esse sistema de membrana aniônica por meio de sua região N-terminal carregada positivamente.

Esses dados dão suporte às nossas descobertas in vitro nas quais o peptídeo crotAMP14 desencadeia rigidez nas membranas das células cancerígenas. Além disso, os mapas de densidade parcial em POPC/Chol e POPC/POPE/POPS indicam que o peptídeo é inserido mais profundamente na membrana cancerígena do que a membrana zwitteriônica. Nesta última, a maior parte do peptídeo está na interface de água no sistema POPC/Chol. Também vale a pena notar que o peptídeo crotAMP14 interage principalmente com a membrana POPC/POPE/POPS por meio de ligações de hidrogênio e salinas envolvendo resíduos carregados positivamente da região N-terminal. O principal mecanismo de ação dos PACs ocorre por meio de suas

interações com os lipídios das membranas celulares, ocasionado a ruptura celular (GHALY et al., 2023).

Em um estudo que investigou o perfil de espectrometria de massa in situ de diferentes linhagens de células de câncer de mama, foi demonstrado que os níveis de fosfatidilinositol (PI(36:1)), PC(36:1) e PC(36:2) estão aumentados em células MDA-MB-231 quando comparados com outras cinco linhagens de células (HE; GUO; LI, 2015). Essa variação no perfil com níveis significativamente elevados de lipídios monoinsaturados podem estar associada ao grau de malignidade dessas neoplasias (HE; GUO; LI, 2015). Além disso, alterações no perfil lipídico no câncer podem ocorrer em diferentes estágios de progressão (DADHICH; KAPOOR, 2022). Embora o crotAMP14 tenha uma carga líquida positiva relativamente alta (+8), experimentos mostraram que a produção *in silico* de ACPs com atividade anticâncer melhorada não requer necessariamente um aumento máximo na carga e hidrofobicidade (MA et al., 2020). Em vez disso, essas propriedades restringem e influenciam umas às outras até que um equilíbrio seja alcançado que alcance uma atividade anticâncer melhorada (MA et al., 2020). Algo semelhante foi adotado no processo redesenho físico-quimicamente guiado do crotAMP14 (OLIVEIRA et al., 2020).

8 CONCLUSÕES

Os dados aqui apresentados lançam luz sobre como os peptídeos com atividade antimicrobiana, principalmente contra bactérias Gram-negativas, podem ser explorados como agentes antitumorais. Embora o peptídeo crotAMP14 tenha alterado o perfil de expressão gênica das células MDA-MB-231, a expressão não resultou em morte celular por apoptose, como visto em ensaios de ativação de caspase. O crotAMP14, por ser um peptídeo catiônico α-helicoidal, se insere na membrana plasmática de células neoplásicas por meio de interações eletrostáticas, devido a diferenças de cargas positivas (peptídeo) e negativas (membrana celular). Como resultado dessa interação, há um aumento na rigidez da membrana plasmática, sem aumento da oxidação, indicando a possível formação de poros e morte celular por necrose. Em células saudáveis essa interação não ocorre devido ao fato de que a membrana plasmática das células saudáveis possuir uma carga líquida neutra, devido a presença de fosfolipídios zwiteriônicos. Desse modo, o crotAMP14 não consegue

assumir sua estrutura 3D e, consequentemente, perturbar a membrana plasmática dessas células. A investigação da atividade antitumoral em potencial dos AMPs catiônicos α-helicoidais permite o desenvolvimento de novos medicamentos, incluindo derivados de peptídeos, para tratar o câncer de mama. Esses tratamentos podem ser mais eficazes e ter menos efeitos colaterais para pacientes com câncer de mama. Além disso, entender o mecanismo de ação dos ACPs é crucial porque lança luz sobre novos alvos farmacológicos e permite o desenvolvimento de novas moléculas capazes de erradicar células por meio de interações eletrostáticas.

9 PERSPECTIVAS

A elucidação do mecanismo de ação antitumoral do crotAMP14 contra células neoplásicas de câncer de mama triplo negativo gerou mais perguntas do que resposta (e perguntas foram e sempre serão a locomotiva do processo científico). Entre essas perguntas podemos destacar algumas como “o crotAMP14 também atua do mesmo modo em outras linhagens de câncer de mama e de outros tipos?”, “em modelos in vivo o crotAMP14 ainda é capaz de erradicar células neoplásicas?” e “como o peptídeo se comporta em modelos 3D tumorais como organoides?” Todas as respostas para essas perguntas podem ser respondidas futuramente por todos aqueles que, assim como eu, se interessam por essa seara microscópica e os dados aqui gerados poderão fornecer algumas pistas para a compreensão do mecanismo de ação antitumoral de peptídeos antimicrobianos.

As próximas etapas do projeto consistirão na realização de microscopia eletrônica de transmissão (MET) para avaliar como o peptídeo se organiza estruturalmente na membrana plasmática das células-alvo. Avaliar se o mecanismo antitumoral do crotAMP14 é semelhante para outras linhagens de câncer de mama com graus de agressividade variados. Além disso, uma última proposição deste trabalho será a investigação da atividade desse peptídeo em organoides a fim de avaliar se a morte celular se mantém, bem como se o crotAMP14 pode alterar algumas propriedades do microambiente tumoral mimetizados pelas estruturas 3D, produzidas com células de MDA-MB-231 e fibroblastos humanos.

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ANEXOS

ARTIGOS E CAPÍTULOS DE LIVROS PUBLICADOS

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Article

Screening of the Skin-Regenerative Potential of Antimicrobial Peptides: Clavanin A, Clavanin-MO, and Mastoparan-MO

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Abstract: Skin wound healing is coordinated by a delicate balance between proinflammatory and anti-inflammatory responses, which can be affected by opportunistic pathogens and metabolic or vascular diseases. Several antimicrobial peptides (AMPs) possess immunomodulatory properties, suggesting their potential to support skin wound healing. Here, we evaluated the proregenerative activity of three recently described AMPs (Clavanin A, Clavanin-MO, and Mastoparan-MO). Human primary dermal fibroblasts (hFibs) were used to determine peptide toxicity and their capacity to induce cell proliferation and migration. Furthermore, mRNA analysis was used to investigate the modulation of genes associated with skin regeneration. Subsequently, the regenerative potential of the peptides was further confirmed using an ex vivo organotypic model of human skin (hOSEC)-based lesion. Our results indicate that the three molecules evaluated in this study have regenerative potential at nontoxic doses (i.e., 200 μM for Clavanin-A and Clavanin-MO, and 6.25 μM for Mastoparan-MO). At these concentrations, all peptides promoted the proliferation and migration of hFibs during in vitro assays. Such processes were accompanied by gene expression signatures related to skin regenerative processes, including significantly higher *KI67*, *HAS2* and *CXCR4* mRNA levels induced by Clavanin A and Mastoparan-MO. Such findings translated into significantly accelerated wound healing promoted by both Clavanin A and Mastoparan-MO in hOSEC-based lesions. Overall, the data demonstrate the proregenerative properties of these peptides using human experimental skin models, with Mastoparan-MO and Clavanin A showing much greater potential for inducing wound healing compared to Clavanin-MO.

Keywords: antimicrobial peptides; Clavanin A; Clavanin-MO; Mastoparan-MO; skin regeneration

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1. Introduction

Normal wound healing involves coordination between the immune response and the processes of cellular migration, proliferation, matrix deposition, and tissue remodeling [1]. During skin wound healing, an excessive inflammatory response induced by opportunistic pathogens, and metabolic, or chronic diseases can lead to nonhealing wounds [2,3]. For instance, an increase in the proinflammatory response, resulting from the deregulation

of several key proinflammatory cytokines, such as IL-1β and tumor necrosis factor–α (TNFα), prolongs the inflammatory phase, and has been related to elevated activity of metalloproteinases that impair cell migration and proliferation process, thus delaying the wound repair [3,4]. This deterioration in healing conditions and the presence of nonhealing wounds increases treatment costs, causes aesthetic damage, or even puts the lives of patients at risk, which makes it necessary to search for new therapeutic options that favor skin tissue regeneration [1,4].

In the search for new proregenerative treatments, antimicrobial peptides (AMPs) arise as a group of molecules that present a broad spectrum of antimicrobial activity, accompanied by immunomodulatory and regenerative properties that promote cell proliferation, and angiogenesis, which favors the wound healing process [5,6]. The AMPs Clavanin A (VFQFLGKIIHHVGNFVHGFSHVF-NH2) and its synthetic derivative Clavanin-MO (FLPIIVFQFLGKIIHHVGNFVHGFSHVF-NH2) present strong activity against both Gram-negative and Gram-positive bacteria and can modify the expression of immune system components that regulate the inflammatory response [7–9]. Clavanin-MO can modulate innate immunity by stimulating leukocyte recruitment and the production of immune mediators such as GM-CSF, IFN-γ, and MCP-1, favoring an increase in the levels of anti-inflammatory cytokines (IL-10) and repressing the expression of proinflammatory cytokines (IL-12 and TNF-α), which suppresses the damage caused by an excessive inflammatory response [9]. Likewise, the peptide Mastoparan-MO (FLPIIINLKALAAALAKKIL), a less toxic synthetic variant of the natural AMP Mastoparan-L, can increase leukocyte migration while suppressing the expression of proinflammatory factors such as TNF-α, and IL-6. These immunomodulatory properties enhance the immune responses required to eliminate infections and reduce the damage caused by an excessive inflammatory response [10,11].

In the present study, we examined the ability of three antimicrobial peptides (Clavanin A, Clavanin-MO, and Mastoparan-MO,) to stimulate cell proliferation, and migration, as well as encourage the activation of genes associated with tissue regeneration in a model of primary human dermal fibroblasts (hFibs). Likewise, using an organotypic human ex vivo skin model (hOSEC), we analyzed the possible induction of wound healing generated by these antimicrobial peptides.

Our results indicate that treatment with Clavanin A, Clavanin-MO, or Mastoparan-MO peptides did not generate changes in the cellular viability of primary skin cells, suggesting low cytotoxicity of these molecules. Likewise, treatment with these AMPs can increase cell proliferation, migratory capabilities, and the induction of gene expression related to tissue repair processes in hFibs. Finally, using an ex vivo human skin culture system, we determined that Clavanin-MO and Mastoparan-MO can potentially facilitate wound healing. These results suggest that these antimicrobial and immunomodulatory peptides have skin-regenerative potential, constituting an exciting alternative for the development of further treatments for skin injuries.

2. Results

2.1. Cytotoxicity Screening of AMPs

We analyzed the cytotoxic potential on skin cells of three well-characterized antimicrobial peptides (Table 1). At 24 h of exposure, the peptides Clavanin A and Clavanin-MO did not produce any significant change in cell viability compared to control when the cells were exposed to doses as high as 200 μM (Figure 1A,B). However, Mastoparan-MO induced a significant and progressive reduction in cell viability, observed starting from 25 μM ($p < 0.001$), and reaching more than 50% at 100 μM ($p < 0.001$) (Figure 1C).

Table 1. List of antimicrobial peptides used during this study and their immunomodulatory properties.

Name	Immunomodulatory Activity	References
Clavanin A	Clavanin A and Clavanin-MO present anti-inflammatory activities in murine macrophage-like cells stimulated with LPS	[7–9]
Clavanin-MO	Clavanin A and Clavanin-MO can increase the production of IL-10 and reduce the expression of proinflammatory IL-12 and TNF-α Clavanin-MO can induce the migration of leukocytes In mice, treatment with Clavanin-MO increased the expression of GM-CSF, IFN-γ, and MCP-1 during the early stages of infection with *E. coli* and *S. aureus*	
Mastoparan-MO	Mastoparans can inhibit expression of Toll-like receptor 4 (TLR4), TNF-α, and interleukin-6 (IL-6) Mastoparan-MO can induce leukocyte migration to the site of infection in an in vivo model Treatment with Mastoparan-MO caused a decrease in proinflammatory cytokines IL-12, TNF-α, and IL-6	[10,11]

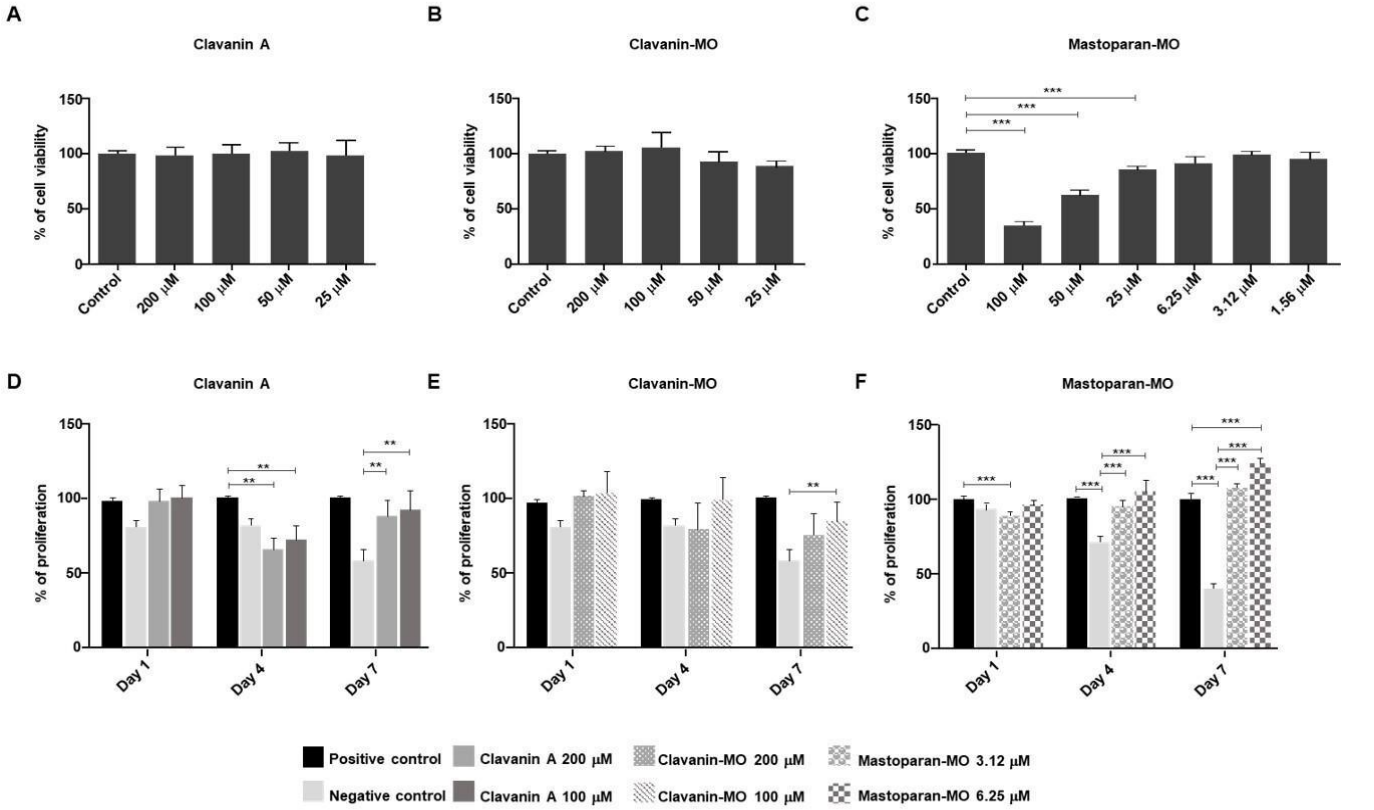

Figure 1. Cytotoxicity screening of AMPs. Cytotoxic potential of Clavanin A and Clavanin-MO at 200, 100, 50, and 25 µM ((**A**,**B**) respectively), and Mastoparan-MO at 100, 50, 25, 6.25, 3.12, and 1.56 µM (**C**), was tested in human primary dermal fibroblasts (hFibs). The mean ± SD of three independent experiments is presented. Effects on cell proliferation of Clavanin A and Clavanin-MO at 200 or 100 µM ((**D**,**E**), respectively) and Mastoparan-MO at 6.25 or 3.12 µM (**F**), were determined at 1, 4, and 7 days by MTT assay. The mean, along with the standard deviation, is shown for three independent experiments. In both cases, the statistical analysis was carried out through one-way ANOVA and Tukey post hoc tests. Asterisks indicate significant differences (** $p < 0.01$; *** $p < 0.001$) relative to the control.

This peptide, however, did not generate a significant change in cell viability when the cells were exposed to concentrations below 25 μM. As cytotoxicity controls, we evaluated the peptides Polybia-MPII and EcDBS1R6, which induced a significant reduction in cell viability ($p < 0.05$) at all concentrations tested (Figure S1). Based on these results, for further experiments, we used Clavanin A and Clavanin-MO at 100 and 200 μM, while Mastoparan-MO was used at 6.25 and 3.12 μM.

2.2. Proliferative Potential

At 7 days of treatment, both Clavanin A (100 μM and 200 μM) and Clavanin-MO (100 μM) were able to induce a significant increase in cell proliferation compared to the control of the solvent vehicle ($p < 0.01$ compared to the untreated control; Figure 1D,E). This trend can also be observed on days 1 and 4 of the treatment with Clavanin-MO at 200 μM. Likewise, the treatment with Mastoparan-MO at 3.12 and 6.25 μM induces a significant increase in cell proliferation (compared to the control of the solvent vehicle) at 4 and 7 days of treatment ($p < 0.001$; Figure 1F). After seven days of treatment, it is possible to observe that the Mastoparan-MO treatment at 6.25 μM can induce a significant increase in cell proliferation compared to the positive control (supplemented with 10% FBS) ($p < 0.001$; Figure 1F). The analysis of the proliferation kinetics (Figure 2A–C) reveals that at 4 days, the treatment with Clavanin A or with Mastoparan-MO at any of the tested concentrations produces a significant increase in cell replication rate compared with the control of the solvent vehicle (Clavanin A at 100 μM or 200 μM, $p < 0.05$; Mastoparan-MO at 3.12, $p < 0.05$ and at 6.25 μM, $p < 0.01$). In the same way, the analysis of the proliferative potential (Figure 2D–F) that was carried out by calculating the population doubling time (PDT) shows that except for cells treated with Clavanin-MO at 100 μM, all treatments generated a significant reduction in the population doubling time compared to the vehicle control (Clavanin A at 100, 200 μM, and Clavanin-MO at 200 μM, $p < 0.05$; Mastoparan-MO at 3.12 μM, $p < 0.01$, and Mastoparan-MO at 6.25 μM, $p < 0.05$).

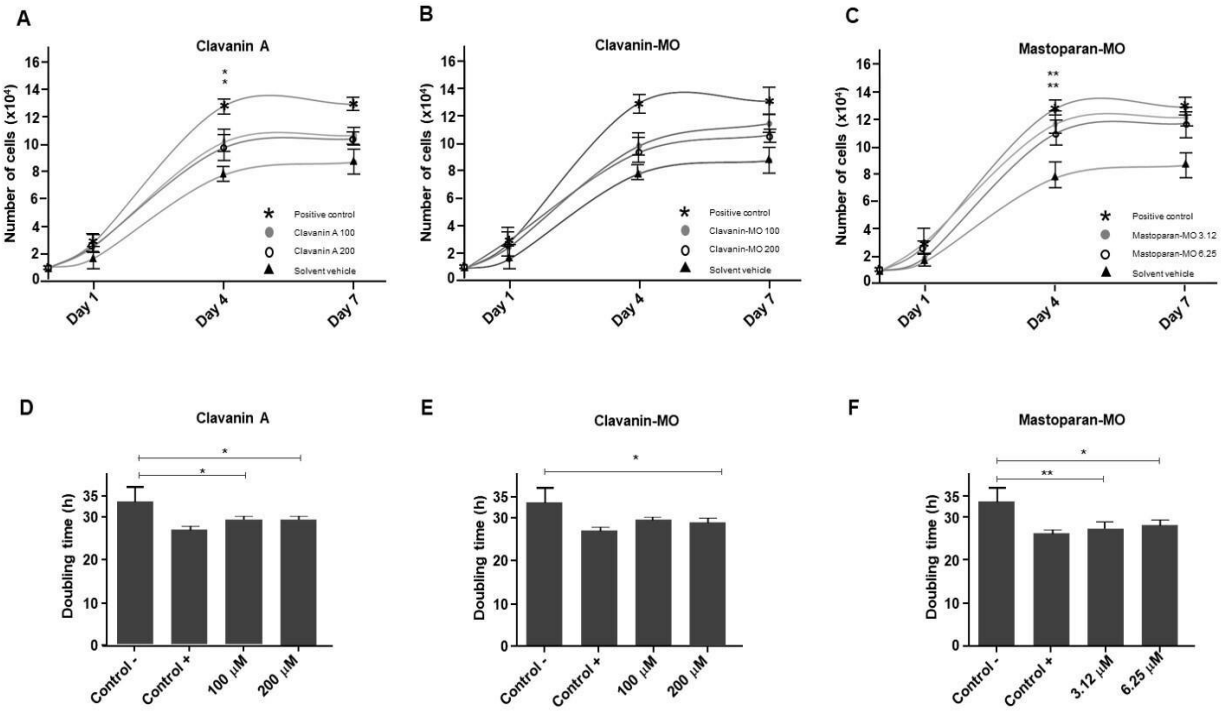

Figure 2. Analysis of proliferation kinetics. Human primary dermal fibroblast (hFibs) were incubated with Clavanin A ((**A**,**D**); 200 or 100 μM), Clavanin-MO ((**B**,**E**); 200 or 100 μM), or Mastoparan-MO ((**C**,**F**); 6.25 or 3.12 μM). Proliferation kinetics (**A**–**C**) were determined at 1, 4, and 7 days by counting

the total number of cells. To determine the proliferative potential of the cells, the population doubling time (PDT) was calculated at 4 days (**D**–**F**) of peptide treatment. The statistical analysis was carried out by means of a one-way ANOVA and Tukey post hoc tests. Significant differences regarding the control are marked with asterisks (* $p < 0.05$; ** $p < 0.01$).

2.3. Gene Expression Profile

Using qRT-PCR, we analyzed potential changes in gene expression of proregenerative factors in cells treated with the peptides (Figure 3). This analysis was conducted with cells treated with the peptides at concentrations of 200 µM for Clavanin A and Clavanin-MO, and 6.25 µM for Mastoparan-MO, as at these concentrations, the peptides showed a significant increase in cell proliferation and a significant reduction in population doubling time (PDT), which suggests a possible proregenerative effect of these molecules. The obtained results revealed that the treatment with Mastoparan-MO generated significant upregulation in expression levels of *FGF2*, *KI67*, *ELN*, *HAS2*, and *CXCR4* transcripts compared to the vehicle control ($p < 0.01$; Figure 3A–C,E,F). Likewise, treatment with Clavanin A can induce a significant upregulation in the expression levels of *KI67*, *HAS2*, *CXCR4*, *CXCR7*, and *BCL2* ($p < 0.01$; Figure 3B,E–H). In contrast, treatment with Clavanin A also generated a trend towards downregulation of *FGF2* and *MMP1* expression (Figure 3A,D). Clavanin-MO treatment promoted a significant upregulation in the expression of *FGF2* ($p < 0.05$ compared to the vehicle control), *ELN*, *MMP1*, *HAS2*, and *CXCR7* ($p < 0.01$ compared to the vehicle control; Figure 3A,C–E,G).

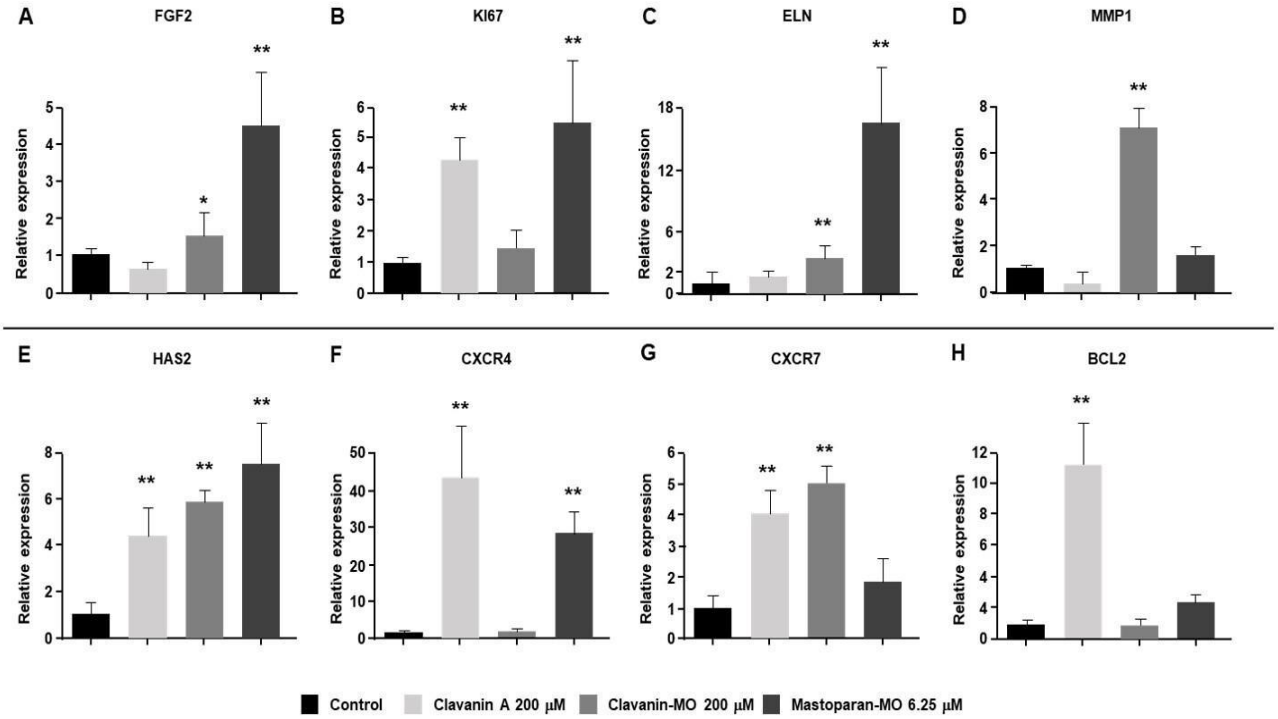

Figure 3. Changes in the expression of proregenerative genes. Relative mRNA expression profile of Human primary dermal fibroblasts (hFibs), that were treated with Clavanin A (200 µM), Clavanin-MO (200 µM), or Mastoparan-MO (6.25 µM), was analyzed. The mRNAs assessed correspond to (**A**) fibroblast growth factor 2 (*FGF2*), (**B**) marker of proliferation Ki-67 (*KI67*), (**C**) elastin (*ELN*), (**D**) matrix metalloproteinase 1 (*MMP1*), (**E**) hyaluronic acid synthase 2 (*HAS2*), (**F**) C-X-C chemokine receptor type 4 (*CXCR4*), (**G**) C-X-C chemokine receptor type 7 (*CXCR7*), and (**H**) B-cell lymphoma 2 protein (*BCL2*). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a ubiquitous control. Analysis of the mean ± SD of three independent experiments is presented. Statistical analysis was carried out by means of a one-way ANOVA and Tukey post hoc tests. Significant differences regarding the control are marked with asterisks (* $p < 0.05$; ** $p < 0.01$).

2.4. Cellular Migration

The treatment with both Clavanin A and Clavanin-MO at 200 μM for 24 and 48 h can induce a significant increase in the percentage of cell migration compared to the control of the solvent vehicle without FBS ($p < 0.01$ compared to the untreated control) (Figure 4A,B,D). Likewise, Mastoparan-MO treatment at 6.25 μM (Figure 4C,D) induced a significant increase in cell migration regarding the solvent vehicle control at both 24 and 48 h. At 48 h of treatment, the cells treated with Mastoparan-MO at 6.25 μM show an increase in cell migration that is greater than that observed in the positive control supplemented with FBS ($p < 0.01$; Figure 4C,D).

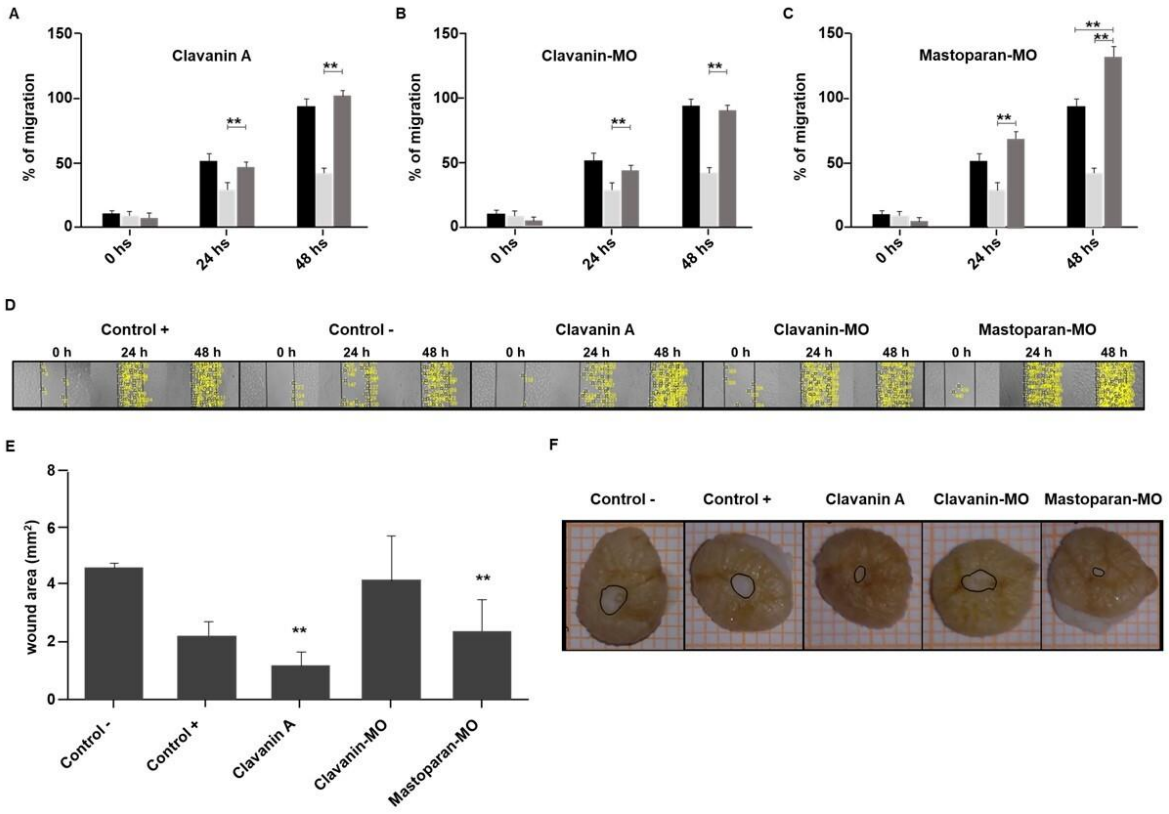

Figure 4. Analysis of fibroblast migration and wound healing in an ex vivo skin model. Human primary dermal fibroblasts (hFibs) were incubated with (**A**) Clavanin A at 200 μM, (**B**) Clavanin-MO at 200 μM, or (**C**) Mastoparan-MO at 6.25 μM, and the cell migration percentage was determined by image analysis. As controls, basal medium supplemented with FBS (positive control, black bars), and basal medium without FSB (negative control, light gray bars). In (**A**–**C**), the dark gray bars correspond to the cells exposed to each of the corresponding peptides. The mean ± SD of the percentage of cells that migrated in three independent experiments at 0, 24, and 48 h is presented. The statistical analysis was carried out by means of one-way ANOVA and Tukey post hoc tests. Significant differences regarding the control are marked with asterisks (** $p < 0.01$). In (**D**), it is possible to observe representative images of hFib migration at 24 and 48 h of peptide treatment (Clavanin A and Clavanin-MO at 200 μM, Mastoparan-MO at 6.25 μM), yellow dots represent the cells that invaded the scratched area. Wound healing assay in human skin explants exposed to: Clavanin A (200 μM), Clavanin-MO (200 μM), or Mastoparan-MO (6.25 μM) (**E**,**F**). Quantification of the area of re-epithelialization and lesion closure in the skin explants at 7 days of treatment was quantified using the ImageJ software (version 1.53 i). The analysis of the mean ± SD of the wound area of three independent experiments is represented (**E**). The statistical analysis was carried out through one-way ANOVA and Tukey post hoc tests. Significant differences regarding the control are marked with asterisks (** $p < 0.01$). In (**F**), it is possible to observe representative images of the closure of the wounds in the human skin explants at 7 days of treatment.

2.5. Wound Healing in hOSEC

The effect of the three AMPs on the wound healing process was analyzed using an ex vivo organotypic model of human skin (hOSEC) (Figure 4E,F). The results indicate that treatment with Clavanin A and Mastoparan-MO at 200 and 6.25 μM, respectively, could induce a significant reduction in the lesion area relative to the negative control of minimal regeneration, treated with the medium supplemented only with FBS (Figure 4E,F) ($p < 0.01$). In contrast, skin explants treated with Clavanin-MO at 200 μM did not show a significant reduction in the lesion area compared to either of the two controls in this experiment.

3. Discussion

Growing evidence indicates that several AMPs can generate an immunomodulatory effect that promotes cell attachment, proliferation, and infiltration, thereby facilitating tissue regeneration and wound healing. This suggests that these types of antimicrobial molecules may have the potential to develop proregenerative treatments [12–14]. In this work, using both a culture system of primary human fibroblasts (hFibs) and an ex vivo organotypic model of human skin (hOSEC), we analyzed the proregenerative potential of three antimicrobial peptides (Clavanin A, Clavanin-MO, and Mastoparan-MO).

Although Clavanin A and Clavanin-MO have been previously reported to exhibit moderate cytotoxicity against monocyte/macrophage-like cells (EC50 < 50 μM) [15], our results indicate that in hFibs, the treatment with these two calvanins did not generate any significant cytotoxic effect, even at the 200 μM dose. Likewise, even though in RAW 264.7 cells, Mastoparan-MO does not present cytotoxicity at doses of up to 200 μM [10], the absence of cytotoxicity in hFibs could only be observed when the cells were treated with this peptide at concentrations below 25 μM, suggesting that cytotoxicity may depend on the cell type tested [16].

Other peptides that present immunomodulatory properties, such as human beta-defensins 2 and 3 (hBD-2 and hBD-3), can stimulate skin cell proliferation, possibly through the expression of fibroblast growth factor receptor 1 (*FGFR1*), and the enhancement in the phosphorylation of FGFR1, JAK2, and STAT3, which are proregenerative factors that promote wound healing, angiogenesis, and fibroblast activation [12,17–19]. Our results indicate that the three AMPs (Clavanin A, Clavanin-MO, and Mastoparan-MO) can induce an increase in the cell proliferation rate of hFibs, which indicates that these peptides, especially Mastoparan-MO, may have properties that facilitate tissue repair through the induction of key genes for cell proliferation such as *FGF2* and *KI67* [20,21].

In this sense, it has also been observed that some antimicrobial peptides, such as the synthetic A-hBD-2 or the LL-37 peptide (the only member of the human cathelicidin family), have the ability to facilitate the wound healing process by stimulating cell migration in both skin cells and mesenchymal stem cells [14,22]. Likewise, Synoeca-MP, an AMP with a broad antimicrobial spectrum, has also been shown to be useful in skin repair when combined with host-defense peptides IDR-1018 [23].

Although the three antimicrobial peptides tested in this study have the potential to induce an increase in cell migration, treatment with Mastoparan-MO can induce a greater increase in the cell migration rate of hFibs. Likewise, upregulation of the *CXCR4* chemokine receptor suggests that the peptides tested can accelerate the migration of epidermal cells through signaling mediated by *CXCL12*, a signaling pathway that has been implicated in the process of wound repair and regeneration [24–26].

Other bioactive peptides, such as *Crotalus adamanteus* toxin-II (CaTx-II) and the PR-39 peptide, can induce cell proliferation, which is accompanied by the synthesis of key constituents of the extracellular matrix such as collagen and proteoglycans [27,28]. In this study, we showed that the three peptides tested during this study can generate upregulation of *HAS2*, while Clavanin-MO and Mastoparan-MO induce an increase in the expression of the *ELN* gene, suggesting that these AMPs can facilitate the remodeling of the extracellular matrix (ECM), which is a crucial step in regeneration and wound repair [29,30].

The hOSEC model stands out as an ex vivo system that closely mimics the physiological conditions of human skin. Currently, this alternative model for analyzing the safety and efficacy of various compounds for wound treatment, avoiding the use of animals, is a valuable tool in the validation of compounds for topical and transdermal applications [31]. Ex vivo models that use human skin explants from elective plastic surgery provide a valuable tool to analyze the effect of various molecules in the early stages of the healing process. These models consistently allow for the maintenance of the cell populations present in native skin for at least 7 days of culture. Furthermore, hOSEC models preserve the structure of the extracellular matrix, which contains collagen, elastin, glycosaminoglycans, and other molecules. This enables the results to be more easily extrapolated to the effects of substances on in vivo human skin [32]. hOSEC models have been used to show that specific collagen peptides of fish and porcine origin have the potential to increase collagen and glycosaminoglycan content in the skin, which favors tissue regeneration processes [33]. Similarly, several ex vivo skin models have been used to evaluate the antimicrobial activity and potential skin irritation effects of the AMPs PXL150 and DPK-060. In these models, these AMPs have shown the potential to reduce the colonization of *S. aureus*, highlighting the value of ex vivo models for assessing the effects of peptides on tissue regeneration and skin repair [34,35].

Here, we used lesioned hOSEC models to determine the wound healing potential of Clavanin A, Clavanin-MO, and Mastoparan-MO. Our results showed that Clavanin A and Mastoparan-MO have significantly accelerated wound closure, compared to the non-treated control. Such results are partly explained by the direct effects of both peptides on fibroblasts, which presented higher proliferation and migration upon Clavanin A and Mastoparan-MO treatments. The modulation of genes related to regenerative processes, such as *KI67*, *HAS2* and *CXCR4* might also be involved, since such genes were significantly increased by both peptides. For Clavanin A, the modulation of *CXCR7* and *BCL2* might complement the underlying mechanism of action, while the mechanism of action of Mastoparan-MO involves *FGF2* and *ELN* modulation. Despite promoting fibroblast migration and reducing fibroblast doubling time, Clavanin-MO only promoted a trend towards wound closure acceleration in lesioned hOSEC samples.

The proregenerative potential of various AMPs is associated with their ability to modulate the inflammation process, which is key in the restoration of normal tissue architecture during wound repair [36]. Peptides such as IDR-1018, which exert their action through regulation of the inflammatory response, can induce the repair of skin wounds [13,23,37]. It has been observed that both Clavanin A and Mastoparan-MO are capable of modulating innate immunity by stimulating leukocyte recruitment to the site of infection and production of immune mediators GM-CSF, IFN-γ, and MCP-1 while suppressing excessive inflammatory response by increasing the synthesis of anti-inflammatory cytokines such as IL-10 and repressing the levels of proinflammatory cytokines IL-6, IL-12, and TNF-α. This suggests that the ability of Clavanin A and Mastoparan-MO to modulate inflammation can be related to the proregenerative properties observed during the ex vivo assay [7–11,38].

It has been observed that combining antimicrobial peptides with immunomodulatory peptides can stimulate proregenerative processes in both monolayer models of human skin cells and 3D culture models of skin equivalents, through the modulation of the inflammatory process [23]. Additionally, it has been observed that the combination of various collagen peptides isolated from *Salmo salar* and *Tilapia nilotica* skin, which have an immunomodulatory effect on the innate immune response, has the potential to accelerate wound healing [39]. Clavanin A and Mastoparan-MO share similar properties of promoting fibroblast migration and proliferation, in addition to modulating common transcripts, such as *KI67* and *HAS2*. Nevertheless, the peptides also presented complementary properties, such as the modulation of *CXCR7* and *BCL2* by Clavanin A, and *FGF2* and *ELN* by Mastoparan-MO. It is possible that such peptides, when combined, could have a dual antibacterial, regenerative, and immunomodulatory effect, like that observed in analyses conducted with the combination of platelet-rich plasma with β-lactams, which simultane-

ously reduce MRSA infection in skin wounds while facilitating the regeneration process in the skin [40].

Another interesting perspective for Clavanin A and Mastoparan-MO peptides as potential molecules that promote tissue regeneration in skin wounds is their incorporation into nanomaterials. This can enhance their stability and activity by protecting them against degradation and controlling their release [41]. Nanoparticles or nanofiber membranes can also improve the solubility of the peptides and provide targeted delivery specificity. In this context, the use of biomaterials based on natural polymers such as collagen, chitosan, and hyaluronic acid offers controlled release aligned with the wound healing process. The effective development of new biomaterials containing these AMPs must consider solubility, stability, and controlled release, as well as the development of biodegradable formulations to promote immunomodulation, reduce toxicity, and improve re-epithelialization, which is crucial for optimal wound healing outcomes [41,42].

4. Materials and Methods

4.1. Peptide Obtention and Quantification

Synthetic peptides used in this work were provided by Peptide 2.0 Inc. (Chantilly, VA, USA). The molecular mass and purity of all peptides were analyzed by matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-ToF MS) on an AutoFlex Speed instrument (BrukerDaltonics, Billerica, MA, USA). Briefly, each peptide was diluted in deionized water, and 1 µL of each solution was mixed with 10 mg/mL α-cyano-4-hydroxycinnamicacid saturated matrix solution, prepared in H2O:ACN:TFA (50:50:0.3, *v*:*v*:*v*). Peptides were plated and dried on a MALDI target plate, and their monoisotopic masses were determined using the reflector mode with external calibration, using the Protein Calibration Standard II for mass spectrometry (Bruker Daltonics, Billerica, MA, USA).

4.2. Cell Culture

Human primary dermal fibroblasts (hFibs) were obtained from healthy donors, and provided by CellSeq Solutions (Belo Horizonte, MG, Brazil). The cells were cultured in a controlled environment (5% CO_2, 37 °C, and 95% humidity). The growth medium employed was DMEM (Gibco, USA), supplemented with 10% *v*/*v*. fetal bovine serum (FBS) (Gibco, Waltham, MA, USA) and 1% *v*/*v*. penicillin/streptomycin solution (1000 U/mL) (Invitrogen, Grand Island, NY, USA).

4.3. Peptide Treatments

Cytotoxicity was evaluated in hFibs that were treated with 200, 100, 50, and 25 µM Clavanin A or Clavanin-MO for 48 h. In the case of Mastoparan-MO treatment, the cells were treated at 100, 50, 25, 6.25, 3.12, and 1.56 µM. In all experiments, fresh aliquots of the peptides were prepared in deionized water immediately before use. As cytotoxicity controls, the cells were treated under the same conditions using the peptides Polybia-MPII (INWLKLGKMVIDAL-NH2) and EcDBS1R6 (PMKKLFKLLARIAVKIPVW) at concentrations of 100, 50, 25, and 12.5 µM. Regarding the assessment of cell proliferation, population doubling time, and cell motility, the experimental design includes a positive control group (DMEM plus 10% FBS) and control of the utilized solvent vehicle (basal media without FBS). The treatment with the peptides was carried out in the solvent vehicle. All assays were conducted independently in triplicate.

4.4. Cell Viability and Cell Proliferation

Cell viability and proliferation were assessed using an MTT assay kit (Sigma Chemicals Co., St. Louis, MO, USA). Briefly, 1×10^4 cells were seeded in 96-well plates and incubated for 24 h for cell attachment, presenting approximately 30% of cell confluence. This guaranteed that cells would not reach 100% confluency until 7 days, which was the longest period of observation. Cell viability was determined at 48 h of peptide treatment,

while cell proliferation was indirectly assessed at 1, 4, and 7 days of treatment. For this, 10 µL per well of MTT solution (5 mg/mL) was added to the cultures, and the plates were incubated for 4 h in the dark. Subsequently, DMSO (200 µL per well) was added and incubated for 30 min. A microplate reader (Bio-Tec PowerWave, Santa Clara, CA, USA) was used to determine the optical density at 595 nm. Reference wavelength was taken at 630 nm.

4.5. Proliferation Kinetics and Population Doubling Time

Monitoring of cell proliferation rate was performed in a 6-well plate with a density of 1×10^4 per well. The culture medium was changed every 2 days. The growth curve of cells was obtained by harvesting and counting the number of cells per well at 1, 4, and 7 days of treatment. The population doubling time (PDT) during the logarithmic growth phase was calculated according to Díez et al. (2015), using the following formula [43]:

$$\text{PDT} = \text{Culture Time (in hours)}/\text{Population doubling number (PDN)}$$

$$\text{PDN} = \text{Log N/No} \times 3.31$$

where N is the number of harvested cells at the end of the growth period, and No is the number of seeded cells.

4.6. Cell Migration Assay

The effect of peptides on the migratory capability of hFibs was determined by the wound scratch assay according to Liang et al. (2007) [44]. Briefly, cells were seeded in 6-well plates and cultured until confluence. Scratches were made with 200 µL tips, and cell debris was removed by PBS washing before peptide treatments. Wound closure was photo-documented at time 0 and every 24 h using a Zeiss Primo Vert microscope (Carl Zeiss, Heidelberg, Germany), until the scratch was visually closed by any experimental group. The cells that invaded the scratched area were counted using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). Positive migration control at 48 h was used for data normalization. Samples were analyzed as independent triplicates.

4.7. Gene Expression Analysis

Gene expression profiles of peptide-treated cells and controls were evaluated by qRT-PCR after 4 days of treatment. Target gene sequences were obtained from Genbank (https://www.ncbi.nlm.nih.gov/genbank/ (accessed on 15 January 2022)), and the primer sets and probes were designed following the standard criteria defined by the SYBRTM using Primer Express® Software v3.0.1 (Thermo Fisher Scientific, Waltham, MA, USA). Information about the primers used for qRT-PCR analysis is shown in Table S1. Genes assessed were *BCL2*, *CXCR4*, *CXCR7*, Elastin (*EL*), *βFGF*, *Ki67*, *MMP1*, and *VEGF*. The *GAPDH* gene was used as a control. Total RNA isolation was performed using TRIzolTM Reagent (Thermofisher, USA) following the manufacturer's instructions, and the amount and quality of RNA were determined using a NanoDrop 1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit (Thermofisher, USA). Amplification reactions were performed on StepOne Plus equipment (Applied Biosystems, Waltham, MA, USA) using standardized reagents for real-time PCR (SYBR™ Green Master Mix, Thermofisher, USA) added from primer sets specific to each gene, or using Taqman probes (TaqMan™ Universal PCR Master Mix, Thermofisher, USA). StepOne Software v2.3 was used to determine the Ct values, and the results were analyzed by the 2-ΔΔCT analysis method.

4.8. Ex vivo Organotypic Model of Human Skin (hOSEC) Culture and Wound Healing Assay

Skin explants were obtained during routine elective cosmetic surgery performed on healthy patients. Ethical approval of this research was granted by the University of Brasília Research Ethics Committee (protocol number 30175020.0.0000.5558). Tissue samples were

collected following informed consent. Explants were washed in phosphate buffer solution (PBS, Thermo Fisher Scientific, USA), supplemented with 1% v/v. of 10,000 U/mL of penicillin/streptomycin solution (Invitrogen, Grand Island, NY, USA), and the hypodermis was removed. Skin explants were cut with a 6 mm full-thickness punch biopsy, and kept in air–liquid interface at 37 °C, 5% CO_2, and 95% humidity in DMEM supplemented with 10% v/v. fetal bovine serum (FBS) (Gibco, USA), and 1% v/v. of 10,000 U/mL of penicillin/streptomycin solution. The wounds were then created within the punch biopsy (2 mm circular biopsy punch). The lesioned skin fragments were placed in metallic support systems and treated with basal medium supplemented with 10% FBS. Control samples were treated with either PBS (negative control), or fibrin gel (positive control for regeneration), prepared by mixing 2.5 µL of fibrinogen at 40 µg/mL, 7.5 µL of water, and 10 µL of thrombin at 25 U/mL. The experimental group samples were treated with the peptides dissolved in the tissue culture medium. Wound healing was observed for 7 days using a Zeiss Primo Vert microscope (Carl Zeiss, Heidelberg, Germany). The change in the lesion area was determined using the ImageJ software (National Institutes of Health, USA).

4.9. Statistical Analysis

The experiments were conducted across a minimum of three separate biological replicates and at least two technical replicates. Data analysis was performed with GraphPad Prism® Software, version 7.02 (San Diego, CA, USA, 2017). A p-value of less than 0.05 was considered significant.

5. Conclusions

In the present study, we analyzed the proregenerative potential of three antimicrobial peptides (Clavanin A, Clavanin-MO, and Mastoparan-MO). The three peptides analyzed showed relatively low toxicity for hFibs and demonstrated the ability to induce cell proliferation and migration in this kind of cell. Interestingly, the Mastoparan-MO peptide exhibited the greatest proregenerative potential among the three molecules analyzed, inducing cell proliferation and migration events that exceeded those observed in the positive control used in this study. Additionally, our molecular analysis revealed a proreparative gene expression profile induced by treatment with these peptides. The use of an hOSEC model of lesioned skin demonstrated that both Clavanin A and Mastoparan-MO could induce a significant reduction in the lesion area compared to the minimal regeneration control. Taken together, the obtained results suggest that the AMPs used in this study have the potential to facilitate skin regeneration. Thanks to their antibacterial and immunomodulatory properties, these molecules could serve as the basis for developing new pharmacological approaches for treating difficult-to-manage wounds.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms25136851/s1, Figure S1: Cytotoxicity controls.; Table S1: Primer sequences used for gene expression analyses by quantitative real-time PCR.

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Institutional Review Board Statement: The study was conducted following the Declaration of Helsinki, and ethical approval of this research was granted by the University of Brasília Research Ethics Committee (protocol number 30175020.0.0000.5558).

Informed Consent Statement: To develop the ex vivo Organotypic Model of Human Skin (hOSEC) experiments, informed consent was obtained from all donors involved in the study.

Data Availability Statement: The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Conflicts of Interest: The authors declare no conflicts of interest.

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Capping motifs in antimicrobial peptides and their relevance for improved biological activities

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N-capping (N-cap) and C-capping (C-cap) in biologically active peptides, including specific amino acids or unconventional group motifs, have been shown to modulate activity against pharmacological targets by interfering with the peptide's secondary structure, thus generating unusual scaffolds. The insertion of capping motifs in linear peptides has been shown to prevent peptide degradation by reducing its susceptibility to proteolytic cleavage, and the replacement of some functional groups by unusual groups in N- or C-capping regions in linear peptides has led to optimized peptide variants with improved secondary structure and enhanced activity. Furthermore, some essential amino acid residues that, when placed in antimicrobial peptide (AMP) capping regions, are capable of complexing metals such as Cu^{2+}, Ni^{2+}, and Zn^{2+}, give rise to the family known as metallo-AMPs, which are capable of boosting antimicrobial efficacy, as well as other activities. Therefore, this review presents and discusses the different strategies for creating N- and C-cap motifs in AMPs, aiming at fine-tuning this class of antimicrobials.

KEYWORDS

capping motif, antimicrobial peptides, metallo-AMPs, amino acid motifs, secondary structure

1 Introduction

The affinity of antimicrobial peptides (AMPs) to the microorganism's membrane have been associated with the net charge and amphiphilicity (Tossi et al., 2000). In most cases, short AMPs sequences with cationic and hydrophobic side chains undergo a coil-to-helix transition from aqueous environments to membrane-like condition (*e.g.*, bilayer phospholipids) (Li et al., 2022). These cationic α-helix AMPs usually as stable or unstable pore formers. The unstable pores can generate a reorganization of the membrane surface and consequently reorient proteins and receptors, resulting in a change in the transmembrane potential, causing an imbalance and cell death (Łoboda et al., 2018; Donaghy et al., 2023). However, just as helices can be formed and incorporated within the membrane to form pores, so can several different types of amphipathic structures be formed, not necessarily imposed by a strict primary or secondary structural organization

(Tossi et al., 2000). As well as helical amphipathic, resulting in part from punctual interactions of the residues within a given sequence, other environmental factors such as pH conditions, ionic strength, and the presence of divalent cations are responsible for AMP activity effectiveness (Donaghy et al., 2023).

In addition to their membranolytic activity on microbes, α-helix peptides can cross the plasma membrane, through the spontaneous translocation mechanism. They engage with intracellular targets, including nucleic acids such as DNA and RNA, preventing protein synthesis, and bind to other molecules, such as enzymes and proteases, affecting important cellular functions (Łoboda et al., 2018). Simultaneously, they exhibit minimal side effects in mammalian cells (Alexander et al., 2017; Li et al., 2022). Implementing strategies to ensure that AMPs establish α-helices to improve their interaction with biological targets, particularly microbes, is crucial in the development of active peptides. The stability of the secondary α-helix is maintained by intramolecular hydrogen bonds formed between the amide hydrogen and carbonyl oxygen atoms in the peptide backbone at positions *i* and *i+4* (Kanbayashi et al., 2022). The emergence of helicity stems from the amino acid residues' primary sequence and their interaction with the surrounding environment (dos Santos Cabrera et al., 2019). The capping motifs, which have a propensity to generate α-helices in a peptide backbone, are well-documented features present in the secondary structures of proteins and peptides (Kier et al., 2010). These strategies have been a starting point and are used to improve AMPs' biological activities.

Capping motifs are extremely important for the stabilization of the structure and, consequently, for the activity of AMPs (Park et al., 2004). Specifically, peptides that form α-helices, N-cap, and C-cap motifs, formed by a set of amino acids in sequence located in the terminal parts of the secondary structure, influence the stability of the helical structure. This stabilization results from additional and unconventional hydrogen bonds, as they present dihedral angles different from those found inside the helix (De Rosa et al., 2022). In other words, the capping phenomenon occurs through the contributions of hydrogen donor and acceptor groups, accompanied by hydrophobic interactions at the ends of the helix, as well as polar residues in the helix (Aurora and Rose, 1998). The participating amino acids experience a loss of free energy, favoring the folding and stability of the helical structure (Serrano and Fersht, 1989).

For example, the presence of a Lys residue at the *C*-terminus is responsible for stabilizing helix formation in polyalanine peptides with seven or more residues (Zabuga and Rizzo, 2015). N-capping motifs have been shown to promote amphipathic helical peptides' interaction with hydrophobic surfaces, dramatically altering the hydrophobicity characteristics of individual amino acid residues (Spicer et al., 2014). Recently, an unusual N-capping motif was identified and formed by an asparagine-lysine-proline (Asn-Lys-Pro) motif. This motif is present in the PaDBS1R7 peptide and has a role in the hybrid structural formation (coil/N-cap/α-helix), contributing to a diverse range of biological activities (Cardoso et al., 2022).

Therefore, in the following topics, we will address the importance of the capping motifs for the stabilization of the secondary structures of AMPs, as well as how it can influence the activity of these molecules. Furthermore, we will discuss motifs composed of amino acid sequences and explore unusual modifications on terminal regions that facilitate helical structuring and enhance activity. Additionally, the capping effects due to unusual groups that initiate the nucleation of peptides as helices will be explored. We will also cover coordination interactions between AMPs and metals, resulting in macrostructures that enhance peptide activity. Employing these motifs is a promising approach for the development of more effective AMPs.

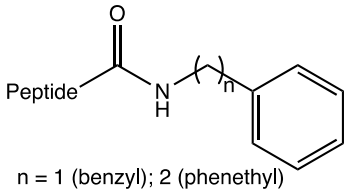
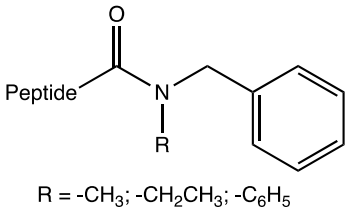
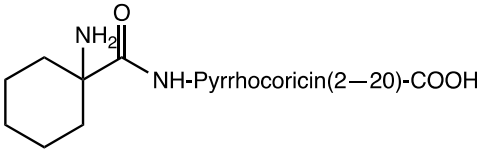
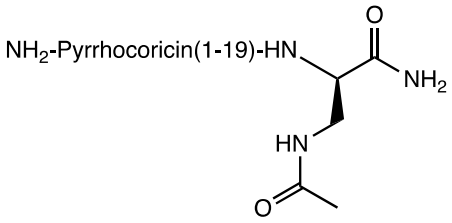
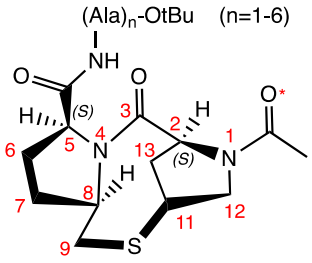
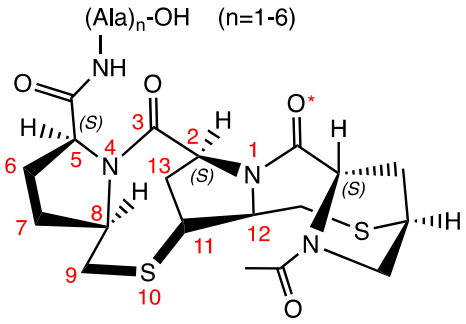
2 Improving the structure of AMPs to enhance their activity

In spite of recent progress, the translational clinical development of AMPs faces challenges, leading to delays in current design strategies (Jiang et al., 2021). A key strategy involves introducing capping motifs, taking advantage of changes in structural features within peptides to promote nucleation and stabilization of α-helix structures. This approach is crucial because helices play a pivotal role as secondary structures, influencing the activity of AMPs through molecular recognition.

The importance of helical structure allows AMPs to be continuously exploited for potential individual use or in combination with established antibiotics, especially in the new era of treating multidrug resistant bacteria affecting both human and animal health (Lei et al., 2019; Fu et al., 2023). Highlighting their advantages, AMPs offer a slower emergence of resistance, rapid lethal action, and effective control of biofilms, positioning them as optimal candidates for treatment of drug-resistant pathogens (Magana et al., 2020). Furthermore, these peptides have shown immunomodulatory effects, either by diminishing the inflammatory response triggered by endotoxins, inducing the synthesis of pro-inflammatory factors, or eliciting the secretion of cytokines (Lei et al., 2019).

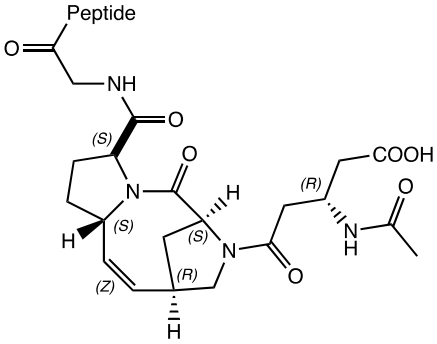
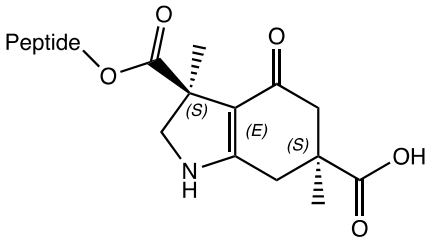
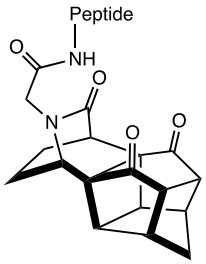
Different chemical modifications, including specific substitutions and/or residue additions to the primary AMP sequence can be used, along with computational approaches to analyze physicochemical and structural properties from the combinatorial library, thus providing analogs with improved activity (Ong et al., 2014; de Oliveira et al., 2023). Among the possible strategies, we can cite the substitution of specific residues, total or specific change of the stereochemistry of amino acids, as well as *N*- and *C*-terminal modification, cyclization, and stapling (de Oliveira et al., 2023). Other classic ways to improve the activity and performance of AMPs are the insertion of unusual amino acids, tricyclic groups, and modifications on the scaffold to generate peptidomimetics (Petri et al., 2022). Hybrid computational methods, such as the combination of different experimental data with molecular dynamics simulations (Mondal et al., 2023), can be employed to predict information to improve secondary structures in peptides. Additionally, artificial intelligence algorithms, such as machine and deep learning (Jiang et al., 2023; Yue et al., 2024), as well as geometric deep learning (Fernandes et al., 2023), contribute to this advancement. This approach has recently

TABLE 1 Structure, capping type and interaction of some unusual groups described in the literature for AMP optimization.

Template/ group/name	Structures	Capping type	Interactions Cap/ Function	Helix type	References
N-monosubstitutions into *C*-terminal amidations		C-cap	Resistance to proteases	-	Svenson et al. (2008)
N,N-disubstitutions into *C*-terminal amidations		C-cap	Resistance to proteases	-	Svenson et al. (2008)
Chem		N-cap	Resistance to proteases, as well as induction of helix formation by chair conformation due to the cyclohexane structure	hybrid 3_{10}-α helices	Pasupuleti et al. (2009)
Dap (Ac)		C-cap	Resistance to proteases	-	Pasupuleti et al. (2009)
Two prolines linked by a thiomethylene unit (-CH_2-S-)		N-cap	H-Bond between NH(Ala-1) and C=O* (acetamide) (*i*, *i*+3); C=O in the C-3 and NH(Ala-2) (*i*, *i*+2) and NH(Ala-3) (*i*, *i*+3). Observed by NOE and ROESY experiments	hybrid 3_{10}-α helices	Kemp et al. (1991)
Three prolines linked by two thiomethylene units (-CH_2-S-)		N-cap	H-Bonds between: NH(Ala-1) and C=O* (Pro) (*i*, *i*+3); Oxygen in the C-3 and NH(Ala-2) (*i*, *i*+2) and NH(Ala-3) (*i*, *i*+3). Observed by NOE and ROESY experiments	hybrid 3_{10}-α helices	Kemp and Rothman (1995)

(Continued on following page)

TABLE 1 (*Continued*) Structure, capping type and interaction of some unusual groups described in the literature for AMP optimization.

Template/ group/name	Structures	Capping type	Interactions Cap/ Function	Helix type	References
ProM-5		N-cap	H-Bonds between: NH (Gly) and carboxylate; C=O of *N*-acetyl-β-homo-Asp and NH(amino acid-1) (*i, i+4*); C=O in the first proline and NH(amino acid-2) (*i, i+3*). Observed by CD and 2D NMR experiments	α-helix	Hack et al. (2013)
S,S-9-O		N-cap	Induction to α-helical conformation (between 50% and 75% helicity). Observed by CD	α-helix	Austin et al. (1997)
Complex bicyclic		N-cap	H-Bond between NH (first amino acid) and the last of the carbonyls	α-helix	Mahon and Arora (2012)

Dap (Ac): N-acetyl-2, 3-diamino propionic acid; Chem: 1-amino-cyclohexane-carboxylic acid.

become a valuable tool to understand the structure-activity relationship of AMPs and to design the next-generation of peptides with improved properties.

With these strategies, it is possible to solve problems such as susceptibility to proteases, but also in specific cases, three-dimensional structuring can be favored, such as α-helix, seeking to improve molecular interactions of AMPs with biological targets such as proteins, receptors, nucleic acids, and other biomolecules of interest related to infectious diseases caused by microbes.

3 Capping motif effects due to unusual groups for AMP optimization

The insertion of specific amino acid motifs into N-cap or C-cap regions has been observed to induce the formation of AMP helices, consequently enhancing their activity against molecular targets. This phenomenon is attributed to the intramolecular interactions among the residues within the peptide backbone (Aurora and Rose, 1998), which provide the necessary balance of rigidity and flexibility to facilitate the structural conformation of a helix (Babii et al., 2017). Natural amino acids play a significant role in enhancing the performance of AMPs, as the side chains within peptides and proteins contribute to molecular recognition through steric and electronic effects (King et al., 2021), but other modifications involving unusual groups can also contribute to their performance.

The capping motif phenomenon in linear peptides also encompasses terminal modifications (Table 1). These modifications can prevent chain degradation by reducing the vulnerability of peptide bonds to proteolytic cleavage (Behrouz et al., 2023; Ding et al., 2023). Furthermore, specific modifications at the ends of sequences employ unconventional conformational motifs, forming intramolecular H-bond pairs to enhance both the structural characteristics and activity of AMPs (Aurora and Rose, 1998).

Among the most commonly employed modifications are *N*-terminal acetylation and *C*-terminal amidation strategies, aimed at enhancing the conformational stability and availability of active AMPs, while maintaining or enhancing their antimicrobial potency (Mura et al., 2016; Mwangi et al., 2023). These are crucial, especially considering the susceptibility of AMPs to degradation by exopeptidases (Magana et al., 2020). For instance, the *N*-terminal acetylation can facilitate helix nucleation through interactions with the side chain or backbone hydrogen donor of Arg in Ac-Leu-Leu-

Arg motifs (Chang et al., 2000); as well as the *N*-terminal amidation in modelin-5 (Lys-Leu-Ala-Lys-Lys-Leu-Ala-Lys-Leu-Ala-Lys-Leu-Ala-Lys-Ala-Leu), contributes to stabilizing helix formation, leading to enhanced levels of amphiphilic helix at a lipid interface, and increasing the efficacy approximately 10-fold in tests against *Enterococcus coli* when compared to peptide not amidated (Dennison and Phoenix, 2011).

In short peptides (of the type Arg-X-Arg-Y, where X is the 4-phenylphenylalanine residue), stability and resistance against trypsin increase when *N*-monosubstitutions (*N*-benzyl and *N*-phenethyl) and *N,N*-disubstitutions (*N*-methyl-N-benzyl; *N*-ethyl-*N*-benzyl, and *N,N*-dibenzyl) are introduced into *C*-terminal amidations (in Y). It should be noted that monosubstituted derivatives with *N*-phenethylamide groups presented good stabilities in various peptides that have X with different aromatic residues (Svenson et al., 2008).

Pyrrocoricin is an AMP from insects that excels against gram-negative bacteria. The peptide has the sequence Val-Asp-Lys-Gly-Ser-Tyr-Leu-Pro-Arg-Pro-Thr(X)-Pro-Pro-Arg-Pro-Ile-Tyr-Asn-Arg-Asn, where X is the disaccharide radical 2-(acetylamino)-2-deoxy-3-O-β-D-galactopyranosyl-α-*D*-galactopyranosyl anchored to the oxygen of Thr-11. Pyrrocoricin analogues were synthesized by Otvos et al. (2000) using the linear sequence without the disaccharide moiety and making changes to the *N*- and *C*-termini to improve its resistance to proteases. Additional amino acids were added at the *N*-terminus, along with acetylation (Ac-Lys-, Ac-Lys-Val-Asp-Lys-, Ac-Arg-), as well as the addition of the Chem group (1-amino-cyclohexane-carboxylic acid). At the *C*-terminus, the Dap (Ac) group (*N*-acetyl-2,3-diamino propionic acid) was added. The results indicate that modification at either end of the termini resulted in a decrease in the antibacterial efficacy of the parent pyrrocoricin. Among the peptides modified at the *N*- or *C*-termini, those with the Chem group at the *N*-terminal and the Dap (Ac) group at the *C*-terminal appear to retain some of the antibacterial activity of the parental pyrrocoricin. An analogue with both modifications showed high potency against bacteria and a lack of toxicity *in vivo*. The effects of protecting groups at the *N*- and *C*-termini play a crucial role in the stability of the peptide in the presence of proteases (Otvos et al., 2000). Peptides containing unusual *N*-terminal amino acids, such as 1-aminocyclopentane-1-carboxylic acid (Acc5) and 1-aminocyclohexane-1-carboxylic acid (Chem group), necessarily adopt folded structural conformations, in the 3_{10}/α-helical regions of the conformational space (Santini et al., 1988; Valle et al., 1988). In the case of the Chem residue, the cyclohexane structure provides a perfect chair conformation, with the amino group in the peptidic bond located axially and the carboxylate group in the equatorial position. This arrangement favors interactions at the beginning of the *N*-terminus and promotes a helical structure in the backbone (Paul et al., 1986; Valle et al., 1988).

To enhance their resistance to high salt concentrations, Chu et al. (2013) incorporated 1 to 3 repeats of β-naphthylalanine (NaI) at the *C*-terminus of the Trp-rich synthetic S1 AMP (Ac-Lys-Lys-Trp-Arg-Lys-Trp-Leu-Ala-Lys-Lys-NH_2). They observed that all three peptides (S1-NaI, S1-NaI-NaI, and S1-NaI-NaI-NaI) were more potent than the unmodified peptide, and the ones with two and three NaI residues still retained their antibacterial activities even with the addition of 300 mM NaCl. Moreover, the peptide with three NaI residues maintained almost 100% integrity after 8 h in bovine calf serum (Chu et al., 2013). Previously, the insertion of motifs containing 2 and 5 Trp into the *C*-terminus of kininogen-derived AMPs, including Lys-Asn-Lys-Gly-Lys-Lys-Asn-Gly-Lys-His (KNK10), Gly-Lys-His-Lys-Asn-Lys-Gly-Lys-Lys-Asn-Gly-Lys-His-Asn-Gly-Trp-Lys (GKH17), and His-Lys-His-Gly-His-Gly-His-Gly-Lys-His-Lys-Asn-Lys-Gly-Lys-Lys-Asn (HKH17), resulted in an enhanced antimicrobial effect against microbes (*S. aureus*, *E. coli*, and *C. albicans*). Additionally, the modified peptides exhibited robust stability against proteolytic degradation by staphylococcal aureolysin V8 proteinase and human leukocyte elastase (Pasupuleti et al., 2009; Schmidtchen et al., 2009). Hydrophobic residues, such as Trp, Phe or β-naphthylalanine, are compelling choices for the terminal positions in AMPs due to their bulky, aromatic, and polarizable nature. These residues interact with the phospholipid membrane, serving as anchors for the peptide. When incorporated into highly cationic AMP sequences, this anchoring effect promotes the formation of membrane defects and facilitates rupture (Pasupuleti et al., 2009; Chu et al., 2013).

Although some unusual groups located at the peptide's termini can prevent this degradation, their insertion can also favor helicity, as is the case of cyclic proline mimetic motifs, which can favor the nucleation of the α-helix when inserted in the *N*-terminal region. As examples, there are templates with two and three prolines, each linked to the other by a thiomethylene unit (-CH_2-S-) in the *N*-terminus of polyalanine peptides. Both bicyclic templates serve as powerful motifs for initiating helix formation. These motifs form an H-bond (*i, i+3*) between the NH(Ala-1) group and the carbonyl group of acetamide (for the template with two prolines) and the Pro (in the template with three prolines); as well as two additional H-bonds formed between the C=O group of the first Pro and the NH groups of Ala-2 (*i, i+2*) and Ala-3 (*i, i+3*) (Kemp et al., 1991; Kemp and Rothman, 1995).

A synthetic tricyclic motif, denominated ProM-5 was synthesized through the stereoselective introduction of a vinylidene bridge into a diproline unit. ProM-5 acts as a powerful structure for the nucleation of α-helix formation in a linear polypeptide chain when incorporated as an N-cap. The vinylidene bridge restricts the flexibility of the 8-membered ring to adopt a favorable conformation by the addition of an *N*-acetyl-β-homo-Asp residue located in the *N*-terminus in the last proline. This conformation allows and induces the formation of intermolecular interactions throughout the peptide chain (H-bonds: NH (Gly) and carboxylate; C=O of *N*-acetyl-β-homo-Asp and NH (amino acid-1) (*i, i+4*); C=O in the first proline and NH (amino acid-2) (*i, i+3*)) (Hack et al., 2013).

Another case involves using semi-rigid structures such as hexahydroindol-4-one (3*S*,6*S*)-diacid (S,S-9-O), which exhibits an unusually induced (between 50% and 70%) α-helix formation, as observed by circular dichroism (CD) when the ester-linked attached peptide chain is present. By contrast, the helix did not form when the peptide chain is coupled through an amide linker, since a 3_{10}-type hydrogen-bonding pattern (*i, i+3*) is expected in the structure in this condition (Austin et al., 1997).

Alternative approaches involve template motifs designed to elongate and replicate spacing by incorporating groups of atoms containing carbonyls, thus resembling the arrangement found in other amino acid sequences. For instance, we can cite only one enantiomer of a complex bicyclic structure, with three carbonyl groups, which presents an increase in the peptides' helicity anchored at the *N*-terminus (Mahon and Arora, 2012).

Other strategies are post-translational modifications, for example, *N*-methylation and *N*-alkylation (Petri et al., 2022), sulfonation, and the addition of phosphate groups, which can be carried out using known amino acid residues to gain chemical diversity, but also to improve activity or availability (King et al., 2021). The bioconjugation strategy is employed making use of natural amino acids with nucleophilic chains such as lysine and cysteine (Hoyt et al., 2019), but also non-canonical amino acids with bioorthogonal groups, such as azides, ketones, and alkynes, which allow subsequent chemical modification (Lang and Chin, 2014).

Stapling has been designed to establish a connection between side chains of natural or non-natural amino acids, forming at a certain space a cyclic structure that stabilizes the molecule, preserving a certain conformation and structure, while limiting its flexibility (Chu et al., 2015; Fairlie and Dantas de Araujo, 2016). This strategy has used modified amino acids containing side chains with terminal vinyl groups that allow, through Ru catalysts, a cyclic structure containing the new double bond, a bridge produced by olefin metathesis reactions. These cross-links can be added at positions *i, i+3*, and *i, i+4* to obtain one helical turn, but also between positions *i, i+7*, bridging two helical turns (Chu et al., 2015; Cromm et al., 2015). To favor helical structures using olefins side chains, bridges are installed at positions *i, i+3* (with six or eight carbon atoms connector); and another at position *i, i+7*, involving a cross-link with 11 carbon atoms. In order to achieve this, it is necessary that the building blocks have an *R* configuration at position *i* and an *S* configuration at position $i + n$. For the *i, i+4* bridge, the most used architecture involves an eight-carbon cross-link derived from two *S*-configured building blocks (Chu et al., 2015). The bioconjugation of cysteines into peptides has also been used for the optimization of them as well as proteins. The sulfhydryl groups from cysteines and an appropriate bifunctional linker allow the cross-link, carried out in solution with unprotected peptides, through two steps. The first step involves one cysteine reacting with the linker to form a linear mono thioether intermediate, followed by an intramolecular ring closure involving the second cysteine and the linker to give the stapling. The advantage of using cysteines for stapling, in relation to amino acids with vinyl groups as radicals, is their easy incorporation into heterologously produced peptide sequences (Fairlie and Dantas de Araujo, 2016). While this strategy is commonly used to establish peptide sequences as helices for enhancing protein-protein interactions (Timmerman et al., 2005; Verdine and Hilinski, 2012; Cromm et al., 2015), stapling insertion can also serve as initiators and stabilizers, creating capping effects, when positioned at the beginning and end of a given peptide sequence. Several studies describing the introduction of staple have found an improvement in some properties, such as providing high levels of helical, strong protection from proteolytic degradation (Shi et al., 2013), improves the bioavailability (Bird et al., 2010), robust cell-penetration and increase in target affinity (Verdine and Hilinski, 2012; Findeisen et al., 2017; Verhoork et al., 2019).

As well as in defensins and other cysteine-rich peptides, which have motifs such as α-helices and antiparallel β-sheets typically stabilized by disulfide bonds (Cociancich et al., 1993; Ehret-Sabatier et al., 1996; Zhu and Gao, 2013), it is possible to generate cyclic and synthetic peptidomimetics, with cysteines and form disulfide bonds to link the sequence through sites, either head-to-tail or head-to-center, favoring or not a specific structure. Cyclization generally helps stabilize the secondary structure and preserve a specific bioactive conformation due to confinement within a rigid structure. However, in certain cases such as some grafted peptides, this constraint may lead to a reduction in antimicrobial activity as it hinders their ability to interact and insert into pathogen membranes or intracellular targets (Rezende et al., 2021). Therefore, the design of this type of peptide can include some intrinsic variables found in the sequences of AMPs with disulfide bonds. The function of such bridges is to maintain different stable motifs within the tertiary structures, since it is believed that these conformations are important to perform a certain recognition function in specific receptors, playing a beneficial role for the organism that produces them (Hogg, 2009).

The exchange of some functional groups for bioisosteres in N- or C-capping regions in linear peptides also leads to improved molecules that can both improve physicochemical characteristics and enhance activity (Ding et al., 2023; Zhan et al., 2023). Furthermore, non-peptide fractions coupled to N-cap tripeptides are active against viral serine proteases, which result in excellent inhibitors of the aforementioned enzyme (Nitsche et al., 2012; 2013).

4 Modulation of α-helix structures by amino acid motif interactions into *N*- and *C*-terminus

According to data in the Protein Data Bank (PDB), α-helices constitute 57% of experimentally identified proteins, as reported on the RCSB PDB website (https://www.rcsb.org/). These helices are a predominant secondary structure commonly found in globular proteins (Wang et al., 2022), and play a pivotal role in molecular recognition (Bajpayee et al., 2023). In many proteins, the α-helix motif serves as a recognition domain by directly binding to other macromolecules (Guharoy and Chakrabarti, 2007). Using these helical structures as a basis for optimizing AMPs could serve as an additional feature, because this approach might potentially broaden its impact by influencing intracellular receptors, thereby improving its efficacy. This is particularly significant since numerous AMPs appear to operate through interaction with microbial membranes, and not through protein-like receptors.

The motivation to comprehend the process of α-helix formation stems from the aspiration to design and develop stable and uniquely folded α-helices, demonstrating novel biological functions and/or therapeutic applications (Acharyya et al., 2019). In a short peptide

sequence, nucleation is a higher energy step and involves the organization of the initial three amino acids into a helical turn, facilitated by specific stabilizing interactions (Mahon and Arora, 2012).

Other non-covalent interactions such as metal-ligand, designed host-guest interactions, salt bridges, cation-π interactions, and π-π stacking are important to the α-helix stabilization, which can be achieved due to the presence of appropriately spaced residues in the peptide chain (Yin, 2012). The spatial distribution of amino acid residues along the folded peptide can form discrete portions, which have hydrophobic or hydrophilic properties (Kabelka and Vácha, 2021). In addition to the fact that helical structures are often established in solution for many peptides, a significant portion of them present the α-helical structure and are enhanced in contact with target membranes (Koehbach and Craik, 2019).

A study investigating the influence of salt bridges on helix formation found that the polyalanine peptide AEP ($(Ala)_9$-Arg-$(Ala)_3$-Glu-$(Ala)_4$-Arg-$(Ala)_2$) stabilizes its α-helix structure through a salt bridge between the side chains of residues Arg-10 and Glu-14 at *i* and *i+4* positions, respectively (Hong et al., 2011). However, the replacement of Glu-14 with Arg in AP ($(Ala)_8$-Arg-$(Ala)_4$-Arg-$(Ala)_4$-Arg-$(Ala)_2$) did not increase the stability of the α-helix, as it remained similar to the α-helix of the original AEP, but with some different contributions (Hong et al., 2011). The difference between the helices shows that in the α-helix length distribution AEP is exposed to more numerous but shorter length α-helix segments, which means that AEP has an increased concentration of α-helix-turn-α-helix conformations (Hong et al., 2011).

In addition, certain motifs are crucial for AMPs' secondary structure formation, such as the nucleolin Thr-Pro-Ala-Lys-Lys motif, in the peptides TP1 (Ac-Gly-Ala-Thr-Pro-Ala-Lys-Lys-Ala-Ala-Gly-NH_2) and TP2 (Ac-Gly-Ala-Thr-Pro-Ala-Lys-Lys-Ala-Ala-Ala-Thr-Pro-Ala-Lys-Lys-Ala-Ala-Gly-NH_2). At high pH and in the presence of trifluoroethanol, both peptides adopt a helical structure, likely stabilized via N-capping, with the threonine and proline sequence initiating short helical segments in the motif. Analysis of the nuclear Overhauser effect (NOE) spectra indicates that the helix starts from Pro. However, Thr interacts with the side chain of the first Lys through two NOE interactions at (*i, i+2*): NH(Thr) to H-β (Lys), and NH(Thr) to NH(Lys). Consequently, the rest of the structure is stabilized by the uncharged side chain of Lys (Xu et al., 1995).

Recently, we reported the effect of the Asn-Lys-Pro motif as an N-cap in the peptide PaDBS1R7 (Pro-Met-Ala-Arg-Asn-Lys-Pro-Lys-Ile-Leu-Lys-Arg-Ile-Leu-Ala-Lys-Ile-Phe-Lys). It was observed from nuclear magnetic resonance (NMR) data that Asn-5 has a crucial role in stabilizing the α-helix, as a coil/N-cap/α-helix structural scaffold, through hydrogen bonds formed between the side chain of Asn and amino acid in the main chain (Lys6, Pro7, Ile9 and Leu10). The N-cap effect is mainly driven through Asn-5(NH in the side chain)/Lys-6(NH), (*i, i+1*), and Asn-5(NH in the side chain)/Leu-10(NH), (*i, i+5*). This peptide eradicated *Pseudomonas aeruginosa* biofilms as well as showing decreased bacterial counts by 100–1000-fold *in vivo* using a skin abscess mouse model (Cardoso et al., 2022).

Examination of both the ^3JNHαH coupling constant and the NOE experiments demonstrated that the X-Leu-Leu-Arg-Ala motif, originating from the *N*-terminal segment of the leucine zipper (LZ)-like domain of the HIV envelope gp41 glycoprotein—where X denotes a group or amino acid residue capable of forming in van der Waals interactions, hydrophobic interactions and/or hydrogen bond with an arginine—functions as the nucleating core for the helix in four synthetic decapeptides (Chang et al., 2000). Understanding the role of amino acid residues in the *N*-terminal region is important for the development of α-helical peptides. Asparagine is considered the best residue for the *N*-terminal region to stabilize the helix, while glycine is good, and glutamine is the worst residue for this position (Chakrabartty et al., 1993). In fact, researchers suggest using helicogenic residues to stabilize helical conformations, such as the introduction of Asp and Lys to form lactam bridges (*i, i+4*) (Barazza et al., 2005; Mimna et al., 2007).

In particular cases, stereochemical aspects also play a significant role in the ability to form α-helical peptides. For example, the replacement of *N*-terminal capping amino acid residues Lys-Leu-Thr of peptide QK (Ac-Lys-Leu-Thr-Trp-Gln-Glu-Leu-Tyr-Gln-Leu-Lys-Tyr-Lys-Gly-Ile-NH_2), a vascular endothelial growth factor (VEGF) mimetic short helical peptide, with the corresponding *D* enantiomers (^{D}Lys-^{D}Leu-^{D}Thr) negatively affected its ability to structure into an α-helix, which is fundamental to its biological activity (De Rosa et al., 2022).

Another important factor for helix formation is the size of the side chain of amino acid residues such as Arg, for example,. Tests over Ala-based peptides with *N*-terminal acetylation and *C*-terminal amidation were conducted to evaluate the effect of side chain length of Arg and Arg analogues ((*S*)-2-amino-6-guanidinohexanoic acid (Agh), (*S*)-2-amino-4-guanidinobutyric acid (Agb) and (*S*)-2-amino-3-guanidinopropionic acid (Agp)), as N-cap and C-cap. The results demonstrated that all four peptides were unfavorable for N-capping. The C-capping parameter followed the trend Agp < Agb < Arg < Agh, showing more favorable C-cap energetics with increasing side chain length (Cheng et al., 2012). By contrast, the propensity for helix formation showed a tendency Agp < Agb > Arg > Agh, highlighting the singularity and importance of the Arg side chain and analogs for helix formation, in the C-cap region (Cheng et al., 2012).

Various peptides were synthesized based on repetitions of the sequence $(Arg\text{-}Leu\text{-}Leu\text{-}Arg)_n$, (n = 2–5), because this motif is considerate a α-helix former. Among these, the peptide $(Arg\text{-}Leu\text{-}Leu\text{-}Arg)_5$ exhibits this characteristic and has minimum inhibitory concentrations (MICs) between 1 and 4 mM against microorganisms (gram-negative bacteria, gram-positive bacteria, and fungi); however, its effectiveness is reduced 32-fold in high salt conditions (100 or 200 mM NaCl). Posteriorly, the Ala-Pro-Lys-Ala-Met and Leu-Gln-Lys-Lys-Gly-Ile motifs were added to the repetitions, in the *N*- and *C*-terminals, respectively. These last motifs have amphipathic characteristics, a positive net charge, and show interactions between the residues that allow nucleation peptides. The *N*-terminal motif (Ala-Pro-Lys-Ala-Met) presents a hydrophobic interaction between the side chains of Ala and Met (*i, i+4*). Meanwhile, the *C*-terminal Leu-Gln-Lys-Lys-Gly-Ile motif forms two H-bonds: one between the Leu amide group and the Ile carbonyl group (*i, i+5*), and another between the amide of the Gln side chain and the carbonyl group of Gly (*i, i+3*). Interestingly, with the addition of two capping motifs into the two peptide repetitions (Ala-Pro-Lys-Ala-Met-Arg-Leu-Leu-Arg-Arg-Leu-Leu-Arg-Leu-

Gln-Lys-Lys-Gly), the integrity of the MIC values (0.5 and 2 μg.mL^{-1} for gram negative, gram-positive bacteria, and yeast) was observed at NaCl concentrations equal to 200 mM, and its helicity was not compromised. These motifs have the property of stabilizing the helix, maintaining the structural integrity and antimicrobial activity of the peptide at high salt concentrations (Park et al., 2004).

The synthetic peptide (Leu-Leu-Lys-Lys)$_2$-NH$_2$ was modified by insertion of a Cys residue at the *C*-terminus and in both terminal regions. Those modifications resulted in an enhanced α-helix structure and significantly increased antibacterial activity against *B. subtilis* (125 mg.L^{-1} for both), compared to the parental peptide (500 mg.L^{-1}). Additionally, the presence of thiol groups in the modified peptide enhances its antimicrobial potency against gram-positive bacteria and yeast. Moreover, it broadened its activity spectrum to include gram-negative bacteria, exhibiting potency levels of >500 mg.L^{-1} against *E. coli* and 63 mg.L^{-1} against *P. aeruginosa* for (Leu-Leu-Lys-Lys)$_2$Cys, and 225 mg.L^{-1} against *E. coli* and 125 mg.L^{-1} against *P. aeruginosa* for Cys-(Leu-Leu-Lys-Lys)$_2$-Cys. Notably, the latter peptide exhibited enhanced pore formation (Wiradharma et al., 2011). Furthermore, this peptide effectively eradicated clinically isolated carbapenem-resistant *Acinetobacter baumannii* in mouse models of peritonitis and pneumonia infections (Huang et al., 2012), and it also exhibited activity *in vitro* against susceptible and multidrug-resistant clinical isolates of *M. tuberculosis* (Khara et al., 2014).

The *C*-terminal region is also important for stabilizing the α-helix and, consequently, maintaining activity, particularly when a polar side chain may be taking place. In a study by Kallenbach and Gong, peptides (based on the parental sequence Tyr-Met-Ser-Glu-Asp-Glu-Leu-Lys-Ala-Ala-Glu-Ala-Ala-Phe-Lys-Arg-His-Gly-Pro-Thr) were stabilized with an *N*-terminal region containing a classical Ser-X-X-Glu capping box (Ser-Glu-Asp-Glu motif), while several different capping motifs were tested near the *C*-terminal region to evaluate stabilization capacity (Kallenbach and Gong, 1999). The capping box, Ser-X-X-Glu, was identified by Harper and Rose, as a helix initiator component in the *N*-terminus of proteins (Harper and Rose, 1993). This motif involves two hydrogen-bonding interactions between the side chain and the main chain: (*i, i+3*). The first occurs between the Ser side chain and the amide of Glu, and the second occurs between the Glu side chain and the amide of Ser (Harper and Rose, 1993). Among substitutions in the three last amino acids at the *C*-terminus, an exceptionally strong interaction occurs when an Asn-18 residue is present in the Asn-Pro-Thr motif, interacting through its amide group in the side chain with the carbonyl group of the Phe-14 three residues away, forming a 3_{10}-helix. This interaction (*i, i+4*) produces a greater propensity for helicity in this case. By contrast, the side chain of Gly-18 in the Gly-Val-Pro motif, also in the *C*-terminus, forms a double main chain–main chain H-bond with Phe-14 (Gly-18/Phe-14; (*i, i+4*)) and Lys15 (Gly-18/Lys-15; (*i, i+3*)). Here, the Pro-20 enhances the structure through nonpolar interaction of its side chain by interacting with the aromatic ring of Phe-14, collectively stabilizing the *N*-terminal capping box (Kallenbach and Gong, 1999).

The *C*-terminal Lys can structure a peptide with three amino acids as a helix. This was demonstrated by Zabuga and Rizzo (2015), when they detected a helix in the sequence Ac-Phe-Ala-LysH$^+$. In this case, the Lys side chain forms three hydrogen bonds, where each hydrogen of the ammonia group bonds to the carbonyls of the main chain, similar to what occurs in polyalanine helices (Zabuga and Rizzo, 2015).

Evidence suggests that edge-face, offset-stacked or face-to-face stacked aromatic interaction also plays a crucial role in the α-helical monomeric structure. Edge-face and offset-stacked geometric interactions are preferred between two phenylalanine residues located *i* and *i+4*, respectively, whereas fully stacked geometry is observed when phenylalanine - pentafluorophenylalanine are in a similar position. In both cases, the interactions can stabilize helix formation in a prototype polyalanine peptide, but the interactions are stronger when the residues, in the same positions, are more towards the *C*-terminal region. The prevalence of helical structures and, consequently the diverse activities observed in AMPs, is significantly influenced by the interaction between rotamer populations of the aromatic chains. The interaction, particularly at the *C*-terminal region, enhances the motif's effectiveness in promoting α-helices through the C-capping effect (Butterfield et al., 2002; Koehbach and Craik, 2019).

Although capping motifs can give us answers about the nucleation of the formation of α-helical structures, there are other factors that influence the stability of these conformations so that the role of complexes formed by interactions with metal ions can also generate another part of this answer.

5 Modulation of activity of AMPs by formation complexes with divalent ions

The role of metal ions cannot be underestimated, since studies with different peptides, proteins, and non-peptide molecules demonstrate their activity in intermolecular interactions within cellular physiological processes. Additionally, they play a role in enhancing the activity of molecules in the immune system. In the last decade, several studies of complexes formed by motifs of AMPs with divalent metal ions, such as Zn^{2+} and Cu^{2+} (Łoboda et al., 2018), have demonstrated the role of these ions in boosting the antimicrobial activity of these substances. This subfamily within the AMPs is called metallo-AMPs and has attracted attention because it has allowed the importance of this complex in the immune system to be demonstrated. Such potentiation may be related to the fact that the binding of an AMP to the metal removes the availability of the microbe to the ion, causing it to suffer due to the deficit of the metal. Alternatively, this complexation could improve the structure or charge of the peptide (Łoboda et al., 2018).

The classification of the relationships between metal cations and AMPs has been described as falling into three classes, explaining the modes of action. However, it is essential to note that this classification is not unique, and there may be other forms. The system was primarily based on Zn^{2+}, as the relationship between Zn^{2+}-AMPs has provided sufficient information to establish their synergistic relationship. Hence, class I is distinguished by the modulation of AMP activity through metal ion binding. In this class, cations can act as a cofactor that either enhances or inhibits the antimicrobial activity of AMPs. In class II, AMPs may regulate cation availability within the host. This implies that AMPs can either enable the availability of ions if the host restricts them from the microbe or increase the concentration of the cation within the

pathogen, leading to toxicity. In class III, cations may indirectly influence the activity of an AMP, and the existence of an ion-AMP complex is not a requirement. In this scenario, the cation enhances the activity of an AMP by inhibiting the microbe's responses (Donaghy et al., 2023).

Among the mechanisms mentioned earlier, we can highlight class I, which is the most commonly observed in certain AMPs and Zn^{2+} ions. Dermcidin-derived peptides belong to a family resulting from the proteolysis processes of dermcidin (DCD), a protein expressed by sweat glands. These peptides play a role in the skin's immune defense system, and within them, DCD-1 and DCD-1L are the major ones. Both peptides have demonstrated efficacy against both gram negative and gram-positive bacteria (*E. coli*, *Enterococcus faecalis*, and *S. aureus*), as well as yeasts like *C. albicans*, in buffer solutions with conditions of pH, ionic strength, and sodium, potassium, magnesium and chlorine ions, which simulate human sweat conditions (Schittek et al., 2001). Structurally, these two peptides present a wide range of residues with polar groups (-OH) and donors of protons (-COOH), the latter being acidic groups that confer a total charge of −2 at neutral pH. Both peptides adopt an undefined random conformation in aqueous solution; however, DCD-1L adopts an α-helix structure in the presence of surfactants that mimic the cell membrane of gram-negative bacteria (Steffen et al., 2006; Paulmann et al., 2012). The mechanism of action of dermcidin-derived peptides is attributed to the formation of oligomerization (composed of dimers and trimers), a structure believed to be important in antibacterial activity. This oligomerization structuring has been observed in an *in vitro* experiment involving DCD-1L in human sweat, and it seems that Zn^{2+} ions play a crucial role in stabilizing the oligomeric structure and in the boosting activity. Activity reduction becomes apparent upon the addition of a chelating agent, such as EDTA (Paulmann et al., 2012).

Dermcidin-derived peptides, with DCD-1L (Ser-Ser-Leu-Leu-Glu-Lys-Gly-Leu-Asp-Gly-Ala-Lys-Lys-Ala-Val-Gly-Gly-Leu-Gly-Lys-Leu-Gly-Lys-Asp-Ala-Val-Glu-Asp-Leu-Glu-Ser-Val-Gly-Lys-Gly-Ala-Val-His-Asp-Val-Lys-Asp-Val-Leu-Asp-Ser-Val-Leu) as a significant constituent, exhibit a mechanism of action attributed to class I. In this process, Zn^{2+} ions play a crucial role in structuring oligomers, forming "ion channels" or pores in bacterial membranes (Paulmann et al., 2012). The structuring was carried out in a solution of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE)/1-palmitoyl-2-oleoyl-sn-glycero-3-phospho (1′-rac-glycerol) (POPG) in a molar ratio 3:1. The elucidation by X-ray diffraction (XRD), solid-state nuclear magnetic resonance (ssNMR), and molecular dynamics (MD) shows that DCD-1L forms a trimer of Zn^{2+} bridge dimers. Each dimer is formed by paired peptide helices oriented in head-to-tail directions and coordinated to Zn^{2+} ions as the central atom via Glu-5, Asp-9 (*i, i+4* and *i, i+9* motifs at *N*-terminus), and His-38′ and Asp-42' (*i, i+38′* together with *i, i+42′* in *C*-terminal). For the formation of trimers, stabilization of the dimers occurs through the pairs Asp-24/Asp-28 and Asp-24'/Asp-28′ in the center of the helix, which coordinate with Zn^{2+}. In total, six polypeptide chains form a hexameric channel that creates a pore resembling a barrel inside the membrane. The Zn^{2+}:DCD-1L complex was observed to form in a 1:1 ratio, where all Zn^{2+} ions neutralize the charge of the hexamer (Figure 1) (Song et al., 2013).

Likewise, certain motifs in AMPs that exhibit binding to transition metals have been reported. Generally, such interactions are attributed to residues with neutral, basic, or acidic polar side chains in AMPs, which contain heteroatoms capable of coordinating with these metals. As an example, we can cite the His-Gly-Phe-Ser-His motif found at the *C*-terminus, between amino acids 17–21 of the peptide clavanin A (Clav-A: Val-Phe-Gln-Phe-Leu-Gly-Lys-Ile-Ile-His-His-Val-Gly-Asn-Phe-Val-His-Gly-Phe-Ser-His-Val-Phe-NH_2) (Duay et al., 2019). Clav-A is a representative within the group of peptides known as clavanins, isolated from the tunicate *Styela clava*. Cationic, amphipathic and rich in His, it is an α-helix-forming peptide, and has a broad spectrum of antimicrobial action against bacteria and fungi. It distinguishes itself by its wide-ranging efficacy against methicillin-resistant *Staphylococcus aureus* (MRSA) at pH 5.5. At physiological pH (pH 7.4), it shows less effectiveness, but it also exhibits activity under high salt conditions. These data suggest that Clav-A has different modes of action under different pH and salt conditions (Lee et al., 1997). In the presence of Zn^{2+} ions, the activity of Clav-A is increased 16-fold, and it seems that this increase is derived from the stabilization of this ion in the His-Gly-Phe-Ser-His motif, where His-17 and His-21 are located (*i*, *i+4*) and each one is responsible for coordination. Molecular modeling on the Zn^{2+}-Clav-A system in membrane environments demonstrates that the mechanism of action may be attributed to the coordination, providing strong electrostatic interactions between the complex (His-Gly-Phe-Ser-His motif at the *C*-terminus of Clav-A) and the lipid layer, leading to membrane dissociation (Duay et al., 2019).

At physiological pH, Clav-A can independently engage with the membrane of gram-negative bacteria, subsequently leading to membrane distortion and changes in cellular functionality, such as the inhibition of division in *E. coli* (Juliano et al., 2020). In contrast, at acidic pH, Clav-A seems to exhibit two mechanisms of action. The first involves binding to ionophores, deactivating their function, and facilitating the transfer of ions between the cytoplasm and the extracellular environment. This enables Zn^{2+} ions to enter the cell, causing Clav-A to act similarly to indolicin, which inhibits DNA synthesis. Additionally, there may be a translocation of the peptide, Zn^{2+}, and/or Zn^{2+}-Clav-A complex across the membrane (Juliano et al., 2017). In the latter case, the Zn^{2+}-Clav-A complex may influence cytoplasmic DNA through a mechanism proposed by Juliano et al. (2020). The mechanism, investigated through quantum mechanics/molecular mechanics (QM/MM) calculations, suggested that Zn^{2+}, in the Zn^{2+}-Clav-A complex, acts as a Lewis acid, activating the P-O bonds in DNA. A hydroxyl group from water then nucleophilically attacks the electrophytic phosphorus, cleaving the P-O bond activated by Zn^{2+}. The breakage of the phosphoester bond hydrolyzes bacterial DNA (Juliano et al., 2020).

A complex of divalent copper (Cu^{2+}) and nickel (Ni^{2+}) ions in amino-terminal motifs, called (ATCUN), has been found in specific AMPs. These motifs are thought to be essential in boosting the oxidative action mechanism (Alexander et al., 2017). One such examples is ixosine, a peptide isolated from the tick *Ixodes sinensis*, which exhibits a similar effect on the lipid membranes of bacteria. The mechanism also appears to be class I, according to the classification by Donaghy et al. (2023), through a strategy employed by the tick in using Cu^{2+} ions as part of the activation of its immune defense against bacterial infections. In the case of ixosine (Gly-Leu-His-Lys-Val-Met-Arg-Glu-Val-Leu-Gly-Tyr-Glu-Arg-Asn-Ser-Tyr-Lys-Lys-Phe-Phe-Leu-Arg), the first three amino acids (Gly-Leu-His) are the motif responsible and that can

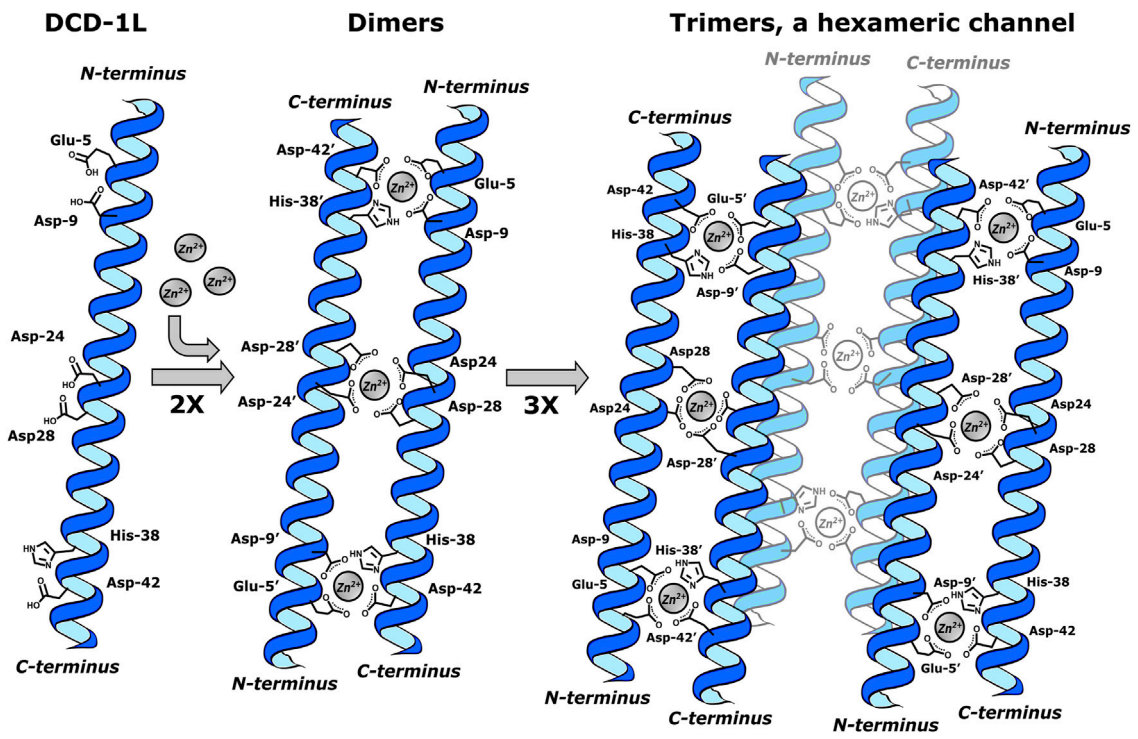


FIGURE 1
DCD-1L forming a trimer of Zn^{2+} bridge dimers, a hexameric channel. Dimers are formed by paired peptide helices (2X) oriented in head-to-tail directions and coordinated to two Zn^{2+} ions through Glu-5, Asp-9 (*i, i+4* and *i, i+9* motifs at *N*-terminus) and His-38′ and Asp-42′ (*i, i+38′* together with *i, i+42′* in *C*-terminal). The formation of trimers occurs through the Asp-24/Asp-28 and Asp-24′/Asp-28′ in the center of the helix, which coordinate with one Zn^{2+}ion. This last interaction stabilizes each dimer in the trimer. This figure was created using Inkscape, version 1.3.0.

coordinate to Cu^{2+} and Ni^{2+}. Similar motifs like NH_2-AA_1-AA_2-His, are found in proteins such as albumins and protamines, which coordinate these two metals through the terminal amino group, the -NH- group of AA_2, and the imidazole ring of histidine (Melino et al., 1999; Libardo et al., 2016). In ticks, it seems that the Cu^{2+}-ixosine complex plays a role in mediating molecular oxygen-dependent lipid peroxidation of phospholipids to produce reactive oxygen species (ROS). The authors theorize that these ROS leads to the intramolecular formation of aldehyde-type carbonyl compounds, derivatives capable of reacting with amino groups of other AMPs, such as ixosine B, for example, to form Schiff bases. This allows the binding of AMPs in the membrane, forming helices as they interact closely with the membrane to create pores (Figure 2) (Libardo et al., 2016).

Using an *in silico* approach, our group previously redesigned the AMPs, CM15 (Lys-Trp-Lys-Leu-Phe-Lys-Lys-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-NH_2), and citropin1.1 (Gly-Leu-Phe-Asp-Val-Ile-Lys-Lys-Val-Ala-Ser-Val-Ile-Gly-Gly-Leu-NH_2), by the addition of ATCUN motifs (Gly-Gly-His or Val-Ile-His) at their *N*-terminus. It is noteworthy that both motifs, when inserted into CM15, were shown to enhance activity against carbapenem-resistant *Klebsiella pneumoniae* (KpC+ 1,825,971) by 4-fold for Gly-Gly-His-CM15 and 8-fold for His-Ile-Val-CM15. By contrast, modification of both ATCUN motifs in citropin1.1 resulted in a 3-fold decrease in antimicrobial activity when tested against both gram negative and gram-positive bacteria. CD spectra for CM15 peptides containing both ATCUN motifs showed an increase in helicity in the absence of Cu^{2+} (Agbale et al., 2019).

Histatins are small peptides released by the parotid and submandibular salivary gland in both humans and primates. These peptides are generally cationic and rich in histidine, playing a key role in antimicrobial activity against bacteria and fungi, wound healing and the buccal immune system (Tay et al., 2009; Puri and Edgerton, 2014). Among the most potent is Histatin 5 (Hst-5: Asp-Ser-His-Ala-Lys-Arg-His-His-Gly-Tyr-Lys-Arg-Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr), resulting from the proteolytic degradation of histatin 1 and 3. Hst-5 is active against *Cryptococcus neoformans, Aspergillus fumigatu, Candida albicans* and other yeasts (Helmerhorst et al., 1999). It also exhibits activity against multiple-drug-resistant pathogens (ESKAPE: *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), causing hospital or nosocomial infections (Du et al., 2017). Hst-5 presents part of an ACTUN motif (Asp-Ser-His) in its *N*-terminal as well as the His-X-X-X-His-His motif in its *N*-terminal and the His-Glu-X-X-His motif in its *C*-terminal for Zn^{2+} coordination. Apparently, the union of Zn^{2+} and Cu^{2+} in the respective motifs confers stabilization on the α-helix structure (Cragnell et al., 2019; McCaslin et al., 2019), and data from several studies on the metal-Hst-5 complex suggest significant consequences for increased activity. In the work by Melino et al. (1999), it was observed that the Zn^{2+}-Hst-5 complex exhibits a catalytic effect on the fusion of negatively charged lipid vesicles via a mechanism of action that involves electrostatic interactions mediated by the formation of dimers (Melino et al., 1999). Additionally, Zn^{2+} ions have shown to increase the bactericidal

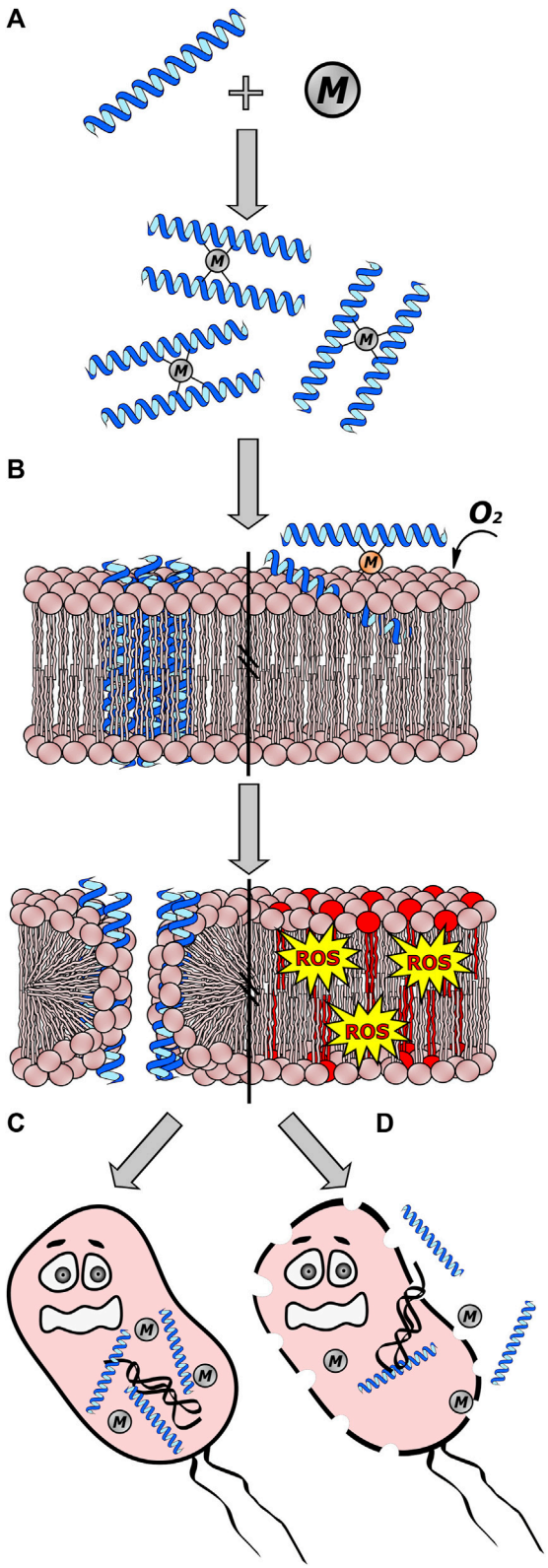


FIGURE 2
Interaction between antimicrobial peptides (AMPs) and metal ions (M). In class I interactions, certain cations can either enhance or inhibit the antimicrobial activity of AMPs by acting as a cofactor. **(A)** Some metallic ions bind to AMPs through specific amino acids, resulting in the formation of dimers. **(B)** The M-AMP complex can then interact with the cell membrane, forming pores or evolving into more complex structures by joining dimers to create channels. Additionally, certain complexes like Cu^{2+} ions and AMPs with the ATCUN motif can lead to the production of reactive oxygen species (ROS) through oxygen-dependent lipid peroxidation of phospholipids, ultimately causing cell membrane disintegration. **(C)** These channels formed allow the passage of AMPs and/or metal ions into the cell, where they can interact with intracellular targets. **(D)** If AMPs fail to interact with intracellular targets, pore and channel formation can destabilize the cell membrane, resulting in cell death and the leakage of cellular material. This figure was created using Inkscape, version 1.3.0.

activity of histidine-rich peptides against *E. faecalis* (Rydengård et al., 2006).

The formation of ROS has been proven through several experiments where the complex is formed between Cu^{2+} and peptides containing ATCUN, such as those of Hst-5. For example, by mass spectrometry, oxidized derivatives of a peptide analogue of Hst-5 (P1: Asp-Ser-His-Ala-Lys-Arg-Ala-His-Gly-Tyr) were detected after the addition of ascorbic acid to a 1: 1 stoichiometry solution of Cu^{2+}:P1 complex. After 5 min of exposure to the reductant, it was possible to identify the presence of adduct ions with an increase of 16 and 32 Da in the mass of P1 (Cabras et al., 2007). Additionally, the same copper complex, using Hst-5 or Hst-8 (Lys-Phe-His-Glu-Lys-His-His-Ser-His-Arg-Gly-Tyr), revealed an increase in ROS production, including hydrogen peroxide, at physiological concentrations of ascorbic acid in *vitro* experiments. Here, Houghton and Nicholas (2009) observed the production of hydrogen peroxide using the Amplex Red assay, by incubating at 20°C for 60 min the peptide stock solutions (Hst-5 or Hst-8) containing copper chloride and buffered ascorbate. The Amplex Red assay is based on the oxidation of 10-acetyl-3,7-dihydroxypenoxazine, which is catalyzed by horseradish peroxidase (HRP) in the presence of H_2O_2 to produce a red fluorescent oxidation product, which is monitored at 570 nm (Houghton and Nicholas, 2009). This finding further supports the effects of ROS production on bacteria killing (Libardo et al., 2016), and control of the metal-peptide complex in candidiasis infections. In *C. albicans*, mitochondria produce superoxide dismutase (Sod5) dependent on Cu/Zn/Mn, which is induced under conditions of oxidative stress. The enzymatic activity of yeast can be a target due to the transmetalation of such ions mediated by Hst-5 (Martchenko et al., 2004). The complex between Zn^{2+}-Hst-5 is similar to that formed by DCD-1L and ClavA, with 2: 2 stoichiometry, where each Zn^{2+} ion coordinates with four histidine residues, two of which are in a polypeptide chain of Hst-5. Some studies suggest that Hst-5 activity in *Candida* species is dependent on Zn^{2+} concentration since the low ratio (0.5:1 or less) of the metal in the complex has been shown to have improved antifungal activity in comparison with the peptide alone. This improvement can be attributed to cellular reorganization induced by Zn^{2+}, decreasing the ability to adhere to the cell wall of the host (Norris et al., 2020). Nevertheless, at higher ratios (1:1 or higher), Zn^{2+} ions seem to impede the antifungal activity of Hst-5 against *C. albicans* (Campbell et al., 2022). According to Stewart et al. (2023), both higher and lower proportions of Zn^{2+} in the Zn^{2+}-Hst-5 complex do not exert an effect on improving or suppressing the effect on multiple species of *Streptococcus*, which normally colonize the

oral cavity. Additionally, Hst-5 does not cause Zn^{2+} starvation in this genus, as it does not compete for Zn^{2+} binding with the Zn^{2+} uptake protein AdcAI (Stewart et al., 2023). The above study, along with others, suggests that Hst-5 may play a role in the development of certain species of oral cavity microorganisms, exerting selective antimicrobial activity and maintaining microbial communities regulated through Zn^{2+} ion concentrations (Norris et al., 2021; Campbell et al., 2022; Stewart et al., 2023). With all this evidence, Hst-5 cannot be classified as belonging to class II; instead, it appears to be able to act in class I or III.

Calcitermin (Val-Ala-Ile-Ala-Leu-Lys-Ala-Ala-His-Tyr-His-Thr-His-Lys-Glu) corresponds to the last 15 residues at the *C*-terminus of calgranulin C, a member of the S100 family of antibacterial proteins produced by neutrophils, monocytes, and keratinocytes (Gottsch et al., 1999; D'Accolti et al., 2023). This AMP shows activity against several pathogens (*E. coli, P. aeruginosa, S. aureus, S. epidermidis, E. faecalis, C. albicans,* and *L. monocytogenes*), depending on the conditions (Cole et al., 2001). For example, at neutral pH (7.4), it does not show activity, but at acidic pH (5.4), it is active against *E. coli, P. aeruginosa*, *E. faecalis,* and *C. albicans* (Cole et al., 2001; Bellotti et al., 2019). In the presence of Zn^{2+} ions, its activity increases against *E. coli* and is effective against *L. monocytogenes* (Cole et al., 2001). Coordination of two metals, Zn^{2+} and Cu^{2+}, occurs effectively at acidic pH, using His-9, His-11, His-13, and different amino acid residues for each metal. In the case of the Zn^{2+} complex, the carboxylate group of the side chain of Glu-15 participates, but with Cu^{2+}, the other ligand is the *N*-terminal group of Val-1. This coordination improves the activity of the peptide against *C. albicans*, as the MIC decreases to 1 $\mu g.mL^{-1}$ for both complexes. Meanwhile, the complex with Cu^{2+} maintains activity against *S. aureus* in the same way as non-complexed calcitermin. CD experiments for the Cu^{2+}-Calcitermin complex, and Cu^{2+} with three other analogs (substitution of each His with an Ala in the parental Calcitermin), reveal that the metal in all complexes helps to adopt a helical-like structure in the presence of membrane-mimicking sodium dodecyl sulfate (SDS) (Bellotti et al., 2019).

Another peptide that relies on the presence of divalent ions is Bacitracin. Isolated as a mixture of cyclic dodecapeptides from *Bacillus* species, it contains both *D*- and *L*-amino acids and is synthesized by nonribosomal peptide synthases (Economou et al., 2013). Among these peptides, bacitracin A is the main constituent, exhibiting the highest effectiveness against bacteria. It is selective for gram-positive bacteria and shows limited activity against gram-negative bacteria. It has been classified as class I according to Donaghy et al. (2023) due to its binding with Zn^{2+} ions, forming a complex that enhances the activity of the cyclopeptide (Donaghy et al., 2023). The Zn^{2+}-Bacitracin complex binds to a lipid intermediate (undecaprenyl pyrophosphate), an important transporter in the biosynthesis of the bacterial cell wall. The binding of the complex to the intermediate interrupts the flow of peptidoglycan precursors, leading to the inhibition of cell wall formation and, consequently, negatively affecting bacterial development (Economou et al., 2013). The stoichiometry of the complex formed between Zn^{2+}-bacitracin-undecaprenyl pyrophosphate has been reported as 1:1:1. These data were suggested by crystallization experiments performed on the Zn^{2+}-bacitracin-geranyl pyrophosphate complex. In this complex, Zn^{2+} coordinates mainly with the *N*-terminal region of this AMP, and geranyl pyrophosphate is also involved. Specifically, bacitracin engages both geranyl pyrophosphate and Zn^{2+} ions in the coordination process. In this coordination, bacitracin employs the terminal amino group, the nitrogen on the thiazoline ring, and the carboxylate of Glu-4. By contrast, the lipid participates use the oxygens of the pyrophosphate group, which, together with a water molecule, assume an octahedral geometry. In this case, unlike the previous examples, the coordination of Zn^{2+} by the His present in the bacitracin macrocycle is not observed (Economou et al., 2013).

Kappacin (106–169) is the non-glycosylated and phosphorylated form of the caseinomacropeptide (CMP) derived from bovine milk k-casein (the *C*-terminal fragment between residues 106 and 169). This AMP exhibits activity against the oral opportunistic pathogen *Streptococcus mutans* and demonstrates a membranolytic effect observed in experiments with artificial liposomes, exhibiting pH-dependent behavior. At acidic pH (6.5), it has the ability to permeabilize liposomes; however, at neutral pH (7.2), it exhibits little effect on them, as evidenced by the lack of antibacterial activity. However, the addition of a metal: Kappacin (106–169) ratio (2:1) for Ca^{2+} or (1:1) for Zn^{2+} results in improved antibacterial activity at neutral pH. Divalent metal cation binding assays and Scatchard analyses indicated that Kappacin (106–169) has two binding sites for the metal. Proton nuclear magnetic resonance (^{1}H-NMR) experiments on the Kappacin (138–158) fragment, conducted in the presence of Ca^{2+} ions and 30% tetrafluoroethylene (TFE), suggest that the peptide adopts a specific conformation in this environment (Dashper et al., 2005). The sequence Kappacin (138–158) (Ala-Val-Glu-Ser-Thr-Val-Ala-Thr-Leu-Glu-Asn-Ser-Pro-Glu-Val-Ile-Glu-Ser-Pro-Pro-Glu) is rich in Glu spaced (*i, i+4*) at the *C*-terminus, which may explain the coordination with divalent ions, thus potentially modulating the peptide structuration.

A new field, known as metallo-AMPs, has emerged from the interaction between divalent metal ions and specific residues within AMPs, warranting further exploration. As mentioned in the observed cases, this interaction acts by increasing effectiveness against microorganisms and arises from the coordination of divalent ions along the structure of AMPs, enhancing the secondary structure and performing various functions in the face of microbial targets. This interaction is expected, as both metallic ions and AMPs are considered essential components of the immune system in various organisms.

6 Concluding remarks and prospects

Capping motifs play an important role in modulating the antimicrobial activity, selectivity, and protease resistance in AMPs. Beyond direct antimicrobial properties, presumably by promoting the structuring of peptide sequences in the α-helix, such as specific sequences and divalent cations, capping motifs may influence other desirable properties, such as *in vivo* stability and biocompatibility. Cationic amino acids, such as Lys and Arg, have demonstrated efficacy as capping motifs because charge at the *N*- or *C*-terminus helps enhance interactions with negatively charged microbial membranes while reducing cytotoxicity against mammalian cells. Some internal interactions of the side chains of these residues, as well as unique and unusually structured motifs, within the peptide chain, contribute to the nucleation and structuring of α-helices, which are crucial for biological activity.

However, further research is needed to fully elucidate the structure-activity relationships between capping motifs and the AMP's mechanism of action. The results from studies of molecular dynamics simulations and biophysical techniques, in which it was explored how specific capping motifs alter AMP secondary structure, oligomerization, membrane perturbation abilities, and the capping motifs' performance against a wider range of clinically relevant and multidrug-resistant pathogens, can be used in new approaches based on artificial intelligence and deep machine learning, to generate new structural data, as well as new active peptides.

Through continued progress in understanding capping motif-mediated effects, it may be possible to customize AMPs for specific infection types or drug delivery applications, helping to address the pressing need for novel anti-infectives, particularly in the face of escalating antimicrobial resistance crises worldwide. Capping motifs offer a promising design element for to developing next-generation AMP therapeutics with improved efficacy, safety, and pharmacological profiles.

Author contributions

JB-V: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing–original draft, Writing–review and editing. ML: Writing–review and editing. MM: Writing–review and editing. MC: Writing–review and editing. OF: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Supervision, Validation, Visualization, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The LL-37 domain: A clue to cathelicidin immunomodulatory response?

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ABSTRACT

Host defense peptides (HDPs) are naturally occurring polypeptide sequences that, in addition to being active against bacteria, fungi, viruses, and other parasites, may stimulate immunomodulatory responses. Cathelicidins, a family of HDPs, are produced by diverse animal species, such as mammals, fish, birds, amphibians, and reptiles, to protect them against pathogen infections. These peptides have variable *C*-terminal domains responsible for their antimicrobial and immunomodulatory activities and a highly conserved *N*-terminal pre-pro region homologous to cathelin. Although cathelicidins are the major components of innate immunity, the molecular basis by which they induce an immune response is still unclear. In this review, we will address the role of the LL-37 domain and its SK-24, IV-20, FK-13 and LL-37 fragments in the immunity response. Other cathelicidins also share structural and functional characteristics with the LL-37 domain, suggesting that these fragments may be responsible for interaction between these peptides and receptors in humans. Fragments of the LL-37 domain can give us clues about how homologous cathelicidins, in general, induce an immune response.

1. Introduction

Although it is a crucial mechanism during rapid bacterial replication events, adaptive immunity requires considerable time to initiate a response to attack by pathogens [1]. However, organisms that do not have an adaptive system or that require a rapid response protect themselves through non-specific response mechanisms, which occur through the production of host defense peptides (HDPs), initiating infection control [1]. HDPs are widely recognized for their antimicrobial activity, being active against infectious bacteria (Gram-positive and Gram-negative, including bacterial biofilm), fungi, viruses, and other parasites. In recent decades, these peptides have been explored as immunomodulators due to their ability to induce an immune response [2–6] (Fig. 1).

HDPs are short bioactive polypeptides found in virtually all living beings as part of the first line of defense [7–11]. Among the various ways to modulate an immune response, the following can be mentioned: (i) the production of anti- and pro-inflammatory cytokines; increased expression of chemokines; (ii) the neutralization of lipopolysaccharides (LPS) and endotoxins; (iii) the regulation of the production of reactive oxygen (ROS) and nitrogen (NOS) species; (iv) antigen presentation stimulus; and (v) activation of leukocyte differentiation [12]. Furthermore, in addition to modulating the host's immune and inflammatory responses, HDPs act concomitantly as antimicrobials, eradicating bacteria and other pathogens that cause infections [13–15].

Cathelicidins and defensins are the two subgroups of HDPs produced by mammals as part of the innate immune system. Humans synthesize many classes of defensins but, interestingly, only a single one of the cathelicidins [16]. Cathelicidins are short-cationic peptides that play a role in host responses toward several highly conserved microbe-associated molecular patterns (MAMPs) [17]. Cathelicidins are mainly found at high concentrations in neutrophil-specific granules, which can be released upon neutrophil activation [18]. Among the cathelicidins produced by mammalians, human LL-37 (4.5 kDa), which is composed of 37 amino acid residues with diLeucine at the *N*-terminus, is the most well-characterized peptide [19,20]. LL-37 plays an important

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role in the human innate immune system, being produced against bacterial (both Gram-positive and Gram-negative) and viral infections [21,22].

LL-37 was initially described in 1995 by three independent research groups through deduction from the myeloid bone marrow cDNA library and isolated from neutrophils [23,24]. The hCAP18 *N*-terminal consists of a 37 amino acid-long peptide, called LL-37, which in addition to having bactericidal activity against several microorganisms, also prevents immunostimulatory effects of bacterial wall molecules (e.g., LPS), protecting against lethal endotoxemia [25]. To control inflammation, LL-37 acts as both a pro- and anti-inflammatory factor. In the first case, it includes the negative regulation of interleukin (IL)-10, the positive regulation of IL-8, IL-12p40, and IL-1β, the induction of type I interferons (IFNs) in plasmacytoid dendritic cells (pDCs) and keratinocytes and mast cell degranulation and the release of inflammatory mediators. As an anti-inflammatory, LL-37 inhibits the formation of the

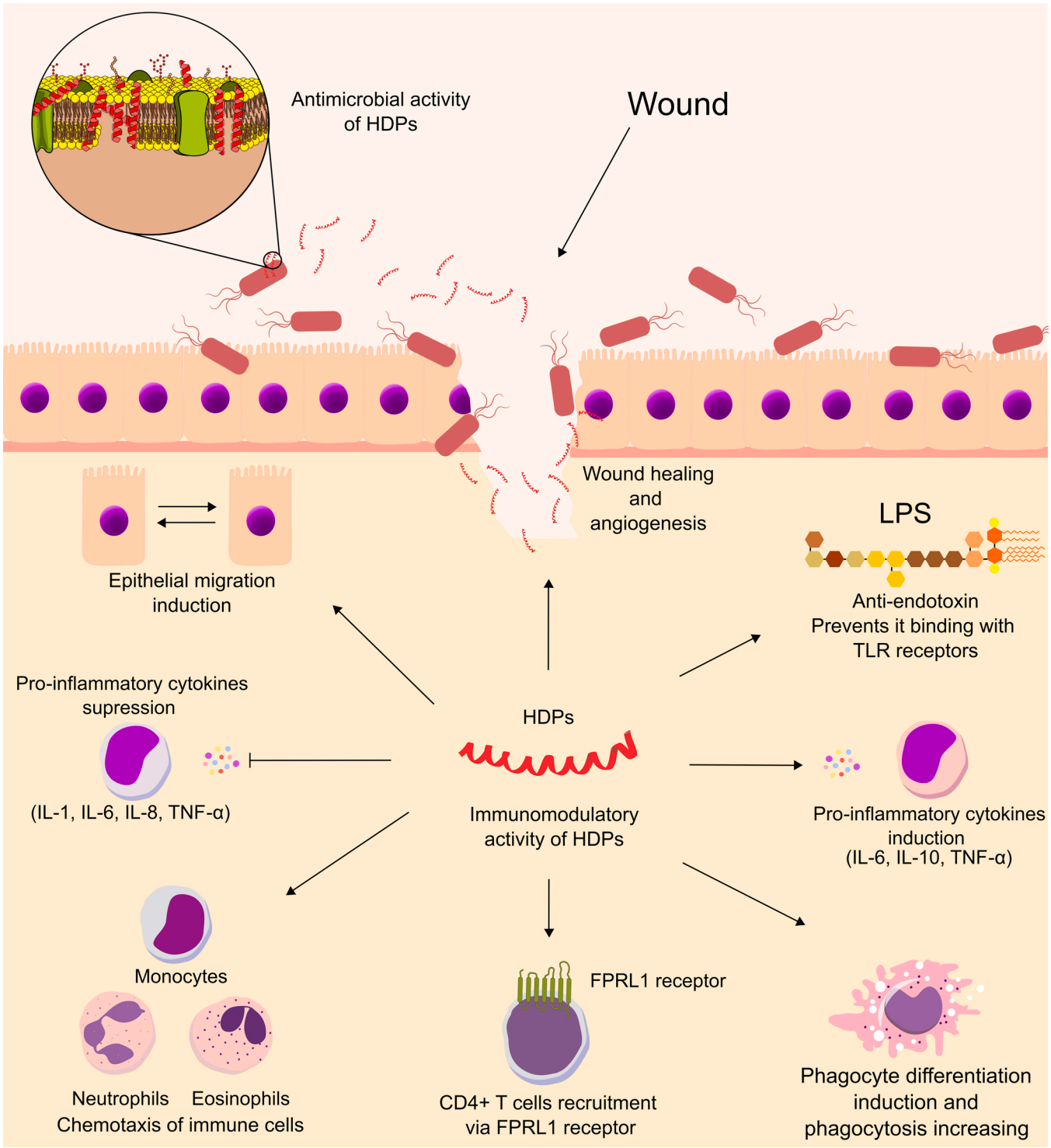

Fig. 1. Representation of antimicrobial and immunomodulatory activities of host defense peptides. In addition to acting directly on the plasma membrane of bacteria, these peptides can trigger a series of immune responses in the host. They can induce the migration of epithelial cells, facilitating healing, as well as inducing or suppressing the production of pro-inflammatory cytokines, and recruiting cells of the immune system.

AIM2 inflammasome, as well as suppressing IFN-γ, tumor necrosis factor (TNF)-α, IL-4, and IL-12 [26].

In recent decades, evidence has been growing regarding the immunomodulatory activity of HDP LL-37. However, the properties that make it a host defense peptide, capable of inducing an immune response, are still unclear. Therefore, in this review we will try to shed light on the possible structural characteristics of LL-37 that are responsible for its immunomodulatory activity. The most likely factor is the presence of a domain responsible for the immunomodulatory response. To clarify this question, we will try to define what makes LL-37 a host defense peptide. We will examine which intrinsic characteristics, such as primary structure and physicochemical properties, could result in the mechanism of action capable of inducing a response from the host's immune system.

2. The human cathelicidin LL-37

Human-produced LL-37 is a member of a group of peptides called cathelicidins [27]. Cathelicidins are one of the most important groups of mammalian HDPs [28], produced by humans and other vertebrates as a major part of their immune system [29]. These HDPs consist of large precursors (94–114 amino acids), a highly conserved *N*-terminal region, which contains a signal peptide and a pro-domain called cathelin (cathepsin L inhibitor)-like domain (CLD), while the *C*-terminal region exhibits a highly variable antimicrobial domain [30–32]. These peptides can be stored in the secretory granules of neutrophils and macrophages as inactive precursors (pre-propeptides) and released extracellularly as mature peptides, after being cleaved by neutrophil elastase [33].

The 18 kDa human cationic antimicrobial protein (hCAP18) is encoded by the *cAMP* gene on chromosome 3 and has several functions in controlling an invading pathogen by directly targeting the microbial membrane in addition to playing important roles in the innate immune system [34–37] (Fig. 1). hCAP18 is initially expressed as an inactive precursor protein, which is processed by proteolytic cleavage to the bioactive peptide LL-37 by exposure to specific serine proteases like proteinase 3, kallikrein 5, and kallikrein 7 [38,39]. LL-37 (NH_2-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-COOH) is a positively charged peptide (+6), at neutral pH, with high content of basic and hydrophobic amino acid residues [40]. In water solution, LL-37 shows disordered conformation, but in contact with salt (HCO_3^-, SO_4^{2-} or $CF_3CO_3^-$), pH variation, and peptide concentration induce LL-37 to adopt a helical structure [41].

LL-37 is produced in several cell types, including macrophages, neutrophils, natural killer cells, and epithelial cells from the skin, intestine, airways, and ocular surface [42]. *In vitro* and in vivo studies have evaluated the capacity of cathelicidin to cause the immune response. According to these, LL-37 and its synthetic derivatives (IDR-1 and IDR-1002) proved able to repress the synthesis of pro-inflammatory cytokines (IL-1, IL-6, IL-8, and TNF-α) by peripheral blood mononuclear cells (PBMC) [43–45]. Besides that, LL-37 also acts in the pro-inflammatory responses induced by LPS, TLR2/4, and TLR9, preventing the release of TNF to activated downstream pathways. Currently, the role of LL-37 in ATP-dependent P2X7R ion channels is highlighted, as this is a key component in the neuroinflammatory cascade [46]. Further, LL37 also prevents the translocation of subunits NF-kB p50 and p65, which are pro-inflammatory gene expression factors [45]. In addition, LL-37 can act, directly or indirectly, to promote the attraction of a variety of leukocytes, including neutrophils, eosinophils [47], monocytes and CD4 + T cells via the FPRL1 receptor [48].

The use of LL-37 in the wound healing process has demonstrated an increase in angiogenesis, stimulating the expression of IL-18 by keratinocytes, and also inducing the production of IFN-γ [49]. This peptide activates the production of the chemokine stromal cell-derived factor 1-α (SDF-1α) and vascular endothelial growth factor-α (VEGFα), which improve wound healing and epithelial cell migration [50]. After the activation of SDF-1α and VEGFα, the endosomal dsRNA uptake is started by a TLR-3-mediated mechanism that raises the expression of heparin-binding EGF-like growth factor (HBEGF) and fibroblast growth factor (FGF2) by dermal keratinocytes and fibroblasts [51].

Regarding LL-37 bactericidal activity, a combinatorial analysis, involving microbiological, spectroscopic, biophysical, and computational approaches, has demonstrated that the *C*-terminal VPRTES tail of LL-37 is important to the mode of attachment to the lipid bilayer, and residues 17–29 are responsible for killing bacteria through membrane disruption [52]. Currently, a study suggests the notable role of LL-37 in protecting patients against SARS-CoV-2. This molecule has showed direct interaction with the receptor binding motif (RBM) fragment responsible for angiotensin converting enzyme 2 (ACE2) contact, as LL-37 has high structural similarity to the ACE2 *N*-terminal helix [53]. In the same way that a motif may be related to the antimicrobial activity of LL-37, other domains/motifs (Fig. 1) may be responsible for the immunomodulatory activity of this peptide.

In the next sections, we will try to discuss what exactly *makes* the LL-37 peptide a host defense peptide. We will discuss the relationship between structure and function, as well as the presence of domains present in this peptide that can give us clues about its immunomodulatory activity. In addition, these reflections can point us towards the development of new synthetic peptides capable of inducing an immune response while causing bacterial death through membrane lysis.

3. Structural and functional analyses of LL-37-related HDPs

The biological activity of HDPs is directly related to several features, including the degree of helicity, charge, hydrophobicity, molecule size, amphipathicity, and solubility [54]. All these features are the result of the arrangement of amino acid residues in the primary structure of HDPs. The study of the relationship between structure and function is complex, so that the presence of some amino acid residues arranged, for example, in the N-terminal region of HDPs may be related to their potential activities [55]. In addition, another study suggests that different degrees of helix adopted by LL-37, in the presence or absence of polysorbate 20, in physiological buffer, can differentially modulate the antiviral and antibacterial activity, which may be greater or lesser for viruses or bacteria [56].

LL-37 can be considered as a model peptide to elucidate the relationship between structure and function of primate homologous cathelicidins. The KR-12 (corresponding to residues 18–29 of LL-37), exhibited antibacterial activity without being toxic to human cells [57]. In a later study, it was demonstrated that the substitution of certain residues (Gln5→Lys; Asp9→Ala or Asp9→lys) of KR-12 increased, by up to 8-fold, the spectrum of antibacterial activity, without being toxic to healthy human cells [58]. Another intermediate region (13–31 residues) of LL-37 was identified, through libraries of overlapping sequences, which acts as an active domain that modulates the immune response through TLR. The data suggest that physical-chemical properties such as cationicity and hydrophobicity are essential for the response of this domain [59].

Through the use of a systematic scan allowed alanine residues, of a bioequivalent version of LL-37, to be substituted in order to elucidate which residues are essential for the immunological recognition of U1 dsRNA. From this study, Kulkarni and co-workers (2021) coined the term "innate immune ventting", in which the presence of LL-37 in tissue or even cell culture allows non-inflammatory nucleic acid fragments to become innately pro-inflammatory [60]. These examples cited above demonstrate the potential that HDP LL-37 has for the development of new drugs. However, studies linking activity and function of LL-37 itself and its domains may provide clues regarding the essential (structural) elements for its immunomodulatory activity. In the next topics, several examples will be discussed.

3.1. Structural and functional analyses of LL-37

The enormous structural and functional diversity of cathelicidins

represents a challenging task in understanding the relationship between the structure and function of these HDPs [61]. However, in some recent studies, a domain shared by several α-helical cathelicidins has been reported [62]. Since the discovery of CAP18, from rabbit leukocytes [63], an analogous domain of human LL-37 was detected by oligonucleotides designed from rabbit CAP18 cDNA [24]. This functional domain is a portion of 20 amino acid residues (106−125) from the *C*-terminal region of rabbit LL-37, recognized as an AMP for binding to LPS [63], and although not all cathelicidins have this domain, it is shared by the superfamily member pfam12153 (Fig. 2), in combination with pfam00666 [62].

LL-37, the most studied cathelicidin [64], was submitted to primary structure modifications for activity identification. Studies performed with LL-37 demonstrated that its known activities were completely or partly impaired or altered when the elucidation of another activity is attempted by specific mutations [65]. Something similar was observed for the synthetic peptides IDR-1002 and IDR-HH2, derived from LL-37, when their primary structure was altered [65]. From the substitution of specific residues, the overlapping of fragments of LL-37 was created, which resulted in favoring the immunomodulatory and antibiofilm activity, or vice versa, depending on the fragment [66]. In the same way, some of the multiple activities of LL-37 were altered when its fragments were investigated in order to identify the active regions [64].

The challenge of studying cathelicidins is predicted when the structural diversity is analyzed [67]. Particularly, LL-37 is a molecule involved in numerous physiological processes such as immunomodulatory [68], antibacterial and antibiofilm [16], antiviral [69], antifungal [64], and autoimmune diseases [70], among others [12], leading to tissue homeostasis by regenerative processes, regulating proinflammatory responses, or acting as an inhibitor of cancer progression [71]. It is a molecule able to cause opposing effects simultaneously in different microenvironments in the host organism and resident

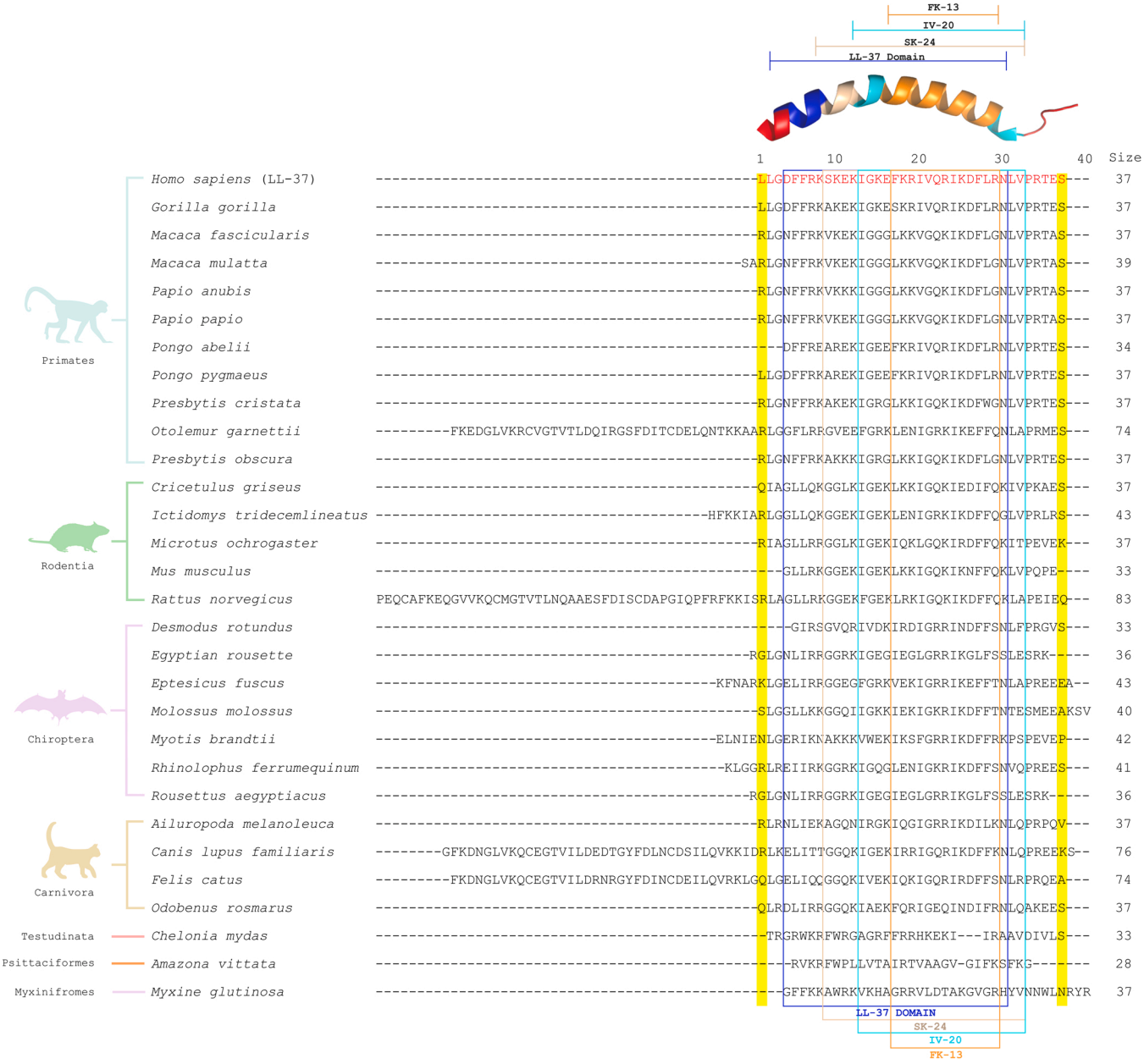

Fig. 2. Alignment of various cathelicidin sequences. At the top right is the three-dimensional structure of LL-37 (PDB: 2K6O). Above it, there are overlapping domains delimited by bars of different colors. On the left of the figure, there are the different species, grouped by Order, in which the cathelicidins were analyzed with LL-37. The colored rectangles in the primary sequences delimit the LL-37 domains discussed in the text.

microorganisms; it has inflammatory and anti-inflammatory activities [68]; it is a molecule that facilitates virulence [72], and simultaneously, it has an antibiofilm capacity [73].

Due to its multifunctional potential, LL-37 could be cataloged as a molecule with diverse capacities in which biological activities are not structurally superimposed. For example, structurally, there is no correlation between antimicrobial and immunomodulating activities [74]. This fact will be hypothesized by the primary structure composition named "chemical space" as a way to explain that the activity landscapes are both independent and overlapping in this molecule type [65]. This is a chemical space that could be determined by a window of some more frequent, but not less outstanding residues (Ala, Arg, Gly, Ile, Leu, Lys, Phe, Trp, Tyr, and Val) among the amino acids.

The challenge is that this amino acid group is shared with the relevant amino acids for antimicrobial activity [75], for example. Particularly, for LL-37, several of these residues are necessary to target bacterial structures [76]. When the primary sequence of LL-37 is analyzed, two principal patterns of structure/function relationship are observed, which are: structural overlapping of activity with the possibility of enhancing one of them by contiguous amino acid substitution; and, when the activities do not structurally overlap [65]. It is therefore not surprising that it is difficult to study the structure/function of cathelicidins.

3.2. LL-37's physicochemical features shared between immunomodulatory and antimicrobial activities

Some studies indicate physicochemical parameters that are important for cathelicidins, such as hydrophobicity, amphipathicity, and cationicity [61,77]. Hydrophobicity, for example, was determinant in LL-37 when it interacted on the receptor. Some points localized on the hydrophobic face of α-helical LL-37 were crucial for scavenger receptor (SR) B1 interaction, but not for the pathogenic U1 RNA ligand [60]. However, the aforementioned cathelicidin features are shared with the general properties of AMPs [78], even with the features of the LL-37 domain [59,64]. The structural conformation of cathelicidins has key features necessary for their diverse activity. The rabbit CAP18 domain, for example, showed a rigid α-helical conformation when interacting by coulombic and hydrophobic forces with diphosphoryl groups and with fatty acyl chains from lipid 2, respectively [79]. Similarly, LL-37 adopts an α-helical conformation and a random *C*-terminal when in contact with hydrophobic environments [64]. This cathelicidin has its hydrophobic path divided by a serine in the ninth position, which is responsible for LL-37/LPS oligomerization [80]. This serine showed to be relevant for LL-37 activity against *E. coli* [81]. Even so, as previously mentioned, LL-37 can adopt helix structuration when in contact with a salt solution [41]. This fact could be responsible for helping the molecular oligomerization between these molecules in physiological conditions. Nonetheless, this advantage could affect both antimicrobial and immunomodulatory activities, as helicoidal structures favor LL-37/LL-37 oligomerization by hydrophobic interactions [82].

Recently, an LL-37 fragment (SK-24) showed a stabilized α-helical conformation with potent antimicrobial activity, superior to that of LL-37. This fragment, included in the LL-37 domain, showed to be the minimal portion of LL-37 for effective oligomerization in PBS [83]. Interestingly, it could be responsible for the ability of LPS-oligomerization, detected in LL-37 [84], to interfere in the immune response. LL-37 interacts with membranes by aromatic (F5, F6, F17, and F27) and cationic residues, which are localized in an amphipathic manner [80]. Specifically, arginine in the 23rd position interacts with phosphatidylglycerols [57,85]. However, the amphipathic α-helical characteristic in LL-37 is not restricted to membrane contact or LPS interaction. A simulation, by docking experiments, of the (IV-20) fragment interacting on FPR-2 receptors showed that it held the helical structure and hydrophobic interactions when contacting the FPR-2 receptor [86].

3.3. LL-37 domain and fragments with immunomodulatory properties

The LL-37 domain has 27 residues and a link to LPSs [24,62]. Usually, cathelicidins are known for acting on several pathogenic ligands such as lipooligosaccharides (LOS), capsular polysaccharides (CPS), lipoteichoic acid (LTA), and ssDNA molecules CpG-ODN, viruses (dsRNA and ssRNA) [77], and host ligands (deleterious DNA) [17], affecting the host immunomodulatory activity. The association between these molecules (LOS, CPS, LTA, dsRNA, ssRNA, and deleterious DNA) and cathelicidins can inhibit or facilitate the interaction of these complexes with cognate cathelicidin receptors [87]. However, it has been well characterized that cathelicidins interact with a wide number of host receptors responsible for immunomodulation [12,77,88,89]. It is surprising that the LL-37 domain can harbor most LL-37 activities. A study detected a minor fragment (IV-20) in LL-37, which is the minimum for FPR-2 receptor interaction [86]. This fragment is a portion located at 13 and 32 residues from LL-37. Previously, this LL-37 portion had been shown to suppress the TLR2/1 and TLR4 receptors' pro-inflammatory activity [59].

Although TLR2/1, TLR4 and FPR-2 receptors were responsible for preventing pro-inflammatory cytokine release and mediating neutrophil activation, respectively [86], those activities are only one part of the functional repertory produced by LL-37 [68]. Nevertheless, IV-20 fragment integrity seems to be the key for LL-37/receptor interaction. In another study, the KR18–37 fragment showed null signaling by TLR3 receptors [90] . Similarly, a minor portion of IV-20, the 17–29 (FK-13) fragment, also lost its immunomodulatory activity [91]. However, the FK-13 fragment was sufficient to interact with MRGX2 receptors, producing degranulation and Ca^{2+} mobilization in human mast cells [91] (Fig. 3). FK-13 showed to be the minimal fragment for inhibiting anti-human immunodeficiency virus type 1 [92]. The 18–29 (KR-12) fragment also showed to be sufficient for antimicrobial activity on *E. coli* [57], and antimicrobial activity with anti-inflammatory activities in vivo [93]. These findings show that most LL-37 activities are contained in the same portion (4–32 residues), as previously explained in the structure/function section.

Interestingly, all fragments with activity on the receptors mentioned are part of the LL-37 domain responsible for interacting with LPS [24,62]. Although studies showed a balance between antimicrobial and immunomodulatory activities of LL-37 and its fragments [74], a common mixture of features of LL-37 govern its diverse activities. Indeed, the antimicrobial activity of LL-37 was maintained, even by the FK-13 fragment [91], and another number of LL-37 fragments [64]. Even so, the activity of LL-37 could be superimposed. It was identified that LL-37 modulates anti-inflammatory responses via TLR4 by membrane interaction [94]. LL-37 could promote the reorganization of host cholesterol-containing membrane domains to prevent TLR4/MD-2 from assembling and later activating the TLR4 receptor [84].

4. LL-37's role in skin diseases

The skin is the first barrier for pathogen invaders. The cutaneous barrier is divided by both structure and function: the epidermis includes the microbiome and chemical and physical barriers; while the dermis shares the physical and the immune barriers [95]. The microbiome barrier is the outermost layer, wherein a diverse community of pathogens predominate [96]. Several skin diseases affect human health, such as acne vulgaris, atopic dermatitis, psoriasis, rosacea, and wound healing. Each one has its molecular disorder particularities, but all HDPs play a role in pros or cons in disease development [26].

Acne vulgaris is a chronic inflammatory disease in the pilosebaceous unit, which is characterized by inflammation, increased sebum production, abnormal keratinization, and colonization with *Propionibacterium acnes* [97]. LL-37, like other HDPs, can aggravate this disease by immune cell recruitment and activation and by the release of proinflammatory cytokines [98]. However, these peptides can kill *P. acnes*

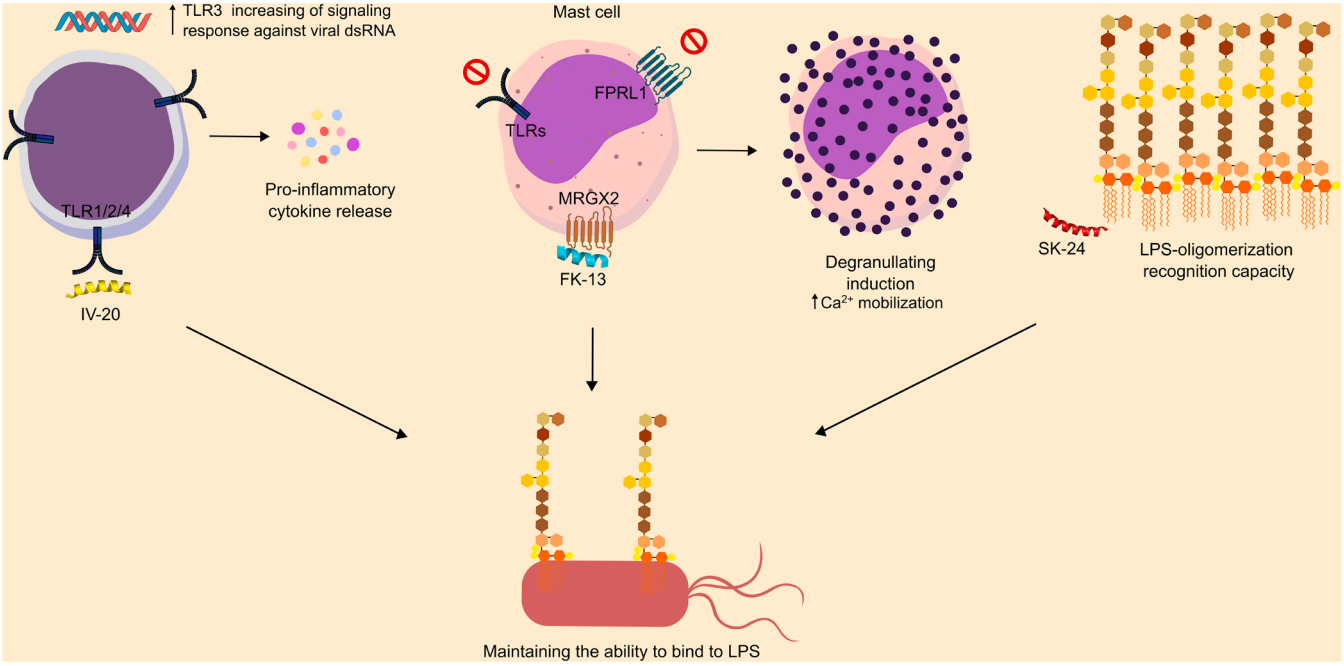

Fig. 3. Schematic representation of the immunomodulatory, pro-inflammatory and LPS-binding activity of domains IV-20, FK-13 and SK-24 derived from LL-37.

and inhibit inflammatory activities stimulated by bacterial ligands [99,100]. Thus, HDPs were proposed as prominent candidates for acne vulgaris [26].

Atopic dermatitis is a chronic inflammatory disorder caused by both immune dysregulation and constant skin alteration [101]. In this disease, LL-37 levels are observed to decrease noticeably [102]. It is noteworthy that LL-37 has relevant functions, such as controlling pathogenic microorganisms. So, the lack of LL-37 could explain the recurrent infections in wound healing related to this disease [26]. Specifically, in atopic dermatitis, LL-37 appears to be a promising candidate because it promotes a reduction in itching, restores the tight junction barrier, and suppresses Th2-mediated inflammation [95]. Unfortunately, LL-37 stimulates inflammatory mediators released from mast cells, which make that molecule disadvantageous for patients with atopic dermatitis if overexpressed [26]. LL-37 interacts with MRGX2 receptors [103].

Psoriasis is a T-cell mediated disease characterized by chronic inflammatory disorder [104]. In psoriasis, LL-37 is over-expressed by keratinocytes after stimulation by pro-inflammatory cytokines [105]. If, on the one hand, LL-37 inhibits the release of inflammatory IL-1β by blocking inflammasome activation [106,107], on the other, it produces inflammation responses promoted by IFN-α production from plasmacytoid dendritic cells [108]. Similarly, LL-37 promotes keratinocyte proliferation [109] and increases type I IFNs in them [107]. Also, LL-37 stimulates immune cell migration [110]. These are multiple activities that lead to disease disorders.

Rosacea is a chronic inflammatory cutaneous disease manifested mainly on the face, which exhibits inflammatory lesions, erythema, and telangiectasia [111]. Unfortunately, the altered and massive expression of LL-37 in this disorder [112] prevents it from being a promising candidate [26]. In this disease, LL-37 and its fragments, generated by kallikrein 5 proteolytic activity [113], have been seen to be relevant to the disease's progress [26].

Wound healing is a complex process of tissue remodeling and repairing, which includes factors such as angiogenesis, granulation tissue formation, hemostasis, inflammation, matrix remodeling, re-epithelialization, and wound contraction [114,115]. In wound healing, LL-37 promotes angiogenesis and vascularization and stimulates the proliferation and migration of immune cells [116]. So, this peptide could favor the wound healing process for patient recuperation, pinpointing LL-37 as promising pharmacological tool [26].

As observed among diverse diseases, LL-37 plays a role in the pros and cons of their development. However, the design of LL-37 fragments could be a determinant for solving some challenges. In particular, to overcome the proinflammatory nature of LL-37, appropriate fragments could be designed. LL-37 fragments that allow an anti-inflammatory and specific antimicrobial activity on *P. acnes* could be a solution to treat acne vulgaris. For atopic dermatitis, a solution could be the use of designed LL-37 fragments, but not FK-13, as previously mentioned, because FK-13 acts on MRGX2 receptors. Meanwhile, for wound healing, due to the pro-inflammatory nature of LL-37, the fragments designed with only anti-inflammatory features are suggested. On the other hand, for psoriasis and rosacea diseases, it is not possible to address any prominent fragment. In psoriasis, this event is due to a lack of clarification about the pro- or anti-inflammatory role of LL-37 [26]. For rosacea, both LL-37 and naturally synthetized fragments favor the disease's progress.

5. Conclusions

The literature highlights the role of cathelicidins interacting on a wide number of targets, but until now, key portions of LL-37 responsible for the LPS ligand and some receptors/interactions can be identified. That LL-37 fragment, whose activities were identified, belongs to the domain initially identified in rabbit CAP18. Although this domain appears to be shared with other cathelicidins, structural and functional features could serve as a basis to understand/research these cathelicidins only in the human receptors researched. The literature shows that there have been intense efforts to identify complex molecular mechanisms that involve immunomodulation by cathelicidins. However, there is a lack of SARs studies to identify these ligand/(pathogenic ligand/receptor) interactions. Exhaustive research will be necessary because of the multiple activities versus complex features found in cathelicidins and the great variety of receptor features. LL-37 has been shown to be a molecule with multifunctional properties allowing its regulatory capacity. It can act directly or indirectly to reach the objective; it shows contrary activities when it activates the pro-inflammatory/anti-inflammatory action and induces virulence/antibiofilm

mechanisms for maintaining homeostasis. Understanding the role of the LL-37 domain in the immunity response is crucial for the clinical application of cathelicidins to treat patients. Identifying, enhancing, or modulating specific activity in LL-37 by specific mutations is challenging, but possible. This, together with the analyses of LL-37 fragments, will allow them to be site-directed to the specific targets, avoiding unwanted activities to treat diseases involved with the immune system.

Conflict of interest

The authors declare that there are no conflicts of interest.

Data Availability

No data was used for the research described in the article.

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From exploring cancer and virus targets to discovering active peptides through mRNA display

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ABSTRACT

During carcinogenesis, neoplastic cells accumulate mutations in genes important for cellular homeostasis, producing defective proteins. Viral infections occur when viral capsid proteins bind to the host cell receptor, allowing the virus to enter the cells. In both cases, proteins play important roles in cancer development and viral infection, so these targets can be exploited to develop alternative treatments. mRNA display technology is a very powerful tool for the development of peptides capable of acting on specific targets in neoplastic cells or on viral capsid proteins. mRNA display technology allows the selection and evolution of peptides with desired functional properties from libraries of many nucleic acid variants. Among other advantages of this technology, the use of flexizymes allows the production of peptides with unnatural amino acid residues, which can enhance the activity of these molecules. From target immobilization, peptides with greater specificity for the targets of interest are generated during the selection rounds. Herein, we will explore the use of mRNA display technology for the development of active peptides after successive rounds of selection, using proteins present in neoplastic cells and viral particles as targets.

Abbreviations: 10Fn3, The 10th type III domain of human fibronectin; ABT, 2-(aminoethyl)-amidocarboxybenzyl thioester; ACE2, Angiotensin-converting enzyme 2; aFx, Amino-flexizyme; AIDS, Acquired immunodeficiency syndrome; Akt kinase family, Protein kinase B; AMPs, Antimicrobial peptides; AXL, Tyrosine-protein kinase receptor UFO; BAX, BCL-2 Associated X, Apoptosis regulator gene; BCL-2, B-cell lymphoma 2; Biotin-Phe-CME, N-biotinylated-phenylalanine-cyanomethyl ester; *C-Myc*, Cellular Myelocytomatosis gene expression; CASP-3, Cysteine-aspartic acid protease 3 (Caspase-3); CASP-8, Cysteine-aspartic acid protease 8 (Caspase-8); CASP-9, Cysteine-aspartic acid protease 9 (Caspase-9); CBT, 4-chlorobenzyl thioester; CCR5, C-C chemokine receptor type 5; CD14, Cluster of differentiation 14 protein; $CD4^{+}T$, T cells with a cluster of differentiation 4 protein; CD81, Tetraspanin (a cell surface protein) - Cluster of differentiation 81; *CDK-4*, Cyclin-dependent kinases-4 gene expression; *CDK-6*, Cyclin-dependent kinases-6 gene expression; cDNA, Complementary deoxyribonucleic acid; CME, Cyano methyl ester; COP1, Constitutional morphogenic protein 1; COVID-19, Coronavirus disease of 2019; *COX-2*, Cyclooxygenase-2 gene expression; CXCR4, C-X-C chemokine receptor type 4; DBE, DiBasic Ester; DC-SIGN, Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; DENV, Dengue virus; dFx, Dinitro-flexizymes; DGEs, Differentially expressed genes; DLC1, Deleted in liver cancer 1protein; DNA, Deoxyribonucleic acid; DNLS, Deregulated *de novo* lipid synthesis; E2s, Ubiquitin-conjugating enzymes; E3, E3 Ubiquitin ligase; E6AP, E6-associated protein; EC_{50}, Half maximal effective concentration; eFx, Enhanced flexizyme; eIF4E, Eukaryotic translation initiation factor 4E; EWI-2, Member of an immunoglobulin subfamily that comprises proteins with a conserved Glu-Trp-Ile (EWI) motif-2; ExoN, Exoribonuclease; FIT, Flexible *in vitro* translation; GAG, Glycosaminoglycan; GBM, Glioblastoma; gp120, Glycoprotein on the HIV envelope number 120; gp41, Glycoprotein on the HIV envelope number 41; GRP78, 78 kDa glucose-regulated protein; GSCs, Glioma stem cells; H1N1, A strain of influenza; HECT, Homologs of the C-terminal domain; HER2, Human epidermal growth factor receptor type 2; HIV, Human immunodeficiency virus; HIV-1, Human immunodeficiency virus type 1; HSP70, Heat shock proteins 70; HSP90, Heat shock proteins 90; HTRF, Homogeneous time-resolved fluorescence; HYNIC, Hydrazinonicotinamide; ICG, Indocyanine green; MCF-7, A breast cancer cell line (Michigan Cancer Foundation-7); MDA-MB-231, A triple-negative breast cancer cell line isolated from M.D. Anderson in Metastatic Breast - 231; MDA-MB-453, A triple-negative breast cancer cell line isolated from M.D. Anderson in Metastatic Breast - 453; MDM2, Murine double minute 2; MERS-CoV, Middle East respiratory syndrome coronavirus; MERTK, Proto-oncogene tyrosine-protein kinase MER; mRNA, Messenger ribonucleic acid; N7-MTase, Guanine-N7-methyltransferase; nAbs, Neutralizing antibodies; NIR-II, Near-Infrared window two; NRPs, Non-ribosomal peptides; nsp14, Non-structural protein 14; NSPs, Non-structural proteins; NTA, Nitrilotriacetic acid; ORF, Open reading frame; p53, Tumor protein with 53 kDa; p53, Protein 53; *P68*, Prototypic multifunctional protein (ATPase and RNA helicase) gene expression; PD-1, Programmed cell death protein 1; PD-L1, Programmed cell death ligand 1; PURE, Protein synthesis using recombinant elements; RaPID, Random nonstandard Peptides Integrated Discovery; RBD, Receptor binding domain; RBM38, RNA binding motif protein 38; SARS-CoV-2, Severe acute respiratory syndrome coronavirus-2; SAvB, Streptavidin-coated magnetic beads; SCD, Stearoyl CoA desaturase; TAM receptors, TYRO3, AXL and MERTK; TAP, Totipotent "all-in-one" peptide; tRNA, Transfer ribonucleic acid; USP38, Ubiquitin-specific peptidase 38; USP7, Ubiquitin-specific peptidase 7; UTR, Untranslated Region; Wnt, Wingless/Integrated; ZIKV, Zika virus; VEGFR-2, Vascular endothelial growth factor receptor 2.

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Contents

1. Introduction

At first glance, cancer and viral infections have nothing in common – and, indeed, the differences run deep. However, from a treatment point of view, knowledge about both families of diseases can be exploited to develop alternative therapies. This is because both present several targets (either because they are present in an overexpressed form on the surface of neoplastic cells, in host cell receptors specific to viruses, or because they have specific target proteins for both diseases). A wide range of upregulated molecular targets (transmembrane proteins) on the surface of neoplastic cells, surrounding stroma, and tumor endothelium have been characterized for tumor cells (Sergeeva, Kolonin, Molldrem, Pasqualini, & Arap, 2006). These receptors could be explored and used in cancer treatment because this disease is the second leading cause of death worldwide (Tran et al., 2022), with approximately 10 million deaths in 2020 alone (Sung et al., 2021).

Another global public health problem is infections caused by viruses. In 2019, the world witnessed a pandemic caused by coronavirus disease (COVID-19), which is provoked by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) (Bakhiet & Taurin, 2021; Kirtipal, Bharadwaj, & Kang, 2020). The human immunodeficiency virus (HIV), responsible for the acquired immunodeficiency syndrome (AIDS), is another important virus with global impacts. The syndrome continues to kill hundreds of thousands per year worldwide, although there has been a decrease in the number of deaths related to HIV (Frank et al., 2019; Ghosn, Taiwo, Seedat, Autran, & Katlama, 2018). Arbovirus, diseases transmitted by hematophagous arthropod bite (Higuera & Ramírez, 2019), such as dengue (DENV), chikungunya (CHIKV) and zika (ZIKV), have been widely studied in recent years due to their global dissemination and epidemiological impacts (Jones et al., 2020), resulting in increased rates of morbidity and mortality (Mercado-Reyes et al., 2019). Every year, approximately 390 million people worldwide are infected with dengue (Kutsuna, Saito, & Ohmagari, 2020). Although the number of deaths is lower when compared to cancer, arboviruses are responsible for a greater number of comorbidities that can last for weeks or even years (Burt et al., 2017). A critical step in the infectious process of viruses involves both recognition and interactions with receptors exposed on the target cell membrane since these receptors play fundamental roles in viral pathogenesis, tissue tropism, and host range (Maginnis, 2018).

Blocking disease-related markers present on cell surfaces (both cancer cells and/or healthy cells), on viruses, as well as other key proteins, can be considered a crucial step for the development of new therapeutic approaches, such as *in vitro* display technologies (Sergeeva et al., 2006). Such approaches involve three main steps for the display and selection of bioinspired peptides: (i) the production of a library (a collection of variants to be tested); (ii) biopanning, which consists of several rounds of enrichment of variants that bind to the desired target (genotype-phenotype linkage); and (iii) the functional screening and characterization of selected variants using appropriate assays (Galán et al., 2016). Currently, there are several types of display technologies, such as virus/phage display (Guliy, Evstigneeva, & Dykman, 2023; Jaroszewicz, Morcinek-Orłowska, Pierzynowska, Gaffke, & Węgrzyn, 2022; Ledsgaard et al., 2022; Zhang, Tang, Chen, & Liu, 2022), (bacterial, yeast, and mammalian) cell surface display (Huang et al., 2020; Shibasaki & Ueda, 2023; Wang et al., 2021), enzyme surface display (Davenport & Hallam, 2022), and *in vitro* display technologies (Ribosomes, mRNA and covalent DNA display) (Sergeeva et al., 2006).

When compared to other display techniques, mRNA display is more advantageous because it provides a powerful means for the selection of proteins and peptides through an effective "reverse translation" (Kamalinia et al., 2020; Wang & Liu, 2011). Although both ribosome display and mRNA display can interrogate very large libraries of peptide variants, the advantage of mRNA display is that the mRNA itself is covalently linked to the peptide, facilitating the selection process, rather than using a ribosome to connect the mRNA and its peptide in a noncovalent ternary complex (Blanco, Verbanic, Seelig, & Chen, 2020). Additionally, when compared to phage display, mRNA display (and its variants such as transcription-translation coupled to puromycin ligand association (TRAP)), which do not require bacterial transformation, produce libraries on the order of 10^{12}–10^{13} diversity, which is somewhat higher than phage display (10^{9}–10^{11}) (Umemoto, Kondo, Fujino, Hayashi, & Murakami, 2023).

mRNA display is a useful technique for developing new therapies against cancer, as well as viral infections. Regarding antitumor treatments, mRNA display can be employed to refine peptides to bind more specifically to important targets (Shiheido, Takashima, Doi, & Yanagawa, 2011). Obviously, mRNA display is widely explored for the development of new peptides with activity against neoplastic lineages through interaction with specific molecular targets (Yang et al., 2015). Through several rounds of enrichment using a 10-amino acid mRNA display library, the H10 peptide was generated. This peptide binds to Anterior Gradient 2 (AGR2), a protein whose level of extracellular (eAGR2) increases, provoking a poor prognosis in cancer patients (Garri et al., 2018). Another example of using mRNA display for the production of new antitumor peptides is the signal peptide-based affinity matured ligand (SPAM) peptide, an 18-residue linear peptide that is nonhomologous to known programmed death ligand 1 (PD-L1). PD-L1 is a pivotal immune checkpoint ligand that provides an escape mechanism from immune surveillance when overexpressed in the cells (Kamalinia et al., 2020).

Regarding the use of mRNA display for the treatment or inhibition of viral infection, studies focus on specific proteins on the viral surface (Tanaka et al., 2022). Furthermore, it is possible to combine the mRNA display technique with others, such as high-throughput sequencing (mRNA-HST), to identify peptide mimotopes that play a crucial role in neutralizing monoclonal antibodies (mAb) (Guo et al., 2015). Another advantage of using this technique is the possibility of examining protein-protein interactions (PPIs). By utilizing the mRNA display method with a uniform distribution library (md-LED), researchers were able to gain insight into the interactions of the influenza virus NS1 protein (Du et al., 2020). Consequently, in addition to selecting

specific peptides for important targets, mRNA display enables the study of PPIs, which can be further explored to construct protein-protein interaction networks in massively parallel experiments (Du et al., 2020). Throughout this review, we will address mRNA display technology for the treatment of diseases such as viral infections and several types of cancer, targeting important receptors and membrane proteins, both in the process of carcinogenesis and in the process of virus entry into host cells, as well as other proteins related to diseases.

2. mRNA display technology

Although the mRNA display was first reported in 1997 (Nemoto, Miyamoto-Sato, Husimi, & Yanagawa, 1997; Roberts & Szostak, 1997), it was the contributions made by David Wilson, Anthony Keefe, and Jack Szostak, in 2001 (Wilson, Keefe, & Szostak, 2001), that led to true revolutions in the selection and evolution of peptides and proteins (Gold, 2001). mRNA display is a robust *in vitro* experiment similar to combinatorial chemistry that allows the selection of peptides and proteins with desired functional properties from libraries of trillions of nucleic acids (DNA and RNA) variants (Fig. 1) (Liu, Barrick, Szostak, & Roberts, 2000; Morioka, Loik, Hipolito, Goto, & Suga, 2015; Newton, Cabezas-Perusse, Tong, & Seelig, 2020). The main characteristic of this method is that the nascent polypeptide chain remains covalently linked to the 3' end of its coding mRNA, allowing for the direct amplification of each protein (Golynskiy & Seelig, 2010; Wang & Liu, 2011). The protein-mRNA covalent complex is formed through puromycin, an antibiotic protein synthesis inhibitor, by mimicking the ribosome substrate (the 3' end of an aminoacyl-tRNA) (Galán et al., 2016; Huang, Wiedmann, & Suga, 2019).

mRNA display technology comprises several different steps. It begins with an acquisition of a library of random DNA sequences from different sources (either chemically synthesized or of genomic origin) (Keefe, 2001). In the first step of the process, the DNA library is converted into mRNA through transcription by the RNA polymerase enzyme (Golynskiy & Seelig, 2010). Then, each of the mRNA molecules is enzymatically linked to a synthetic spacer, which consists of a DNA strand containing puromycin at its 3' end (second step) (Nemoto et al., 1997; Roberts & Szostak, 1997). Puromycin mimics the aminoacyl portion of the transfer RNA (tRNA) and, during the *in vitro* translation process, enters the site called "A" of the ribosome, attaching itself *via* a peptide bond to the nascent polypeptide chain (Wilson et al., 2001). After the incorporation of puromycin at the *C*-terminus of the peptide, a phenotype

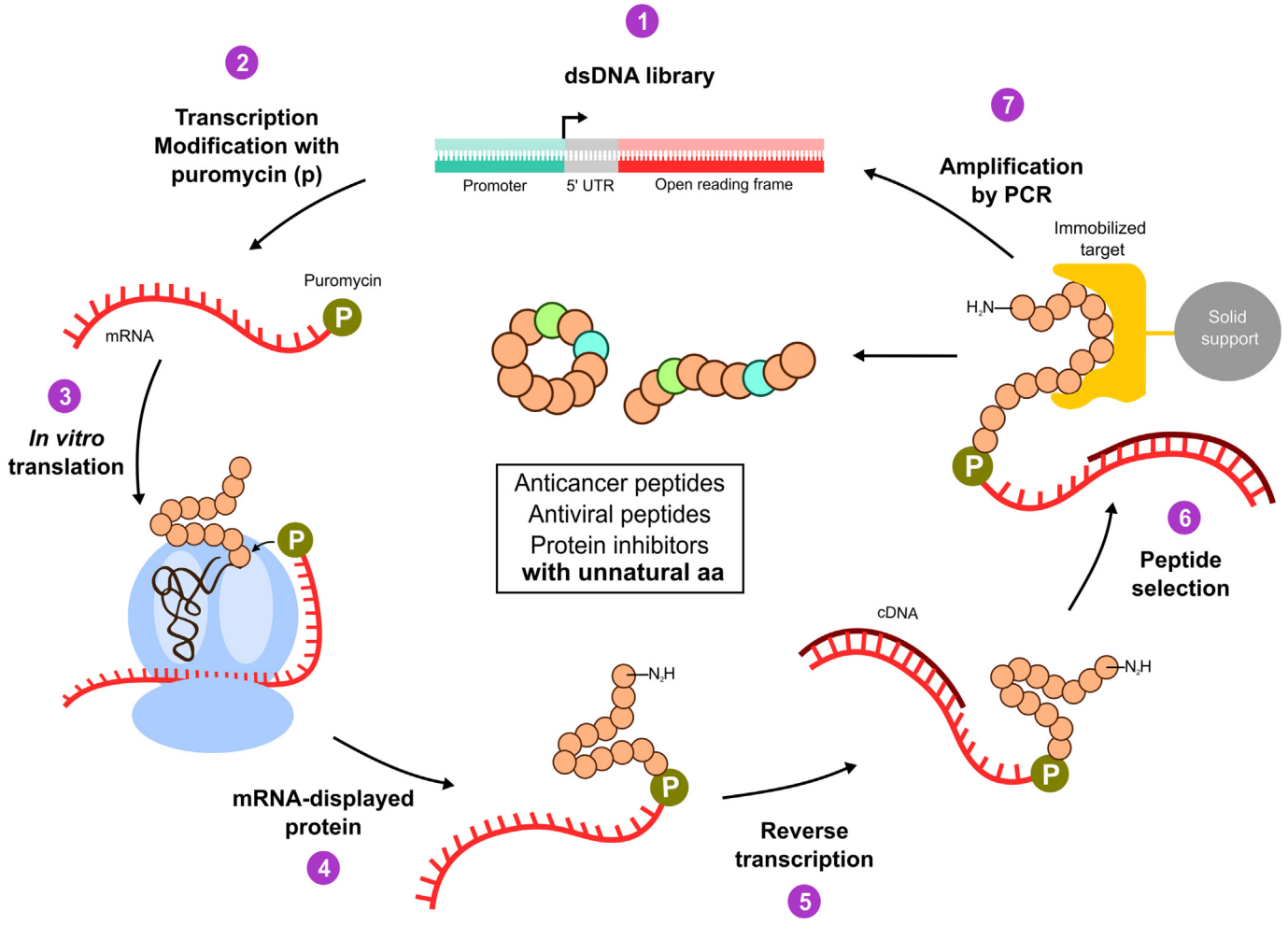


Fig. 1. Formation of a mRNA-displayed protein and mRNA display cycle. (1) dsDNA library. DNA library is obtained from biological extraction or synthetic methods, and modified for insertion of promoter in 5' end. (2) Transcription and ligation. DNA is amplified and transcribed to start the cycle. mRNA pool is obtained and modified with puromycin at its 3' end. (3) *In vitro* translation. After finishing reading the mRNA-ORF, the ribosome stops at the poly(dA) junction, where the modified tyrosine of puromycin is recognized by the ribosome, which catalyzes the transfer of the nascent peptide to the non-hydrolyzable modified tyrosine of puromycin at the 3' end of the mRNA display template. (4) mRNA-displayed protein. The mRNA is now covalently attached to the corresponding translated peptide *via* the puromycin, having obtained a stable molecular conjugate referred to as an mRNA–peptide fusion. (5) Reverse transcription. cDNA is produced to stabilize the mRNA by complementarity. (6) Selection. It is the process of immobilization of nascent proteins, based on interaction with the target, with subsequent isolation. (7) Amplification and mutagenesis. cDNA is amplified and some variations can be made by exchanging base pairs in the sequence to start the cycle again. This figure was created using Inkscape, version 1.3.0.

(peptide)-genotype (mRNA) connection is created, facilitating the selection and evolution of biomolecules of desired interest (Peacock & Suga, 2021). When compared to ribosome display, the mRNA-puromycin conjugates are more stable due to the covalent link between the mRNA coding and the displayed protein (Nemoto et al., 1997; Roberts & Szostak, 1997).

Both cell lysate and protein synthesis using recombinant elements (PURE system) translation systems, as well as other reconstituted mixtures of individually purified components, can be used for the *in vitro* translation reaction (third step) (Peacock & Suga, 2021). In the fourth step, the library preparation is completed. Puromycin-conjugated mRNA is commonly reverse-transcribed to generate a library of molecules containing the mRNA/cDNA-puromycin-peptide. Complementary DNA (cDNA) acts to protect the mRNA against non-specific interactions that may occur during the polypeptide selection process (Wilson et al., 2001). Isolation of high-affinity ligands through repeated attempts at using mRNA display has resulted in the production of several combinatorial or semi-combinatorial peptide libraries (Jaroszewicz et al., 2022). In the last step of mRNA display, the *in vitro* peptide selection takes place through interaction tests with immobilized targets (Wilson et al., 2001).

All these steps, from the DNA library transcription into mRNA, through its binding with puromycin, *in vitro* selection against immobilized targets to cDNA production (if done after the selection of the peptides), are commonly referred to as a round of selection (Josephson, Ricardo, & Szostak, 2014), which can be repeated many times to produce more specific molecules. The mRNA display technique makes it possible to incorporate unnatural amino acids into the polypeptide chain, increasing the diversity of molecules that can be generated (Huang et al., 2019).

2.1. Diversifying peptides by inserting unnatural amino acids

In some fields of importance, when compared to peptides and small molecules, peptides indicated high activity and selectivity, low toxicity, and no accumulation, even though they are unstable in biological fluids due to rapid degradation by proteinases (Sabatino et al., 2016). Cyclic peptides have been considered advantageous in combating challenging targets where interactions such as protein-protein, protein-nucleic acid, and transcription factors occur. Cyclic peptide structures with at least 12 atoms have been classified as macrocyclic and have been observed to provide an appropriate organization for the radical groups, as well as allowing for a semi-rigid structure for the scaffolding of this molecule type. These characteristics enable macrocyclic peptides to adopt specific conformations against targets, granting them affinity for biological receptors, as well as resistance to protease degradation. Moreover, when compared to small molecules, macrocyclic peptides possess the therapeutic benefits of larger biomolecules, including high potency, reasonably low selectivity, and low production costs (Huang et al., 2019). Another category of peptides is antimicrobial peptides (AMPs), which are produced by the innate system of living organisms. AMPs have been employed as multifunctional molecules due to their reported activities. (Scavello, Amiche, & Ghia, 2022).

Both types of peptides could be explored as a starting point for the search for peptides against therapeutic targets for diseases such as cancer and viral infections. The exploration of cancer and virus targets using methodologies such as mRNA display, and the utilization of DNA or RNA sources that encode AMPs, can be useful in generating cyclic and acyclic peptides. In such peptides, structural modifications at the level of modified side chains, *N*-methylated amino acids, and the configuration of the alpha-carbons will allow the generation of highly diverse peptide libraries for drug discovery. The introduction of amino acids with modified chains, containing functional groups that react with each other, is a benefit of mRNA display technology as it enables the production of cyclic peptides through a spontaneous formation step prior to the selection process (Huang et al., 2019). Thus, the formation of non-biological polymers (with unnatural amino acids) represents a significant advantage as it greatly expands the range of potential ligand targets, as well as the biological stability of these molecules (Sergeeva et al., 2006).

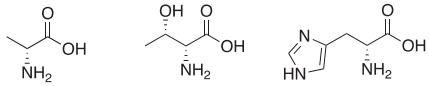
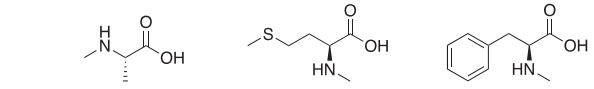
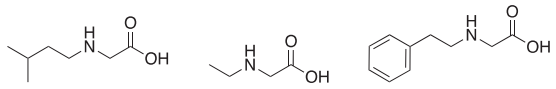
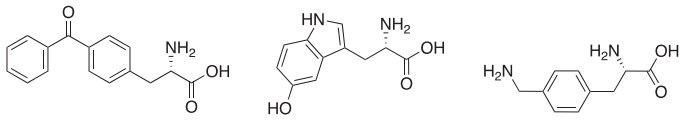
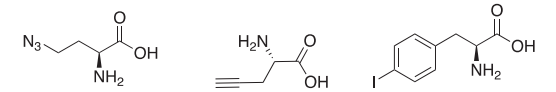
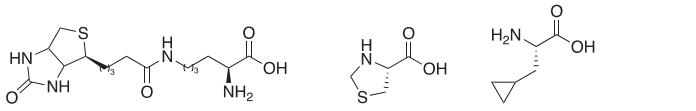
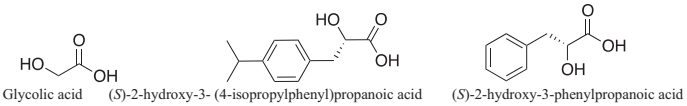
Since 2003, several amino acid residues have been introduced to the mRNA display platform, including modified α-amino acids (*D*-stereochemistry, *N*-acyl, and *N*-alkyl), as well as β-amino acids and γ-amino acids (Murakami, Kourouklis, & Suga, 2003). Additionally, other amino acids such as α-hydroxy acids, α-mercapto acids, and thio acids have successfully been incorporated into translated peptides using the Flexible *in vitro* Translation (FIT) System (Tables 1 and 2). This system utilizes flexizymes integrated with a custom-made *in vitro* translation apparatus, enabling genetic code reprogramming (Goto, Katoh, & Suga, 2011).

In the mRNA display methodology, flexizymes are a class of ribozymes (flexible ribozymes) that can incorporate unnatural amino acids into tRNAs. Through a catalyzed reaction, they can aminoacylate the 3' end of the tRNA by reading the corresponding anticodon. Flexizymes tolerate a plethora of different tRNAs as an aminoacyl-acceptor, as well as an amino benzyl ester as an aminoacyl-donor part (Murakami et al., 2006; Ohuchi, Murakami, & Suga, 2007). The production of aminoacyl-tRNAs containing unnatural amino acids can be done in an *in vitro* selection step, and this complex (aminoacyl-tRNAs) can be used so that the ribosome can add the respective unnatural amino acids in the translation process. This new tRNA aminoacylation tool is an advantage for the methodology of peptide synthesis in cell-free translation systems. This innovation in the form of *in vitro* translation reprogramming, which uses flexizymes, was developed by Suga and colleagues in 2006 (Murakami et al., 2006). They applied an *in vitro* selection, not precisely to select proteins, but to select mRNA sequences that functioned as flexizymes and incorporated unnatural amino acids in the 3' end of the same sequence, which resulted in selective hydrolysis of the mRNA at the corresponding aminoacyl-tRNA (Ohuchi et al., 2007).

After generating selection cycles, the authors were able to produce flexizymes highly capable of mediating the aminoacylation of tRNAs. This was done by creating an open reading frame (ORF), in which the 5' region contained 70 nucleotides randomly, and from there, the end of the 3' region had a tRNA sequence. For the selection cycles, the reaction between the mRNA pool and the *N*-biotinylated-phenylalanine-cyanomethyl ester (Biotin-Phe-CME) was performed. After its aminoacylation reaction, which was performed using a streptavidin resin, the first generation of flexizymes (r24mini) was generated. r24mini was used for randomization evolution to obtain a new ribozyme with lower substrate selectivity but high aminoacylation activity, obtained through a cycle of mutagenesis and the exchange of the end of the 3' region for a microhelix. In this way, they were able to obtain aminoacylation in the presence of δ-(*N*-biotinyl)amino-α-(*S*)-hydroxybutanoic acid DBE (DiBasic Ester), giving rise to dinitro-flexizymes (dFx) (Ohuchi et al., 2007). Then followed the enhanced flexizyme (eFx), which favors Cyano Methyl Ester (CME)-activated amino acids with an aromatic group or bulky hydrophobic group close to the carbonyl site or 4-chlorobenzyl thioester (CBT)-activated general amino acids; and the amino-flexizyme (aFx) that prefers 2-(aminoethyl)-amidocarboxybenzyl thioester (ABT)-activated hydrophobic amino acids. The dFx, eFx, and aFx can undergo aminoacylation in all tRNAs with a 3′-terminal CCA motif when provided with a suitable amino acid substrate (Fig. 2).

The first flexizyme (r24mini) generation showed the function of *cis*-regulation (intramolecular action), but also *trans*-regulation (intermolecular action) on the ribosome. Because the generation showed *trans*-regulation, it cannot be used as a versatile catalyst because it prefers the particular tRNA used in selection. However, dFx came to accept a wide range of DBE esters, from unnatural α-*L*-amino acids, α-*N*-methyl *L*-amino acids, α-*N*-acyl *L*-amino acids, α-*D*-amino acids, β-amino acids, as well as α-hydroxy acids. This made dFx useful for choosing, in an almost unlimited number of ways, amino acids in any desired tRNAs (Ohuchi et al., 2007). In this way, the authors were capable of

Table 1
Some examples of unnatural amino acids incorporate into the nascent peptide through the FIT System in mRNA display technology. DBE (DiBasic Ester), CME (CyanoMethyl Ester), and CBT (4-ChloroBenzyl Thioester) are the leaving groups used in the autocatalytic aminoacylation reaction.

Type of unnatural amino acids	Structure and name	Leaving group	References
D-Amino acids	 *D*-alanine *D*-threonine *D*-histidine	DBE/CME/CBT	(Goto, Murakami, & Suga, 2008; Murakami, Ohta, Ashigai, & Suga, 2006)
N-Methyl amino acids	 *N*-methyl alanine. *N*-methyl methionine *N*-methyl phenylalanine	DBE/CME/CBT	(Kawakami, Murakami, & Suga, 2008a)
N-substituted glycines	 *N*-isopentyl glycine *N*-ethyl glycine *N*-phenethyl glycine	DBE	(Kawakami, Murakami, & Suga, 2008b)
Aromatics and heterocycles	 *p*-benzoyl phenylalanine 5-hydroxy-tryptophan. 4-aminomethyl-phenylalanine	CME	(Murakami et al., 2003; Yamagishi, Ashigai, Goto, Murakami, & Suga, 2009)
Reactive groups	 *L*-azidohomoalanine Propargylglycine *p*-iodo-*L*-phenylalanine	DBE or CME	(Murakami, Ohta, Ashigai, & Suga, 2006; Sako, Morimoto, Murakami, & Suga, 2008)
Aliphatics	 *ε*-*N*-biotinyl-lysine *L*-thiazolidine-4-carboxylic acid (*S*)-2-amino-3-cyclopropylpropanoic acid	DBE or CME	(Kawakami, Ishizawa, & Murakami, 2013; Hartman, Josephson, Lin, & Szostak, 2007; Murakami, Ohta, Ashigai, & Suga, 2006)
α-hydroxy acids	 Glycolic acid (*S*)-2-hydroxy-3- (4-isopropylphenyl)propanoic acid (*S*)-2-hydroxy-3-phenylpropanoic acid	DBE/CME	(Murakami, Ohta, Ashigai, & Suga, 2006; Ohta, Murakami, Higashimura, & Suga, 2007)

synthesizing unnatural peptides using flexizymes in a cell-free translation system, reallocating the codons of natural amino acids to unnatural amino acids, and directing peptide synthesis at will.

Taking advantage of flexizymes' ability to add unnatural amino acids, Yamagishi and colleagues in 2011 presented a new way of synthesizing macrocyclic peptides with *N*-methylated amino acids using translation machinery through the reprogramming of the genetic code (Yamagishi et al., 2011a). Coupled with mRNA display, this was called the Random nonstandard Peptides Integrated Discovery (RaPID) system. This system allows the rapid selection of strong ligands in an arbitrarily chosen therapeutic target.

The *in vitro* selection step can be divided into two categories. The first is selection by catalysis, which is of great interest because the chemistry of molecular recognition and catalysis in living systems is largely done by polypeptides. In this category, peptides and/or proteins generated in fusion are separated by interaction with fixed molecular targets in a matrix (Keefe, 2001), in addition to having information about their biological functionality. It is common for peptides and/or proteins isolated *via* mRNA display to maintain the binding property with targets in a similar way to when they are in the fused molecule (Liu et al., 2000), which makes this methodology a fast and powerful tool for the screening of biologically active polypeptides when there is no *in vivo* selection strategy, or it is too difficult to design. However, if the objective is the synthesis of biologically active peptides, a disadvantage in selection is that mRNAs may interact to form aptamers with the target proteins, rather than the fused-generated proteins or peptides (Liu et al., 2000), This selection by aptamers constitutes the second category of *in vitro* selection. To reduce this problem in the search for catalytic interactions made by peptides, it is convenient to make a previous selection before cDNA synthesis, thus having information about the formation of mRNA aptamers (Liu et al., 2000).

Selection by catalysis is faster and easier to perform in the laboratory (Keefe, 2001), and is the most widely used category to detect active cyclic peptides. For large fused proteins, which may have enzymatic activity, it has been established that the general scheme for selection by catalysis is bond formation, where the reaction product formed is the sole criterion of the bond formed, allowing the isolation of new activities without knowledge of the reaction mechanism. It can also be modified to look for enzymes that catalyze bond-breaking reactions or products, or reactions that involve covalent modifications (Seelig, 2011).

For low molecular mass peptides, the purpose of selection is to separate sequences that catalyze a reaction from those that do not. One way to achieve separation is to arrange selection so that library members that catalyze the desired reaction covalently attach to the substrate once the substrate is covalently attached to a tag (such as biotin). Therefore, the tag can be used as a basis for separating library members that do not catalyze the reaction by immobilization, for example, employing

Table 2
Cyclopeptides containing natural and unnatural amino acids with anticancer or antiviral activity identified by mRNA display technology.

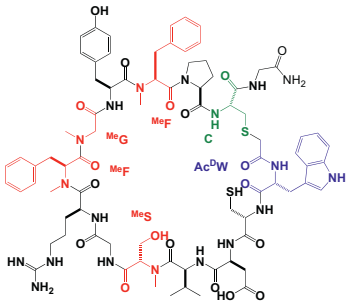
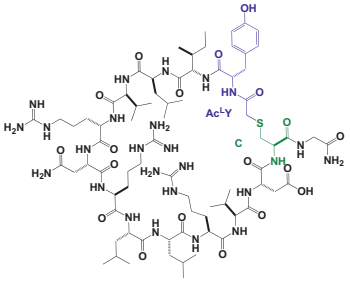
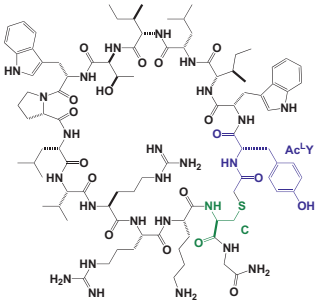
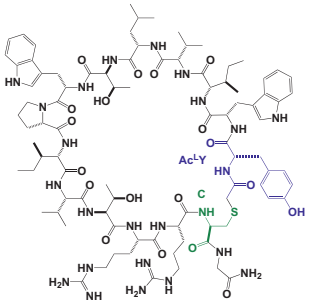
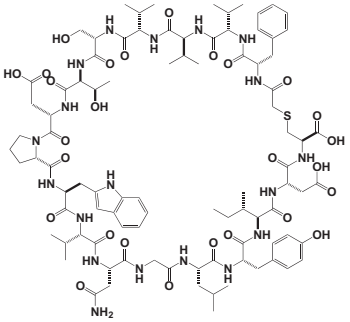
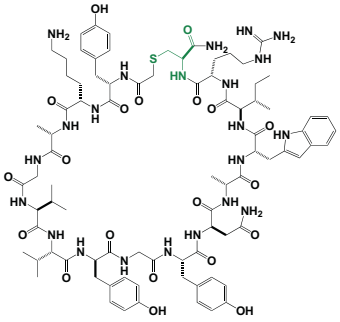
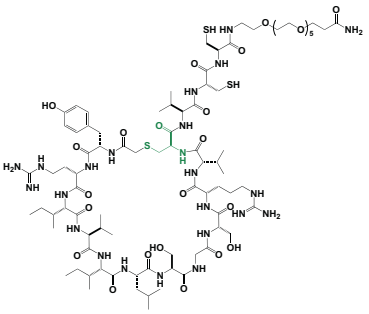
Peptide name	Structure	Target	References
CM_{11}-1		The peptide bound E6AP and inhibited polyubiquitination in cell lysates	(Yamagishi et al., 2011a)
Pakti-L1		Selective inhibitor of Akt2 isoform	(Hayashi, Morimoto and Suga, 2012)
Pakti-L2		Inhibitor of Akt1/2/3	(Hayashi, Morimoto and Suga, 2012)
Pakti-L3		Inhibitor of Akt1/2/3	(Hayashi, Morimoto and Suga, 2012)
L1		Inhibitor of VEGFR-2	(Kawakami et al., 2013)

Table 2 (*continued*)

Peptide name	Structure	Target	References
Cyclic peptide 4		Bind to RBD of S-glycoprotein	(Norman et al., 2021)
Cyclic peptide 5		Bind to RBD of S-glycoprotein	(Norman et al., 2021)

C: Cysteine, ^{Me}F: *N*-methyl-*L*-phenylalanine, ^{Me}G: *N*-methyl-*L*-glycine, ^{Me}S: *N*-methyl-*L*-serine, Ac^{D}W: *N*-(acetyl)-*D*-tryptophane, Ac^{L}Y: *N*-(acetyl)-*L*-tyrosine.

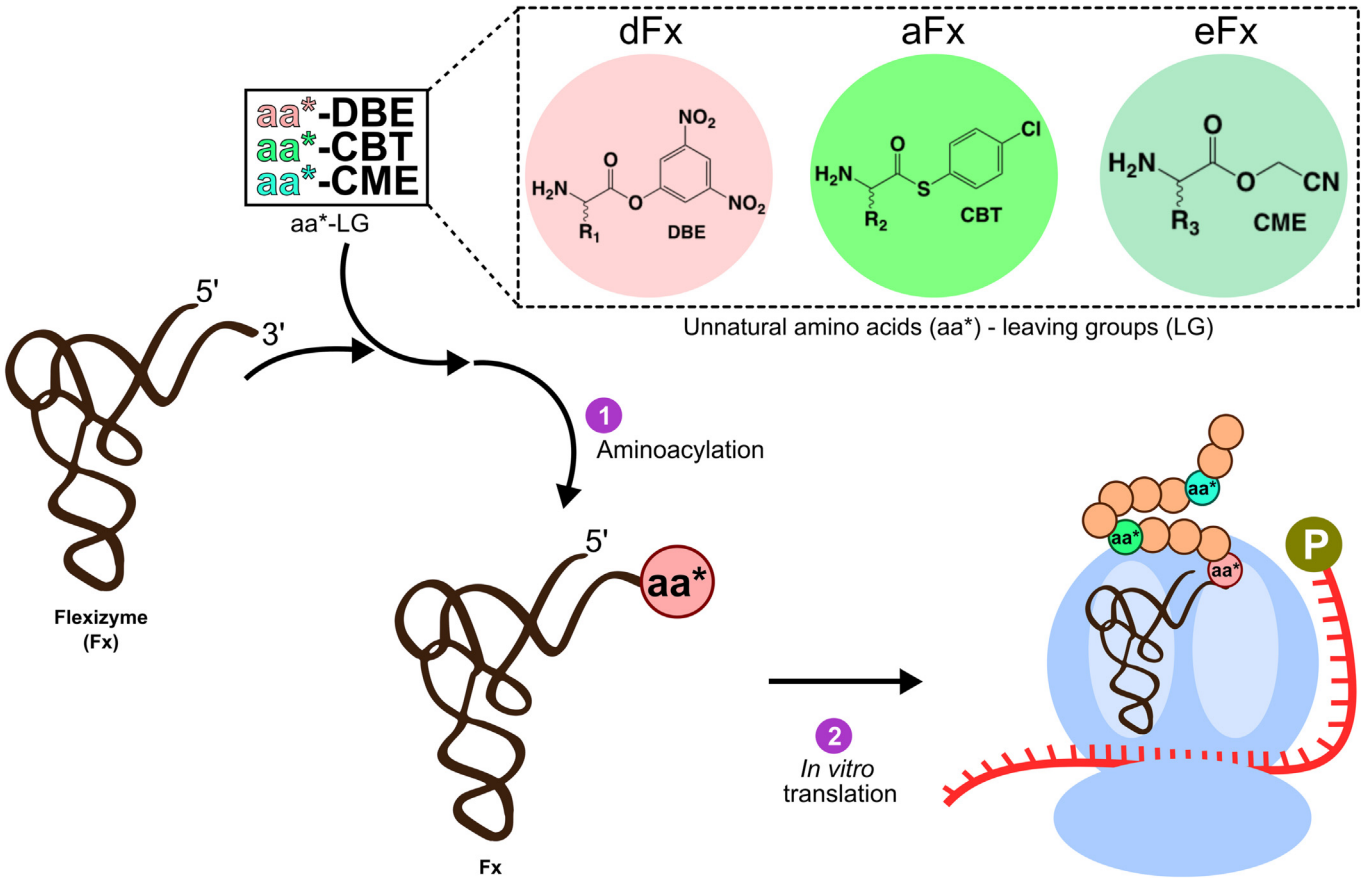


Fig. 2. Flexizyme autocatalytic aminocylation reaction and amino acid incorporation into the peptide growing on the ribosome. (1) Aminoacylation. Flexizyme-catalyzed aminoacylation using as substrate aa*-DBE, aa*-CBT or aa*-CME to give charged tRNAs. DBE (DiBasic Ester), CME (CyanoMethyl Ester) and, CBT (4-ChloroBenzyl Thioester) are the leaving groups in the substrate. (2) *In vitro* translation. aa*-tRNAs in combination with the machinery of translation and allowing peptide mRNA display libraries production. This figure was created using Inkscape, version 1.3.0.

affinity chromatography (binding biotin to an immobilized streptavidin) (Lipovsek & Plückthun, 2004). Substrate immobilization before incubation with the library can also be done in an alternative manner. Other mechanisms can be the one in which the entire library is immobilized, and only those that manage to catalyze a specific reaction can be released, isolating them in the free form (Keefe, 2001). After the recovery or production of cDNAs, depending on the case, they are amplified by PCR, where the amplification reaction can generate errors and, therefore, new sequences that can be used to increase the diversity of the library (Lipovsek & Plückthun, 2004).

3. Biological sources of nucleic acids with potential use in mRNA display

In 2005, griffithsin (GRFT) was isolated from red algae, *Griffithsia sp.*, found in New Zealand coastal waters (Lee, 2019; Mori et al., 2005). It is a dimeric protein of 121 amino acid residues (~13 kDa), with a domain-switched structure (Ziółkowska & Wlodawer, 2006). The cyanovirin-N (CV-N) lectin, which has 101 amino acid residues in its primary structure (~11 kDa), was isolated in 1997 from a culture of cyanobacteria (*Nostoc ellipsosporum*) (Botos et al., 2002; Boyd et al., 1997). Scytovirin (SVN) is a protein composed of 95 amino acid residues (9.7 kDa), which was isolated from aqueous extracts of the cyanobacteria *Scytonema varium* (Bokesch et al., 2003). GRFT, CV-N, and SVN have antiviral activity (EC_{50}) against HIV-1 when tested at 0.04, 0.1, and 0.3 nM, respectively (Balzarini, 2006). Therefore, the genome of marine algae and cyanobacteria can be a promising source for the production of DNA libraries to be explored through the mRNA display technique.

Proteins from the viral envelope are highly glycosylated, with approximately half of the total molecular mass of gp120 consisting of *N*-glycans, with a small and variable contribution of *O*-glycans (Huskens & Schols, 2012). Lectins interact with portions of these carbohydrates in different ways, which inhibits the binding between the virus protein and the host cell's plasma membrane receptors (Botos & Wlodawer, 2005; Koharudin & Gronenborn, 2014). In recent years, several lectins, including GRFT, CV-N, and SVN, have been isolated from cyanobacteria and red algae (Li, Zhang, Chen, Wei, & Chen, 2008). The native and recombinant forms of these lectins have broad antiviral activity against both laboratory-adapted primary isolates and strains of HIV-1 (Alexandre et al., 2012).

Azurin is a 14 kDa redox-active protein containing copper secreted by the bacterium *Pseudomonas aeruginosa* (Huang et al., 2020; Nar et al., 1992; Yaghoubi et al., 2020). Recently, this protein has aroused interest in the development of new treatments (Gammuto, Chiellini, Iozzo, Fani, & Petroni, 2022), since the penetrating peptide Azurin-p28, a sequence made up of 28 amino acids (Leu50-Asp77) of cupredoxin azurine, demonstrated strong anticancer activity in clinical trials (Warso et al., 2013). This peptide binds to the p53 protein (both wild-type and mutant) inhibiting its ubiquitination, mediated by COP1, preventing proteasomal degradation (Lulla et al., 2016). Furthermore, molecular docking analyses and dynamic simulations attribute an antiviral activity to azurin and its azurin-p28 derivative against SARS-CoV-2 (Sasidharan et al., 2021), and H1N1 influenza (Sasidharan et al., 2021). There are still many other proteins and peptides that can be prospected from bacteria such as bacteriocins, enzymes (including arginine deiminase and *L*-asparaginase), and non-ribosomal peptides (NRPs) (Karpiński & Adamczak, 2018).

Another promising source for obtaining DNA libraries is the skin secretions of frogs because, in recent decades, several peptides with antitumor potential have been identified from them. Brevinin-1RL1 is a peptide of 24 amino acids isolated from *Rana limnocharis,* which shares a 7-member ring intramolecular disulfide bridge domain located at the *C*-terminus. This peptide has preferential cytotoxicity against neoplastic cells, inducing caspase-dependent necrosis and apoptosis (Ju et al., 2021). HN-1, a host defense peptide produced by *Amolops hainanensis*, is capable of inhibiting local tumor growth and metastases in a xenograft human breast tumor model (Qiao et al., 2019). Although many peptides produced by these organisms act by destabilizing the plasma membrane, such as dermaseptin-PS1 from *Phyllomedusa sauvagei* (Long et al., 2019), exploring the genes and specific sequences of amino acids present in these animals may allow the development of new active molecules against cancer and viruses, through mRNA display. Table 3 summarizes these peptides and other, active agents against cancer and virus targets.

Recently, it was reported that ubiquitin-specific peptidase 38 (USP38) represses ZIKV infection by binding to the envelope protein (E) *via* its *C*-terminal domain and attenuating its K48-linked and K63-linked polyubiquitylation (Wang et al., 2021). By binding to the E protein, the deubiquitinase gene USP38 can be used to produce a mRNA display library, aiming not only to inhibit ZIKV but also DENV, since both use the E protein to invade host cells.

4. Peptides libraries and molecular targets for the development of alternative cancer treatments

Despite the development of new treatments, cancer remains a leading cause of death worldwide, and the development of new therapies for its treatment is an area of intense research. Peptides have been considered a promising class of molecules with anticancer activity due to their ability to selectively target cancer cells and induce cell death. Libraries of peptides generated by mRNA display have been used to identify peptides with anticancer activity against a variety of cancer types. Several pivotal targets for cancer are discussed below and represented in Fig. 3.

An example is the macrocyclic *N*-methyl-peptide that was selected as an anti-E6AP, and these are strong inhibitors of polyubiquitination of proteins such as p53. The p53 protein is an important protein that acts as a key transcription factor in the correction of cancer-related mutagenesis. Proteasomes are the enzymatic complexes responsible for the degradation of proteins. This system is regulated by the enzymatic catalysis of the enzymes ubiquitin-E1 activator, ubiquitin-conjugative E2, and ubiquitin E3 ligase. Many ubiquitin E3 ligase families are responsible for the specific conjugation of polyubiquitin to a given protein (*e.g.*, the protein p53), leading to its proteolysis. Faulty regulation of protein proteolysis is, in many cases, due to the E3 ligase family, causing failures in signal translation, which can develop into disorders such as cancer. Homologs to the C-terminal domain of E6AP (HECT) belong to the E3 family, and its *N*-terminal domain interacts with the E6 protein, forming the E6AP-E6 complex, which provides the specific activity of E3 ligase, transferring polyubiquitins to p53 and promoting its degradation in an E6-dependent pathway. The E6-independent pathway also promotes the degradation of tumor-associated proteins (Yamagishi et al., 2011a).

From the arbitrary reallocation of codons from natural to unnatural *N*-methylated amino acids, associated with the reconstituted *E. coli* cell-free system and the use of flexyzymes, it was possible to generate an mRNA model capable of producing fusion peptide libraries used in a later selection step. In the selection step of the RaPID system, the HECT domain of E6AP was proposed as a biological target against the fused-generated peptides. The HECT domain was expressed as Avi-$(His)_6$-GB1-HECT, and the *N*-terminal part was biotinylated and immobilized with streptavidin-coated magnetic beads (SAvB) (Yamagishi et al., 2011a).

The selection made it possible to identify a library of *N*-methylated peptides of high complexity, quickly and reliably, due to the randomness of unnatural residues, in conserved regions. The similarity in composition, location, and type of *N*-methylated residues in the sequences suggests that there are specific interactions between the residues and the HECT domain of E6AP. The kinetic and dissociation constants of some selected peptides were calculated, finding a strong and marked activity against HECT; one of them, CM_{11}-1 (Table 2), containing

Table 3
Some anticancer and antiviral peptides, identified in various ways, act on targets related to cancer and viruses.

Peptide	Source	Amino acids sequences	Secondary structure	Activity	Biological target	References
Griffithsin (GRFT)	*Griffithsia* sp	XSLTHRKFGGSGGSPFSGLSSIAVRSGS YLDAIIIDGVHHGGSGGNLSPTFTFGS GEYISNMTIRSGDYIDNISFETNMGR RFGPYGGSGGSANTLSNVKVIQING SAGDYLDSLDIYYEQY	Predominantly β-sheet	Antiviral (HIV-1)	High-mannose oligosaccharides in gp120 protein of HIV-1	www.rcsb.org; (Balzarini, 2006; Lee, 2019)
Cyanovirin-N (CV-N)	*Nostoc ellipsosporum*	LGKFSQTCYNSAIQGSVLTSTCERTN GGYNTSSIDLNSVIENVDGSLKWQP SNFIETCRNTQLAGSSELAAECKTRA QQFVSTKINLDDHIANIDGTLKYE	Predominantly β-sheet	Antiviral (HIV-1)	High-mannose oligosaccharides in gp120 protein of HIV-1	www.rcsb.org; (Balzarini, 2006; Botos et al., 2002)
Scytovirin (SVN)	*Scytonema varium*	GSGPTYCWNEANNPGGPNRCSNN KQCDGARTCSSSGFCQGTSRKPDPG PKGPTYCWDEAKNPGGPNRCSNSK QCDGARTCSSSGFCQGTAGHAAA	Mixture	Antiviral (HIV-1)	High-mannose oligosaccharides in gp120 protein of HIV-1	www.rcsb.org; (Balzarini, 2006)
Azurin-p28	*Pseudomonas aeruginosa*	LSTAADMQGVVTDGMASGLDKD YLKPDD	α-helix	Anticancer	Binds to p53 and inhibits COP1 binding	www.rcsb.org; (Lulla et al., 2016; Nar et al., 1992; Warso et al., 2013)
Brevinin-1RL1	*Rana limnocharis*	FFPLIAGLAARFLPKIFCSITKRC	Cyclic peptide with intra-molecular disulfide-bridged	Anticancer (Induced tumor cell proliferative inhibition)	Activation of caspases	(Ju et al., 2021)
HN-1	*Amolops hainanensis*	FALGAVTKLLPSLLCMITRKC	α-helix	Anticancer (Malignant cells proliferation and tumor growth inhibition)	Activation of caspases	(Qiao et al., 2019)
Dermaseptin-PS1	*Phyllomedusa sauvagei*	ALWKTMLKKLGTVALHAGKAAL GAVADTISQ-NH_2	Not adopt structure	Anticancer (antiproliferative glioblastoma U-251 MG cells)	Disrupted cell membranes; induced apoptosis through mitochondrial-related signal pathway in U-251 MG cells	(Long et al., 2019)
SKACP003	Synthetic peptide designed *in silico* from AMPs database with anticancer activity	FPLPCAYKGTYC	–	Anticancer (apoptotic mechanisms in human breast cancer cell lines)	Destroyed the β-catenin protein of human breast cancer cell lines	(Selvarathinam, Thekkumalai, Perumalsamy, & Vilwanathan, 2021)
CopA3	Synthesized as *D*-form from defensin-like peptide coprisin from Korean dung beetle (*Copris tripartitus*)	$^{D}L^{D}L^{D}C^{D}I^{D}A^{D}L^{D}R^{D}K^{D}K$-$NH_2$	α-helix	Anticancer (suppressed tumor development of colorectal cancer line cells)	Inhibits the binding of p53 to the MDM2 protein	(Dey, Sharma, Vadlamudi, & Kang, 2023)
P152	Obtained from the fragments of the EWI-2 protein	CFMKRLRK	–	Anticancer	Minding to CD81, preventing MDA-MB-231 cell migration	(Suwatthanarak, Usuba, Kuroha, Tanaka, & Okochi, 2023)
A11	Annexin A1 (ANXA1)-derived peptide	EYVQTVKSSKG	–	Anticancer (Inhibition the growth of cancer cells in immune-competent mice)	Targeting PD- L1 degradation	(Yu et al., 2023)
TAP	Synthetic peptide obtained by "3D-molecular-Evolution" screening	TPE-SKDEEWHKNNFPLSPNTYYEDQG	α-helix	Anticancer	Blocking the PD-1/PD-L1 axis	(Zhang et al., 2023)

(continued on next page)

Table 3 (*continued*)

Peptide	Source	Amino acids sequences	Secondary structure	Activity	Biological target	References
	strategy					
^{99m}Tc-HP-Ark2	Synthetic peptide designed	ArK dimer (^{D}K^{D}L^{D}R^{D}L^{D}E^{D}W^{D}N^{D}R-NHCO $(CH_2)_7$NHCOCH(NH_2) $(CH_2)_2$CONH$(CH_2)_7$ CONH^{D}R^{D}N^{D}W^{D}E^{D}L^{D}R^{D}L^{D}K)	–	Anticancer/probe	Targeting HER2	(Shi et al., 2023)
[^{99m}Tc]Tc-HYNIC-LY	Synthetic peptide designed	[^{99m}Tc]Tc-HYNIC-$(Ser)_3$-LTVPWY	–	Anticancer/probe	Targeting HER2	(Biabani Ardakani et al., 2023)
Herceptide	Screened from phage display	RSLWSDFYASASRGP	–	Anticancer/probe	Targeting HER2	(Cao, Li, Shi, Liu, & Cheng, 2023)
H10	Screened from mRNA display	MKMQVRIYLV	–	Anticancer	Targeting AGR2	(Garri et al., 2018)
SPAM	Screened from mRNA display	MPIFLDHILNKFWILHYA	–	Anticancer	Targeting human PD-L1	(Kamalinia et al., 2020)

TPE: Tetraphenylethylene, HYNIC: Hydrazinonicotinamide, ^{99m}Tc: Technetium-99 m.

Ac^{D}W from the insertion of *N*-(2-chloroacetyl)-*D*-tryptophane (ClAc-^{D}W), was the most abundant after the selection cycles. An additional test was performed for the most active peptide (CM_{11}-1), by observing HECT ubiquitination inhibition. This peptide inhibits thioester bond formation in HECT at a concentration of 1 μM, observed by comparing different concentrations of the same peptide and similar peptides (Yamagishi et al., 2011a).

A similar study implemented the RaPID system to search for selective peptides against the Akt kinase family (Hayashi, Morimoto & Suga, 2012). The Akt family consists of serine/threonine kinases that play a role in the regulation of various signal translation pathways. In humans, they consist of three isomorphs (Akt1, Akt2, and Akt3), which are a target because their defective regulation generates changes in apoptosis, cell proliferation, and metabolism. The high amounts of Akt1 and its activation in some cancers are clues to disease development. Akt2 is believed to be associated with the development of some malignant phenotypes in cancer, and Akt3 (expressed in the brain) is also known to contribute to cancer development by activating growth factors through a mechanism of signal transduction (Hayashi, Morimoto & Suga, 2012).

By constructing two libraries of thioester-macrocyclic peptides that contained *L* or *D N*-(2-chloroacetyl)tyrosine (ClAc^{L}Y or ClAc^{D}Y) from the template AUG-(NNK)n-UGC, where the codons AUG and UGC were assigned to ClAc^{L}Y or ClAc^{D}Y, and cysteine residue respectively, Hayashi and colleagues generated peptides of different conformation due to the configuration of the initial amino acids (Hayashi, Morimoto & Suga, 2012). Independent selection of libraries was performed on Akt2, which was immobilized by the presence of hexaHis in the *N*-terminal part, on Ni^{2+}-NTA magnetic beads. In both libraries, peptides with cycle members between 11 and 15 were found, some of which had the same amino acid residues in the central region in a randomized form. Among them, the peptide Pakti-L1 (Table 2), with 14 amino acid residues, belonging to the ClAc^{L}Y library, was found 28 times in the selection step and was a highly selective inhibitor against Akt2 isoform, with an IC_{50} of 110 nM. Unlike Pakti-L1, which is active only against Akt2, Pakti-L2 and Pakti-L3 (other cyclopeptides from the same library) are active against Akt1/2/3. Furthermore, Pakti-L2 and Pakti-L3 are selective against Akt kinases, however, they are less specific with their targets when compared to the cyclopeptide Pakti-L1. On the other hand, both peptides' libraries did not show similar sequences, despite having started from the same mRNA sequence, a factor attributed to the ^{L}Y-initiator in the ClAc^{L}Y libraries, which allows a different conformational sequence space when compared to of ^{D}Y-initiator. This leads to different amino acid sequences forming different conformations (Hayashi, Morimoto & Suga, 2012).

The vascular endothelial growth factor receptor 2 (VEGFR-2) belongs to the VEGFR family, which are transmembrane tyrosine kinase receptors that regulate the formation of blood and lymphatic vessels. VEGFR-2 has a major role in mediating VEGF-induced responses and is considered the earliest marker of endothelial cell development, as well as directly regulates tumor angiogenesis. Suga and collaborators show the selection of thioether-macrocyclized peptides against VEGFR-2. The most potent selected peptide, L1 (Table 2), blocked VEGF-induced human umbilical vein endothelial cell (HUVEC) proliferation with an IC_{50} of 60 nM and inhibits the angiogenesis of HUVEC in a tube formation assay (Kawakami et al., 2013). Other works that show the application of mRNA display technology for the generation of peptide libraries, as well as the selection against immobilized targets, are Goto, Ito, Kato, Tsunoda, & Suga, 2014 and Iwasaki et al., 2015.

Antitumor peptides destroy the plasma membrane of neoplastic cells through electrostatic interactions (Drayton et al., 2021); however, other different alternatives can be explored through interactions of cancer-related proteins and AMPs, by use of mRNA display technology. Indeed, the plasma membrane lipid bilayer, directly or indirectly, constitutes a target that can be druggable (Lirussi et al., 2023). Deregulated

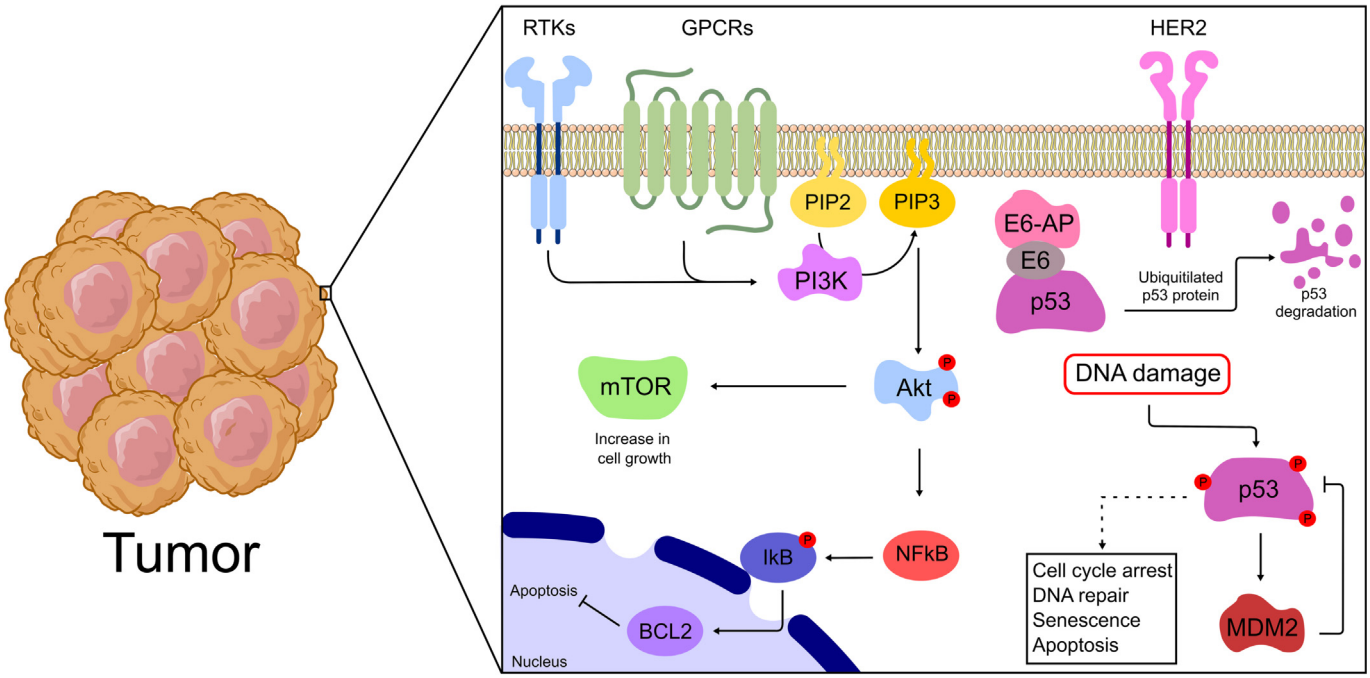


Fig. 3. Targets present in neoplastic cells that can be exploited for the development of antitumor peptides. These targets can be the receptors present in the plasmatic membrane (RTKs, GPCRs, HER2), or intracellular targets such as the proteins involved in signaling life PI3K/Akt/mTOR, or p53 and its antagonist MDM2. Furthermore, the BCL2 protein can be exploited for immobilization, as well as E6. RTK: receptor tyrosine kinases; GPCR: G protein-coupled receptor; PIP2: phosphatidylinositol 4,5-bisphosphate; PIP3: phosphatidylinositol (3,4,5)-trisphosphate; ubiquitin ligase E6-AP (E6-associated protein). This figure was created using Inkscape, version 1.3.0.

de novo lipid synthesis (DNLS) in glioblastoma (GBM) presents a druggable potential. Therefore, the mRNA display technology can be exploited in the development of peptides capable of acting on target enzymes in the process of forming the plasmatic membrane of neoplastic cells. In addition, other cancer receptors or proteins key to cancer pathogenesis could be explored for the development of new drugs by mRNA display screening.

In recent years, several peptides that bind to a specific cancer target protein have been described, such as the synthetic peptide SKACP003 (FPLPCAYKGTYC, Table 3) (Selvarathinam et al., 2021). This peptide inhibits the growth of MDA-MB-231, MDA-MB-453, and MCF-7 breast cancer cell lines through apoptotic mechanisms by selectively targeting the cytoplasmic β-catenin protein. Additionally, SKACP003 induces a decrease in BCL-2 while increasing the expression of BAX, CASP-3, CASP-8, and CASP-9 (caspases) gene (Selvarathinam et al., 2023). Furthermore, when cells were exposed to the peptide, there was a reduction in the expression of Wnt/β-catenin targeting genes such as *C-Myc*, *P68,* and *COX-2*, as well as a significant downregulation of *CDK-4* and *CDK-6* (Selvarathinam et al., 2023). CopA3 (^{D}L^{D}L^{D}C^{D}I^{D}A^{D}L^{D}R^{D}K^{D}K-NH_2, Table 3) is a 9-mer dimeric *D*-type peptide with antitumor activity, active against human colorectal cancer cells. This peptide can bind specifically to the MDM2 protein, suppressing the ubiquitination of p53, a process mediated by MDM2. Consequently, CopA3 significantly decreases the interaction between p53 and its suppressor (MDM2), increasing the concentration of p53 in the nucleus, resulting in cell death by apoptotic mechanisms (Dey et al., 2023).

Recently, using an approach called peptide matrix technology, the peptide P152 (CFMKRLRK, Table 3) was obtained from the fragments of the EWI-2 protein, one of the main partners of CD81 (Suwatthanarak et al., 2023). P152 binds preferentially to the extracellular loop presented in the CD81, preventing the migration of MDA-MB-231 cells, which is one of the stages of metastasis (Suwatthanarak et al., 2023).

The A11 peptide (Table 3) derived from annexin A1 (ANXA1), has demonstrated the ability to decrease the stability and levels of programmed cell death ligand 1 (PD-L1), degrading it through the ubiquitin-proteasome pathway. A11 competes with PD-L1 deubiquitinase USP7 for PD-L1 binding, promoting the ability of T cells to kill breast cancer cells in *in vitro* tests, in addition to inhibiting tumor immune evasion in xenograft tumors in mice (Yu et al., 2023). Using a "3D-molecular-evolution" screening strategy, Zhang and colleagues developed a totipotent "all-in-one" peptide (TAP, sequence TPE-SKDEEWHKNNFPLSPNTYYEDQG, where TPE is a tetraphenylethylene motif, Table 3), that also acts on the PD-1/PD-L1 axis by self-assembly property. Once the PD-1/PD-L1 axis is blocked, the complex formed by RNA binding motif protein 38 (RBM38) and eukaryotic translation initiation factor 4E (Rbm38-eIF4E) is inhibited, activating p53 (Zhang et al., 2023).

Furthermore, the mRNA display technology can contribute to developing probes that can be used for cancer diagnosis through molecular imaging of specific target expression, such as human epidermal growth factor receptor type 2 (HER2). In recent work, Shi and colleagues developed a molecular probe targeting HER2 called ^{99m}Tc-HP-Ark2 (dimer of ^{D}R^{D}N^{D}W^{D}E^{D}L^{D}R^{D}L^{D}K, Table 3). The results demonstrated that, when compared to the previous probes, it was able to increase its effectiveness for the clinical detection of HER2 expression (Shi et al., 2023). Using a similar approach, the small peptide LTVSPWY was labeled with Technetium-99 m (^{99m}Tc) by an ethylenediaminediacetic acid/tricine mixture and HYNIC as co-ligands, to produce [^{99m}Tc]Tc-HYNIC-LY (Table 3). This complex is safe and viable for the identification of HER2-positive lesions in patients with primary breast cancer, making this peptide a valuable non-invasive approach to HER2-targeted therapy (Biabani Ardakani et al., 2023). Another example is the HER2-targeted herceptide peptide (RSLWSDFYASASRGP, Table 3), screened from phage display, which was further conjugated to the near-infrared (NIR) fluorescent dye indocyanine green (ICG) to obtain a probe ICG-Herceptide, for used in NIR fluorescence imaging (Cao et al., 2023).

Computational strategies can also be employed for studying targets in neoplastic cells with the aim of developing mRNA display libraries.

Bioinformatics tools can be applied to identify new targets with potential pharmacological applications in the treatment of various types of cancer. From the analysis of data obtained through RNA-seq, a list of differentially expressed genes (DGEs), as well as some biomarkers and druggable targets, was identified in gastric cancer through analysis and functional enrichment using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tool (Yang, Bhat, Qazi, & Raza, 2023). Among these, a lower expression of the DLC1 gene was observed, which represents higher recurrence risks and shorter survival rates in patients with gastric cancer. Thus, DLC1 is a promising molecule for the development of specific druggable targets for subsets of gastric cancer (Yang et al., 2023). Therefore, mRNA display technology can be employed for the development of specific probes for both the diagnosis and treatment of various types of cancer. Furthermore, the examples mentioned here can be leveraged for the development of mRNA display libraries.

5. Peptides libraries and molecular targets in viruses: Potential infection inhibitors

Viral infections are a significant global health concern, and the development of new antiviral therapies is an area of active research. Peptides have emerged as a promising class of antiviral agents due to their ability to inhibit viral replication and entry into host cells. Due to the presence of proteins in the viral capsid responsible for the entry of the virus into the host cell, these targets can be exploited to prevent viral infection (Fig. 4).

Recently, six cyclic peptides with affinity dissociation constant (K_D) ranging from 15 to 550 nM, binding to the receptor binding domain (RBD) of S-glycoprotein, were selected from three distinct libraries, each containing over one trillion molecules, through mRNA display. Among them, two cyclopeptides, 4 and 5 (Table 2), were the most active against SARS-CoV-2 RBD, with K_D values of 15 nM and 96 nM, respectively. Specifically, the cyclopeptide 4 demonstrated a higher affinity with RBD, binding to a cryptic binding site, and displacing a β-strand near the *C*-terminus (Norman et al., 2021). In addition, other targets of SARS-Cov-2 can be explored using this technique, such as nonstructural protein 14 (nsp14). nsp14 is a bifunctional protein, as it contains the 3'-to-5' exoribonuclease (ExoN) and guanine-N7-methyltransferase (N7-MTase) domains. Recent data suggest that this protein is essential for the replication of both Middle East respiratory syndrome coronavirus (MERS-CoV) and SARS-CoV-2 (Mers-cov, Ogando, Zevenhoven-dobbe, Van Meer, & Der, & Bredenbeek, 2020), which allows the treatment of both diseases through mRNA display.

From a library based on 88 amino acid residue variants of the 10th domain of human fibronectin type III (10Fn3), antibody-like proteins were developed that bind to the SARS-CoV nucleocapsid, modulating the *in vivo* virus replication in a domain-specific way (Liao et al., 2009). However, the growing number of SARS-Cov-2 variants capable of evading the humoral protection conferred by exposure to the virus itself or immunization (attenuated virus, mRNA vaccine, *etc.*) requires the search for broadly reactive neutralizing antibodies (nAbs), something that is already being pursued (Tanaka et al., 2022). The examples demonstrate that the mRNA display technique is important not only because it allows the design and selection of candidate molecules but also a rapid response to pandemics, both for treatment and diagnosis.

SARS-Cov-2 enters human cells through the interaction between its capsid protein, S-glycoprotein (spike protein), and the receptor angiotensin-converting enzyme 2 (ACE2) present on the surface of host target cells. One way to combat the coronavirus is to inhibit its anchorage to its receptor, thus neutralizing the interaction between S-glycoprotein and ACE2 (Saxena et al., 2022). This glycoprotein is an important target to be explored through the mRNA display technique, aiming at the development of polypeptides with antiviral or diagnostic potential.

DENV and ZIKV belong to the *Flavivirus* genus (*Flaviviridea* family), characterized by the presence of an approximately 11 kb long, positive-strand RNA (+ ssRNA) linear virus molecule, which contains a single ORF encoding a 3400 amino acid polypeptide flanked by two non-coding regions (5' and 3' UTR) at the ends of the genome (de Zanotto & de Leite, 2018). There are four DENV serotypes (DENV1, DENV2, DENV3, and DENV4) capable of epidemic spread (Oliver, Carr, & Smith, 2019). DENV infects different cell types, including mast cells, epithelial cells, muscle cells, endothelial cells, dendritic cells, hepatocytes, monocytes, and bone marrow cells (Guzman, Gubler, Izquierdo, Martinez, & Halstead, 2016). Several putative receptors have been proposed for DENV, such as heparan sulfate, a type of glycosaminoglycan (GAG), the lipopolysaccharide (LPS)-CD14 complex, the heat shock proteins HSP70/90, the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), and the chaperone GRP78, as well as the tyrosine-protein kinase receptor UFO (unidentified function) (AXL), which mediate the entry of the virus into host cells (Nanaware, Banerjee, Bagchi, Bagchi, & Mukherjee, 2021). Additionally, other receptors such as phosphatidylserines (PS), T cells immunoglobulin (Ig) and mucin (TIM) receptors, the tyrosine-protein kinase receptor (TYRO3), AXL, and the proto-oncogene tyrosine-protein kinase MER (MERTK) (the TAM receptors) act as primary receptors or co-receptors (Nanaware et al., 2021). The viral capsid proteins orchestrating virus-host interactions during entry are M and E. The E protein has three domains (DI–DIII), with DIII being responsible for binding to host receptors. To date, many mutations in this domain have been identified, affecting its binding to the receptor (Guzman et al., 2016). ZIKV is also capable of infecting various types of cells and has been found in the placenta, brain, eye, testicles, uterus, vagina, and body fluids such as blood, tears, saliva, urine, semen, and cervical mucus, which increases the risk of transmission through sexual contact (Ferraris, Yssel, & Missé, 2019). Similar to DENV, ZIKV also has several identified receptors, including DC-SIGN, TIM-1, and TAM receptors (Ferraris et al., 2019). These receptors facilitate the entry of ZIKV into monocytes, macrophages, neural progenitor cells (NPCs), and fetal cells (Mwaliko et al., 2021). Among these receptors, AXL is suggested to play a pivotal role in mediating ZIKV entry and is involved in cellular autophagy, which enhances viral replication in human skin fibroblasts (Hamel et al., 2015). CHIKV belongs to the *Alphavirus* genus (*Togaviridae* family), and, like the other two arboviruses mentioned, has a single-stranded RNA genome with positive polarity, with a size of 11.5 kb (Oliver et al., 2019). It contains two ORFs, one encoding a nonstructural polyprotein that is processed into four nonstructural proteins (NSPs), and the other encoding a structural polyprotein expressed as a subgenomic RNA molecule (Higuera & Ramírez, 2019). CHIKV interacts with mammalian cell receptors, including prohibitin (PHB), matrix remodeling-associated protein 8 (MXRA8), glycosaminoglycan (GAG) (as CHIKV can enter GAG-free cells), DC-SIGN, and TIM1/4 (Khongwichit, Chansaenroj, Chirathaworn, & Poovorawan, 2021). The E2 protein is considered the major antibody neutralization target and is credited with the ability to bind to cell receptors (Schnierle, 2019).

The HIV replicative cycle is divided into early and late phases. The early phase begins with the virion binding to the cell surface and concludes with the integration of the viral complementary DNA (cDNA) into the host genome (Kirchhoff, 2013). HIV-1 infects $CD4^+$ T cells and macrophages through the interaction between gp120 and its main surface receptor, CD4 (Engelman & Cherepanov, 2012). However, the virus requires C-C chemokine receptor type 5 (CCR5) and the C-X-C chemokine receptor type 4 (CXCR4) co-receptors to enter the cell. Different variants of HIV can interact with one or other chemokine receptors, but some can bind to both (Deeks, Overbaugh, Phillips, & Buchbinder, 2015). After the second interaction, a hydrophobic region on gp41 is exposed and inserted into the plasma membrane, allowing the cellular and viral membranes to come into close proximity and create a fusion pore (Suárez, Rocha-Perugini, Álvarez, & Yáñez-Mó, 2018). Once fusion occurs, the mature capsid is released

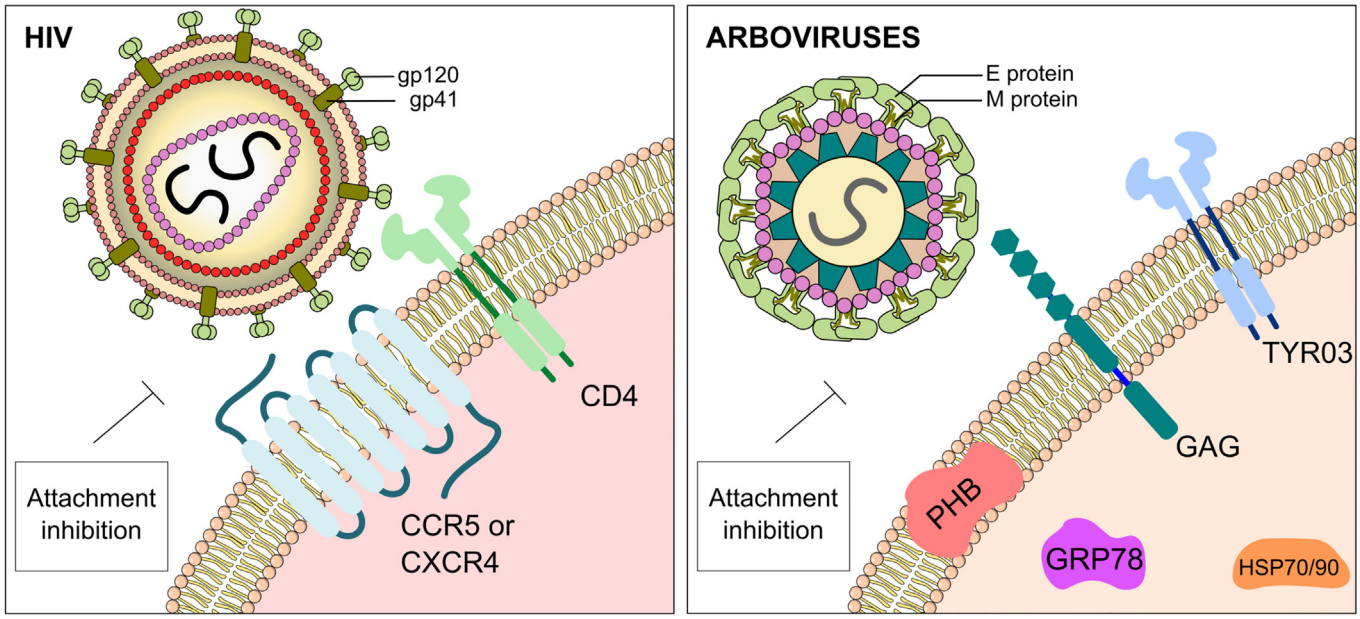


Fig. 4. Targets present on the surface of virions that can be exploited for viral infection by inhibiting virus entry into host cells. On the left side of the panel, the human immunodeficiency virus is represented, which has the glycoproteins gp120 and gp41, responsible for the entry of the virus into cells through interaction with CD4, CCR5 or CXCR4 receptors. On the right side of the panel arboviruses are represented, which can interact with both membrane receptors (GAG, TYR03) and intracellular proteins (HSP70/90, GRP78). CD4: cluster of differentiation 4; CCR5: C-C chemokine receptor type 5; CXCR4: C-X-C motif chemokine receptor 4; PHB: prohibitin; GAG: glycosaminoglycans; TYR03: protein tyrosine kinase 3; HSP70/90: heat shock proteins 70 and 90; GRP78: glucose-regulated protein 78. This figure was created using Inkscape, version 1.3.0.

into the cytoplasm of the newly infected cell (Campbell & Hope, 2015). From this point on, the p51 enzyme initiates the synthesis of double-stranded DNA (dsDNA) through reverse transcription of the virus's ssRNA, while the capsid migrates along microtubules toward the nucleus (Novikova, Zhang, Freed, & Peng, 2019). The viral cDNA is associated with proteins p12 (protease), p51 (reverse transcriptase), p32 (integrase), and the viral protein R (Vpr), forming the preintegration complex (PIC) (Archin, Sung, Garrido, Soriano-Sarabia, & Margolis, 2014; Nisole & Saïb, 2004). The PIC crosses the nuclear pore complex (NPC) in a capsid protein (CA)-dependent process, and the viral cDNA (provirus) is integrated into the host cell's chromosome (Barré-Sinoussi, Ross, & Delfraissy, 2013). The late phase begins after the transcription of the provirus, translation of proteins, assembly, and release of viral particles (Kirchhoff, 2013).

6. Prospects and concluding remarks

Throughout this article, several examples have been given regarding the potential that mRNA display technology holds for the development of peptide-based drugs, particularly those with anticancer and antiviral activity. One of the main advantages of mRNA display is that it provides the selection of highly specific peptides for targets of clinical interest. Therefore, this technology has unparalleled potential to revolutionize the development of new medicines aimed at treating various types of cancer, as well as preventing viral infections, in addition to several other diseases in which interactions between proteins and receptors occur. Furthermore, mRNA display can also be used in imaging, allowing significant advances both in studying the spatial localization of proteins (and other targets) *in situ* and in tracking protein binding sites. In this way, mRNA display technology can be an important ally in precision medicine, enabling personalized diagnoses and treatments, since essential targets in disease progression can be targeted by peptides capable of acting specifically on these targets. Another advantage of this powerful tool is its ability to improve the activity of bioinspired peptides, enhancing their activity against neoplastic cells, as well as inhibiting the entry of viruses into host cells. Furthermore, with the increasing accumulation of data generated by high-throughput sequencing of RNA and DNA and other "multi-omics" approaches, new potential targets have been elucidated, which can be explored through mRNA display for the development of new bioinspired drugs. Therefore, mRNA display is a technique that promises to revolutionize the way both the search for new medicines and the diagnosis and treatment of complex diseases are carried out today.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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Feature

Challenges and advances in antimicrobial peptide development

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Microbial resistance is a major concern for public health worldwide, mainly because of the inappropriate use of antimicrobials. In this scenario, antimicrobial peptides (AMPs) have emerged as a potential therapeutic alternative means by which to control infectious diseases, because of their broad spectrum of action. However, some challenges can make their clinical application problematic, including metabolic instability and toxicity. Here, we provide a clear description of AMPs as promising molecules for the development of unusual antimicrobial drugs. We also describe current strategies used to overcome the main difficulties related to AMP clinical application, including different peptide designs and nanoformulation.

Keywords: antimicrobial peptides; microbial resistance; chemical design; nanoformulation

Introduction

Antimicrobial compounds discovered during the early 1920s significantly reduced morbidity and mortality from infectious diseases, and became a milestone in pharmacology.[1] Penicillin revolutionized the medical treatment of infections caused by Gram-positive bacteria, such as *Staphylococcus* and *Streptococcus*, and the arrival of streptomycin allowed the control of TB.[2] Since then, different groups of antibiotics have been discovered,[3] such as sulfonamides, β-lactams, aminoglycosides, quinolones, cyclic peptides, tetracyclines, macrolides, amphenicols, and nitroimidazoles, among others.[4] Nevertheless, according to the guidelines of the Clinical & Laboratory Standards Institute (CLSI), many antibiotics are no longer effective in treating infections.[5] Thus, novel antimicrobials are urgently needed against pathogens of medical importance represented by the acronym 'ESKAPE': *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.[5–8] The WHO classified these pathogens as critical, high, and medium priority according to the urgency for new treatments. The critical priority group includes carbapenem-resistant *A. baumannii* and *P. aeruginosa,* along with extended-spectrum β-lactamase (ESBL) or carbapenem-resistant *K. pneumoniae* and *Enterobacter* spp. The high-priority group list includes vancomycin-resistant *E. faecium* (VRE) and methicillin and vancomycin-resistant *S. aureus* (MRSA and VRSA).[5]

During the search for innovative antimicrobial therapies to combat resistant pathogens, AMPs have emerged as a potential approach.[9] Also known as host

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defense peptides (HDPs), these molecules represent one of the oldest innate defense mechanisms in living organisms and have wide diversity in terms of composition, structure, and biological effects. [10] They show activity against Gram-positive and -negative bacteria and other pathogens, such as fungi, viruses, and parasites.[6] AMPs can also exert potent antibiofilm activity against multiresistant bacteria.[11,12] This is of relevance to clinical medicine because biofilms cause 80% of nosocomial infections, and are one of the biggest global health concerns.

A biofilm is formed by a community of microorganisms adhered to biotic surfaces, such as epidermal cells, mucosal surfaces, or to medical devices.[13] They are surrounded by a self-produced extracellular polymeric substance (EPS), which includes exogenous DNA, lipids, polysaccharides, and proteins.[14,15] Biofilms exert different degrees of antimicrobial resistance compared with bacterial planktonic cells of the same species, being capable of reducing drug penetration and diffusion via their intrinsic structural properties.[15,16] Mechanisms of action by which AMPs can act against biofilms include: (i) suppression of cell signaling against stressful environments by blocking the synthesis of guanosine tetraphosphate (ppGpp) and pentaphosphate (pppGpp) signal nucleotides; (ii) negative regulation of gene expression involved in biofilm formation. They also trigger the negative regulation of genes involved in the transport of binding proteins and increase the regulation of the expression of icaR genes, responsible for inhibiting biofilm production;[17] and (iii) membrane disruption of already-formed biofilms by membranolytic mechanisms of action, leading to destruction of the cell membrane.[14] They can also have antitumor activity or modulate the immune response.[9] In addition, AMPs have a low tendency to accumulate in tissues, and are generally free of adverse effects.[18]

Several AMPs are in clinical and preclinical trials against different infections,[19–21] and some have US Food and Drug Administration (FDA) and European Medicines Agency (EMA) certifications for market entry and clinical application.[22,23] Thus, there is no doubt that AMPs remain promising compounds for developing alternative therapies toward microbial infections and other diseases.[4] Although the global market for AMPs is growing, it is still small compared with the antibiotic market, which was worth ~US$42.53 billion in 2021. The total revenue from antibiotics is expected to grow at a compound annual growth rate (CAGR) of 4.5% from 2022 to 2029, reaching almost US$60.48 billion in 2029.[24] The global AMP market was valued at US$5 million in 2022 and is estimated to reach ~US$7 million by the end of 2029, growing at a 7% CAGR from 2023 to 2029.[25] Currently, more than 60 peptides have been approved by the FDA and more than 400 are in clinical trials.[26] Only seven of those peptides have reached the market, and are used mainly as topical medications and, in some cases involving serious infections, as injectables.[27]

Despite their favorable features, AMPs are usually unstable in the gastrointestinal tract and other body fluids, have poor absorption and distribution and fast metabolic degradation and excretion, which result in low bioavailability.[28] Here, we describe the development value of AMPs for clinical applications and the most promising strategies to design and deliver novel AMPs to improve their physical and chemical properties with the aim of overcoming the difficulties associated with the introduction of AMP oral and systemic therapy.

Drawbacks and attempts in AMP therapeutic development

Despite the great potential of AMPs and their diverse therapeutic applications, only a few have entered clinical practice.[18] Unfortunately, along with their positive features, AMPs have some drawbacks (Box 1). The biological functions of AMPs are directly associated with their 3D structure.[29] However, the sequence–structure–function relationships are not well understood.[30] Different physicochemical properties, such as hydrophobicity, hydrophobic moment, net positive charge, amphipathicity and helical propensity, have been associated with the structure–function relationships underlying AMP biological function.[29] In addition, these features provide numerous possible peptide sequences, generating great structural and functional diversity. To develop AMPs with broad-spectrum activity and to overcome these drawbacks, AMPs need to be redesigned.[31]

Box 1 Drawbacks in AMPs therapeutic development. One of the main limitations associated with the clinical use of AMPs is their low bioavailability. Generally, these molecules have difficulty crossing biological membranes, such as the cell membrane, intestinal mucosa or blood-brain barrier, which can lead to poor absorption at the site of action. Therefore, topical use, intravenous, intramuscular or subcutaneous administration are the most common applications.[7,23,24] Chemical reactions such as hydrolysis by gastric acid or digestive enzymes also reduce absorption and therefore bioavailability. Rapid removal from the circulation by high hepatic and renal clearance is also a significant obstacle.[5,21,22] The low selectivity is another issue associated with the clinical apllication. Generally, the low target specificity of these molecules is associated with cytotoxicity, which may exert hemolytic activity.[20] Furthermore, interactions with different targets or receptors may occur, leading to side effects and possible risk of immunogenic effects.[11]

Among the computational approaches developed so far for the prediction of AMPs, there are conventional predictors based on machine learning, such as Antimicrobial Peptide Collection (CAMP), iAMP-2L, and iAMPpred, which apply machine learning algorithms to extracted features of peptide sequences to identify AMPs.[32] Such tools require prior knowledge of AMP structure and mechanism because they use conventional machine learning methods and have predesigned features.[33] One of the main approaches to designing AMPs is to rationally design new peptides through curated insights into antimicrobial activity, such as the Joker algorithm. This algorithm makes it possible to insert patterns into sequences to quickly produce new AMP sequences.[34]

There are also methods based on deep learning, which use encodings of original sequences as input. In some cases, the sequence information obtained from sequences by some third-party tools is added, features are extracted, and classification labels are produced through a

neural network structure.[32] Deep learning methods generally outperform conventional methods in many bioinformatics tasks.[33] There are studies that use a machine learning approach that selects physicochemical characteristics within a given domain of knowledge in a nonlinear way, in addition to information on amino acid replacement frequencies, to rationally design AMPs.[34]

Deep learning models developed for AMP prediction include bidirectional long-term memory recurrent neural networks (RNNs), (Bi-LSTM) for AMP prediction, and the Deep-AmPEP30 online server, which applies a convolutional neural network (CNN) for AMP prediction, among others. However, these servers have certain restrictions, such as the size of peptides or specificity, such as the development of AMPs against a certain microorganism.[33] AMP design has been a field of great interest for the development of antimicrobial agents with boosted spectrum activity, improved *in vivo* half-life and decreasing cytotoxicity or hemolytic activity against mammalian cells.[35–38] There are different chemical modifications, such as specific substitutions and/or additions to the amino acid sequence (Figure 1), that can be used in combination with analysis of AMP physicochemical properties and computational approaches for AMP prediction to boost the spectrum activity and also overcome AMP limitations.[39]

Amino acid substitution strategies

Most AMPs comprise cationic, hydrophilic, and hydrophobic amino acids arranged in a molecule that can assemble into an amphipathic structure.[40] Generally, only a few amino acids appear to be essential for antimicrobial activity. The cationic amino acids lysine and arginine (Lys/Arg) have a positive charge that enables interaction with the anionic surface of the microbial cytoplasmic membrane. Tryptophan (Trp) has a key role in the formation of the amphipathic helical structures of AMP. Single or multiple substitutions

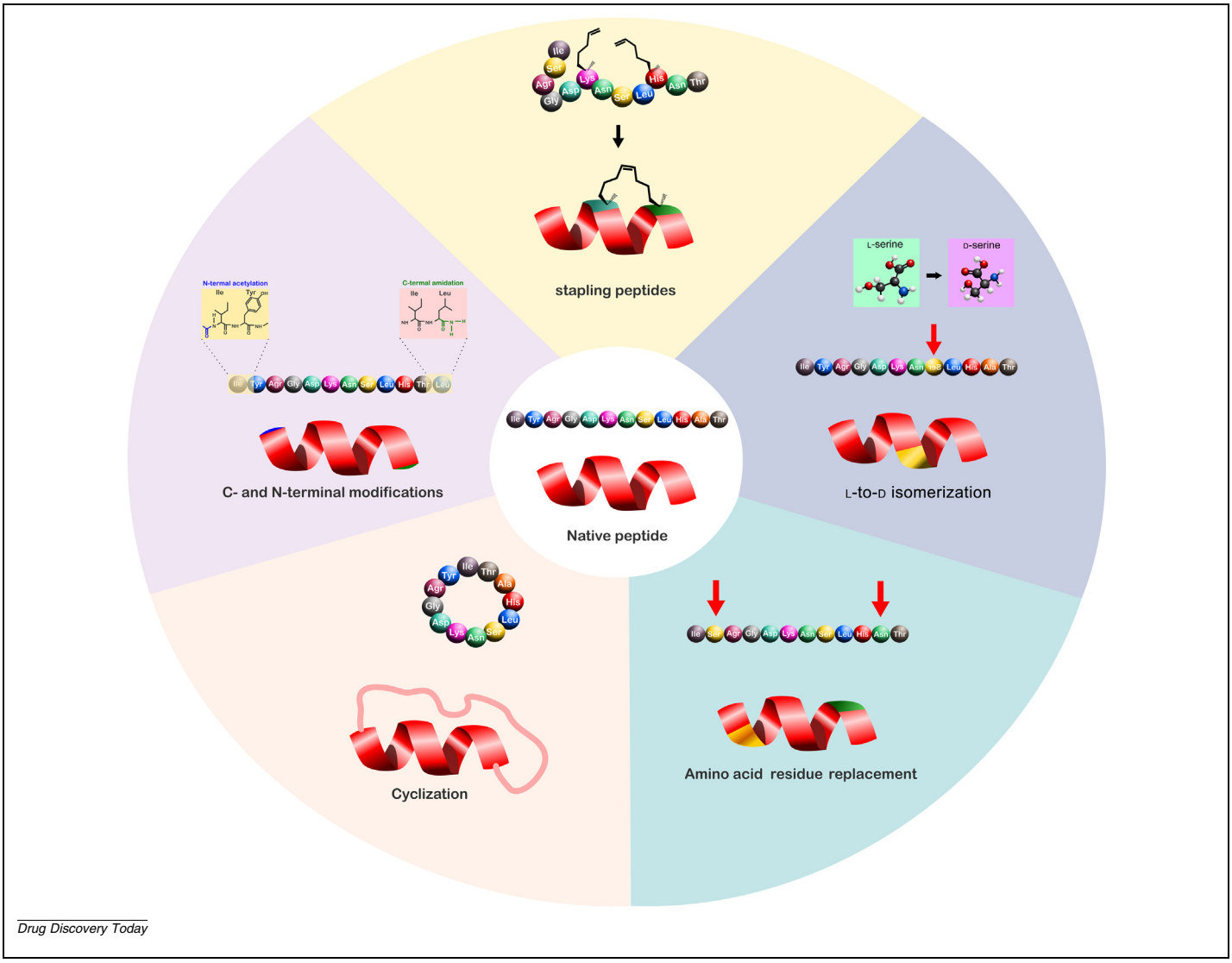

FIGURE 1

Chemical design strategies to improve antimicrobial peptide (AMP) stability. In the center, the native peptide; in pink, the C and N-terminal modifications; in yellow, stapling peptides (the sticks represent the stapling reaction); in purple, L- to -D isomerization; in green, amino acid residue replacement; and finally, in beige, the cyclization. The red arrows indicate the position of the amino acid modifications.

using these amino acid residues could improve the antimicrobial activity, increase the half-life, and decrease the hemolytic activity. Therefore, amino acid substitution has been widely used in AMP optimization.[41]

Natural amino acids have close analogs that can effectively replace crucial residues to modify rigidity and peptide conformation.[42,43] The AMP melittin, isolated from bee venom, has strong antimicrobial activity.[10] However, it also has high hemolytic activity and, therefore, its clinical use is limited. Previous studies have shown that melittin Trp at position 19 is significantly involved with its hemolytic activity. Therefore, replacing Leu → Trp in this position decrease the hemolytic activity, whereas replacing Pro → Trp in the 14th position improved the α-helical conformation and also reduced the hemolytic effects compared with the parental peptide. These sequence substitutions for Trp, one of the most hydrophobic residues, appear to promote peptide–peptide interactions induced by Trp–Trp interactions, resulting in a decrease in the hemolytic activity of the analog compared with melittin activity.[44]

L- to -D heterochiral isomeration strategies

Most amino acids can form two (L- and D-) optically active (enantiomeric) forms, which have different physiological and biological activities.[41] The incorporation of amino acid residues in the D-amino acid configuration is capable of significantly improving the metabolic stability of biologically active peptides, because human and microbe proteinases exclusively recognize L-amino acids (L-AAs), rather than D-amino acids (D-AAs).[35,36,45] D-AAs promote increased resistance to host proteinases, which generally cleave L-AA peptide bonds, and can be used to change one or more of them, or even the entire sequence.[10] This strategy was used in the AMP protonectin, primarily isolated from the venom of the wasp *Agelaia pallipes*, with an amino acid sequence of ILGTILGLLKGL-NH_2. The peptide demonstrated potent antibacterial and antifungal activity with a membrane-active mode of action but, unfortunately, it is susceptible to protease.[46] All the amino acid residues were replaced with the corresponding D-amino acids, and the analog demonstrated greater stability against trypsin, chymotrypsin, and human serum compared with the original peptide, comprising L-amino acids. In addition, the D-enantiomer of protonectin (D-prt) exhibited strong antimicrobial activity against bacteria and fungi.[46]

Another amino acid sequence, polybia-CP AMP (ILGTILGLLKSL-NH_2), was also initially isolated from *Polybia paulista* wasp venom.[47] This AMP has antibacterial and antifungal activity, in addition to antitumor action. Nevertheless, polybia-CP is susceptible to proteinases, which can impair its pharmacokinetics.[47] The replacement of all L-amino acids by D-amino acids provided resistance to trypsin and chymotrypsin degradation without changing the antimicrobial activity.[47] The PDGu(7)-block-PBLK(13) it is an enantiomeric block co-β-peptide comprising two β-lactam monomers: a protected D-glucose (DGup) β-lactam and a protected cationic L-lysine (BLKp) β-lactam, block copolymerized in one shot. This co-β-peptide is bactericidal against MRSA. It acts on replication and, unlike classical antibiotics, is active against persistent MRSA cells and biofilms. Furthermore, it is non-cytotoxic and nonhemolytic *in vitro*.[48]

N- and C-terminal modification strategies

Another important issue is that different amino acid residues at the N- or C-termini directly influence proteolysis and further peptide degradation. Residues, such as Met, Ser, Ala, Thr, Val, and Gly, at the N-terminus are more resistant to degradation in the plasma, whereas Pro, Glu, Ser, and Thr are more susceptible. Modifications of the N- and C-terminal sequences are widely used to modify and improve AMP properties, such as enhancing the bioavailability without changing the specificity and affinity of targeting.[42,49] Typical modifications include amidation (C-terminus), acetylation and methylation (N-terminus).[41] Generally, the N-terminal acetylation of an AMP increases its helicity and prevents enzymatic degradation, whereas its C-terminal amidation enhances its structural stability and antimicrobial activity.[50] L1A AMP (IDGLKAIWKKVADLLKNT-NH_2) is a mastoparan-like peptide, which exhibits selective antibacterial activity against Gram-negative bacteria without being hemolytic. Peptide *N*-acetylation (ac-L1A) showed that the helical content of the peptide and also its lytic activity increased in anionic lipid vesicles. N-terminus acetylation has a significant effect on the secondary structure of the peptide and its insertion into the bilayer, favoring lipid core bilayer perturbation by L1A, causing higher membrane disruption.[51]

Cyclization and stapling strategies

Cyclic peptides (cyclotides) can be found in a variety of plants and have a head-to-tail cyclized backbone and three conserved disulfide bonds, creating a cyclic cysteine knot motif.[52] This structure confers greater plasma and gastric fluid stability, oral bioavailability and a variety of biological activities.[41] Furthermore, cyclotide structures exhibit considerable topological plasticity, because changing the number and type of amino acids in the loops does little to distort the overall shape, making them particularly amenable to molecular grafting strategies.[52]

Therefore, cyclization design is also often used to improve antimicrobial efficacy, increase the stability of peptides and cell selectivity, and further reduce host toxicity.[36,42,53] One of the methods used to generate cyclized peptides is the formation of disulfide bonds (heterodetic cyclization through a non-amide). Heterodetic cyclization occurs via the oxidation of cysteine (Cys) side-chain thiols, which creates single or multiple disulfide bridges. In addition, cyclic peptides can be produced through amide bonds (homodetic cyclization through an amide).[41] Lactamization can be used for the formation of stable cyclic AMPs. This strategy involves the formation of intramolecular peptide bonds to produce homodetic cyclic peptides. Different modes for the formation of additional peptide bonds include head-to-tail, side chain-to-head/tail, and side chain-to-side chain.[41]

The AMP ZY4 is a cyclic peptide stabilized by a disulfide bridge developed based on the cateliciding-BF sequence, a peptide isolated from *Bungarus fasciatus* snake venom, which exhibits strong and rapid antimicrobial activities. The designed cyclic peptide showed excellent activity both *in vitro* and in an animal model against *P. aeruginosa* and *A. baumannii* by permeabilizing the membrane.[54] Cyclic derivates of the immunomodulatory and antibiofilm

innate defense regulator peptide (IDR)-1018 were designed based on three different synthetic strategies: head-to-tail cyclization, side-chain-to-tail cyclization, and a disulfide bond cross-linkage. All three strategies enhance proteolytic stability and a decrease *in vitro* and *in vivo* aggregation. Side-chain-to-tail cyclization showed inflammation suppression capability and significantly reduced bacterial loads in a high-density *S. aureus* murine skin infection model.[55]

Another widely used technique that has the aim of improving proteolytic resistance and increasing potency and cellular permeability is peptide stapling.[56] This strategy has been used to support short peptides in stable secondary structures, which are mainly in α-helical conformations.[57] Generally, a macrocycle peptide is formed by linking two positions in the peptide sequence through various chemical clamping methods, including disulfide bonds, lactam bridges, triazole rings, or hydrogen bond surrogate approaches.[57] The all-hydrocarbon peptide stapling strategy [46] is one of the most recognized techniques for peptide development. It is performed through ring-closing metathesis between olefin-bearing amino acids and has been widely applied in the production of short peptides with robust pharmacological properties.[58]

One example of stapling was its use with the peptide B1-Leu (B1-L, VKRFKKFFRKLKKLV-NH2), a cathelicidin-BF AMP found in *Bungarus fasciatus* snake venom. This peptide showed anticancer activity against drug-resistant cell lines, such as MCF-7/ADM and K562/ADM, and is considered a potential candidate for cancer therapy.[59] The design and synthesis of various all-hydrocarbon stapled B1-Leu derivatives were proposed to improve the pharmacokinetic properties of the peptide.[59] Researchers found that most stapled derivatives showed a higher helicity level, stronger protease stability, improved antitumor activity, and low hemolytic activity. The optimal derivatives, B1-L-3 and B1-L-6, could be promising lead compounds for the development of novel cancer therapeutics.[59]

Nanoencapsulation for AMP stability and biodistribution improvement

Nanotechnology can be used to minimize the disadvantages of natural and synthetic AMPs. The delivery of drugs through specialized vehicles (Figure 2)

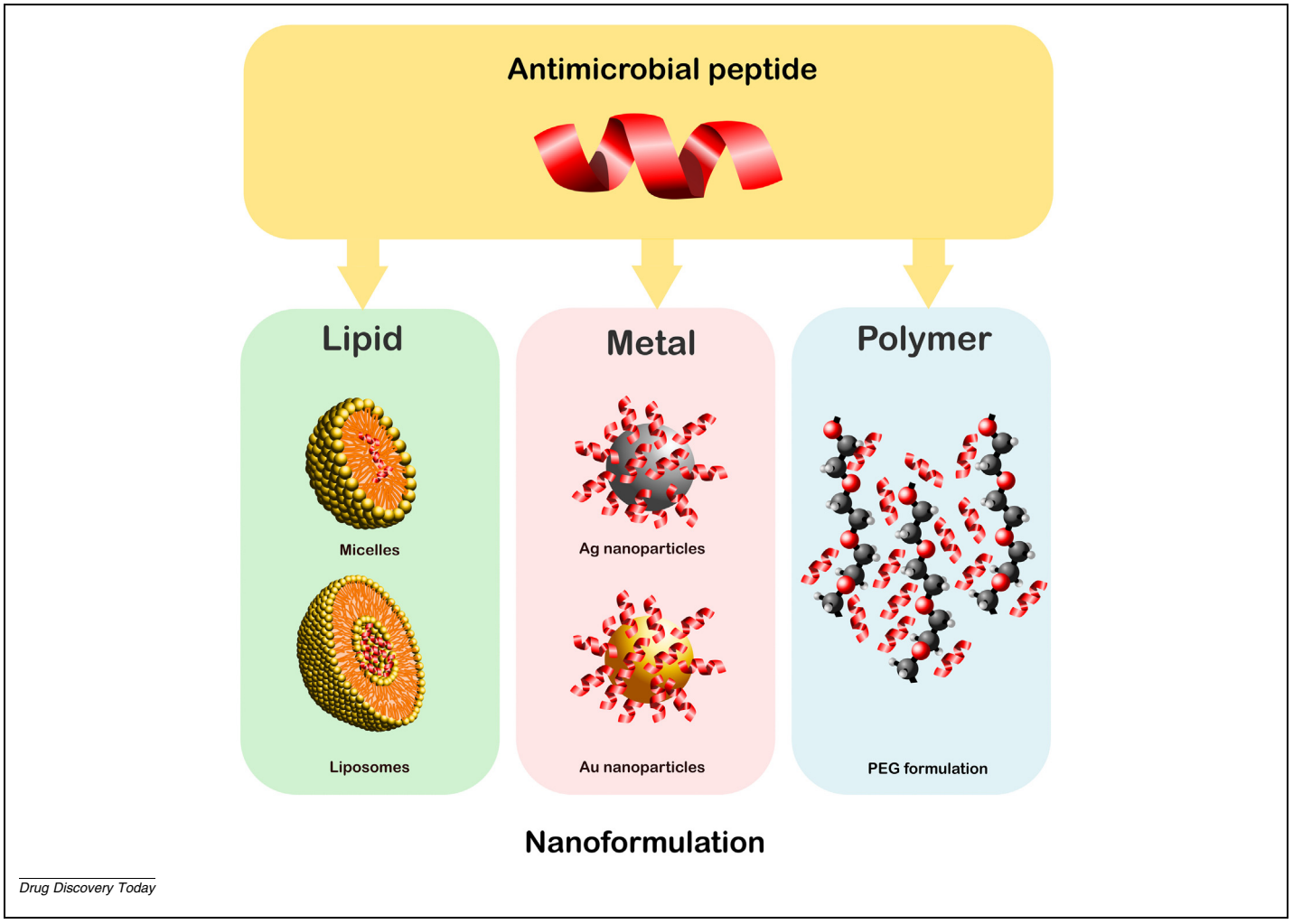

FIGURE 2

Nanoencapsulation techniques for antimicrobial peptide (AMP) stability and biodistribution improvement. In yellow, the AMP; in green, lipid nanoparticles (NPs; micelles and liposomes); in pink, silver (Ag) and gold (Au) NPs; in blue, polymer NPs, poly (ethylene glycol) (PEG) formulation.

can improve the biological properties of the therapeutic molecule,[9] such as an increase in the effectiveness of therapy, direct administration to the target organ, protection of the encapsulated peptide from degradation, and reduction of toxicity and adverse effects.[1,60] The conjugation of AMPs and nanoparticles (NPs) as a type of carrier to deliver AMPs is an effective solution for many problems associated with AMP use.

Metal NPs have a large surface area:volume ratio, which makes it possible to load a wide variety of molecules on their surface.[8,9] Metallic NPs commonly used in the field of infectious diseases are silver (Ag) and gold (Au).[1,61] AMPs conjugated with AuNPs showed greater stability in serum and in the presence of nonphysiological concentrations of proteolytic enzymes compared with soluble AMPs, as well as low cytotoxicity against human cells.[62] The peptide indolicidin, when conjugated with AuNPs, demonstrated greater efficacy in preventing cell adhesion and in destroying biofilms formed by *Candida albicans in vitro* compared with isolated AuNPs and indolicidin, probably because the peptide is protected from degradation by proteases.[63] AgNPs are one of the most studied nanomaterials, because of their own innate antimicrobial activity against Gram-positive and -negative bacteria. Indeed, Ag is a promising delivery system for AMPs and one of the most popular metals used in the synthesis of NPs.[64] Many studies related to the conjugation of AgNPs and AMPs have obtained promising results. The AMP andersonin-Y1 was first modified by adding a cysteine residue to the N terminus (CAY1 peptide) and the C terminus (AY1C peptide), to be later conjugated with AgNPs. Conjugation of AgNPs with a peptide containing cysteine conferred stability to the nanopeptide conjugate. This nanoformulation system increased binding of modified peptides to AgNPs, and also increased their antimicrobial properties against various strains of pathogenic bacteria, including *K. pneumoniae*. In addition, the hemolytic activity of the conjugates was reduced.[65]

In addition to metals, biodegradable polymeric nanosystems can improve cell penetration, intracellular retention, and specific subcellular distribution of antimicrobial agents.[66] In addition, water-soluble synthetic polymers offer improved solubility, biocompatibility and stability, and extended circulation for the payload.[1,9] Several biodegradable polymers have been used, such as poly (lactic acid) (PLA), poly (glycolic acid) (PGA), poly (lactide-co-glycolide) (PLGA), poly (caprolactone) (PCL) and poly (cyanoacrylate) (PCA). As the hydrophilic segment, the most used type is poly (ethylene glycol) (PEG).[1,60] In addition to synthetic polymers, there are many natural polymers, such as DNA, cellulose, or chitosan.[60] PEGylation can promote a drastic reduction in cytotoxicity for a wide variety of AMPs.[60] Selective arginine PEGylation of arginine-rich AMPs can reduce toxicity, provides protection against serum proteinases, and further allows the stable release of the AMP bioactive form.[67] Derivatives of AMP esculentin-1 encapsulated in PLGA NPs coated with poly (vinyl alcohol) demonstrated better transport through an artificial pulmonary mucus and bacterial barrier and exhibited a four to 17-fold increase in activity in a mouse model with *in vivo* lung infection.[68]

Lipid NPs are the most commonly used vehicle for AMP delivery and can vary from liposomes and micelles to liquid crystalline NPs. They generally provide high biocompatibility, safety, and biodegradability, decreasing the chances of cytotoxicity and accumulation that can arise in organs when using other NPs.[9] Liposomes have been used for decades in drug delivery. They are lipid bilayer vesicles, usually comprising phospholipids, or other lipids and membrane components.[9] For example, the liposome LL-37 coated with PEG demonstrated less toxicity and increased antiviral activity compared with free AMP. The formulation demonstrated long-term stability, in addition to a high encapsulation yield, which are favorable physicochemical properties.[69]

Concluding remarks and prospects

Microbial resistance is a significant threat to global health, because it hinders the treatment of infectious diseases, resulting in increased hospitalization, higher medical costs, and elevated mortality. Given that most conventional antibiotics are no longer effective in combating some infectious diseases, new treatment strategies are being investigated. AMPs have great potential as alternative therapeutic agents in this scenario, because of their biological activities against pathogenic and drug-resistant microorganisms. Nonetheless, they have some negative features, such as systemic toxicity, susceptibility to protease degradation, short half-lives, and rapid renal clearance, which limit their clinical progress.

In this regard, chemical modifications and delivery systems have been reported to improve AMP pharmacokinetics. Approaches such as the incorporation of the D-amino acid configuration, N- and C-terminal modifications and cyclization design, are used to improve AMP stability and bioavailability and to decrease their toxicity. AMP nanoencapsulation can also be used to improve AMP biological properties. Metal NPs, polymeric nanosystems, and lipid NPs can protect the encapsulated peptide from degradation and toxicity. They can also reduce adverse effects and improve effectiveness. In the coming decades, all these strategies could be used to develop more potent and 'personalized' drugs for each patient, reducing adverse effects and enhancing their effectiveness.

Declaration of interests

The authors declare that they have no conflict of interest associated with this article.

Data availability

No data was used for the research described in the article.

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Evaluation of the biotechnological potential of peptide Cupiennin 1a and analogs

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Antimicrobial peptides (AMPs) are components in the innate immune system of various organisms, and many AMPs can be found in poisons from animals such as spiders, scorpions, and snakes. The peptide Cupiennin-1a is present in the venom of the spider *Cupiennius salei* and belongs to a group of peptides called cupiennins. The peptide demonstrated high cytotoxic activity against mammalian cells; thus, aiming to solve this problem, seven analogs were designed (R1a, R1b, R2b, R3b, R6b, R8b, and R10b) based on the primary structure of the peptide Cupiennin 1a, reducing its size and substituting some amino acid residues. The antimicrobial results showed that all Cupiennin 1a analogs displayed antimicrobial activity against the tested bacterial and fungal strains. Cytotoxicity tests demonstrated a decrease in the cytotoxic effect of the analogs when compared to the peptide Cupiennin-1a. The antitumor activity against breast adenocarcinoma lines was observed for all the peptides, displaying a better effect against the MCF-7 and MDAMB-231 cell lines. The eight peptides have insecticidal potential, and the original peptide and analogs R6b, R8b, and R10b showed better efficiency even at low concentrations. The rational design of the analogs led to new molecules displaying activities against different cell types and reduced cytotoxicity toward healthy mammalian cells when compared to the original peptide, demonstrating that this was an interesting approach for the development of molecules with biotechnological potential.

KEYWORDS

antimicrobial peptides, rational design, analogs, spider, cytotoxicity

Introduction

Due to the continued emergence of microorganisms that are increasingly resistant to current antibiotics, numerous concerns about existing therapeutic agents for the treatment of autoimmune diseases, and widespread problems with pests in agriculture, many antimicrobial molecules have been studied worldwide. In this context, antimicrobial peptides appear as a new biotechnological tool for therapeutic use because these molecules can be selective, efficient, and fast-acting (Le, 2017).

Antimicrobial peptides (AMPs) are molecules present in the innate immune system of various organisms. These molecules have a considerable variation in the composition of their amino acid residues, forming different secondary structures, contributing to the diverse biological activities they can display. AMPs are low molecular weight molecules with a wide spectrum of activities, and may inhibit bacteria, viruses, fungi, tumor cells, and protozoa (Zhao et al., 2013) and can act as immunomodulators (Boparai and Sharma, 2019). Most AMPs have amphiphilicity characteristics that allow them to be soluble in aqueous environments (Adem Bahar, 2015).

Among the most abundant AMPs in nature, those with cationic alpha-helical structures stand out, as these can disturb the cytoplasmic membrane by osmotic shock, leading to cell lysis. The main AMPs known for this capability are cecropin, magainin, human cathelicidin LL-37, and its derivatives, in addition to proline-rich antimicrobial peptides (prAMPs; Graf et al., 2017; Le, 2017). Physicochemical characteristics and secondary structure contribute to a good selectivity and efficacy on cytoplasmic membranes, making these molecules a target for the generation of new biological agents (Torres et al., 2019).

Among the various animal-derived AMPs with biotechnological potential, we highlight the peptide Cupiennin-1a, also called M-ctenitoxin-Cs1a or Cu-1a, present in the venom of the wandering spider *C. salei*. Described by Kuhn-Nentwig et al. (2002), the peptide presents 35 amino acid residues, with a mostly hydrophobic N-terminal region and a polar/charged C-terminal. It is a cationic peptide with a global charge of +8, an amidated C-terminal, presenting a high potential helix formation in the presence of trifluoroethanol (TFE; Kuhn-Nentwig et al., 2002).

Peptides of the cupiennin group act by cytolytic mechanisms against a broad spectrum of bacteria, such as *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis* (Kuhn-Nentwig et al., 2002), *S. epidermidis, Bacillus subtilis, P. putida*, and *Paracoccus denitrificans* (Haeberli et al., 2000). Thus, it was suggested that the role of cupiennin in the venom of *C. salei* could be to protect the poison apparatus (glands and ducts) against infections, as well as to enhance neurotoxin interaction with intracellular targets (Kuhn-Nentwig et al., 2015). However, Cupiennin-1a (Cu-1a) has a wide range of other biological activities, such as its insecticidal action against flies of the species *Drosophila melanogaster* (Kuhn-Nentwig et al., 2002), and parasitic activity against *Trypanosoma cruzi, Trypanosoma brucei*, and *Plasmodium falciparum* (Kuhn-Nentwig et al., 2013).

This peptide shows high cytotoxicity to human erythrocytes, which makes it difficult to use as a therapeutic agent. Thus, the rational design of potential antimicrobial peptides would be an attempt to optimize toxicity for therapeutic use and the search for new biotechnological activities (Porto et al., 2012). This work aimed to select the Cu-1a peptide sequence as a template for the rational design of 7 molecules, in order to reduce the toxicity of the original peptide without losing the biotechnological potential already described in the literature.

Materials and methods

Synthesis of Cu-1a peptide and its analogs

The antimicrobial peptide Cu-1a was synthesized according to the amino acid sequence GFGALFKFLAKKVAKTVAKQ AAKQGAKYVVNKQME-NH2 (where NH2 indicates an amidated C-terminal) of the peptide isolated from *C. salei* spider venom (Kuhn-Nentwig et al., 2002) by Aminotech Company (Campinas, São Paulo—Brazil). The synthesis was carried out using solid-phase approach and F-Moc methodology (Maggiora et al., 1992). Then, seven analogs were designed based on the deletion and substitution of amino acid residues from the original Cu-1a peptide sequence to maintain the desired characteristics for analogous molecules and subsequently synthesized as previously described for Cu-1a.

Molecular mass analysis, quantification, and helix projection

The masses of the 8 peptides were analyzed by mass spectrometry using a MALDI-TOF/TOF (Matrix Assisted Laser Desorption Ionization—Time of Flight) Autoflex Speed (Bruker Daltonics). Peptides were diluted in ultrapure water and mixed with a solution of α-cyano-4-hydroxycinnamic acid matrix at 10 mg.ml^{-1} (50% (v/v) acetonitrile, 0.3% (v/v) trifluoroacetic acid) in a 1:3 ratio. Subsequently, this mixture was deposited in triplicate on an MTP384 ground steel plate and maintained at room temperature until its total crystallization. The mass/charge ratio (m/z) of the peptides was obtained in the range of 700–5,600 m/z, in reflected, positive mode, after external calibration of the equipment with Peptide Calibration Standard II (Bruker Daltonics). MS/MS spectra were obtained *via* LIFT fragmentation (Suckau et al., 2003), and *de novo* interpretation for amino acid sequence confirmation was performed using FlexAnalysis software (Bruker Daltonics).

To perform the experiments, each peptide was diluted in Milli-Q water and quantified by UV absorption at 205, 215, and 225 nm, using the concentration formula (Murphy and Kies, 1960).

TABLE 1 Concentrations in μM and μg.ml^{-1} used in activity assays against bacteria, fungi, RAW264.7 cells, and tumor cells.

Peptides	Concentration μM	Concentration μg.ml^{-1}*
Cu-1a	33–4.22	128–16
R1a	63.26–7.90	128–16
R1b	67.54–8.44	128–16
R2b	68.62–8.57	128–16
R3b	67.21–8.40	128–16
R6b	70.8–8.85	128–16
R8b	70.43–8.80	128–16
R10b	63.3–8.53	128–16

*These concentrations refer to micromolar ratios between 128 and 16 μg.ml^{-1}.

$A = (225 - 215) \times 0.144.$

$B = 205 \times 0.31.$

$A + B/2 = [\,]\ \text{mg.ml}^{-1}.$

After quantification, peptides were aliquoted at a concentration of 128 μg.ml^{-1}.

The projection of the helical wheel of peptides was performed using NetWheels,[1] developed by the Institute of Biology at the University of Brasília.

Evaluation of antimicrobial activity against bacteria

Susceptibility tests were performed using clinical isolates *of Klebsiella pneumoniae carbapenemase* (KPC 1410503) and methicillin-resistant *S. aureus* (MRSA 3730592). Tests were also performed with ATCC (American Type Culture Collection) isolates of *K. pneumoniae* (ATCC 13883) and *S. aureus* (ATCC 25923).

Two multidrug-resistant clinical isolates and two ATCC isolates were chosen to perform microbial susceptibility testing as models for studies with bacteria susceptible to antimicrobials found in the Gram-Positive and Gram-Negative groups of bacteria.

The broth microdilution method was performed according to the Clinical and Laboratory Standards Institute (CLSI) M07-A10 protocol to determine the Minimum Inhibitory Concentration (MIC; Clinical Laboratory Standards Institute, 2015). The assay was performed on 96-well microplates (TPP, United States), containing 5×10^5 CFU.ml^{-1} of each tested bacterium per well and different peptide micromolar concentrations (Table 1) in a final volume of 100 μl of Mueller-Hinton Broth (MH). Negative control was represented by bacteria incubated in MH medium, while positive control was represented by culture incubated in MH medium with Amikacin. Plates were kept under agitation at 37°C in a BioTek spectrophotometer (PowerWaveTM HT Microplate Reader) with optical density (OD) readings at 595 nm every 30 min for 24 h. Inhibition results were analyzed using GraphPad Prism 8 software by comparing the OD from negative control wells (100%) to those containing peptides. All tests were performed in biological triplicates.

Evaluation of antimicrobial activity against *Candida parapsilosis*

The broth microdilution method was performed according to the Clinical and Laboratory Standards Institute Guideline M27-A3 with modifications to determine the Minimum Inhibitory Concentration (MIC; Clinical Laboratory Standards Institute, 2008).

Initially, the fungus *C. parapsilosis* (ATCC22019) was grown in a Petri dish containing Sabouraud Dextrose Agar medium (Dextrose 40 g/L; Peptic digestion of animal tissue 5.0 g.L^{-1}; and Agar 15 g.L^{-1}) for 48 h. Subsequently, a pre-inoculum was performed from a yeast colony that was placed in 10 ml of liquid Sabouraud medium and incubated for 16 h at 28°C, with a constant rotation of 200 rpm. After the incubation period, 2.5×10^3 CFU.ml^{-1} of fungus per well was incubated with different peptide micromolar concentrations (Table 1) to assess the MIC for each peptide.

Fungal cells incubated with Sabouraud medium were used as negative control, while positive control was represented by fungal cells incubated in Sabouraud medium and amphotericin B (10 μM). Subsequently, plates were incubated for 24 h at 30°C. After this period, the absorbance was read (595 nm) in a BioTek spectrophotometer (PowerWaveTM HT Microplate Reader), and data of antifungal activity with percentage of inhibition of peptides were plotted in GraphPad Prism 8 Software.

RAW 264.7 and human fibroblast cell line culture

The cell line RAW 264.7 was obtained from the Rio de Janeiro Cell Bank (CR108). These cells are macrophages derived from induced tumors in male BALB/c mice infected with Abelson's murine leukemia virus (Raschke et al., 1978). RAW264.7 cells were grown in DMEM medium (Dulbecco's Modified Eagle's medium, composed of $CaCl_2$ (anhydrous) 200 mg.ml^{-1}, $Fe(NO_3).9H_2O$ 0.1 mg.ml^{-1}, KCL 400 mg.ml^{-1}, $MgSO_4$ (anhydrous) 97.67 mg.ml^{-1}, NaCL 6.400 mg.ml^{-1}, $NaH_2PO_4.H_2O$ 125 mg.ml^{-1}) supplemented with 10% (v/v) of bovine fetal serum, 0.5% (v/v) of DMEM amino acid solution, 0.05% (w/v) gentamicin, 0.5% (w/v) of L-glutamine, and 0.5% (w/v) of penicillin/streptomycin (1,000 U.ml^{-1}) in an incubator containing 5% CO_2, at 37°C, and 95% humidity (Raschke et al., 1978; Nguyen et al., 2017).

Human fibroblast (Hfib) cell cultures were cultivated in Dulbecco's Modified Eagle Medium (DMEM; Sigma-Aldrich,

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United States) supplemented with 10% fetal bovine serum (Sigma-Aldrich). Hfib were cultured in a humidified incubator containing 5% CO_2 at 37°C and 95% humidity.

Cytotoxicity tests against RAW 264.7 and Hfib

RAW 264.7 cells were seeded in 96-well plates, at a concentration of 10^4 cells/well, and incubated for 24 h. Each peptide was subsequently added at different micromolar concentrations (Table 1). Positive control was performed by cells in culture medium and negative control, as cell culture in lysis solution (10 mM Tris, 1 mM EDTA, and 0.1% Triton X-100, pH 7.4).

Hfib cell line was seeded onto 96-well plates (1×10^3 cells/well, 100 µl) before treatment and allowed to adhere and grow over the 24 h period. Each peptide was subsequently added at different micromolar concentrations. The results were evaluated using GraphPad Prism 8 software, where a One-Way ANOVA statistical test followed by Bonferroni's multiple comparisons test was performed.

Cell viability analyses were performed in technical and biological replicates, and readings were obtained after 24 h of contact with peptides. At the end of the incubation period, MTT (Sigma-Aldrich, United States) was used to assess the ability of living cells to reduce salt 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide for formazan production. Plates were read at 570 ηm (Mosmann, 1983) and results were expressed in the percentage of living cells. The results were analyzed using GraphPad Prism 8 software where a One-Way ANOVA statistical test was followed by Bonferroni's multiple comparisons test.

Nitric oxide production

After 24 h in the presence of peptides, RAW 264.7 cell supernatants were collected for nitric oxide (NO) evaluation. NO production was assessed by nitrite detection by Griess reaction (Mosmann, 1983), with modifications. One hundred microliters of cell culture supernatant were transferred to 96-well plates. A standard curve of nitrite was performed (200 – 0.097 mM). Then, 100 µl of a 1% (w/v) sulfanilamide solution was added in phosphoric acid 2.5% (w/v) and naphthylethylenediamine 1% (w/v) in phosphoric acid 2.5% (w/v), in the proportion of 1:1 to all wells. After 10 min of incubation at room temperature, plates were read at 490 ηm.

Cytotoxicity tests against cancer cell lines

MDA-MB-231 and MCF-7 cell lines, both mammary adenocarcinomas, were cultivated in Roswell Park Memorial Institute (RPMI) 1,640 medium (Sigma-Aldrich, United States) supplemented with 10% fetal bovine serum (Sigma-Aldrich). Cell lines were cultured in a humidified incubator containing 5% CO_2 at 37°C. Antitumor tests were performed on technical replicates and were assessed by MTT (Sigma-Aldrich, United States) assay, as described previously. Both cell lines were seeded onto 96-well plates (1×10^3 cells/well, 100 µl) before treatment and allowed to adhere and grow over the 24 h period. Each peptide was subsequently added at different micromolar concentrations (Table 1). The results were evaluated using GraphPad Prism 8 software, where a One-Way ANOVA statistical test followed by Bonferroni's multiple comparisons test was performed.

Analysis of insecticidal activity

To evaluate the ability of the peptides to cause lysis and morphological changes in insect cells (Dutra, 2019; Patent: BR 102022 006138 6) as a first screening of the insecticidal activity of the peptides, in this work, the Spodoptera IPLB-SF-21AE _ LVI-Cenargen cell line (Vaughn et al., 1977) was used and different concentrations of the peptides were added to the culture medium.

Cells were maintained at 27°C in TNMFH medium (Grace's insect medium supplemented with lactalbumin hydrolyzate and yeastolate), supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich; Castro et al., 2006).

Cell cultivation was carried out in 10 ml culture bottles, model K11-1050 25cm^2, with 5 ml of culture medium per bottle. For the assays, the SF21 cells were grown in TNMFH medium, added 10% (v/v) of bovine fetal serum, and distributed in plates of 96 wells at the concentration of 2×10^4 cells per well, after counting was performed in the Neubauer chamber. Cell suspension was distributed in a volume of 100 µl per well. Peptides were diluted in TNMFH medium and 100 µl of medium with peptide was added, obtaining concentrations of 10, 5, and 1 µM, reaching the volume of 200 µl at the final concentrations of reaction per well. Cells were placed in incubation at 27°C, for 24 h.

After 24 h, the cells were analyzed for their modifications, morphological alterations, turbidity of the medium, formation of clumps, and/or lysis in the presence of peptides, by observation under optical microscopy (Nikon Eclipse TS100) with 20× and 40× magnification. Characteristics that could represent cell death or cell dysfunction caused by the peptides were observed compared to control cells. Cells were evaluated by cell viability using the MTT test 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide 0.5 $mg.ml^{-1}$ (Sigma-Aldrich). To perform the test, the cell culture medium was discarded, and we added 100 µl of new medium. Ten µl of MTT was added to each well and incubated for 4 h, with the plates protected from light. After 4 h, we added 100 µl of DMSO to each well to dissolve the formazan crystals, and the reading in the microplate reader (Riss et al., 2016) was then performed. The results were analyzed using GraphPad Prism 8 software, where a One-Way ANOVA statistical test was followed by Bonferroni's multiple comparisons test.

TABLE 2 Sequence, number of amino acid residues, monoisotopic molecular weight [M+H+], charge, hydrophobicity (*H*), hydrophobic moment (μ*H*), and isoelectric point (pI) of the seven analog peptides.

Peptide	Sequence	Number of amino acid residues	$[M+H]^+$	Charge	*H*	μ*H*	pI
*Cu-1a	GFGALFKFLAKKVAKTVAKQAA KQGAKYVVNKQME-NH_2	35	3798.63	8	−0.138	0.0226	11.30
R1a	GFGALFKFLAKKVAKTVA**K**-NH_2	19	2023.34	5	−0.2579	3.1033	11.1
R1b	GFGALFKFLAKKVAKTVA-NH_2	18	1895.14	4	0.2778	3.1243	11
R2b	GFGALFKFLAKKVAK**A**VA-NH_2	18	1865.13	4	0.4278	3.2387	11
R3b	GFGALFK**R**LAKKVAKTVA-NH_2	18	1904.32	5	−0.8333	3.8198	11.8
R6b	GFGALFKFLAK**A**VAK**A**VA-NH_2	18	1807.98	3	0.9167	3.3629	10.8
R8b	GFGALFK**R**LAK**A**VAK**A**VA-NH_2	18	1817.21	4	−0.1944	3.9049	11.8
R10b	GFG**K**LFK**R**LAK**A**VAK**A**VA-NH_2	18	1874.13	5	−0.6833	4.3819	11.8

*Data reported by Kuhn-Nentwig et al. (2002), Hydrophobicity (*H*) and Hydrophobic Moment (μ*H*) data were described by the total mean values of each characteristic by the authors. Modifications to amino acid residues made in the primary peptide sequence are marked in blue (Ala) and red (Arg) and green (Lys) along the generated sequence.

Results

Molecular mass analysis and helical wheel peptide projection

The seven analogs were generated excluding part of the C-terminal of the Cu-1a peptide described by Kuhn-Nentwig et al. (2002), producing molecules containing 18 or 19 amino acid residues, amino acid substitution was also performed along the peptide chain. The molecular mass and sequences of the seven synthesized analogs were analyzed by mass spectrometry using MALDI-TOF/TOF, Table 2 shows the results obtained.

Physicochemical characteristics such as cationicity, helicity, hydrophobicity, hydrophobic moment, extension of the peptide chain, and c-terminal amides were taken into account for the rational design of these analogs. The analogs generated are positively charged peptides as well as the Cu-1a peptide. Arginine (R), Lysine (K), and Alanine (A) were the amino acids chosen for substitution along the peptide chain due to their potential to contribute to the formation of idealized amphipathic helixes. These amino acids can also contribute to the bending of peptides, because the interactions between the lateral chains can stabilize helical structures (Deslouches et al., 2013).

Finally, the analogs also presented an amidated C-terminal, as well as the original molecule. There are several ways to visualize structural conformations adopted by peptides, two of which are commonly used by biochemists for two-dimensional visualization of secondary molecule structures such as helical wheels and Wenxiang diagrams (Wadhwa et al., 2018). In this work, we chose two-dimensional visualization using helical wheels with software for the projection of the helical wheel, such as NetWheels.[2]

Figure 1 shows the helical projection of the wheel of the eight synthetic peptides, highlighting in red the basic residues of polar amino acids, in blue, the acidic polar amino acids, in green, the unloaded polar amino acids, and, finally, in yellow, the hydrophobic amino acids. It is also possible to observe that the hydrophilic (red) and hydrophobic (yellow) part that make up each peptide are well distributed. Through these regions, we see that the generated molecules have amphipathic characteristics that can contribute to the biological activity of peptides.

Antimicrobial activity against bacteria and fungi

Antimicrobial assays showed that Cu-1a and its analogs showed activity against bacteria and fungi at different concentrations.

Cu-1a and analogs R1a, R2b, R6b, R8b, and R10b showed the best antimicrobial results, and analog R10b was the most efficient of them against both strains of *S. aureus*. Except for the Cu-1a peptide, there was no change in the concentration required to inhibit the growth of *S. aureus* ATCC25923 and MRSA strain by analogs (Table 3).

For the clinical isolate KPC1410503, the Cu-1a peptide and the analog R10b were efficient at low concentrations, while the analogs R1b, R2b, and R8b presented MIC values at high concentrations. MIC was not found for the analogs R1a, R3b, and R6b against KPC1410503 at the tested concentrations. Regarding the ATCC13883 strain of *K. pneumoniae*, the Cu-1a peptide and the analogs R2b, R8b, and R10b showed better results, while for peptides R1a, R1b, R3b, and R6b, MIC was found at high concentrations.

In general, most peptides showed activity against both strains of *K. pneumoniae*. However, Cu-1a and analog R10b showed the best activity against both strains. It was also observed that a lower

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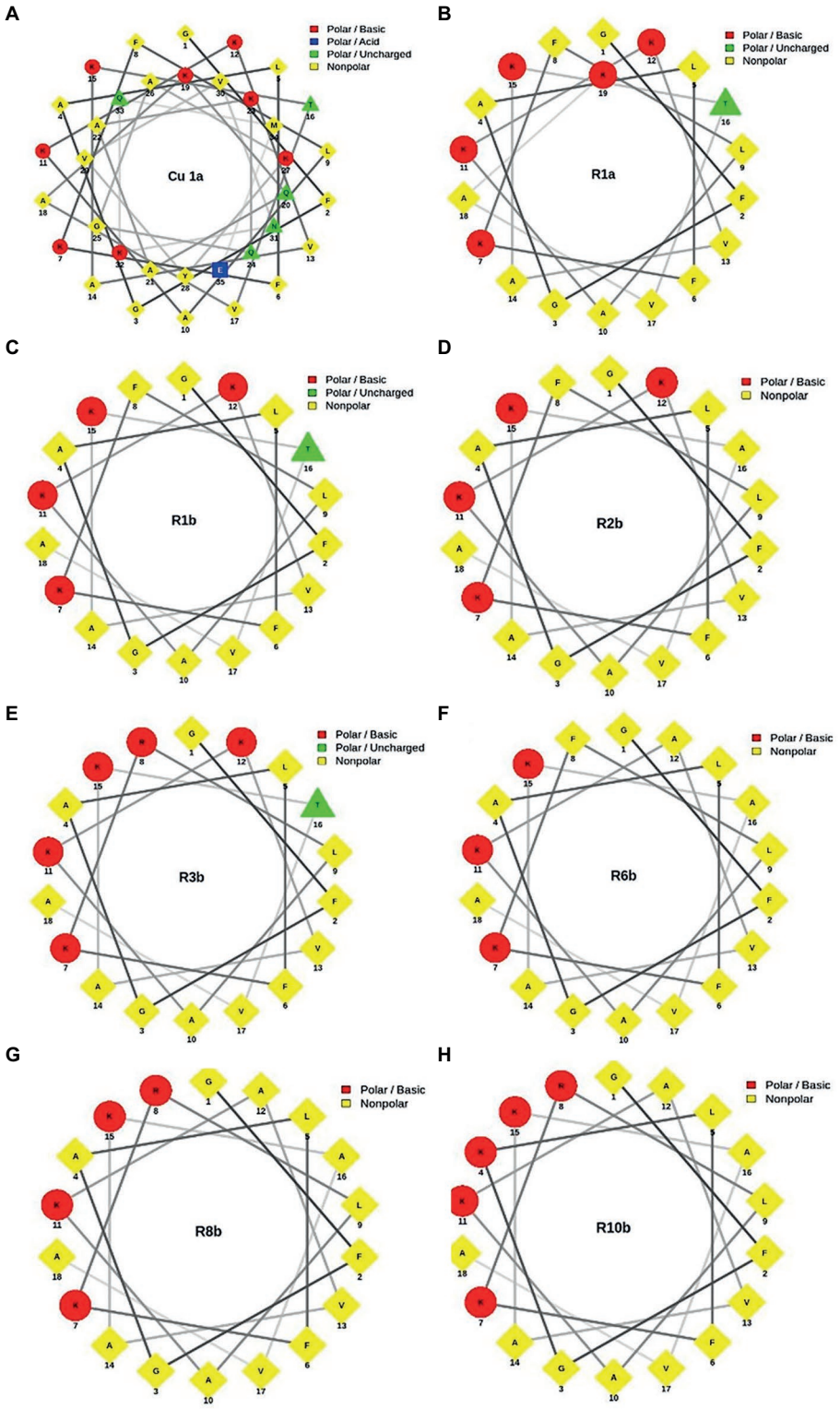

FIGURE 1
Helical wheel projection of analogous synthetic peptides designed based on the Cu-1a peptide sequence. In red, the basic polar amino acid residues; in blue, the acid polar amino acids; in green, the uncharged polar amino acids; and in yellow, the nonpolar amino acids. **(A)** Cu-1a; **(B)** R1a; **(C)** R1b; **(D)** R2b; **(E)** R3b; **(F)** R6b; **(G)** R8b; and **(H)** R10b.

concentration of Cu-1a and its analogs is necessary to inhibit the microbial growth of the ATCC13883 strain compared to the multidrug-resistant clinical isolate KPC1410503 (Table 3).

The Cu-1a peptide and its analogs were able to inhibit the growth of the multidrug-resistant strains KPC1410503 and MRSA3730592, as well as inhibit the growth of the reference

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TABLE 3 MIC values found for peptide Cu 1a and their analogs against bacteria and fungi.

Bacterial strain	Minimum inhibitory concentration (MIC) (μM)							
	Cu-1a	R1a	R1b	R2b	R3b	R6b	R8b	R10b
K. pneumoniae carbapenemase—KPC	16.9	NE	67.54	34.31	NE	NE	35.21	17.07
Methicillin-Resistant S. aureus—MRSA	4.22	15.81	33.77	17.15	67.21	17.7	17.60	8.53
K. pneumoniae (ATCC13883)	16.9	31.63	33.77	17.15	67.21	35.4	17.60	17.07
S. aureus (ATCC25923)	16.9	15.81	33.77	17.15	67.21	17.7	17.60	8.53
C. parapsilosis (ATCC22019)	33.8	15.81	16.88	34.31	33.60	70.8	35.21	34.15

NE, MIC value not found.

ATCC strains of *K. pneumoniae* and *S. aureus*. It is important to note that this is the first report on the antimicrobial effect of the Cu-1a peptide against strains of *K. pneumoniae* and MRSA.

It was also observed that all peptides tested had antimicrobial effects against *C. parapsilosis* ATCC22019 at different concentrations. Peptides R1a and R1b showed better antifungal activity, showing MIC values at low concentrations when compared to Cu-1a peptide and analogs R2b, R3b, R6b, R8b, and R10b (Table 3). These results emphasize the activity of the peptide against bacteria and fungi, in addition to its efficacy against resistant multidrug microorganisms.

RAW 264.7 and Hfib cell viability and NO production

To evaluate the cytotoxicity of all tested peptides, the MTT assay was performed to evaluate cell viability. As expected, the peptide Cu-1a exhibited high toxicity at all tested concentrations (Figure 2A) against RAW264.7 cells. The analogous peptides showed different levels of toxicity against RAW264.7 cells (Figures 2B–H). Changes made in the sequence and size of the Cu-1a peptide were not effective in decreasing the cytotoxicity toward RAW264.7 cells in most of the designed peptides. Analogs R2b, R3b, R6b, and R10b (Figures 2C–H) showed high toxicity against macrophages at concentrations that were effective for the microorganisms evaluated, eliminating these as promising peptides for therapeutic agents like the original peptide. On the other hand, analogs R1a, R1b, and R8B were not toxic to RAW264.7 cells at the tested concentration. These results indicate that R1a, R1b, and R8b analog peptides can be further explored as promising therapeutic agents. Lastly, after 24 h, the NO production was analyzed in cell culture supernatant, and no NO production was observed in the presence of the peptides at all tested concentrations.

Excluding the R3b peptide, all-analog peptides showed cytotoxicity against human fibroblast cells at the highest concentration (Figure 3). In addition to eradicating 100% of the cells at 70.8 μM, the R6b peptides also showed a slight activity at 35.4 μM, reducing the cell viability (Figure 3E). Although the R8b (Figure 3F) and R10b (Figure 3G) peptide analogs were toxic to Hfib cells, killing 100% of them at the highest concentration (70.43 and 68.3, respectively), they were not harmful to these cells at the other concentrations. However, these peptides remain to kill MCF-7 cell lines at the lowest concentrations. Regarding the tests performed with the MDA-MB-231 strain, the R1a analog peptide demonstrated activity against mammary adenocarcinoma cells (at 63.26, 31.63, and 15.81 μM), but R1a did not present a cytotoxic effect, at these concentrations, against Hfib cells, being toxic only at the highest concentration (63.26 μM). The R3b peptide analog was the only one, among all analogs, that did not show any cytotoxic effect on Hfib cells. Furthermore, this peptide showed activity against both MCF-7 and MDA-MB-231 (at the highest concentration tested). The data suggest that these analog peptides can be explored as a potential cancer-fighting molecule in the future.

Cytotoxicity tests against cancer cell lines

The MTT assay was also used to evaluate the cytotoxic effect of peptides against two cellular types of mammary adenocarcinoma, and MCF-7 was chosen as the standard cell (Figure 4) and MDA-MB231 (Figure 5) as a cell resistant to antitumor agents. The peptide Cu-1a showed activity against both tumor cells (Figures 4A, 5A), even at low concentrations. In addition, all analogs demonstrated antitumor potential against MCF-7 in all tested concentrations (Figures 4B–H). They were highly effective, as it is possible to observe cell viability below 50% even at lower concentrations.

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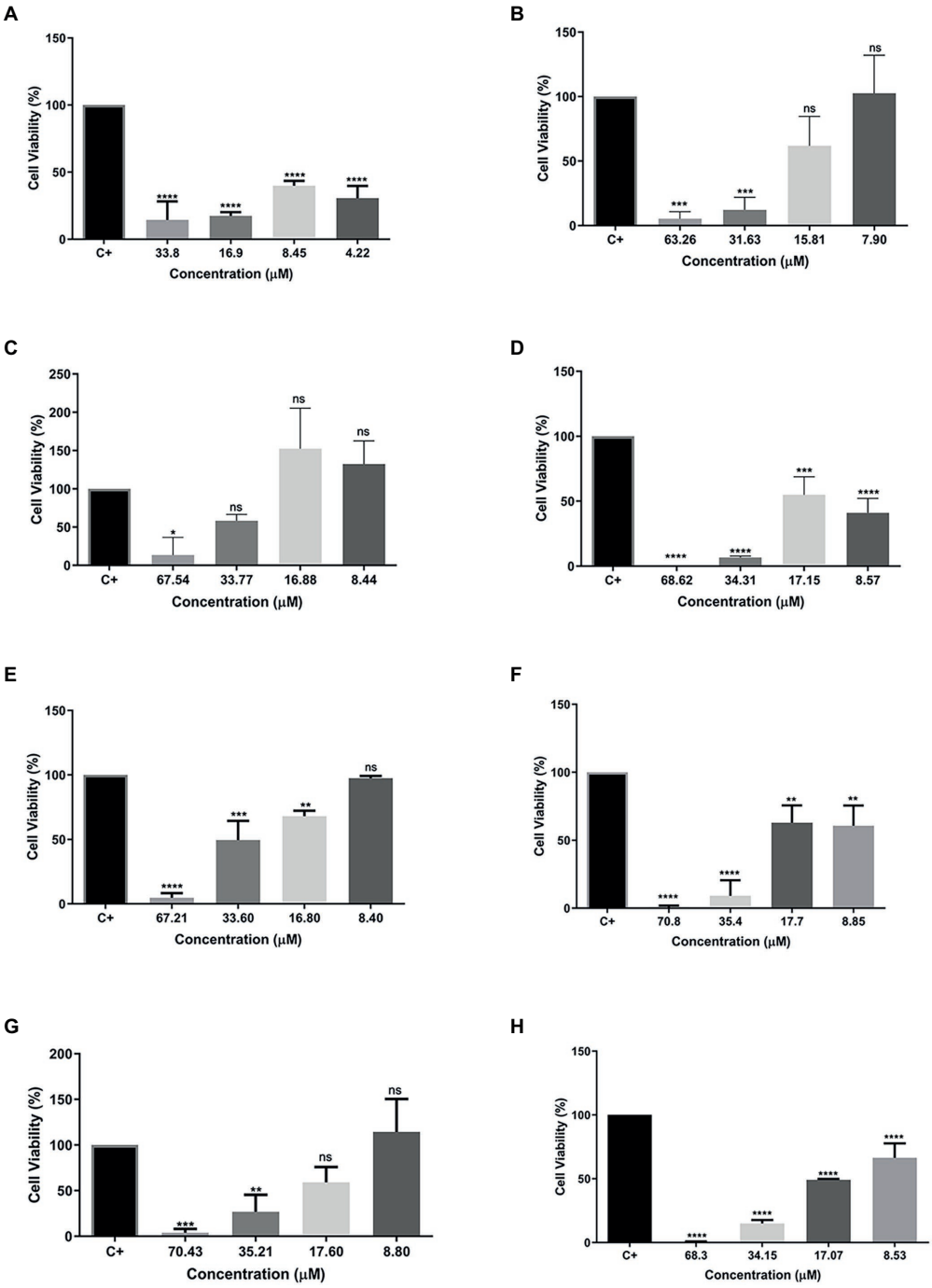

FIGURE 2
Percentage of cell viability in RAW 264.7 cells. **(A)** Cu-1a; **(B)** R1a; **(C)** R1b; **(D)** R2b; **(E)** R3b; **(F)** R6b; **(G)** R8b; and **(H)** R10b. One-way ANOVA—Bonferroni's multiple comparisons test. Each bar represents the mean±SD of cellular absorbance; $N=3$. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, and NS $p>0.05$.

On the other hand, different results were found when peptides were tested against the therapeutic agent resistant to antitumor MDAMB-231 (Figure 5). The peptide Cu-1a continued to show activity against the therapeutic agent-resistant breast cancer cell line (MDAMB-231), even at low concentrations, while the analog peptides demonstrated antitumor activity against this line only at higher concentrations. The data obtained with the two tumor strains show that the analog peptides did not lose their antitumor potential after the removal and replacement of amino acid residues from the original sequence, but they were not able to perform as well as peptide Cu-1a against resistant tumor cells.

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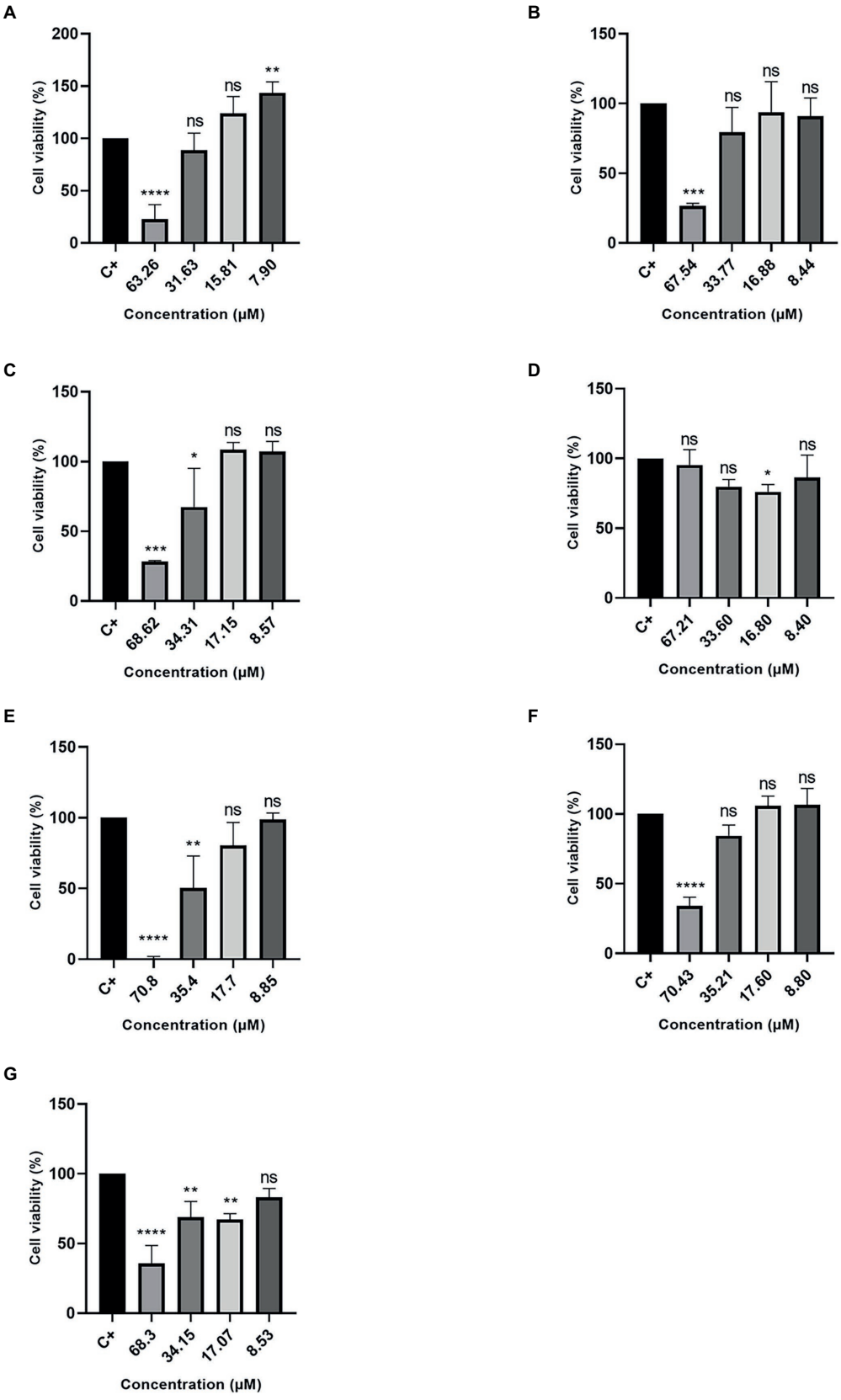

FIGURE 3
Percentage of Cell Viability in HFib cells. **(A)** R1a; **(B)** R1b; **(C)** R2b; **(D)** R3b; **(E)** R6b; **(F)** R8b; and **(G)** R10b. One-way ANOVA—Bonferroni's multiple comparisons test. Each bar represents the mean±SD of cellular absorbance; $N=3$. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, and NS $p>0.05$.

In vitro test against insect cells

The insecticidal activity was tested against line cells of the corn caterpillar *Spodoptera frugiperda* (SF21), an important agricultural pest capable of producing damage in several commercial crops worldwide.

Different concentrations (10, 5, and 1 μM) of all eight peptides were used. Cells were then evaluated for their cell viability after 24 h of contact with the different peptide concentrations. Most of the tested peptides showed cytotoxic activity at all concentrations, except for peptides R1a, R1b, and R3b (Figures 6B–E), which presented low toxicity. The peptide Cu-1a (Figure 6A) and the analogs R2b, R6b, R8b, and R10b (Figures 6D,F–H) were toxic to insect cells in all tested concentrations but were more efficient at higher concentrations. Moreover, peptide R3b (Figure 6E) showed lower efficiency (14% viable cells) only at a concentration of 10 μM. At concentrations of 5 and 1 μM, cell viability was greater than 50%. These results demonstrate the insecticide potential for the peptide Cu-1a and its analogs R2b, R6b, R8b, and R10b, which can be promising for further studies on their insecticidal activity.

Discussion

Rational design of peptides and helical wheel peptide projection

The rational design of peptides consists of several physicochemical parameters previously analyzed for projection and creation of new peptides with antimicrobial activities and reduction of unwanted activities, such as hemolysis or toxicity toward mammalian cells (Ya'u and Nujarin, 2020). Currently, there are three main approaches mostly used for the projection of new peptides: based on templates, physicochemical properties, and *de novo*. Within these approaches, important structural characteristics for antimicrobial peptide activity are analyzed, such as size, hydrophobicity, conformation, charge, and amino acid sequence (Fjell et al., 2012).

The objective of this research was to perform rational design of molecules from a protein with potential for application in different biotechnological areas. For this, we chose the peptide Cu-1a described by Kuhn-Nentwig et al. (2002) because it adapted to all the parameters, we sought for the model molecule. We observed that this peptide has biotechnological potential, but has high toxicity, which causes this peptide to be discarded due to applicability issues. We thus designed seven analogous molecules, aiming to reduce the cytotoxic effect without losing the biological activities described in the literature. Changes were performed in sequence size and amino acid residues, valuing the hydrophobicity, helicity, cationicity, and reduction of cytotoxic activity in analog peptides, since these parameters are important for Cu-1a activity.

For a better visualization of the modifications made in the analog peptides, we chose helical wheels (Figure 1) because they are a type of plot widely used to visualize the structural conformations adopted by peptides. In this type of plot, it is possible to observe secondary structures of the alpha-helix type adopted by some peptides. In addition, this allows the researcher to have a panoramic view of helical peptides, with the amino acid residues interacting with each other and adopting the shape of a perfect circle, which allows the visualization of the hydrophobic and hydrophilic faces of the molecules (Schiffer and Edmundson, 1967; Wadhwa et al., 2018).

Through helical wheels, we can perceive all analogs with a larger hydrophobic face which can contribute to interactions of the molecule with different cell types. Peptide hydrophobicity influences the activity and selectivity of AMPs. Increased hydrophobicity can increase antimicrobial activity, and its reduction leads to decreased antimicrobial effect. In addition, hydrophobicity can lead to a variety of target cells from a peptide (Almsned, 2017). Thus, hydrophobicity and charge were factors considered for the design of synthetic analog peptides.

Antimicrobial activity against bacteria and fungi

Previous literature has described the antimicrobial activity of peptide Cu-1a against ATCC strains of *Escherichia coli, S. aureus, Enterococcus faecalis*, and *Pseudomonas aeruginosa* (Kuhn-Nentwig et al., 2002). However, there was no information regarding its antimicrobial activity against multidrug-resistant clinical isolates such as KPC and MRSA and against fungi. Similar antimicrobial effects against *K. pneumoniae* ATCC strains have been previously described by Kuhn-Nentwig et al. (2002). This peptide also presented an antimicrobial effect against clinical isolates of KPC and MRSA. Antimicrobial activity was observed against these strains and against the yeast *C. parapsilosis* by the analogs developed from peptides. The peptides that showed the best microbial activity were Cu-1a and analog R10b, which obtained MICs found in low concentrations for all tested organisms.

Antimicrobial tests demonstrated that changes in analog peptides when compared to Cu-1a resulted in reduced antimicrobial activity when tested against the clinical isolate of KPC1410503 at lower concentrations, and MIC was observed only at higher concentrations. Many multidrug-resistant strains of KPC have a thicker capsule than strains with little resistance, hindering the activity of antimicrobials that act on the membrane (Nepal et al., 2017). That was the case of the clinical isolate used in this study, but this was not observed for the analog R10b, where the substitution of Ala-4 by Lys-4, Phe-8 by Arg-8, Lys-12 by Ala-12, and Thr-16 by Ala-16 resulted in an increase in hydrophobicity and + 5 load. These modifications were important for the activity of this analog against this strain, and a MIC close to the concentration of the original peptide was found. Thus, we can conclude that the alterations made in the

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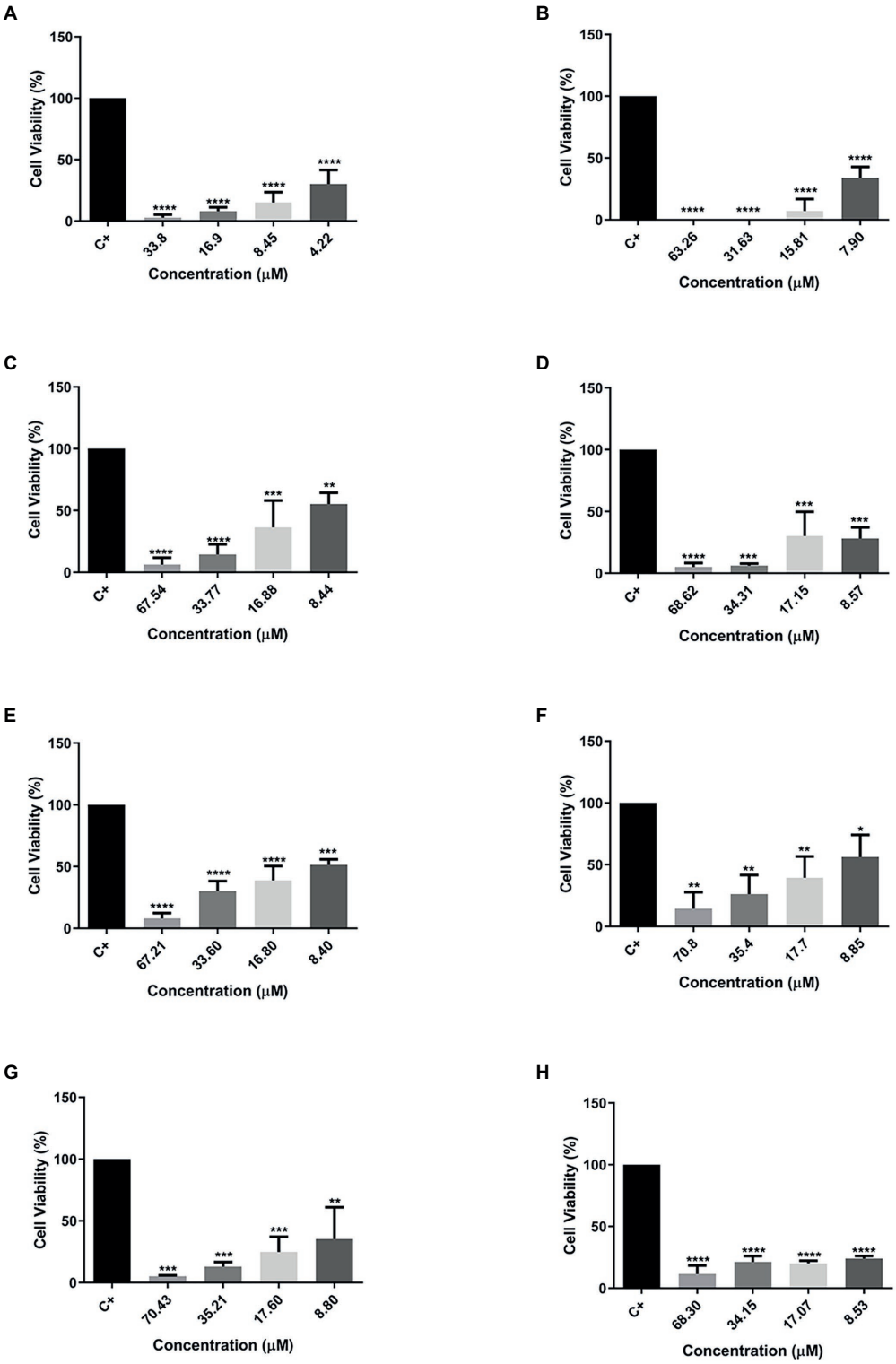

FIGURE 4
Percentage of cell viability in MCF-7 cancer cells. **(A)** Cu-1a; **(B)** R1a; **(C)** R1b; **(D)** R2b; **(E)** R3b; **(F)** R6b; **(G)** R8b; and **(H)** R10b. One-way ANOVA—Bonferroni's multiple comparisons test. Each bar represents the mean±SD of cellular absorbance; $N=3$. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, and NS $p>0.05$.

analog R10b contributed to the selectivity and activity of this peptide in relation to this multidrug-resistant strain of KPC1410503.

When tested against strain ATCC13883 of *K. pneumoniae* whose resistance profile is low and with the absence of a capsule, we observed that the peptides R2b and R8b showed better activity,

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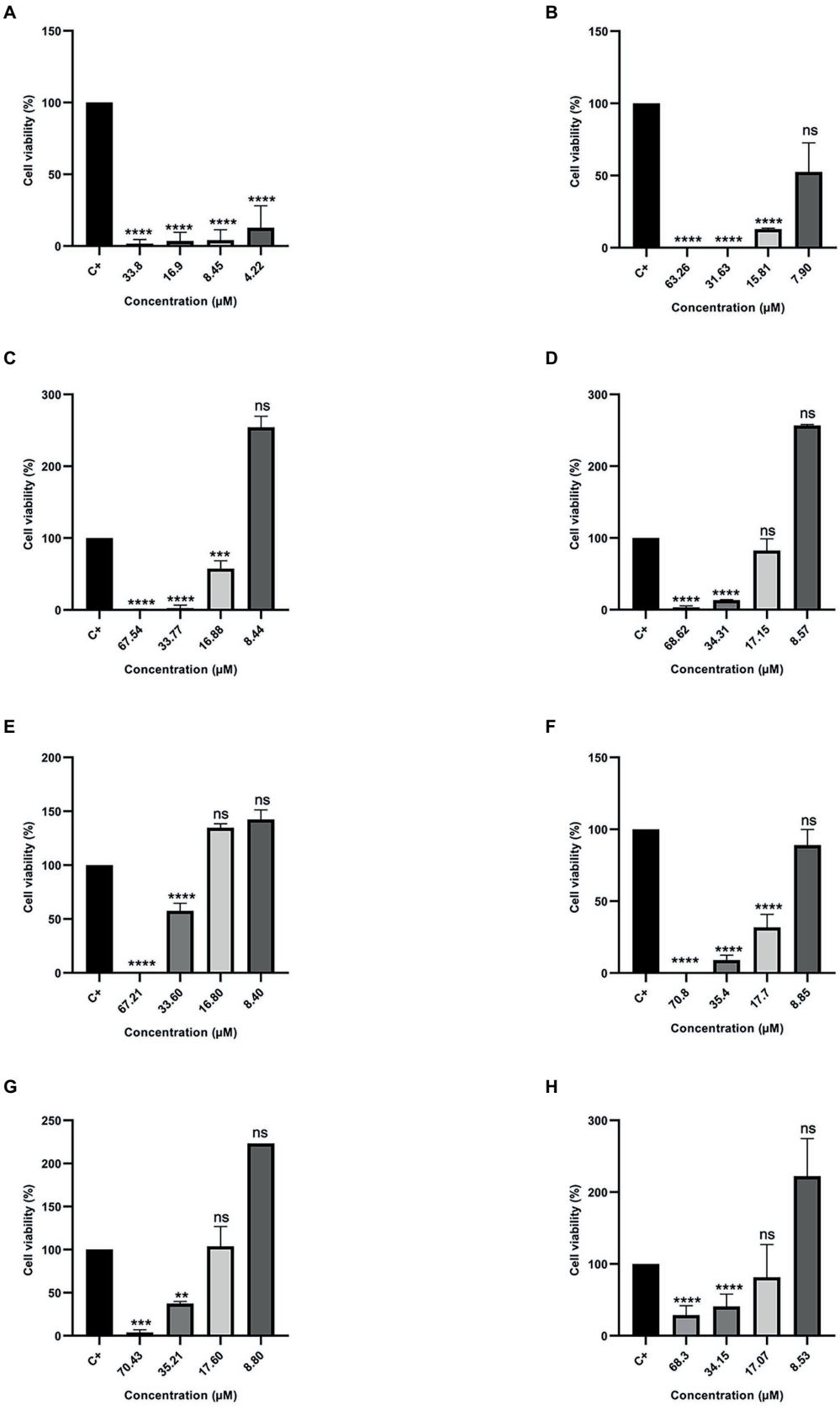

FIGURE 5
Percentage of Cell Viability in MDA-MB231 cancer cells. **(A)** Cu-1a; **(B)** R1a; **(C)** R1b; **(D)** R2b; **(E)** R3b; **(F)** R6b; **(G)** R8b; and **(H)** R10b. One-way ANOVA—Bonferroni's multiple comparisons test. Each bar represents the mean±SD of cellular absorbance; $N=3$. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, and NS $p>0.05$.

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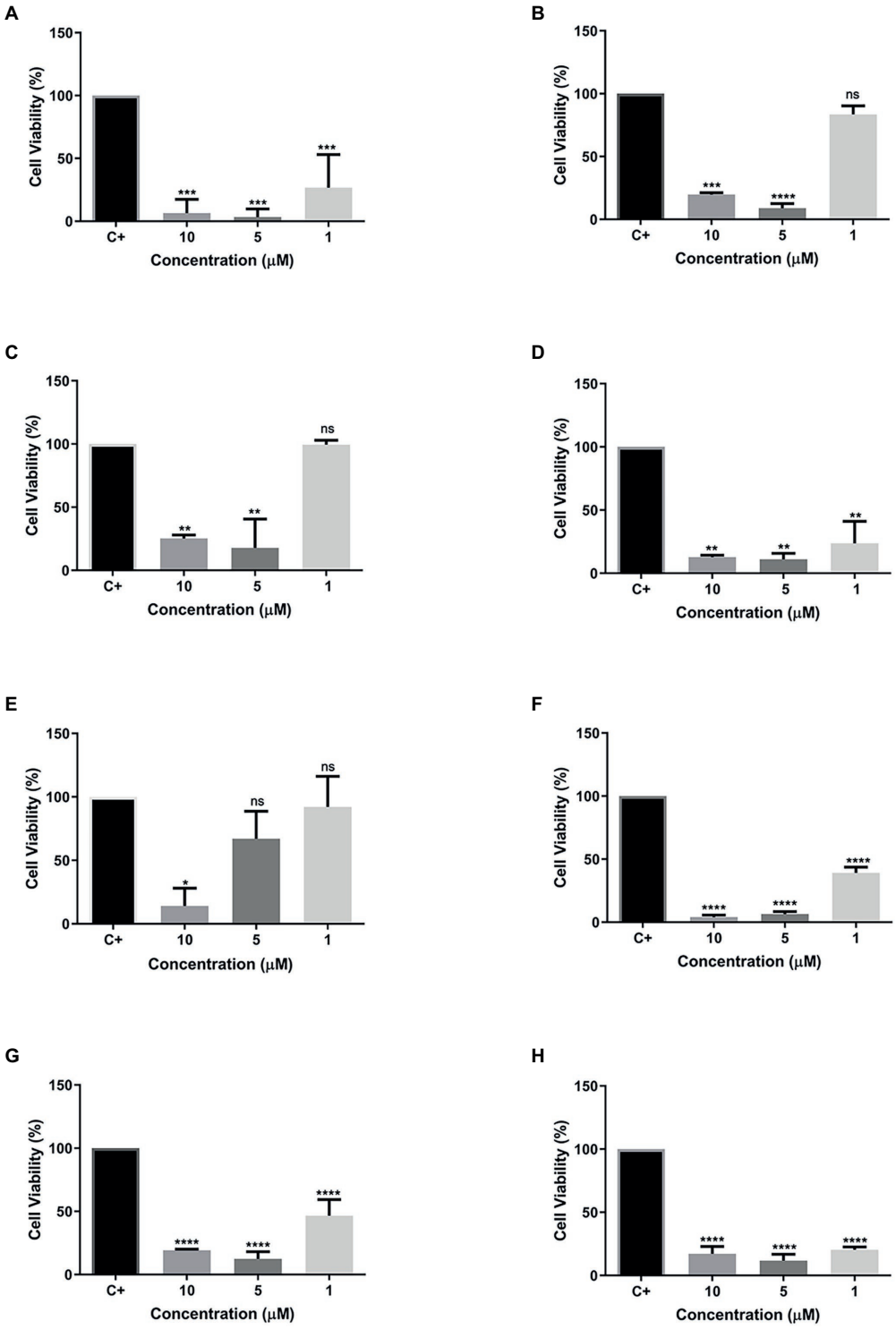

FIGURE 6
Cell viability of synthetic peptides tested against SF21 cell line at concentrations of 10, 5, and 1μM. **(A)** Cu-1a; **(B)** R1a; **(C)** R1b; **(D)** R2b; **(E)** R3b; **(F)** R6b; **(G)** R8b; and **(H)** R10b. One-way ANOVA—Bonferroni's multiple comparisons test. Each bar represents the mean±SD of cellular absorbance; $N=3$. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, and NS $p>0.05$.

with MIC at low concentrations, while the peptide Cu-1a and the analog R10b maintained their activities against this strain. Alterations realized in the analog R10b contributed to its activity and selectivity against multidrug-resistant strains of this bacterial species. It was observed that the alteration of Thr-16 by Ala-16 in the analogs R2b, R8b, and R10b resulted in an improvement in

activity against *K. pneumoniae* ATCC13883. In addition, the substitution of Lys-12 by Ala-12 was important for R8b and R10b activity against this strain. The substitution of Ala-4 by Lys-4 was important to maintain the MIC against KPC1410503 in a non-resistant strain, ATCC13883, which was not observed for analogs R2b and R8b; these did not have this substitution and the MIC for KPC1410503 was only found in high concentrations.

There was no difference in MIC concentration found for the two strains of *S. aureus* when exposed to different concentrations of analog peptides; however, the MIC found for Cu-1a versus MRSA3730592 was better than that observed for the non-resistant strain, demonstrating that this peptide obtained greater selectivity against this strain. The analogs that showed the best activity against both microbial strains were R1a, R2b, R6b, R8b, and R10b, with the analog R10b showing better microbial activity against both strains.

The analog peptide R10 showed better activity against all bacterial strains tested, demonstrating that the alterations performed maintained its activity against gram-positive and gram-negative bacteria, making it the most promising among the analog peptides against bacteria. Lys and Arg are the preferred amino acids during the rational design of molecules with more efficient antimicrobial actions, but the presence of Arg residues is accompanied by high hemolytic properties when compared to Lys-rich peptides (Wiradharma et al., 2011; Ong et al., 2013). We performed the interleaved use of Lys and Arg additions following the analog peptide R10b, which contributed to selectivity against microbial agents, and when tested in RAW cells, it was observed that at the MIC value found for bacteria, there is low toxicity in RAW cells, with more than 50% cell viability at concentrations of 17.07 and 8.53 μM.

When tested against fungi, the analog peptides that stood out were R1a and R1b with MIC at low concentrations, demonstrating that the alterations made in these peptides favored their selectivity against fungal strains. This was not found for the original peptide, which had activity at higher concentrations. In these two peptides, the alterations were only in the last amino acid residue, where in R1a Lys-19, it was maintained and in R1b this residue was removed, but this alteration did not result in loss of activity against fungi, demonstrating that the structural arrangement resulted in greater selectivity against fungi. Substitutions of amino acid residues along the sequence in other analog peptides resulted in the loss of antifungal activity, and activity was observed only at high concentrations.

In a study conducted by Wiradharma et al. (2011), with α-helical AMPs of unnatural origin, these peptides were cationic and amphiphilic and were designed to mimic the behavior of α-helical AMPs of natural occurrence. In this study, the peptide LLKK3 caught our attention because it presented the best activity against *C. albicans*. This peptide is rich in residues of Leu and Lys, and our analogs R1a and R1b have these residues arranged in its sequence. When comparing our data, we observed that, just like the peptide LLKK3, our analogs R1a and R1b have an appropriate hydrophobic-cationic balance to form an α-helical conformation in a membrane-like environment besides having an ideal amphiphilic helical conformation that can be seen in the helical wheel projection. Therefore, it is suggested that these residues collaborate with the structural arrangement adopted by these peptides in fungal cells. Despite maintaining the hydrophobic-cationic balance in the other analog peptides when performing changes in amino acid residues, the structural arrangement undergoes alteration, and this interferes with the interaction of peptides with fungal cells, leading to decreased activity.

Cytotoxicity RAW cells compared to other cell types

To verify the toxic effect of the analog peptides after the modifications, we performed the cell cytotoxicity assay in RAW264.7 cells and observed that the peptide Cu-1a was toxic in all tested concentrations, confirming the cytotoxic effect of this molecule. The first 19 residues from the primary sequence of Cu-1a form the initial part of the toxic N-terminal of Cu-1a (Kuhn-Nentwig et al., 2013), and these were the basis for the generation of the R1a analog. From this, the modifications were made along the sequence for the generation of other analogues, in view of the reduction of toxicity already reported previously in the literature.

The modifications made to the peptide sequences resulted in a reduction in the toxicity observed on RAW264.7 macrophages at lower concentrations. When we removed Lys-19 from the sequence to generate the analog R1b, we observed a reduction in toxicity from 33.77 μM, with 58% of viable cells, and this is not observed in the R1a analog, which presents high toxicity in this concentration range. Substitutions of amino acid residues along the peptide chain reduced the cellular toxicity observed for the analog peptides R2b, R3b, R6b, R8b, and R10b at lower concentrations, and it is possible to visualize cell viability equal to or greater than 50%.

The results of cytotoxic tests demonstrate that the analog R1a could be used in the control of MRSA, ATCC25923 of *S. aureus,* and ATCC22019 of *C. parapsilosis*, because we observed 63% of viable cells in the MIC found for these strains. R1b could be used to control all the microorganisms tested, except for KPC1410503, where high toxicity of this peptide is observed in the MIC concentration found for this strain. For the analog R2b, cell viability was observed above 50% in the MIC found for MRSA3730592, ATCC25923 of *S. aureus*, ATCC13883 of *K. pneumoniae*, and can be used to control these pathogens.

R3b was the only analog that presented MIC at high concentrations against all tested microorganisms, being toxic to RAW264.7 cells in this concentration. R6b could be used only to control the two strains of *S. aureus* tested, and cell viability of 62% was observed in the MIC found for these two strains. From the MIC concentration found for MRSA3730592, ATCC25923 of *S. aureus,* and ATCC13883 of *K. pneumoniae*, cell viability of

59% was observed in RAW264.7 cells when exposed to the analog R8b.

Still analyzing Figure 2 in comparison with Table 3, the analog R10b showed better antimicrobial activity against all bacteria tested when compared to other analog peptides. It was demonstrated that the substitutions performed in the sequence contributed to a greater selectivity of the peptide against bacteria and can be used in the control of these bacteria, since cell viability is observed above 50% in RAW264 cells. This analog also stood out when tested against MCF-7 and Insect Cells SF-21, demonstrating it to be a peptide with various biotechnological applications.

Cytotoxicity Hfib cells

Hfib cells were used to assess the cytotoxic potential of analog peptides (Figure 3). The data showed that, with the exception of the R3b analog (Figure 3D), the remaining peptides were toxic, at some level, to cells when tested with the highest concentration of each analog peptide. However, in the other concentrations tested in this work, the analogs did not show considerable cytotoxicity. Previous tests have shown that cupiennin 1a has hemolytic activity against human erythrocytes at a semi-maximal concentration (EC_{50}) of 24.4 μM (Kuhn-Nentwig et al., 2002). Tests performed against mouse skeletal myoblasts (L-6 cells) demonstrated that the peptide cupiennin 1a is toxic to these cells at a concentration of 0.342 uM (EC_{50}; Kuhn-Nentwig et al., 2011). The concentrations tested in this work are higher than those used in these studies. Thus, it is possible to suggest that cupiennin 1a analog peptides maintain the potential anticancer activity, without being toxic to healthy cells, even at higher concentrations.

Cytotoxicity tests against cancer cell lines

Kuhn-Nentwig et al. (2011) published a study in which they demonstrated that Cu-1a has activity against different human leukemic cell lines, as well as being able to eradicate HeLa cells, both in *in vitro* tests. In this work, we tested the antitumor activity of Cu-1a and its analogs through MTT assays and evaluated cell viability after exposure to different peptide concentrations against two cell lines of breast cancer. MCF-7 was chosen as a strain non-resistant to therapeutic agents, and MDAMB-231 was chosen as resistant strain, to evaluate peptide behavior when exposed to two different cancer strains. We expected to find activity of the peptide Cu-1a and its analogs against cell lines of breast adenocarcinoma. In this work, we were able to confirm the activity against tumor cells for the peptide Cu-1a and its analogs.

The analog peptides that showed the best activity against MCF-7 were R1a and R10b, and it was possible to observe low cell viability in all concentrations tested. On the other hand, only the analog R1a maintained activity when tested with the tumor cell MDAMB-231, a breast cancer cell resistant to therapeutic agents. Although the resistance factor influences the action of AMPs on cell growth, it is necessary to consider the sequence of peptides and how their structural arrangement occurs in cell membranes. In this context, when analyzing the changes made along the R10b peptide sequence with the observed cytotoxicity, it is seen that these changes led to the loss of the activity of this peptide in more resistant tumor cells, which was not observed for the analog R1a that did not undergo such changes in its sequence and maintained its activity.

Although toxicity to MCF-7 is observed for the other analog peptides, the results indicate that they do not have selectivity for resistant tumor cells, as with the R10b analog mentioned above. So, we can infer that changes in amino acid residues along the chain of other analog peptides also resulted in the loss of their activity against the resistant lineage MDAMB-231, where the other analog peptides were toxic only at the highest concentrations. The secondary structure of projected peptides affects the potency, selectivity, and structural orientation of the peptides, depending on the orientation angle, while the arrangement adopted by the peptide produces destabilization of the membrane phospholipids and affects permeability (Hanaoka et al., 2016; Liscano et al., 2020). Therefore, very large changes along the peptide chain can influence these interactions and consequently lead to loss of activity, depending on the cell type to which it is exposed.

In vitro test against insect cells

The insecticidal activity of Cu-1a was demonstrated by injecting the peptide in *Drosophila melanogaster*. Although the methodology and the organism used to assess insecticidal activity are different, we can see that our data corroborate those of Kuhn-Nentwig et al. (2002) on the insecticidal activity shown by the peptide Cu-1a. The authors report that it took 5.9 pmol of peptide per mg of fly to obtain the EC50.

We performed cell viability tests to evaluate the insecticidal effect of Cu-1a and its analogs against the cell line of *Spodoptera frugiperda* (SF-21). The peptide Cu-1a showed high cellular toxicity against the SF-21 strain in all tested concentrations (Figure 5A), thus confirming its insecticidal potential, as described by Kuhn-Nentwig et al. (2002).

The analog peptides showed cellular toxicity against SF-21 (Figures 6B–D,F). The analogs R1a and R1b showed toxicity only when tested at concentrations of 10 and 5 μM and were not toxic with 1 μM peptide. The analog R3b was toxic to cells only at the highest concentration tested. The peptides R2b, R6b, R8b, and R10 had a higher cytotoxic effect on insect cells, and it was possible to observe cell viability below 50% in the lowest concentration tested (1 μM). However, using the methodology used here, *in vivo* tests will be necessary to confirm the results presented here as insecticidal activity of these molecules. However, the results obtained in the cell viability assay give us an indication that these peptides have insecticidal potential.

Conclusion

The analogs that presented the best biological activities against different cell types and that presented the least toxic effect were R8b and R10b. These peptides stand out from the others because, in addition to having activity against bacteria and fungi, they also presented activity against tumor cells MCF-7 and insect cells SF-21 in all concentrations tested. In addition, they showed low toxicity in RAW264.7 and Hfib cells at different concentrations tested. Thus, we can conclude that substitutions performed on these peptides were able to maintain the broad spectrum of biological activity previously reported for Cu-1a, besides having contributed to a reduction in the toxicity. Rational design from potential molecules of natural origin contributes strongly to the development of new molecules with different biotechnological applications.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://page.ucb.br/bc/pesquisador.listaProducoes?idc=39177&id1=106&id2=2018, UCB 34322.

Author contributions

RA, MR, and SD: conceptualization. RA, TD, and ML: methodology. RA, MR, NB, TR, and SD: validation. SD: resources. RA, TR, ML, and NB: data curation. RA: writing—original draft preparation. RA, TR, NB, MR, and SD: writing—reviewing and editing. SD and MR: supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Mini Review

An Overview of the Importance of Bacterial Elements for Plant Genetic Engineering

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Abstract

Plant genetic engineering is one of the most important aspects of biotechnology applied to plant systems. The stable introduction of exogenous genetic material in plant cells is a determining step for obtaining transgenic plants. In this context, bacteria are crucial for the development of transgenic plants. Gene cloning often involves the use of bacterial plasmids and DNA-modifying enzymes synthesized in genetically modified bacteria. In addition, among the several methods of introducing genes into plants, the method that uses *Agrobacterium tumefaciens* continues to be used to obtain genetically modified plants for the agricultural, pharmaceutical and materials industry sectors. This minireview aims to present the basic aspects of bacterial elements related gene manipulation for obtaining transgenic plants.

Keywords: Biotechnology; Plant Genetic Engineering; Gene Cloning

Introduction

As molecular units that contain the genetic information expressed in RNA and proteins, genes were the target of new uses and biological approaches in the early 1980s, from the discovery that certain organisms, notably bacteria, naturally have the ability to transfer genes for plants, changing the phenotype of the latter. The understanding of the main molecular mechanisms that govern the genetic transformation of plants opened precedents for the *in vitro* transfer of genes between different organisms, commonly called transgenics. In this context, we can define transgenia and transgene respectively as i) the incorporation of exogenous and inheritable DNA by living organisms through *in vitro* methods ans ii) genes originating from different organisms (they may even belong to species that do not have sexual compatibility), usually obtained through recombinant DNA technology and introduced naturally or in the laboratory into the genome of recipient species [1].

In this way, transgenic organisms such as bacteria, yeast cells, filamentous fungi, plants and animals, are those that contain one or more genes inserted in their genomes by means other than the sexual route. The cells of these organisms are able to coordinate transcription of transgenes and also the translation of mature mRNAs into proteins correctly folded, assembled and modified after translation. In this case, the new proteins produced - called recombinant proteins - are capable of desirably altering the phenotype of gene receptor organisms in order to achieve a series of practical objectives [1].

Recombinant protein is can be defined as a polypeptide encoded by a transgene and normally synthesized in organisms other than those of its origin. Some recombinant proteins can be encoded by additional copies of transgenes from the host plant itself, introduced *in vitro* into its genome [2].

When the development of transgenic plants, as well as other genetically modified organisms, meets objectives related to food production or improved obtaining drugs, for example, we can say that they are essentially biotechnological products. In this context, Biotechnology can be defined as the various forms of technology that exploit biological sources - usually microorganisms and/or their products and components. The term is also commonly used to define the forms of technology that depend on the use of molecular biology/genetic engineering techniques for the construction of new organisms and/or products for industrial, medical and other applications [3].

The molecular strategies that advocate the recombinant protein biosynthesis, mediated by the enzymatic machinery of higher plants, are the applied examples that arrived on the market and those most frequently used by research groups involving the genetic transformation of plants. A significant portion of the research involving transgenic plants is restricted to fundamental studies of experimental validation, prior to obtaining a product intended for the market. The herbaceous plant *Arabidopsis thaliana* (L.) Heynh. a crucifer evolving closely to the mustard plant [*Brassica integrifolia* (H. West) Rupr.], is inserted in the latter case, since it received the status of model plant for experimenting with plant transgenics in a manner analogous to the position reached by flies of the genus *Drosophila sp*. for the study of population genetics [3].

Whether as a result of the expression of one or more recombinant proteins or by the suppression/blocking of the expression of proteins and metabolic intermediates of the plant itself or of infesting pathogens, the objectives that govern obtaining transgenic plants for the market are mainly focused on obtaining food, medicines and industrial products and reagents [4].

The ability of higher plants to express genes from other organisms (not necessarily close to the evolutionary point of view) and to produce recombinant proteins in satisfactory quantities and in an economically viable manner are the main factors that characterize them as efficient systems for the production of numerous proteins with the most varied applications. The uses of recombinant proteins

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of plant origin are divided into two major groups: endogenous and exogenous. Endogenous use is that which is restricted to the organism of origin of the recombinant protein. Exogenous use is one whose purpose concerns other organisms than that of origin of a recombinant protein [5].

Endogenous uses, that is, in the transgenic plant itself, is usually related to agronomic and recurrent aspects of human food, such as obtaining resistance to forms of biotic stress, such as the attack of insects and pathogens that cause diseases that compromise crops and the storage of agricultural products; obtaining tolerance to different forms of abiotic stress - mainly to the toxic action of herbicides - and the so-called "functional" foods, which have higher yields of production of certain oils, vitamins, amino acids and carbohydrates and which have greater nutritional value and desirable characteristics to the human and animal diet, such as the decrease in antinutritional/allergenic and undesirable factors for the food industry [4-6].

Transgenic plants also function as vehicles for the production of proteins and other biomolecules to be extracted from plant material and purified for their exogenous application, that is, outside the plant in which they were biosynthesized. The destinations for using these true protein bio-factories are numerous, ranging from the pharmaceutical industry to that of cleaning and hygiene materials, including the development of recombinant enzymes for strictly industrial application and also to obtain new materials (biopolymers) with desirable characteristics for the clothing, surgical and potential employment sectors in the security, naval, air and military sectors [6].

Obtaining transgenic plants is based on three broad sets of cellular and molecular techniques: genetic engineering, techniques for transferring genes between different organisms and *in vitro* culture of plant tissues. Genetic engineering is a comprehensive set of concepts, techniques and molecular applications carried out from the first half of the 1970s and developed by the progressive correlation of important information for understanding the structure and function of nucleic acids, obtained over thirty years before, and by the discovery of enzymes capable of modifying the molecular structure of nucleic acids, which allowed their manipulation *in vitro*. Genetic engineering allows the controlled modification of nucleic acid molecules in the laboratory, to generate new combinations of genes or sequences (such as fusion proteins) [7].

Genetic engineering techniques allow the assembly, in the laboratory, of hybrid DNA molecules, containing functional segments of deoxyribonucleic acids from more than one organism structured in an organized way, as in a "molecular mosaic", in which multiple stretches of DNA are assembled in logically and that allows each segment to perform its function correctly [2-4].

The tools used to manipulate the DNA of different organisms are enzymes capable of structurally modifying these molecules and integrating recombinant fragments (containing different combinations of DNA - hence the name recombinant DNA technology) in circular bacterial DNA molecules called plasmids [1].

Plasmid is a bacterial extrachromosomal genetic element, often transmissible from cell to cell, which can be propagated both in the cytoplasm and as an integral part of the bacterial chromosome. These circular DNA molecules are not essential for the bacterium's survival, but contain genes normally associated with important phenotypic traits, such as antibiotic resistance. Its application is recurrent in genetic engineering in the cloning and expression of transgenes [1-4].

The DNA-modifying enzymes mentioned earlier catalyze processes of alteration at the structural level in the molecule, such as restriction and cleavage at specific sequential sites of nitrogenous nucleotide bases and the binding of compatible DNA ends [4,8].

The first of these enzymes was purified from cells of the bacterium *Escherichia coli* and characterized in 1967 by Martin Gellert, of the National Institutes of Health (NIH) and called DNA ligase. Gellert found that this enzyme was able to catalyze the establishment of phosphodiester bonds between different fragments of lambda phage (λ) DNA, linking different parts of the virus's DNA molecule [5,8].

Just a year later, biochemists Stuart Linn and Werner Arber, from the University of Geneva (Switzerland), discovered the catalytic action of DNA restriction enzymes, culminating in the purification and characterization of the first site-specific restriction endonuclease - endonuclease R, later called *Hind* II [4,8].

The use of both classes of enzymes allows for the specific fragmentation of DNA molecules carrying the restriction sites recognized by the endonuclease, in addition to the binding of the ends of different molecules by reconstituting the covalent fodder-type bonds between the phosphate groups and the deoxyribose molecules from both ends, a mechanism effected by the catalytic action of the enzyme DNA ligase [3,8].

The same procedure can be used to insert fragments containing transgenes - including those that confer resistance to antibiotics - in the bacterial plasmids themselves previously isolated in the laboratory [8]. Plasmids containing transgenes are then re-introduced into bacteria that accept them as elements of their own genome. Once the transgenic bacterial cells (carrying the modified plasmid) can replicate under suitable conditions, in a selective culture medium containing the antibiotic against which they are resistant, the plasmids carrying the transgenes also undergo a replication process, resulting in the obtaining of new copies of plasmid DNA. Each replicated bacterial clone contains multiple copies of the plasmid and, similarly, of the transgene itself. This mechanism was called gene cloning [5,8].

Once multiplied on a large scale, the various copies of the plasmids containing the transgenes are separated from the bacterial cells and purified *in vitro*, in order to be free of contaminating molecules, for their later introduction into the nucleus or lumen of plant organelles containing their own genomes, such as mitochondria and chloroplasts [8].

Bacterial plasmids modified by genetic engineering and used in experiments of genetic transformation of plants are also called plasmid vectors of gene expression, since they contain the coding sequences of recombinant proteins properly flanked by different regulatory sequences, as promoters; signal peptides and terminators which, once arranged sequentially and in the same reading phase, constitute the expression cassette to be stably integrated into the plant genome [3,4].

The expression cassette consists of one or more genes and their flanking regulatory sequences. Generally, its constituents are the

promoter of the gene, an open reading phase (the coding sequence) and the DNA sequence corresponding to the 3 'untranslated region of the mRNA, which contains the polyadenylation site in eukaryote. The promoter of a gene is a region of DNA directly involved in the initiation of gene transcription, located upstream, on the same strand and generally close to the coding sequence it regulates. Present at the promoter are the site of initiation of RNA synthesis and the sites and responsive binding elements of RNA polymerase dependent on DNA and transcription factors, as well as regulatory elements such as activators and repressors of transcription [8].

The signal peptide sequence is a small sequence encoding a short peptide chain, directly involved with the post-translational targeting and transport of a protein to organelles or to the secretory pathway of proteins in transit in eukaryotic cells. They can be associated with both the N and the C-terminus of the target protein. Some signal peptides undergo specific enzymatic recognition and cleavage after protein transport. A gene's coding sequence is the portion of nucleic acid that is transcribed into RNA, which may or may not be translated into a protein. The transcription terminator is the region that marks the end of the gene and contains the transcription stop signal [1,2].

DNA cloning techniques were first developed and executed in 1972 by Paul Berg at Stanford University, California, and resulted in the obtaining of a hybrid DNA molecule, containing a stretch of DNA sequence from a Lambda phage (λ) inserted into the genome of the SV40 tumor-causing virus, which could be inserted into mammalian cells [3,5].

Just a year after Berg's achievement, Stanley Cohen, at the same university, was able to develop a method of inserting exogenous bacterial plasmids into recipient *E. coli* cells and, together with Herbert Boyer of the University of California, reported the first *in vitro* construction of a bacterial plasmid containing genes from two other plasmids previously digested with the restriction enzyme *Eco*RI. Once inserted in *E. coli* cells, the hybrid plasmid of Boyer and Cohen was efficient in giving the transgenic bacteria simultaneous tolerance to antibiotics tetracycline and kanamycin [8].

The second premise for obtaining transgenic plants is the transfer of genes of interest from other organisms to plant cells potentially precursor to an organ or an embryo. The genetic transformation of plants can be understood as the controlled introduction of genes from different animal, plant or microorganism species, into a recipient plant genome, regardless of fertilization [1-5].

Despite the development of different techniques of plant genetic transformation, the introduction of genes of interest in higher plants has been carried out conventionally by two processes: through the infection of target tissues by transgenic cells of the bacterium *A. tumefaciens* - method preferably used for the transformation genetics of most dicotyledonous plants - or by bombarding explants with microparticles containing DNA carrying the genes of interest that are superficially adsorbed (a method known as biobalistics) [8].

This last method, in addition to demonstrating less genotypic dependence, is the most used for the genetic transformation of legumes, such as soybeans (*Glycine max*) and alfalfa (*Medicago sativa* L.) and cereals, such as rice (*Oriza sativa*), wheat (*Triticum aestivum*) and corn (*Zea mays*) [9].

The method that uses *Agrobacterium tumefaciens*

Genetic transformation by agrobacteria is based on the capacity that the species *A. tumefaciens*, a bacterium typically from the soil, aerobic, in the form of a bacillus, Gram-negative, non-spore-forming and etiological agent of plant disease known as crown gall, has in transferring part of the DNA of its plasmid Ti (from Tumor inducing) to the genome of cells of host plants [10].

In order to take advantage of the potential for transferring genes from *A. tumefaciens* to higher plants, a methodology for cultivating "unarmed" strains of the bacterium was developed, which had, by double recombination, deleted the oncogenes from a region of 150 to 250kb of the plasmid Ti, called T-DNA. The right and left ends of the T-DNA, essential for the transfer, are kept intact and between them the sequence containing the gene of interest to be introduced into the plant is cloned [8,9].

Another region of the bacterial Ti plasmid, called the virulence region (vir regulon region with approximately 25 genes), encodes proteins that promote the transfer of the T-DNA region containing the genes of interest to the plant cell genome. Thus, it is necessary to carry out a co-culture based on the cultivation of the explant to be transformed together with an unarmed strain of *A. tumefaciens* carrying the plasmid containing the genes of interest to be transferred to the plant [8,9].

The agroinfection method was adopted to obtain the first transgenic plant, developed by researchers from the group of Luis Herrera-Estrella and Patricia Zambryski at the University of Gent (Belgium) in 1984 - a tobacco plant (*Nicotiana. tabacum*) containing a gene bacterial resistance to kanamycin antibiotic [8].

The method of accelerating microparticles covered with DNA

The biobalistic process recommends the acceleration of microprojectiles (from 0.2 to 4.0 μm in diameter) covered with sequences of nucleic acids, at a final speed of 1,500 km/h, on the tissue segments or plant organs to be transformed. The microprojectiles, of gold or tungsten, penetrate non-lethally the cell wall and the cytoplasmic membrane of the pumped cells, lodging themselves randomly in the cell organelles, among which the nucleus, mitochondria and chloroplasts, when then the DNA is dissociated from microprojectiles by the action of intra-organellar liquid content and integration by homologous recombination of the sequence containing the genes of interest to the plant genome [10].

A wide variety of microprojectile acceleration systems have already been developed, most of which share the idea of generating a shock wave carrying enough energy to displace a carrier membrane containing the microprojectiles coated with DNA [10].

The shock wave can be generated by a chemical explosion, by a discharge of helium at high pressure, by the vaporization of a drop of water by electrical discharge with high voltage and low capacitance or vice versa, by a discharge of compressed air or, when the use of a carrier membrane is not required, using a low pressure helium gas discharge. The system that uses helium gas under high pressure is the one that is more efficient in obtaining high transformation frequencies, in addition to being widely used for the genetic transformation of a very large variety of plant species [10-15].

In vitro culture of plant tissues

In most cases, the choice of transgenic plants for the production of any class of protein focuses preferably on the stable genetic transformation and regeneration of complete plants from explants. Explants are segments of tissue or plant organs used to initiate an *in vitro* culture [1,16].

As transgenic plants are regenerated *in vitro* from previously transformed germ or totipotent cells, all cells of the resulting individuals are carriers of the exogenous nucleotide sequence (transgene), being able to transmit copies to their offspring by the same principles that govern heredity in non- transgenic plants [10].

In vitro morphogenesis is based on the totipotence of plant cells and the ability to grow these cells in the laboratory, through the use of nutrient culture media and growth regulators appropriate to modulate plant regeneration. Two models of *in vitro* morphogenesis and its variations are used for the regeneration of plants for their genetic transformation: organogenesis and somatic embryogenesis [10].

Organogenesis stimulates the formation of aerial parts or roots in callus culture (mass of cells of continuous proliferation and more or less disorderly) or other explants, from the neoformation of vegetative or floral stem buds that become stem axes or roots. In this method, the regenerated organs have a multicellular and subepidermal origin and regeneration occurs from meristematic cell groups of the original tissue [10].

The cultivation of embryos with their apical meristematic region preserved or simply from the apical or axillary meristem systems, in the presence of cytokinins in culture medium, is an efficient inducer of aerial part organogenesis, capable of regenerating transgenic plants without the need for passage of the tissue by intermediate stages of de-differentiation, such as callus, for example [10].

Somatic embryogenesis recommends the development of embryos from somatic cells, as a result of an external stimulus (contact with growth regulators, such as 2,4-D - 2,4 dichlorophenoxyacetic acid). This tissue culture method leads to adventitious multi-embryonic formation of explants, and the originated embryos have their own vascular axes, single-cell or few cell histological origin and superficial location [10].

The regeneration process of plants undergoing genetic transformation *via* biobalistic reduces the contact period between the explant and hormonal phyto-regulators, since the tissue is not necessarily submitted to the callus phase, a characteristic that makes the process of obtaining transformants faster and under more intensified control conditions [10].

Another important advantage of the biobalistic system is the reduction of *in vitro* culture time, providing molecular and biochemical tests for the identification of transforming events and proteins with greater precocity. Both forms of plant regeneration *in vitro* allow, from the initial explants, the continuous obtaining of tissues and experimental material through cyclic cultures, which once associated with other tissue culture techniques have fundamental applications and importance in several areas as in the genetic improvement of plants, in phytopathology and in the genetic transformation of plants [17].

As a result of the integrated action between the three sets of the main techniques that constitute the process of genetic transformation of plants, it is possible to build plasmid vectors containing genes of interest, perform their introduction in plant cells and explants, verify the integration of exogenous sequences to the genome of plant species in question and express them stably in successive generations, in order to obtain transgenic plants and recombinant proteins in significant quantities [17].

Conclusion

Transgenic plants can function efficiently as vehicles for the expression of recombinant proteins and, in many cases, promote high levels of accumulation of recombinant production, through different molecular strategies. The action of DNA-modifying enzymes, often biosynthesized by genetically modified bacteria, is essential for the manipulation of DNA fragments containing the genes of interest and other elements of the expression cassette.

A. tumefaciens, like biobalistics, are the main methods of genetic transformation of plants. An interesting aspect of agroinfection is the greater control of the number of copies inserted in the genome of the host plant, thanks to the relationship of biological compatibility between bacteria/plant and the type of integration directed by the end of the expression cassette cloned in the Ti plasmid.

Regardless of the genetic transformation method and the type of regulatory sequences present in the plasmid expression vector, the levels of gene expression are directly related to the choice of the compartment for the accumulation of recombinant proteins, post-translational protein stability and adaptation at the genetic level of sequences capable of minimizing proteolytic degradation in the various stages of modification after translation.

In principle, it takes a long time to optimize the expression of a recombinant protein in any biological production system, especially when different subcellular compartments are available, such as those observed in plant cells.

Once the appropriate combination involving the type of plant receiving the transgene has been reached, the different elements of regulation of gene expression in the expression cassette, the molecular strategy of subcellular protein addressing and the protocols for genetic transformation and tissue culture, the capacity The plant's functioning as a correct, safe and economical agent of biosynthesis of a given recombinant protein is put in check over several stages until effectively exercising direct or indirect influences on the consumer's quality of life.

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BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

Co-occurrence of linear and cyclic pelgipeptins in broth cultures of *Paenibacillus elgii* AC13

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Abstract

Paenibacillus elgii AC13 produces antimicrobial lipopeptides of agricultural and pharmaceutical importance. It secretes four cyclic lipopeptides named pelgipeptins, previously characterized in *P. elgii* B69. These lipopeptides result from the expression of a nonribosomal peptide gene cluster. *P. elgii* AC13 also produced two linear lipopeptides with ratios of [M + H] + 1105 and 1119 m/z. These compounds were previously observed in *Paenibacillus* sp. strain OSY-N, but due to purification difficulties, their characterization was executed using synthetically produced linear pelgipeptins. In the present study, purification was achieved from the supernatants of cultures from three complex media by high-performance liquid chromatography. The partial characterization of linear pelgipeptins revealed the similar antimicrobial activity and cytotoxicity of their synthetically produced counterparts, known as paenipeptins. Cyclic forms were highly stable to changes in pH, temperature, and organic extraction with n-butanol as shown by mass spectrometry (MALDI-TOF); therefore, these steps did not cause the hydrolysis of pelgipeptins. A low-activity thioesterase could also generate the linear isoforms observed; this enzyme catalyzes the cyclization process and is coded in the same gene cluster. Alternatively, the cyclic forms were hydrolyzed by an unknown protease produced during growth in the complex medium used in the present study. Although culture conditions are known to produce pelgipeptins with different yields and amino acid compositions, the occurrence of linear and cyclic forms simultaneously has not yet been reported. A mixture of cyclic and linear pelgipeptins presents a potential advantage of the higher antimicrobial activity of cyclic forms combined with the lower cytotoxicity of linear isoforms.

Keywords *Paenibacillus elgii* · Lipopeptides · Pelgipeptin · NRP isoforms · Antimicrobial agent · MALDI-TOF

Introduction

Pelgipeptins are lipopeptides first described in *Paenibacillus elgii* B69. Currently, they comprise a family of five homologous compounds, or isoforms, of cyclic cationic lipopeptides containing nine amino acids and a fatty acid chain. These lipopeptides are also known for their broad-spectrum antimicrobial activity against various bacteria and soil fungi [1, 2].

Pelgipeptins are synthesized by nonribosomal peptide synthases (NRPSs) coded by the *plp* gene cluster (40.8 kb) identified and characterized in *P. elgii* B69 [3]. The molecular weights of pelgipeptins A, B, C, D [1, 2], and E [4] are 1072, 1100, 1086, 1086, and 1072 Da, respectively. Variations among pelgipeptins A to D also include substitution at the second amino acid position, which may be either a valine or isoleucine. Moreover, pelgipeptins A and D exhibit a lipid tail with a methyl group linked at its penultimate *(iso)* carbon, whereas pelgipeptins B and C show this to the antepenultimate (*anteiso*) carbon. *P. elgii* BC34-6 isolated from red clay soil produces pelgipeptin E [4]. This compound exhibits the same stereochemistry as the amino acid and the ester bond of pelgipeptins A and C, but it is attached to a straight lipid chain.

A study has reported that *Paenibacillus* sp. strain OSY-N synthesizes three lipopeptides belonging to a new

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polypeptide family named paenipeptin A to C [5]. Paenipeptins A and B are linear lipopeptides, whereas paenipeptin C is a cyclic lipopeptide with a m/z ratio of 1133 $[M+H]^+$. Paenipeptins A and B are, in fact, linear counterparts of pelgipeptins B and C, respectively [5]. However, the authors only detected these isoforms in Tryptic Soy Agar (TSA) solid culture medium and not on liquid culture. Due to the low yield of linear paenipeptins from cultures, the synthetic analogs of paenipeptins A and B were used to assess their antimicrobial activity showing the potential to eradicate biofilms. They were thus reported as an alternative strategy to fight multidrug-resistant bacteria or fungi [6].

P. elgii AC13 studied in the present work was isolated from soil samples; its draft genome was obtained previously [7]. Culture supernatants on Nutrient Broth (NB) revealed the presence of ions $[M+H]+1119$ and 1105 m/z in addition to pelgipeptins A to D. These ions exhibited the same mass/charge ratio as the linear counterparts of pelgipeptins B and C, previously reported as paenipeptins A and B described in *P. elgii* OSY-N [5]. The current work aimed to investigate the influence of culture media on the occurrence of linear lipopeptides produced by *P. elgii* AC13. Culture conditions can influence the diversity of lipopeptides produced by bacteria, but it was not yet demonstrated that they could also influence the production of linear and cyclic lipopeptides. This study also investigated whether the linear pelgipeptins purified from culture supernatants presented the same characteristics and properties as their synthetic counterparts.

Methods

Bacteria strains

The antimicrobial compounds were isolated and purified from *P. elgii* AC13 (= CBMAI 2485) isolated from Cerrado soils, the Brazilian savanna [7]. The antimicrobial assays were performed using *Escherichia coli* ATCC11229 and *Staphylococcus aureus* ATCC14458.

Culture conditions

The occurrence of pelgipeptins in culture supernatants was evaluated on the following complex media: (i) Nutrient Broth (beef extract 0.3% and peptone 0.5%), this medium was from BD-DIFCO; (ii) Müller Hinton Broth (MH) (beef extract 0.2%, acid digest of casein 1.75%, and starch 0.15%) was from FLUKA-SIGMA; and (iii) Tryptic Soy Broth (TSB) (triptone 1.7%, bactosoytone 0.3%, glucose 0.25%, NaCl 0.5%, K_2HPO_4 0.25%) was from KASVI. All cultures were executed in triplicates, and each experiment was repeated at least three times.

A spore stock from *P. elgii* AC13 was inoculated on 100 mL of each medium tested at a final concentration of 10^3 spores/mL and incubated at 37 °C with shaking at 200 rpm. The lipopeptides were extracted and purified within 48 h.

Cultures starting from vegetative cells of AC13 were also obtained in NB. This procedure has been commonly utilized in other studies, including our previous work [8]. Briefly, a pre-inoculum was obtained from a single colony from *P. elgii* AC13 that was incubated on NB at 37 °C with shaking at 200 rpm. Growth was monitored by absorbance at 600 nm. Upon reaching an optical density of 0.6 to 0.8, a volume of 1 mL was transferred to 100 mL of sterile NB and incubated for 48 h at 37 °C with shaking at 200 rpm.

Purification and quantification of pelgipeptins

The cultures were centrifuged at $9000 \times g$ for 10 min to remove the cells, and the lipopeptides were extracted from the supernatant with n-butanol and water (1:1; v/v). The organic layer was lyophilized, and the dry content was suspended in ultrapure water. This solution was then filtered (0.22 µm) and purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Shim-pack Shimadzu C_{18} column VP-ODS, 4.6×150 mm, and 4.6 µm particle size (Shimadzu, Japan). Mobile phases were HPLC-grade water and acetonitrile (ACN), both containing 0.1% trifluoroacetic acid (TFA). The solvent gradient was as follows: 0–10 min, 20% ACN, and 10–40 min, 20–60% ACN. The flow rate was kept at 1.0 mL/min, and the elution was monitored by measuring the absorbance at 216 nm using a UV detector (Prominence, Shimadzu, Japan). The fractions containing purified lipopeptides were collected and purified for a second time. Alternatively, a mixture of cyclic pelgipeptins A to D (pelgipeptin mix) was used; this mixture was produced and purified as described by Fulgencio et al. (2021). The purified lipopeptides were quantified by Murphy's method [9].

Pelgipeptins were then identified using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) (Autoflex Speed, Bruker Daltonics). The lipopeptides were mixed with an α-cyano-4-hydroxycinnamic acid solution (10 mg/mL) in a proportion of 1:1, and the spectra were acquired in reflected positive mode (RP 700–3500 m/z) with external calibration using peptide calibration standard II (Bruker Daltonics).

The quantification of the lipopeptide isoforms in the cell-free supernatant from cultures was determined by RP-HPLC. A standard curve was obtained from stock solutions with known concentrations of the lipopeptides. The quantitative chromatography of standards and supernatants after organic extraction was performed using the same column, gradient, and detection method. Purified solutions from several extractions from each culture medium were then concentrated as

follows: NB 100×, MH 25×, and TSB 25×to obtain a better resolution of the chromatographic profile allowing the quantification of the fractions corresponding to the linear and cyclic lipopeptide isoforms investigated in the present study.

Determination of the primary structure of the lipopeptides

The amino acid sequence was obtained using tandem mass spectrometry (MALDI-TOF MS/MS) LIFT method in positive ion mode. The primary sequence of the peptides was determined using FlexAnalysis software (Bruker Daltonics®).

Determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentration assay of purified lipopeptides was measured in 96-well microplates according to the Clinical and Laboratory Standards Institute [10]. The peptide solutions were prepared to final concentrations of 1.5–100 µM. Commercial polymyxin B (Sigma-Aldrich), penicillin G (Sigma-Aldrich), and chloramphenicol (Sigma-Aldrich) were employed as positive controls, and sterile ultrapure water was used as a negative control. The tested bacterial strains and peptide solution mixtures were incubated at 37 ºC for 16 h, and the absorbance (595 nm) was measured once per hour in a BioTek (Winooski, VT, USA) microplate reader. MICs were defined as the lowest peptide concentration that inhibited bacterial growth.

Stability assays

A mixture containing only cyclic isoforms was produced according to Fulgêncio et. al. (2021) and purified by solid-phase extraction [11]. This mixture was used to determine the stability of the pelgipeptins. The antimicrobial stability was evaluated using the agar diffusion method against *E. coli* ATCC ATCC11229 on the MH medium. Temperature stability assays were performed with a solution containing 100 µg/mL of the pelgipeptin mix. This solution was incubated for 12 h in the following temperatures: 40, 60, 80, and 100 °C. The pH assays were executed in a range of values between 2.0 and 12.0. The pelgipeptin mix solution was incubated for 12 h in each pH and then neutralized to pH 7.0.

The antimicrobial activity after stability tests was executed by applying 10 µg of the pelgipeptin mix (after each treatment) on a Whatman 6 mm filter paper on the MH plates pre-inoculated with *E. coli*. After each treatment, the inhibition halo was measured and compared with the control, which was the diameter of the halo observed for the pelgipeptins mix incubated at 37 °C and pH 7.0. In addition, the structural stability of the cyclic isoforms of pelgipeptins after each temperature and pH was investigated using RP-HPLC and MALDI-TOF. The presence of linear isoforms indicated that the treatment caused the cleavage of the cyclic lipopeptides. The structural stability was also evaluated before and after the n-butanol purification step.

Cytotoxic activities of pelgipeptins A, B, C, and D and isoform B'

The cytotoxicity of pelgipeptins A, B, C, D, and isoform B' was evaluated using human fibroblast cells. Initially, fibroblasts were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and incubated at 5% CO_2 and 95% humidity at 37 ºC until the cytotoxicity assays were conducted.

The cell viability tests were performed on technical replicates and were assessed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) (Sigma-Aldrich, USA) colorimetric assay [12]. Briefly, the cells were seeded onto 96-well plates (10^3 cells/well; 100 µL) and allowed to adhere and grow for 24 h. The cells were then incubated with fresh prepared medium and lipopeptide or polymyxin B to final concentrations of 3–50 µmol l^{-1}. After the treatment, 90 µL was added to 10 µL of MTT (50 µg), and then to each well, the cells were incubated for 4 h. Then, 100 µL of dimethyl sulfoxide (DMSO) was added to all wells and mixed to dissolve the dark blue crystals (formazan). Finally, the absorbance was measured on a microplate spectrophotometer at 570 nm. Cells treated with 50 µL of the lysis solution of sodium hypochlorite (2.5%) were used as negative control, and the untreated cells were used as the positive control group.

Statistical analysis

The data for cytotoxicity and pelgipeptin yield were expressed as the mean ± standard deviation (SD) of three independent experiments. Multiple comparisons were performed by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test using GraphPad Prism 9 (GraphPad Software, San Diego, CA). The comparisons between two samples were performed using the *t* test for unpaired data.

Pelgipeptin gene cluster

The putative sequence for the pelgipeptin gene cluster was identified by gene similarly to the *P. elgii* B69 *plp* gene cluster [3] (accession number, JQ745271.1). BLASTn tool [13] was utilized to identify the contigs containing the *plp* genes from the previously obtained draft genome of *P. elgii* AC13

[7]. Genome annotation revealed forty putative NRPS gene clusters also identified in *P. elgii* strain B69. The genes from the *plp* biosynthetic gene cluster of *P. elgii* B69 were aligned against the contigs from the *P. elgii* AC13 draft genome using Geneious 11.1.4 [14]. The missing base pairs between contigs were obtained by PCR amplification followed by Sanger sequencing of the PCR products. The NRP domains were identified using antiSMASH 5.0 [15]. The complete pelgipeptin gene cluster was obtained, and it is deposited under accession number MN577593.

Results and discussion

Paenibacillus elgii AC13 isolated from tropical soils produced four cyclic lipopeptides corresponding to pelgipeptins A to D, described previously from cultures of *P. elgii* B69 [1, 2]. During the maintenance of *P. elgii* AC13 cultures, two other compounds were detected in the supernatant of NB, and these compounds had ions of masses $[M+H]^+$ 1105.7 and 1119.7 m/z. The present study identified these two ions as the linear isoforms of pelgipeptins C and B, named herein pelgipeptins C' and

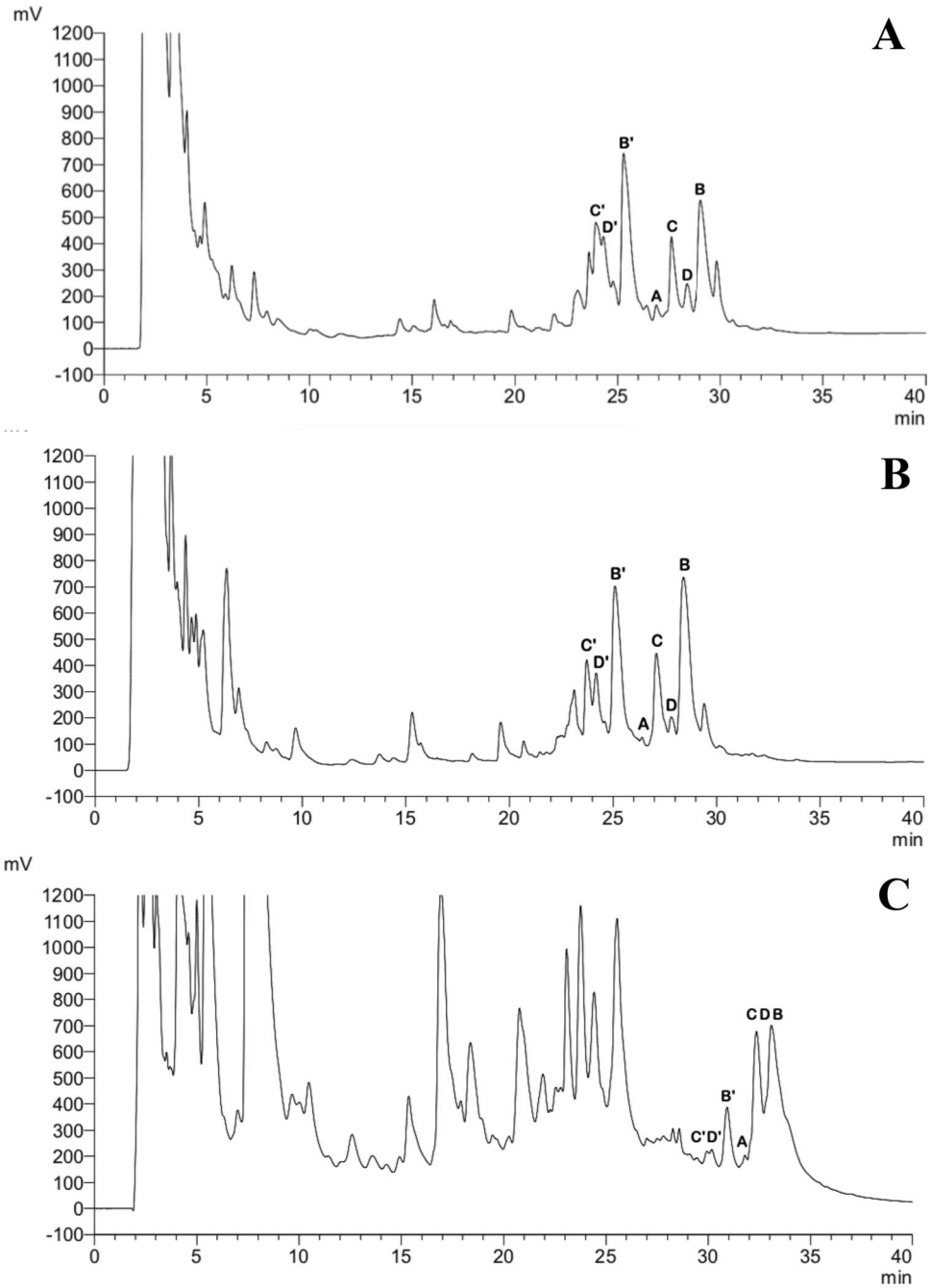

Fig. 1 Chromatographic profile of *P. elgii* AC13 supernatant after organic extraction with butanol, cultivated in **A** Nutrient Broth, **B** Müller Hinton Broth, and **C** Tryptic Soy Broth. C', linear pelgipeptin C; D', putative linear pelgipeptin D; B', linear pelgipeptin B; A, B, C, and D indicate pelgipeptin cyclic isoforms

B' (Table S1). Additionally, they occurred in all complex media tested in the study: MH, NB, and TSB, but there were a few differences in their supernatants.

The RP-HPLC of supernatants obtained from MH and NB resulted in six fractions with a retention time of 24 to 30 min (Fig. 1A and B; Table S1); supernatants in TSB presented a retention time of 30 to 35 min and lower resolution in fractions (Fig. 1C). Fractions C' and B' corresponded to compounds with monoisotopic masses of $[M+H]^+$ 1105.7 and 1119.7, respectively. Moreover, MS/MS predicted sequence of fraction C' was the same as that found for pelgipeptin C (Fig. S1, Table S1), and fraction B' was the same as pelgipeptin B (Fig. S2, Table S1). Thus, the purified lipopeptides obtained from *P. elgii* AC13 cultures in complex media were identified as follows: fractions B' and C' were the linear counterparts of pelgipeptins B and C, respectively.

The remaining fractions contained the antimicrobial cyclic lipopeptides, namely, pelgipeptins A to D (Fig. 1). Fraction D' is likely to be the linear counterpart of pelgipeptin D, but its sequencing was not performed due to difficulties in obtaining the pure fraction. Cyclic and linear forms of pelgipeptin A were also not obtained in sufficient quantities to allow their characterization.

The linear isoforms of pelgipeptins were previously detected in the cultures of *Paenibacillus* sp. OSY-N, and they were named paenipeptins A and B [5]. The linear lipopeptides of OYS-N were only detected in cells harvested from Tryptic Soy Agar plates. Differently from OYS-N, *P. elgii* AC13 produced linear and cyclic pelgipeptins in all the complex media tested. This result may be due to species or strain genetic differences, but the classification of OSY-N is not yet clear, impairing further genomic comparisons. Alternatively, the occurrence of linear pelgipeptins could result from differences in culture growth conditions, as observed in the present study.

P. elgii AC13 cultures in NB derived from vegetative cells or spores did not show significant differences ($P < 0.01$) in the concentration of cyclic or linear lipopeptides. Nonetheless, cultures originating from spores presented higher repeatability and were easier to set up; hence, inoculation using spores was used in subsequent experiments.

Cultivation in MH medium yielded the highest concentration of total isoforms (Fig. 2), which may be due to the abundance and nutrient variety this medium offers. Alternatively, the chemical components of the media could interact with other reagents employed in the purification procedures [16], hindering the recovery of linear forms in some complex media. This result was observed in the supernatant of TSB medium that presented a variety of other undesired compounds in the detriment of linear isoforms (Fig. 1B). Cultures in TSB produced 5.6 times fewer linear isoforms than cyclic pelgipeptins, and it was the lowest yield of linear pelgipeptins obtained from all tested media (Fig. 2). These results are in accordance with the data reported for *Paenibacillus* sp. OSY-N, because linear pelgipeptins (paenipeptins) were not obtained from TSB cultures but only from its solid medium counterpart, TSA [5]. Although it is true that some peptides are only produced on solid media, this may not be the case for the linear forms of pelgipeptins, as demonstrated in the present study.

The reasons for the presence of linear pelgipeptins in our culture conditions are still unclear. The genome of *P. elgii* AC13 revealed the presence of a gene cluster highly similar to the gene cluster responsible for the synthesis of these lipopeptides in *P. elgii* B69 (Table 1) containing a thioesterase domain (*plp*B) [3].

Thioesterases guide the lactone ring formation characteristic of pelgipeptins and other cyclic lipopeptides such as surfactin and iturin [17]; thus, linear forms could result from a low thioesterase activity. Another hypothesis is that the culture conditions or the purification process caused the hydrolysis of cyclic pelgipeptins. However, the cyclic forms of pelgipeptins were highly stable. Hydrolysis was not observed in high temperatures (Fig. S3), and it was only obtained from pH 12 (Fig. S4). In addition, the n-butanol extraction did not cause major structural changes in the purified mix of pelgipeptins (Fig. S5), indicating that this critical purification step did not cause the hydrolysis of this lipopeptide. Finally, an enzymatic activity could be responsible for the linear isoforms of pelgipeptins, but cyclic lipopeptides are generally highly resistant to protease activity. Pelgipeptins were previously reported to be resistant to degradation by pepsin, proteinase K, and trypsin (Wu et al. 2010). The hydrolysis of surfactin was only observed for *Staphylococcus aureus* V8 endoprotease and a novel enzyme from *Streptomyces* sp. MG1 [18, 19].

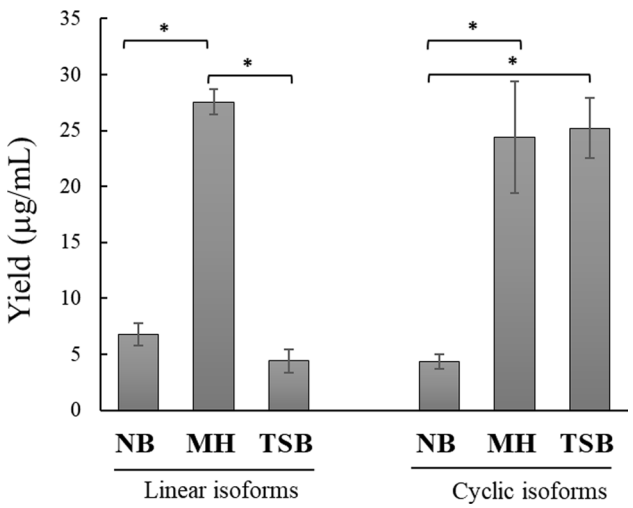

Fig. 2 Yields of linear and cyclic isoforms of pelgipeptins in different culture media. Significance level between samples obtained by Bonferroni's post hoc test after ANOVA. * $p < 0.001$. NB, Nutrient Broth; MH, Muller Hinton; TSB, Tryptic Soy Broth

Table 1 Identification and similarity scores of the deduced amino acid composition of Plp proteins from *Paenibacillus elgii* AC13 and *Paenibacillus elgii* B69[1]

Protein	Similarity	Function
PlpA	100%	Diaminobutyrate-2-oxoglutarate aminotransferase
PlpB	97.2%	Esterase and lipase
PlpC	98.5%	4′-phosphopantetheinyl transferase
PlpD	97.2%	Nonribosomal peptide synthetase
PlpE	96.5%	Nonribosomal peptide synthetase
PlpF	97.7%	Nonribosomal peptide synthetase
PlpG	97.9%	ABC transporter
PlpH	96.9%	ABC transporter

[1]Qian et al., 2012

Antimicrobial activity of purified pelgipeptins

The antimicrobial activity of synthetically produced paenipeptins A and B was tested against 19 bacterial strains [5]. Purified pelgipeptins B' and C' tested here also exhibited a higher MIC than its cyclic counterpart against *E. coli* ATCC11229 and *S. aureus* ATCC14458 (Table 2). As previously reported, pelgipeptin cyclization improved the antimicrobial activity against gram-negative bacteria, but it was not a required step for its activity [5].

The disk diffusion tests revealed that pH and temperature treatments did not suppress the antimicrobial activity of the pelgipeptin mix (containing cyclic pelgipeptins A to D). The inhibition halos of *E. coli* growth were observed in all treatments, except for the treatment at pH 12. The structural analysis of cyclic pelgipeptins submitted to different temperatures and pH conditions also revealed the presence of linear isoforms only for the assays at pH 11 and 12 (Fig. S4). Antimicrobial activity stability of cyclic lipopeptides is already known; for instance, surfactin maintains its surfactant and antimicrobial activity in pHs 5 to 13 and stability after autoclaving [20]. The cell-free extract containing cyclic pelgipeptins was previously reported to only reduce its antimicrobial activity after 2 h at 80 or 100 °C (Wu et al. 2010). Differently from the cell-free extract (Wu et al. 2010), the purified pelgipeptins studied here lost their antimicrobial activity exclusively after high pH treatments. The results presented here showed that this treatment also caused the hydrolysis of cyclic pelgipeptins, thereby producing the less active linear isoforms.

The linear isoforms isolated from *P. elgii* AC13 cultures presented higher MICs and were less toxic to the primary culture of human fibroblasts than the cyclic isoforms (Fig. 3). This result is in accordance with the cytotoxicity of paenipeptins (the synthetically produced linear pelgipeptins) which was tested on defibrinated rabbit blood cells [5]. Therefore, the culture-obtained linear pelgipeptins present similar characteristics as their synthetic counterparts.

The results obtained in the present study verified that the linear isoforms of pelgipeptins C and B occur in the liquid cultures of *P. elgii* AC13 along with their cyclic counterparts. It is common to observe a preferential synthesis of certain molecules to the detriment of others caused by changes in the culture medium composition or incubation conditions [16, 21]. However, this effect was not yet reported to produce the linear forms of cyclic lipopeptides.

In conclusion, the culture medium composition, incubation conditions, and extraction methods can modify the yield and the repertoire of the lipopeptides produced by *Paenibacillus* species. The present study demonstrated that culture media combined with the method of extraction allows the purification of the linear counterparts of the cyclic pelgipeptins produced by *P. elgii* AC13. This knowledge may be useful in further modulation of the quantitative and qualitative production of pelgipeptins, providing a mix of cyclic and linear isoforms from one single culture. Finally, studies on the mechanism of action and the cell toxicity of the linear pelgipeptins were executed using the synthetic linear lipopeptides C or B, named paenipeptins [6]. It would be appropriate to keep the name paenipeptins for these synthetic molecules, making it easier to differentiate them from the linear compounds that co-occur with cyclic isoforms and can be easily obtained from cultures.

Table 2 Minimum inhibitory concentration values (μM) against tested strains exhibited by pelgipeptins cyclic isoforms (A, B, C, and D), linear isoforms (B' and C'), pelgipeptin mix, and reference antibiotics

Strains	Pelgipeptins							Controls		
	A 1073 m/z	*B 1101 m/z*	*C 1087 m/z*	*D 1087 m/z*	*B' 1119 m/z*	*C' 1105 m/z*	*Pelgipeptin mix*	*Ampicillin*	*Chloramphenicol*	*Polymyxin B*
E. coli ATCC11229	6.25	12.5	6.25	6.25	50	> 100	12.5	25	NT	3.1
S. aureus ATCC14458	12.5	12.5	12.5	12.5	> 100	> 100	6.25	NT	12.5	25

NT, not tested. Pelgipeptin mix contains cyclic pelgipeptins A to D

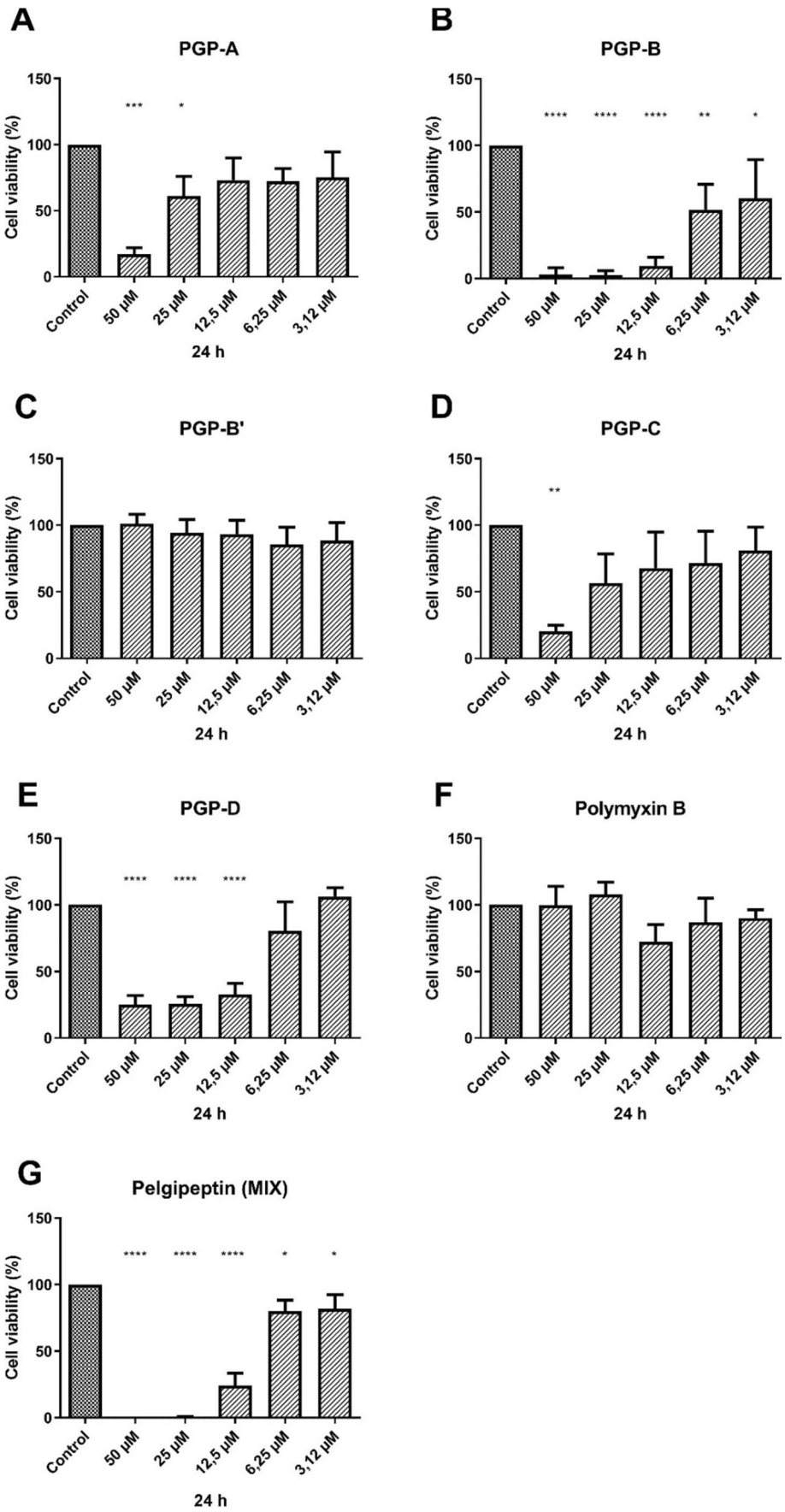

Fig. 3 Cell viability assay performed after the 24-h incubation period with different concentrations of pelgipeptins (PGP) and polymyxin B. Each bar represents the mean ± SD of cellular absorbance (abs), $n = 3$. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$

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Author contribution All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by Thiago Fellipe de Araújo, Daniel Barros Ortega, Rosiane Andrade da Costa, Isadora Emanoela Pereira Costa de Andrade, Débora Luiza Albano Fulgêncio, Marise Leite Mendonça, Flávio Silva Costa, and Michel Lopes Leite. The first draft of the manuscript was written by Cristine Chaves Barreto and Thiago Fellipe de Araújo. The final critical analysis of data was executed by Cristine Chaves Barreto, Marcelo Henrique Soller Ramada, and Rosiane Costa. All authors commented on previous versions of the manuscript.

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Data Availability Pelgipeptin gene cluster full sequence was deposited in the GenBank under number MN577593. *Paenibacillus elgii* AC13 is deposited in the Brazilian Collection of Environmental and Industrial Microorganisms – CBMAI under the number CBMAI 2485. This culture collection is part of the World Federation for Culture Collections. *Paenibacillus elgii* AC13 studies are registered at the SisGen System of the Brazilian government under number A356834.

Code availability Not applicable.

Declarations

Ethics approval Not applicable.

Consent to participation Not applicable.

Consent to publication Not applicable.

Conflict of interest The authors declare no competing interests.

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Artificial intelligence and the future of life sciences

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Over the past few decades, the number of health and 'omics-related data' generated and stored has grown exponentially. Patient information can be collected in real time and explored using various artificial intelligence (AI) tools in clinical trials; mobile devices can also be used to improve aspects of both the diagnosis and treatment of diseases. In addition, AI can be used in the development of new drugs or for drug repurposing, in faster diagnosis and more efficient treatment for various diseases, as well as to identify data-driven hypotheses for scientists. In this review, we discuss how AI is starting to revolutionize the life sciences sector.

Keywords: Artificial intelligence (AI); Machine learning (ML); Big data analytics; Clinical trials; Mobile technologies; Diseases; Life sciences; Patient-centric solutions

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Introduction

For a long time, AI was nothing more than science fiction,[1] being represented in art by films such as *Metropolis*, a German expressionism movie, directed by Fritz Lang in 1927, with one of the first appearances of a robot. Although the nomenclature robot (*robota*) was coined by the Czech playwright Karel Čapek in his 1921 play *R.U.R* (*Rossum's Universal Robots*, English translation),[2] anthropomorphic machines have populated human imagination long before, such as automatons made by Pierre Jaquet-Droz during the 18th century, which are still impressive today. However, AI is no longer a sci-fi product.[3]

The first steps toward the production of machines capable of solving complex problems were taken during the World War II. In 1942, Alan Turing, a brilliant English mathematician working for the British Government, built a machine called 'The Bombe' to crack the Enigma code used by the German army.[4] Eight years later, he published his seminal article with the following question 'Can machines think?'.[5] In this work, he set out to establish a base of cognitive principle, functioning, and function.[6] Turing argued that it was possible to build machines capable of 'imitating' human thought.[7]

However, the term 'AI' was only coined years later by John McCarthy, Marvin Minsky, Nathaniel Rochester, and Claude Shannon at the Dartmouth Artificial Intelligence Conference in 1956.[8–11] After the conference, the AI field experienced an increase in interest from researchers and investors. However, the following years were troubled for the AI field, witnessing periods of massive investment alternated by hiatuses, known as winters, in which investments declined sharply.[1,4] Along with other revolutions, such as the steam engine, scientific and mass production, and digital technology, AI is now considered to be the 'fourth' Industrial Revolution.[12]

AI is practically everywhere, especially in tools used by big tech companies, such as Apple, Amazon, Facebook, Google, Microsoft, and others. It is not unexpected to assume that AI is also revolutionizing life sciences, mainly biomedicine and healthcare, aiming at improving disease diagnosis and patient outcomes, thus reducing healthcare costs.[13] Several researchers have been using AI for numerous topics in biomedicine, such as the identification of peptides capable of modulating inflammation in healthy adults for use in sports nutrition,[14] as well as for breast cancer imaging,[15] drug rational discovery and development,[16,17] cardiology precision medicine studies,[18,19] in the radiology field, and so on.[20–22]

In this review, we address the main advances and impacts of AI in life sciences, with an emphasis on biomedicine and healthcare, which have improved the diagnosis and treatment of patients, directly impacting patient quality of life and reducing healthcare costs. In addition, we also discuss how the future of these areas will be affected by the increasing use of AI technologies, with several challenges ahead.

AI and life sciences

The world has witnessed over the past five decades a real revolution in the area of Information Technology (IT), which has resulted in the production and storage of a huge number of data, not only in the area of technology, but also in other areas, allowing researchers to offer various products and services.[23] Today, big data have unprecedented potential at improving medical outcomes and population health.[24,25] Given the increasing volume and complexity of data from different sources, big data are directly associated with computational resources, which includes information that is structured, semistructured, or even unstructured.[26]

However, what should we do with this deluge of data? Since these are not, by themselves, very informative, it is necessary to use analytical approaches, such as AI algorithms, that are able to transform mere data into actionable analytical knowledge.[27] Although the term 'AI' can have several meanings,[28] it can be understood as the study of processes and practical aspects that allow the development of computer systems capable of performing intelligent tasks, such as decision making, without being explicitly programmed for those tasks.[29]

The most popular approach is the use of machine learning (ML), a subdiscipline of AI, which involves using modern computers and mathematical algorithms to reason and make inferences from the available data set, identifying patterns of interaction between variables and, thus, allowing the model to improve itself and learn from experience.[30–33] Deep learning (DL), a broad subfield of ML, which uses artificial neural networks (ANNs) with several layers to identify patterns in raw data, provides new opportunities to obtain learning models from complex data.[34–37] These advances are reflected in the number of publications on AI in life sciences, which has been increasing since the start of the 21st century (Fig. 1).

Currently, the combination of the expertise of researchers and the potential of machines is aiming to improve diagnoses using imaging in several medical specialties, such as radiology, dermatology, pathology, and ophthalmology.[38,39] According to Hamet and Tremblay, the application of AI in medicine can be divided into two aspects, a virtual one, in which ML is used (more com-

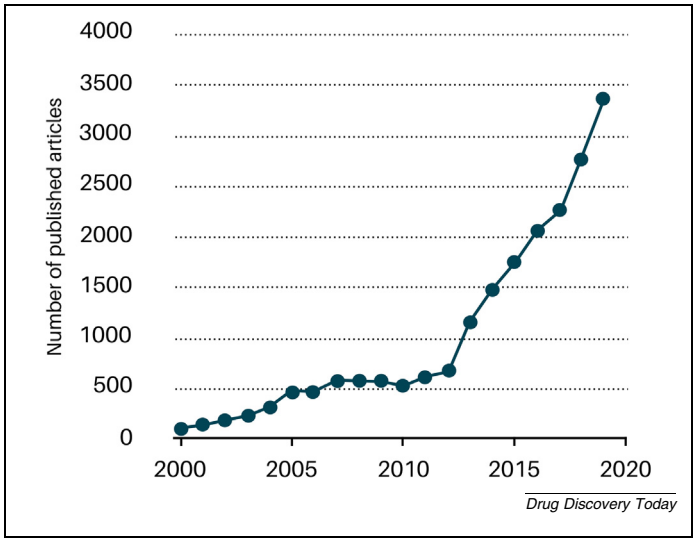

FIGURE 1

Number of publications involving artificial intelligence (AI) and life sciences between 2000 and 2020. The data was obtained through a search of PubMed, using the terms 'Artificial Intelligence AND Life Sciences'. All hits per year were considered in this graphical representation.

monly DL), and a physics one, in which AI is performed by robots.[2] However, AI technologies will likely impact other aspects of emergency medicine and healthcare delivery in the near future.[40,41] Here, we discuss examples of current uses of AI in the biomedical field.

Recent progress in Natural Language Processing (NLP) led by the evolution of model architecture and model pretraining,[42] resulted in 'Transformers', facilitating the building of higher-capacity models and the effective use of this capacity for a variety of tasks. Transformers is an open-source library with the goal of opening these advances to the wider ML community. This library is dedicated to supporting Transformer-based architectures and facilitating the distribution of pretrained models. Transformers is designed to mirror the standard NLP & ML model pipeline: process data, apply a model, and make predictions. Given the increasingly important role of transformers and pretraining in NLP, it is important for those models to be accessible to researchers and end-users.

Transformers were introduced for whole-genome sequence-labeling tasks, leading to a better processing and annotation of long DNA sequences and outperforming recurrent neural networks on natural language sequence-to-sequence labeling benchmarks.[43] Researchers in traditional Chinese medicine are also applying Transformers in their records classification. They explored a wide variety of state-of-the-art DL models and found that Transformers can achieve a better result against all other learning models and state-of-the-art methods.[44] Skeletal bone age prediction based on a deep residual network with spatial transformer has also been performed.[45]

Transformers are being applied to Electronic Health Records (EHRs)[46] to improve the accuracy of predicting future diagnoses to evaluate disease embedding, attention, and interpretability, and, thus, disease prediction. Some say that Transformers will be the future of medicine[47] because they can work side by side with engineering biology, making it possible to reprogram DNA inside harmful microbes. This is turning them into patient-saving drugs and making it possible to build living diagnostics.[47] Transformers are better suited for massively parallel computation on modern ML acceleration hardware and, although there is a range of future topics that one can base on Transformers, these studies only show the impact and future of transformers in DL.[48]

In 2019, Park and colleagues developed a neural network segmentation model (HeadXNet) capable of generating voxel-by-voxel predictions of intracranial aneurysms by computed tomographic angiography (CTA). After applying the model, they demonstrated a significant increase in sensitivity, precision, and reliability among clinicians, optimizing patient care.[49] Sheth and colleagues developed and validated an automated method, based on ML (DeepSymNet), capable of evaluating, using CTA, large vessel occlusion (LVO) and the volume of the ischemic nucleus in patients, with satisfactory results.[50]

In a 2018 study, Jun and collaborators proposed DL 3D black-blood (BB) imaging that uses 3D convolutional neural networks (CNNs) with an autolabeling technique to detect metastatic brain tumors. Although there was a false positive for two patients, the sensitivities were 100% for deeply learned and original BB images in the analysis per patient.[51] In another study, Citak-Er and collaborators assessed the contribution of quantitative characteristics of multiparametric magnetic resonance (MR) in the classification of gliomas based on ML, using three different ML algorithms, including a support vector machine with linear kernel, multilayer perceptions, and logistic regression, with an approach of several regions of interest. This model achieved an accuracy of 93.0%, a specificity of 86.7% and a sensitivity of 96.4% for the classification of gliomas.[52]

Another ML technique, randomized survival (RF) forests, was applied to predict six cardiovascular outcomes compared with standard cardiovascular risk scores. The data generated suggested that ML together with deep phenotyping improved the accuracy of predicting cardiovascular events in an initially asymptomatic population, which could lead to a better understanding of subclinical disease markers without causality assumptions.[53] Also using an ML algorithm, Redlich and colleagues investigated whether factors that were identified by structural MR techniques are capable of predicting the response to electroconvulsive therapy (ECT).[54]

These advances in AI have made possible the accomplishment of several tasks in radiological imaging enabling evaluation of the risks, and the detection, diagnosis, prognosis, and response to different therapies, allowing the discovery of diseases by multiple 'omics' tools.[55] These technologies can be applied to improve imaging techniques already in use, as shown by researchers in the USA, who evaluated whether medical imaging can be used for patients with musculoskeletal (MSK) problems, allowing the increased use of common MSK imaging modalities.[56] In addition, this approach can also be used for liver imaging studies.[57]

Most of the appeal in using AI for drug design is the ability to focus on structural alternatives based on accessing a large volume of data obtained from high-throughput screening (HTS), through implicit model-building processes.[58] AI ends up making the decision or complementing human choices about which structures are the candidates with the greatest potential for therapeutic action and the highest level of specificity.[59] In this way, the focuses of machine intelligence are alternatives to structures designed from pre-existing backbones, the creation of new chemical scaffolds, and the selection of the most promising molecules.[60]

The biggest challenge for drug design using AI is how to avoid expensive HTS to autonomously allow the generation of new putative structures [new chemical entities (NCEs)] with the best possible combination of properties.[23] This problem is challenging and is enhanced by five technical/economic and mental bottlenecks that must be overcome for the design of really effective drugs: obtaining vast data sets; generating putative structures; optimizing choice based on multiple criteria; the shortening of the design–make–test analyze (DMTA) cycle; and a change in the scientific mindset of stakeholders (researchers and business people).[61]

Recently, scientists have been using DL to design more specific drugs with fewer adverse effects.[61–63] DL is a set of ML techniques that use several multilayer neural networks ('deep neural networks') to establish relationships between numerous and complex variables.[61] The main aims of DL relate to ligand-based optimization of compounds, to generate a wide set of can-

didate molecules, and restrict the choice to a few, aiming at rapid synthesis and testing.[64,65]

Over the past 10 years, the most successful efforts at using AI for drug discovery and drug design have been in the areas of imaging and NLP, often associated with DL.[66] This combination of methods allows access to millions of data points for training (large training data sets) complemented with data augmentation of weakly labeled data to funnel the large data input and obtain good generalist models of molecular structures and active chemical scaffolds.[61,67]

Regarding the design of protein structures, Google's DeepMind ML research branch developed, in 2020, the neural network-based algorithm AlphaFold 2, a powerful tool makes effective use of unlabeled data to predict the 3D structure of proteins with greater precision than other algorithms, rivaling in quality traditional imaging methods.[68] AlphaFold 2 make effective use of unlabeled data related to a data set of 170 000 proteins with known structures, and a larger database of amino acid sequences with unknown structures.[68] Google's AI machine makes a great leap in solving protein 3D shapes because the algorithm applies DL to structural and genetic data to predict, with greater precision and accuracy, the distance between pairs of amino acids in a protein, perhaps leading to unimagined advances in the area of protein folding and, perhaps, revolutionizing bioengineering and the way of studying living beings.[68]

Despite reported advances, the use of AI for drug design still presents three major technical obstacles: the design of structures based on sketches obtained from very restricted sets of data; the need to use biological molecules the level of structural and functional knowledge as putative drug targets for which is low; and the difficulty in obtaining large labeled data sets.[60,63,65]

The field of drug design always seeks a holistic approach, in which it focuses on not only the disease, but also the patient's body and mind.[69] Therefore, pharmaceutical research on new AI technologies has received substantial attention in recent decades, because learning architectures have shown more accurate results in predicting properties, which is currently indispensable.[70] Since the 1960s, chemical medicine has been using AI with varying degrees of success to determine important properties of compounds, such as the quantitative structure–activity approach (QSAR), capable of making predictions of solubility, bioactivity, and log P.[29,71–73]

In addition to these limitations, the drug development process requires a long period, ranging from 10 to 15 years, because of the need to identify and validate targets, and perform preclinical and clinical trials.[74] Another limitation is that the failure rate in clinical trials, performed on model organisms, can exceed 90%. All of these factors contribute to the cost of developing new drugs, which can exceed U$ 2.6 billion.[75] The use of AI in classical drug development methods allows a reduction in human intervention during the process, thus reducing inefficiencies and uncertainties during the necessary steps.[76]

In a paper published in 2018, Li and colleagues used a quantitative model of structural relationship, which was based on the radial basis function (RBF) ANN model, trained by the particle swarm optimization (PSO) algorithm, to predict the pKA value of 74 types of drugs. Given that the pKA value is a vital parameter in the design of drugs and pharmacology, the data obtained suggested that the model had a good prediction performance and could be used as a reference to explore other QSARs.[77]

Zilcha-Mano and colleagues used ML to test whether interactions between characteristics of patients using antidepressants before treatment, could better predict who would be most likely

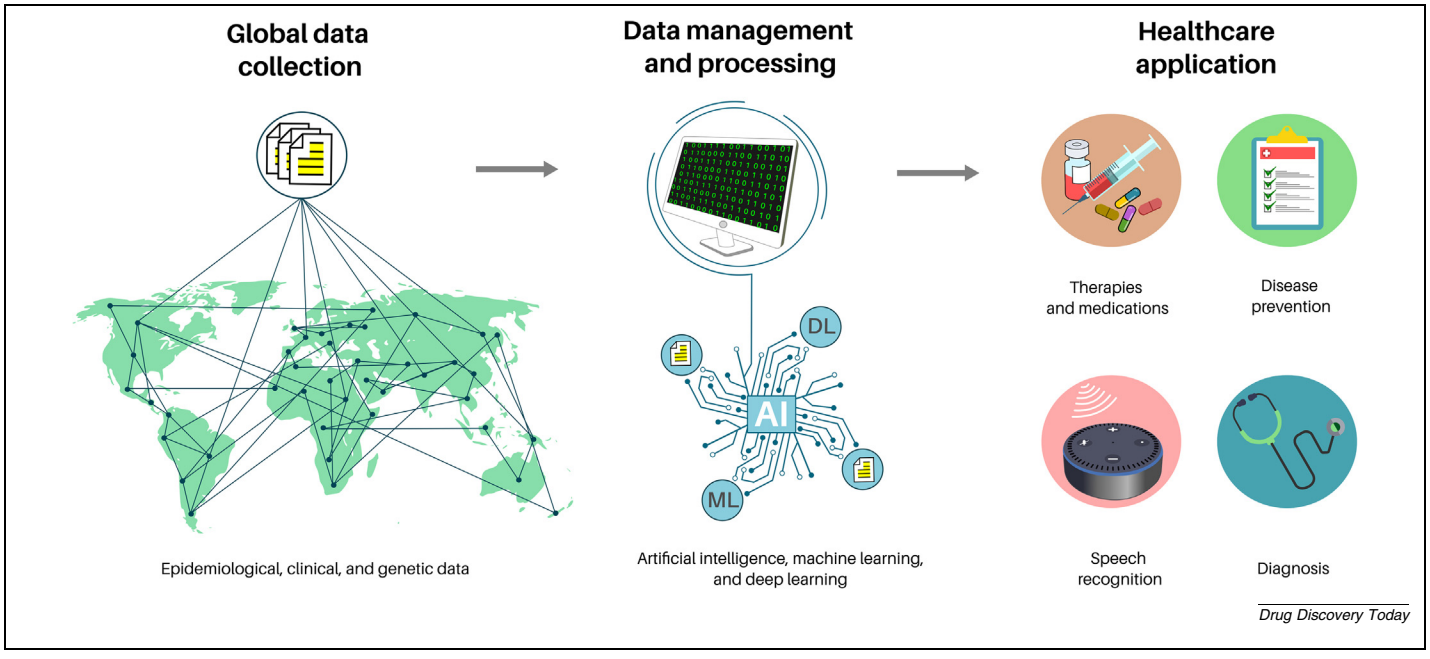

FIGURE 2

Use of artificial intelligence (AI) tools in life sciences and healthcare. Data collected from several patients can be integrated and analyzed using different AI tools to improve the diagnosis, treatment, and prevention of various diseases. In addition, voice and speech recognition can be used by doctors in clinical trials and patients during consultations and clinical follow-up. AI can also help in scientific hypothesis and article writing via dictation.

to benefit from placebo versus medication. The results were interesting, mainly for the group of patients with a higher level of education, in whom the placebo had the greatest response, almost superior to the medication [B = −0.57, t(96) = −1.90, $p = 0.06$].[78]

All the examples mentioned above make it clear that the application of AI, through ML and other technologies, can improve the diagnosis, quality of healthcare, and the development of new drugs based on algorithms capable of enhancing analytical analysis, revolutionizing the field of medicine and life sciences as a whole (Fig. 2).

AI as a tool to combat the COVID-19 pandemic

In late 2019, the first cases of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 2019 (COVID-19), were identified in Wuhan City, Hubei Province, China, although it was originally characterized as an outbreak of pneumonia of unknown etiology.[79,80] At the time of writing, there were more than 150 + million cases and 3.2 + million deaths caused by the COVID-19 pandemic worldwide according to the Coronavirus Research Center from the John's Hopkin's University (https://coronavirus.jhu.edu/map.html) and to the latest weekly epidemiological and operational updates (April, 2021) from the WHO.[81]

The increase in the number of cases of SARS-CoV-2 has overburdened healthcare systems and directly impacted economies worldwide, generating a global crisis.[82] Given the seriousness of the current scenario, a multidisciplinary approach from different scientific fields has been used to combat the pandemic.[83] Several efforts in this direction are being made using computational methods, such as AI and ML, to create strategies for the detection of, and coping with, COVID-19.[84,85]

The forecast for new cases and the number of deaths by COVID-19 is essential for the development of public policies. AI can be used to track and predict how this disease will spread over time and space.[86] In this regard, Silva and colleagues used ML to forecast 1, 3, and 6 days ahead the COVID-19 cumulative confirmed cases, in five Brazilian states and five American states with a high daily incidence, coupled with climatic exogenous variables. From the analyses, they observed that climatic variables, such as temperature and precipitation, influenced the forecast and, in some cases of COVID-19, the climatic variables reached up to 50% of importance in the forecast.[87]

Zhu and colleagues also developed a DL algorithm and a risk stratification scoring system to predict COVID-19 mortality. They compared the performance of the prediction by using the COVID-19 severity score, the CURB-65 score, and the pneumonia severity index (PSI). The data suggested that the models can be used to make clinical decisions in environments with few resources and time constraints.[88] In another article, an ANN was used to provide a patient-specific, point-of-admission mortality risk prediction to inform clinical management decisions at the first opportunity.[89]

Real-time quantitative PCR (RT-qPCR) is a gold-standard method for the diagnosis of COVID-19; however, the test can take up to 2 days to complete, and additional tests are needed to rule out false positives; thus, there is an urgent need for alternative faster and more accurate methods.[90] Given that the epithelial cells lining the human respiratory tract are attacked in COVID-19 use of X-rays, computed tomography (CT) scan images, and AI can be used to investigate the integrity of the lungs of patients with different clinical types of COVID-19.[91–93] One of these approaches was taken by Lin and colleagues, in which they developed the COVID-19 neural network (COVNet), a 3D DL framework to detect COVID-19 using chest CT.[94] Another study used breast CT and AI to explore possible similarities between macrophage activation syndrome (MAS) and severe COVID-19 in patients with pulmonary involvement. Again, the data demonstrated that there might be laboratory and radiological differences between both diseases, opening new avenues for future studies.[95]

AI can also be used to identify drug markers for the development of treatments for COVID-19,[96] to predict SARS-CoV-2 infection from complete blood counts,[97] to analyze the role of climatic and urban parameters in confirmed cases of COVID-19,[98] and to repurpose existing drugs used to treat other diseases, such as baricitinib, an oral Janus kinase (JAK)1/JAK2 inhibitor approved for the treatment of rheumatoid arthritis (RA) tested against COVID-19.[99]

These data suggest that the use of AI is a promising tool in combating the recent coronavirus pandemic and future pandemics. It can be used not only to predict the number of cases, but also in the diagnosis and possible treatment of patients. In addition, AI has been important in the development of COVID-19 vaccines currently in use.

Real-world data and real-world evidence

In the context of life science and healthcare, Real-World Data (RWD) can be defined as data relating to patient health/or healthcare delivery, obtained outside of randomized clinical trials (RCTs).[100,101] These data are collected from a patient's EHR in a hospital or from a health insurance company, from claim processes, product and disease registries, and self-generated patient data (e.g., home-use settings or mobile devices).[102] The RWD sources can provide, in near-real time, a volume of information at the granular or large-scale level, which can be used to support many types of clinical trial design, including RCTs, such as large simple trials, pragmatic clinical trials, and observational studies.[101,102]

The EHR data recorded can be classified as structured data (e.g., International Classification of Diseases codes, laboratory results, and medications) and unstructured data (e.g., physician notes). The unstructured data (most recorded data) require complex processing before performing statistical tests and conducting ML tasks, whereas structured data do not require such processing.[103]

The analysis of RWD produces Real-World Evidence (RWE), which is clinical evidence regarding the usage, and potential benefits or risks, of a medical product.[102] However, to obtain high-quality RWE that is acceptable for regulatory use and other decision-making applications, a large amount of RWD must be collected and converted into an analyzable format with a certain level of data accuracy and reliability ensured, something that humans alone are not able to do. For this purpose, AI capabilities are increasingly being applied to the analysis of RWD.[104]

Three AI capabilities are particularly applicable to generate RWE for the healthcare industry: NLP, ML, and robotic process automation (RPA).[101] NLP is a computerized interpretation and organization of human language within unstructured data (e.g., physician notes or speech recognition) to indicate a specific condition or event. It includes text classification, recognition of syntax, interpretation of word meaning based on location in a sentence, and language translation in a computable, interpretable, and accurate way.[105,106]

Examples of NLP applications include automated HIV risk assessment by extracting key terms in clinical texts or the development of NLP algorithms for asthma ascertainment using EHRs.[107,108] Nayor and colleagues developed and validated an NLP pipeline that accurately and automatically calculates adenoma detection rate and serrated polyp detection rate to evaluate colonoscopy quality parameters.[109]

By contrast, ML includes a variety of predictive statistical and mathematical modeling techniques, which are often layered onto NLP to reinterpret and correct initial assumptions following repeated usage.[101] A 2018 survey showed that 60% of pharma industry respondents were using ML in their RWE programs and 95% expected to use AI for this purpose in the coming years.[104]

Furthermore, ML can be used in RWD for the development of models for risk prediction of diseases, such as atrial fibrillation, or to monitor the health and well-being of patients.[110] For instance, Enshaeifar and colleagues used ML to develop an algorithm to detect urinary tract infections, which are one of the top five reasons for hospital admissions for patients with dementia.[111]

The third AI capability used to generate RWE is RPA, which comprises a software that automates repeated tasks, helping to speed the processes at the same time as reducing human error.[101,112] This software brought a huge development in many sectors by executing specifically defined tasks, but was limited in the sense that it could not adjust to changing conditions or learn from experience. In addition, RPA can be associated with ML, becoming more useful in the analysis of RWD. In this context, RPA can be used to extract field values from unstructured data based on the scripted/programmed set, whereas ML learns from these data to generate RWE.[113]

AI and clinical trials

Clinical trials are essential for the development of new drugs. They are designed to control variability through detailed eligibility criteria and carefully designed clinical protocols performed by specialized research personnel.[102] However, clinical trials take ~ 10–15 years and cost an average of US$1.5–2.0 billion; furthermore, only one in ten compounds entering a clinical trial reaches the market. The two main factors responsible for the failure of clinical trials are patient cohort selection and recruiting mechanisms that fail to bring the most-suitable patients to a trial in time, as well as a lack of technical infrastructure (reliable and efficient adherence control, patient monitoring, and clinical endpoint detection systems) to run a trial. However, AI can be used to overcome these shortcomings.[114]

Both ML and DL can be used to automatically find patterns of meaning in large data sets, such as text, speech, or images (Fig. 2). The human–machine interfaces (HMIs), a direct communication pathway between a human and a device, allow exchange of information between computers and humans, and the NLP can understand and correlate content in written or spoken language. All these methods used in AI can be applied to correlate large and diverse data sets, such as EHRs, medical literature, and trial databases, to improve patient–trial matching and recruitment before a trial starts, as well as for monitoring patients automatically and continuously during the trial.[114]

The Support Vector Machine (SVM) is a ML model that is used to predict diseases based on clinical features.[115,116] It is a useful tool that has the potential for use in cohort selection for clinical trials. In a 2019 pilot study, researchers used SVM and other ML models to predict postpartum depression (PPD) from patients in the Weill Cornell Medicine and New York-Presbyterian Hospital between 2015 and 2017. They used data from EHRs and identified race, obesity, anxiety, depression, different types of pain, antidepressants, and anti-inflammatory drugs during pregnancy as significant predictors. PPD is one of the most frequent maternal morbidities after delivery with serious implications and, currently, there is a lack of effective screening strategies and high-quality clinical trials. Thus, the use of SVM can leverage a large amount of detailed patient data from EHRs, allowing the prediction of PPD and the implementation of effective clinical decision support interventions.[115]

In another example, AI was used to improve the accuracy of screening for valvular and congenital heart disease by auscultation. Thompson and colleagues developed a heart murmur detection algorithm that was quantitatively and objectively evaluated by a virtual clinical trial. As a result, they obtained a highly specific algorithm for detection of the pathological murmurs in the analysed data set, similar to levels reported for specialist auscultation, making it a potentially useful screening tool for heart disease.[117]

One promising approach of AI in clinical trials are the intelligent assistants, such as Amazon's Alexa, Google's Assistant, Microsoft's Cortana, and Apple's Siri. There are reports that highlight their potential use in clinical trials and in the treatment of diseases.[118,119] For instance, they could be used in the management of diabetic foot ulcer (DFU) in five different feature categories: (1) the patient–doctor interface (facilitate scheduling appointments, refiling medications, etc.); (2) patient–caregiver interface (they can support communication between patients and their formal and informal caregivers); (3) alerting/notification (they could give timely alerts or notifications to improve patient lifestyle and/or engage the patient in the prescribed care management plan); (4) self-care (they could answer patient's questions about how to proceed in case of some unusual feature on their foot); and (5) gamification (used to engage the patient in regular self-care by sending notifications about their improvement).[118] In addition, all of these categories could be also used in monitoring patients in clinical trials.

The challenges ahead: Technological issues and privacy

In the big data era, when personal information can be sold and traded in the information market and between stock brokers,[120] a new way of thinking is needed, not only in terms of privacy set-

tings, but how privacy works (privacy model). The provision of personal data (voluntarily or involuntarily, consciously or unconsciously) can be done in numerous ways, such as when making purchases, paying taxes, buying gasoline, exchanging e-mails, sharing photos, among others. With more personal information from users as associations, they can provide a better service, but damage to privacy might be the result of this compilation of data by different entities over time.[121,122]

With regard to privacy in public health research and practice, big data raises three main issues: (1) the risk of inadvertent disclosure of personally identifiable information[123]; (2) the potential to increase the size of the data to make it difficult to determine and identify a data set and to avoid 'deductive disclosure' of personally identifiable information; and (3) the challenge of identifying and maintaining ethical research standards in the face of emerging technologies that can change generally accepted privacy standards (GPS, drones, social media, etc.). Avoiding the disclosure of participants' private information is a fundamental ethical principle required in the USA by the Health Insurance Portability and Liability Act (HIPAA),[124,125] but the inadvertent disclosure of information by health researchers has already occurred repeatedly.[126]

The traditional approach of asking for consent to use and collect data to control, restrict, and limit access to personal information has remained largely unchanged since the 1970s. It is still used now, when people consent to provide their data daily without having read, understood, or thought too much about browsing digital media and digital information environments.[120–122] According to Mai, this approach has been inefficient given the new information and communication technologies, so new solutions and new conceptual approaches are needed to understand privacy in the digital information society.[120]

Mai's article[120] presents three privacy models that can help both in visualizing the problem and in conceiving possible solutions: (1) a digital pan-optical model, in which the basic idea is that surveillance and violation of privacy are conducted by someone 'watching' another person, and the observation is assumed to be uninterrupted and surreptitious; (2) a capture model, in which the focus is on how human activities are constructed in 'languages representing a computer system'; and (3) the privacy datafication model, in which attention is focused on the anonymous creation of new personal information, the reinterpretation and statistical analysis of data, and the commerce of that information.

These different models help us to better understand the perspective of privacy and form the ethical basis for dealing with the challenges created by the production of new knowledge that the analysis of big data and uses of digital media generate.[120,121,127]

Can AI write scientific and biomedical articles?

High-quality publications in high-impact journals are determinants of a researcher's success. In addition, these factors influence current and future job prospects for researchers. However, writing a successful publication requires a multitude of skills, including analysis and data collection, writing, and even ethics.[128] Thus, it is often difficult for researchers to find time to write scientific articles and to get them published. Even people who are fully involved in research, the time dedicated to the writing process can be unproductive and fragmented. In addition, common methods of academic writing in a computer form or on paper can be time-consuming.[129]

Among the methods that can facilitate this process are an outline of the paper before the actual writing and dictation.[128–132] The use of dictation allows the free transfer of ideas to paper, without interrupting thoughts in a more practical way. In addition, the speed of speech is more similar to the speed of thinking compared with typing text on a computer or handwriting.[128] The dictation of scientific articles is recognized as an efficient method for producing first drafts of high-quality articles, reducing the risk of writer's block, and having an appropriate language complexity even when used by inexperienced writers.[133,134]

Pommergard and collaborators showed the feasibility of using voice recognition software (VRS) to transcribe dictated scientific articles, especially when research departments do not have staff who do transcriptions.[133] Andresen and collaborators have used this alternative efficiently for years.[130,131] The process used by this research group initially consists of a detailed outline ('map of the article') followed by the manuscript written by dictation via a smartphone. This methodology provides an overview of all parts of the article, facilitating changes that might be necessary in the final version of the article.[132] In addition to being time efficient, producing high-quality, concise articles, and not requiring substantial academic experience for users, this system can be a way of increasing efficiency in academic writing.[134]

Pharma and biotechnology catching up on data science and AI

Developments in the field of biotechnology are increasingly dependent on the extensive use of big data. Future productions in this area depend on the ability of researchers to master the skills necessary to integrate their own contributions with the range of information available in these databases. The large volumes of data generated and stored in the areas of biotechnology create several opportunities for researchers, as well as for companies that offer producers and services in this area. Currently, it is almost impossible to conduct research in biotechnology without using databases and AI technologies to process and explore the vast collection of data available, whether public or private.[23]

There are several applications of big data in the biotechnology area, including the design of experiments from the automation of steps to the continuous monitoring of the results of that experiment. Other alternatives for drug discovery are scanning databases for promising molecules, predicting the potential for small molecules to bind targets, and optimizing production processes.[135] Until the final commercialization process, new drugs must undergo clinical tests until they receive approval from the relevant authority [e.g., US Food and Drug Administration (FDA)]. This entire process is one of the riskiest and most expensive ventures in the world. Specialists in areas such as biology, pharmacology, chemistry, safety, among others, are needed to find drug candidates under the supervision of the FDA, legal teams, and even other specialists from competing companies.

The average expenditure for the development of a successful drug is ~ US$ 2.6 billion.[136,137]

AI is already used in the pharmaceutical industry in the areas of bioinformatics, biochemical modeling, predictive toxicology, among others.[138] To take full advantage of the AI industry methods, one must change the current approach from a scientific method guided by experts to a partnership between scientists and AI.[136] In this way, the pharmaceutical industry benefits from scientific knowledge of the literature and data in an appropriate manner for decision-making. An important aspect of successful ML for property prediction is access to a large data set, given that, in the pharmaceutical industry, numerous data are collected during the optimization of compounds for various applications, which can be used both for the training of ML models and lead-to-compound optimization.[70] Currently underused AI methods would complement human decision-makers, finding complex data patterns, prioritizing experiments, finding better targets, improving modeling, choosing patients for clinical trials, extracting insights, and even predicting clinical results.[137–139]

Other variants considered by AI are those of molecular variations (point mutations, insertions, and translocations of gene sequences) between individuals and populations, which can be indicative of response to treatment or adverse effects, thus guiding the discovery of new drugs. For example, Vogelstein *et al.* reported the use of EGFR kinase inhibitors and ALK anaplastic kinase inhibitors to treat these variations in *EGFR* and *ALK*, respectively. Such information on genetic variants can be found in databases such as COSMIC (https://cancer.sanger.ac.uk/cosmic), ClinVar (www.ncbi.nlm.nih.gov/clinvar/), and OMIM (www.omim.org).[140,141]

AI in mobile application development: The CogniAction case study

The cognitive and motor development of disabled children is being improved every day with the help of technology. The main impact of this improvement is felt in the pedagogical interactions and in the social inclusion of these children. The constant improvement of mobile devices with touch screens, coupled with the development of software that uses AI, are revolutionizing the way of collecting, storing, distributing, and analyzing data, allowing new discoveries. Accessibility, user interface (UI), and user experience (UX) are present in most research projects and are a reason for investment by most industries that produce technology. Driven by the game market, many scientific applications are based on the principles of gamification and playfulness to achieve their goals. Game accessibility has three classification subgroups: the technical accessibility, which is the basic accessibility of the web; the accessibility of game content, linked to the complexity of the scenarios and game genre; and the accessibility

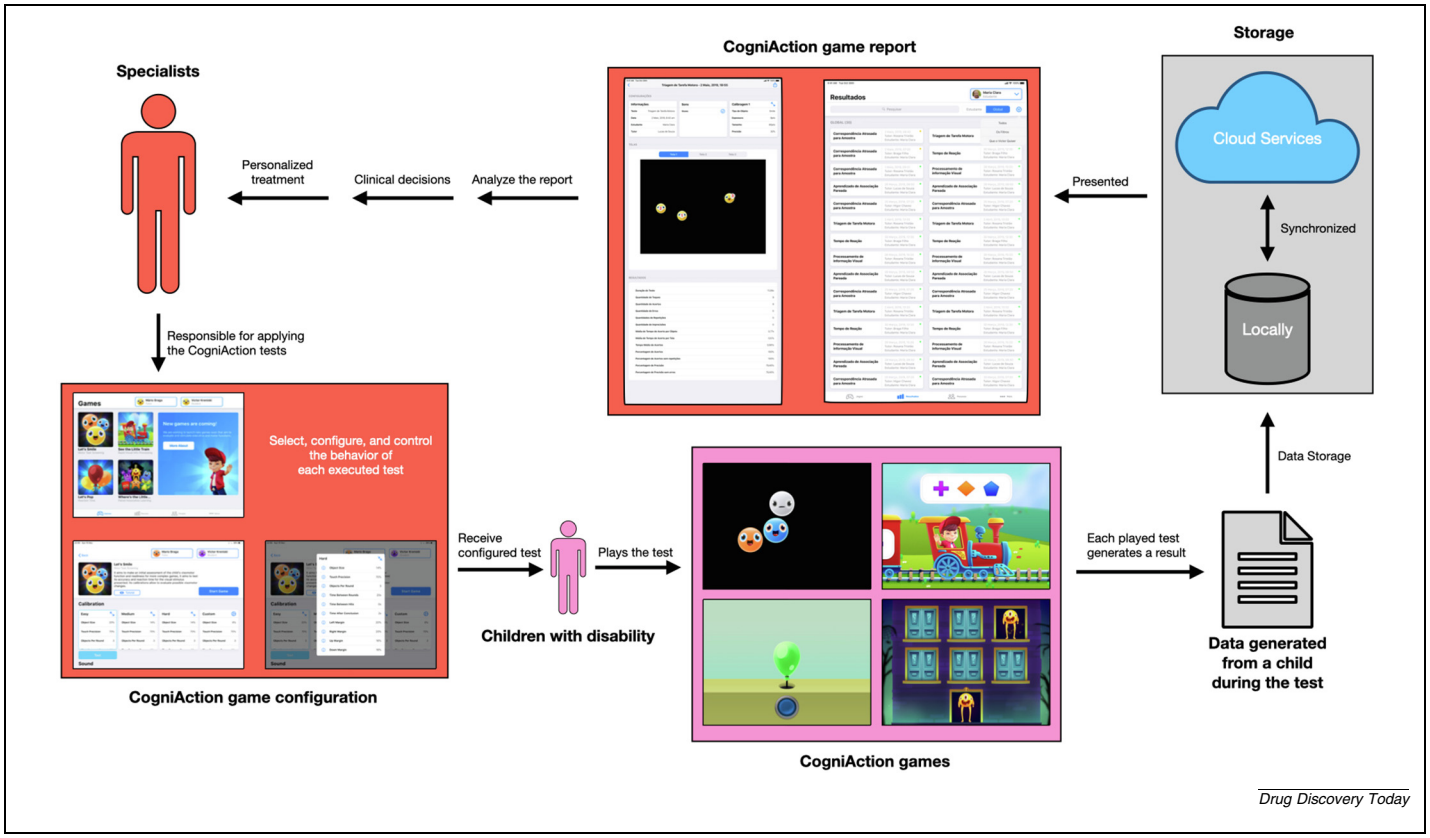

FIGURE 3

Schematic representation and depiction of a pipeline, starting with the specialist using the CogniAction app to collect data generated from users with disability to intervene and create a personalized treatment. The specialist is responsible for creating and configuring the App. The child plays the game and the data generated is collected, covering the behavior of the user during the game. After the game has ended, a result is generated, stored, and synced with a cloud server and/or 'in-house' data server. This allows the specialist to generate a report and make clinical decisions to personalize treatment for the user. In addition, artificial intelligence (AI) tools are used to maximize the data collection and the solution can 'learn' based on the users and improve with time.

of the game interface, the latter is analyzed from the perspective of an appropriate game according to each identified disability (www.iso.org/obp/ui/#iso:std:iso:9241:-171:ed-1:v1:en). From the user's point of view, the interface is one of the most important parts of the relationship established with the device, especially in the case of children with the most diverse disabilities. However, it is not possible to use most serious games for people with disabilities, given that games take advantage of abilities that people with disabilities might not have.[142] The design and playfulness found in most educational games for mainstream children form an appropriate set that assist education professionals in achieving better results in the teaching–learning process. This scenario is also being used to improve the motor and cognitive abilities of children with disabilities.

An example of a case study built and tested by us, exclusively for mobile devices, the CogniAction app, developed for children with Down's Syndrome and neurological problems, shows that it is possible to bring together all the elements that favor cognitive and motor improvement, from conception to use by the end-stage user (Fig. 3). In this case, children, parents, and professionals in special education show that the evaluation of sensory, perceptual, cognitive, and psychomotor functions, with the aid of applications, is essential to identify the neuropsychological profile of children with motor difficulties and neurodevelopment disorders.[143] The application contains tests capable of generating information, based on the child's interaction, making the most of the touch screen. Information, such as intervals between touches, precision of touches on different objects or outside them, quantities of objects on the screen, and size of objects, are translated into data that measure spatial intelligence, visual attention, short-term memory, working memory, speed of manual processing, and visual–motor coordination, allowing monitoring by specialists. The architecture and technological platform chosen for the development of the project enabled the most to be made of the hardware and operating system (OS), resulting in the high quality, and greater compatibility, of the devices involved. By allowing detailed data collection, it is observed that this app makes room for the use of technologies such as AI, ML and others linked to large-scale data analysis. The data collected as a specific result of the game mechanics can be compared with data on the environment and health status of each child and their degree of disability, thus allowing a more accurate analysis of best practices and new perspectives for the use of these solutions. As evidenced in[143], the benefits of the CogniAction application go beyond the simple act of testing neuromotor functions, offering the possibility of contributing to clinical neuropsychology units worldwide, both in high- and low-income countries. Therefore, AI combined with the development of specific applications for people with disabilities also offers an invaluable way to improve their living conditions and social inclusion, treating them with dignity and equity.

Concluding remarks and prospects

AI is no longer just a science fiction present in films and books: it is a reality that can be accessed through voice commands on mobile devices. Advances obtained in the area of AI have revolutionized several fields of human knowledge and we believe that it will now significantly impact the life sciences sector, as being witnessed with the current coronavirus pandemic. However, far beyond predictions of the number of cases or deaths, machines are 'being taught' to recognize patterns that can be used in the early diagnosis of diseases, such as COVID-19. Another example is how gamification associated with mobile devices and AI tools can help improve the lives of people with disabilities (as shown by the CogniAction case study).

In addition, researchers are exploring AI to develop new or repurposed drugs, allowing for new, more accurate drug prescriptions. AI has also been used to overcome two major problems associated with clinical trials: patient cohort selection and recruitment mechanisms. It is also possible, using ML, to collect data, analyze it, and even publish a scientific article via dictation and speech recognition. There is no doubt that AI will continue to make great strides to improve accuracy and lower costs in the healthcare and life sciences sectors.

Declaration of interests

The authors declare that they have no conflict of interest in this article. The CogniAction app is freely available to download from the Apple App store (https://apps.apple.com/us/app/cogniaction/id1525766188).

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Epigenetic Therapies in the Precision Medicine Era

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The elucidation of the 3D structure of DNA revolutionized modern science and created the basis for the field of molecular biology. The advent of DNA sequencing, and further refinement with Next-Generation Sequencing (NGS) techniques, has made possible the enormous accumulation of data, which are useful for understanding the molecular mechanisms associated to various complex and rare diseases. Thanks to these advances, today it is known that several mechanisms regulate gene expression without the occurrence of mutations in the genome, a phenomenom known as Epigenetics and Epigenomics. The main mechanisms involved are DNA methylation, histone modifications, and non-coding RNA transcription. The knowledge of these mechanisms applied to biomedicine has enabled the emergence of several fields, especially precision medicine, which is based on the genetic and epigenetic profiles of patients applied to personalized diagnostics and treatments. In this review, the history of the scientific advances that have enabled the development of precision medicine will be discussed, with a focus in epigenetics. Moreover, several molecules that have been approved for use or have the potential in epigenetic therapies (epidrugs) will also be discussed here, those of which act on targets responsible for maintaining the correct epigenetic pattern or correcting wrong patterns in diseases.

1. Introduction

Questions are (and always will be) the engine that drives science. One of these great scientific questions was "What is the structure of the deoxyribonucleic acid (DNA)?." Fortunately, it was answered in 1953 by Watson and Crick.[1] The elucidation of the 3D structure of the DNA was, undoubtedly, one of the greatest discoveries, if not the greatest, made in the mid-20th century and it allowed the emergence of the field of molecular biology as we know it today. However, this breakthrough could not be possible without the contributions of those who came before (**Figure 1**).

Nowadays, the 3D molecular structure of DNA is no longer a problem, although new evidence about other possible DNA structural conformations is coming out.[2] With the publication of the first draft of the human genome in the early 21st century, the researchers all over the world believed that it could be possible to find the answers to all rare and complex diseases.[3] Unfortunately, even the most modest predictions about finding cures for diseases of clinical concern have not been fully confirmed, and more questions than answers came up.

One of the biggest mysteries of the "post-sequencing era of the human genome" is the fact that only 22 000 to 25 000 genes in the whole genome encode proteins.[4,5] Apparently, most of the human genome is made up of what became known as "junk DNA."[5,6] This term is described by evolutionary biologists as DNA sequences from which gain or loss does not affect the fitness of the bearer.[7] However, although the exact function of a great proportion of these transcripts is not yet understood, we now know that they have important functions.[8–15]

The emergence, and constant improvement, of next-generation sequencing (NGS) technologies, allowed us to demonstrated that from seemingly nonfunctional regions, or "junk DNA," a multitude of noncoding RNAs (ncRNAs) are highly transcribed.[16,17] These technological advances have also

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allowed us to broaden knowledge about other factors that control gene expression—the answers are "above" the DNA sequence as new layers of information are discovered. In addition to noncoding RNAs, histone modifications and DNA methylation are epigenetic mechanisms capable of altering the pattern of gene expression without changes in the nitrogenous bases.[18,19]

If any problem occurs in the epigenetic mechanisms' regulation and maintenance, they can lead to harmful phenotypic variations, causing the development of several diseases,[20] such as many cancer types,[21–26] congenital and adult heart disease,[27] mental disorders, etc.[28] All the knowledge generated by NGS technologies allowed us to think about a new way of treating complex diseases, such as cancer, through precision medicine,[29] through the establishment of specific treatment and diagnosis strategies for a class of patients.[30] Precision medicine will assist us with intensive care[31] and in the development of drugs that will act on the targets responsible for the disease in specific groups of patients.[32]

Throughout this review, we will address the advances that have allowed the emergency of the epigenetic field and epigenetic therapies. Also, an extensive review of the literature will be done to compile the clinical studies that are already being developed to use epigenetic therapies to treat dozens of complex diseases such as cancer, diabetes mellitus, heart diseases, and so on.

2. DNA Sequencing: More Questions Than Answers?

After the elucidation of the 3D structure of DNA, other great scientific question arose: "How to read it?" To answer this question, the initial efforts focused on sequencing RNA species due to their simplicity when compared to DNA.[33] The first steps beginning in the mid-1960s when, for the first time, Robert Holley and coworkers obtained the whole nucleotides sequence of an alanine *transfer* RNA (tRNA) isolated from yeast.[34] Regarding DNA sequencing, in 1968 Wu and Kaiser published the sequence of 12 bases of the cohesive ends of the bacteriophage lambda (λ) DNA.[35]

Nowadays, it is possible to sequence the entire human genome in a few hours due to the development of High-Throughput Sequencing Technologies, also called NGS, available since the early 2000s.[36–39] In addition to the increasingly modern sequencers, constant innovations in data generation have resulted in the exponential decrease in the cost of sequencing entire genomes. These technological advances have led to the breakdown of paradoxes within the field of genetics and the study of complex diseases.[37]

Thanks to the Human Genome Project, today we know that only ≈1.2% of the euchromatin genome is transcribed to protein.[40] However, this does not mean that all the rest of the genome is made of nonfunctional sequences. A growing body of evidence has shown that from the so-called "junk DNA" several noncoding RNAs (ncRNAs) molecules are transcribed. As demonstrated by the Encyclopedia of DNA Elements (ENCODE) project, ≈80% of the human genome is related to biochemical functions such as RNA transcription, transcription-factor-binding, chromatin structure, and histone modification.[41–44] Furthermore, pieces of evidence have been suggested that more than 90% of the genome is transcribed into RNA molecules.[12,45]

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These data gave rise to more questions and efforts to answer them have turned to other layers of information "above" the DNA sequence. Changes in the gene expression pattern that does not involve the replacement of the nitrogen bases throughout the genome are epigenetic events.[46] The "classic" epigenetic mechanisms are DNA methylation and histone modification. However, ncRNAs make up the third epigenetic mechanism.

3. Epigenetics and Its Mechanisms of Gene Regulation

Epigenetics is the study of changes in the chromatin structure inheritable by mitosis or meiosis that does not involve changes in the DNA sequence.[47–49] The epigenetics marks are made by DNA methylation, histone modification, and ncRNAs (**Figure 2**).[50–52] DNA methylation consists of covalently adding a methyl ($-HC_3$) group to the 5′-carbon of the CpG islands.[53,54] This epigenetic mark is mediated by enzymes called DNA methyltransferases (DNMTs).[55,56] There are three different DNMTs, are they DNMT1, DNMT2, and DNMT3 (having DNMT3a and DNMT3b isoforms).[57]

DNMT1 is responsible for maintaining the pattern of methylation in the cell division. It can recognize the hemimethylated strand and add the methyl group to the newly synthesized DNA strand.[58] DNMT2, also known as TRDMT1, are highly

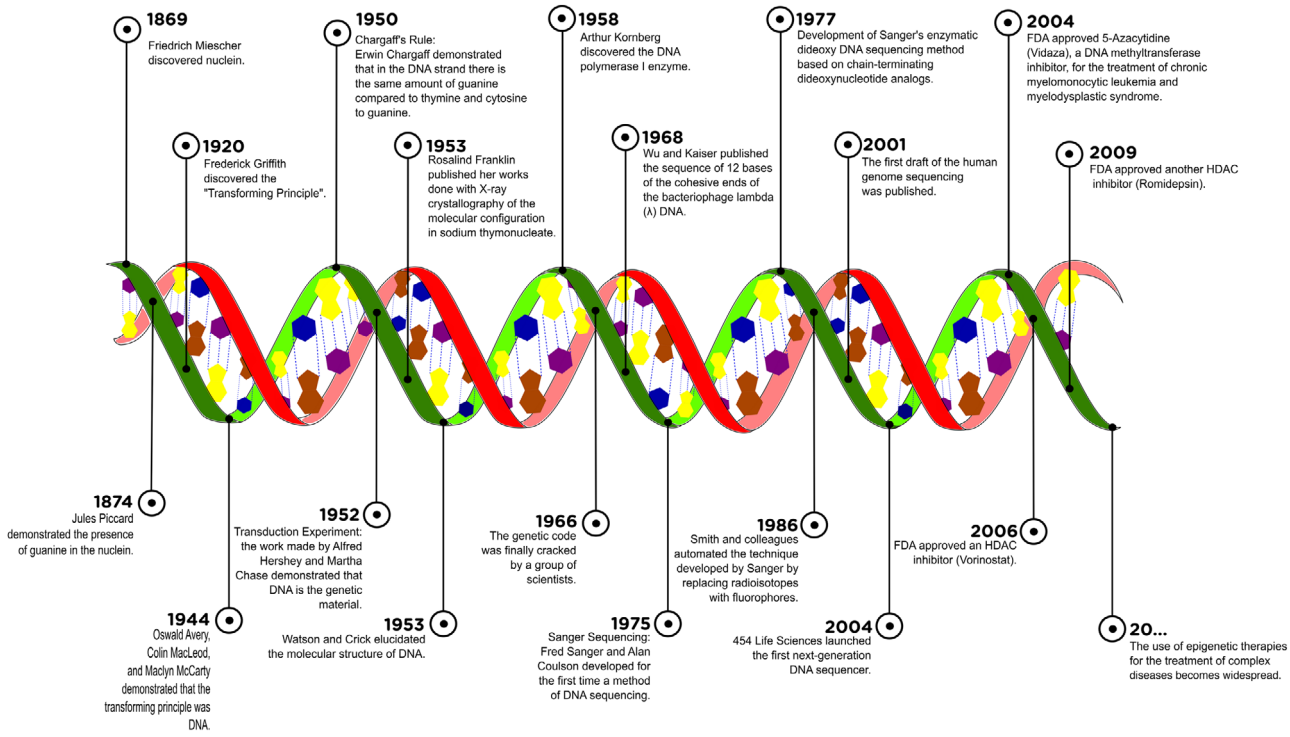

Figure 1. Timeline showing the main breakthroughs made in the biomedicine field that were important to genetics and epigenetics. This figure pointed the main milestones since Miershe's discovery of nuclein in 1869 to the first human genome draft sequencing in 2001. These landmarks were reviewed by Choudhuri (2006) and Dahm (2008). This Figure also depicts the emergence of epigenetic therapies and drugs first approved by the FDA.

conserved proteins, which catalyze methylation at position 38 of different tRNAs instead of genomic DNA.[59–62] DNMT3a/3b are enzymes very similar in structure and function, and it are related to maintenance of the methylation pattern, possibly adding the methyl group at the CpG sites missed by DNMT1. Besides, they are still responsible for *de novo* methylation on unmethylated sites during the development process.[58,63]

DNA methylation can also occur in non-CpG islands, such as CpA, CpT, and CpC, and is also responsible for regulating gene expression in mammal cells.[64–67] However, this kind of methylation is rare, occurring in special cell types as neurons, embryonic stem cells, and oocytes, for example.[68] N^6-methyladenine (m^6A) is the most common base modification identified in eukaryotic mRNAs.[69–72] This epigenetic mark is mediated by the main catalytic components formed by METTL3/METTL14/WTAP, which can be "erased" by FTO and ALKBH5.[73]

This mechanism is well preserved throughout various species of plants, fungi, and animals.[56,74] Abnormalities in the pattern of DNA methylation can trigger the emergence of several complex human diseases. Cancer is an example of complex diseases that may have as one of its origins the change in the epigenetic pattern. Hypomethylated DNA causes chromosomal instability, loss of imprinting and abnormal activation of oncogenes, causing the onset of several types of cancers.[75]

Much more than merely keeping the DNA coiled around the histones, forming the nucleosomes, chromatin also functions as a template for epigenetic information.[76] The chromatin controls gene expression by preventing RNA *pol* from having access to a specific gene.[77] The nucleosome is the smallest chromatin unit and is essential for gene regulation. Each of these units is formed by an octamer protein called histone, in which it is formed by subunits H2A, H2B, H3, and H4 in pairs, coiled by ≈147pb forming 1.7 turns around it.[78,79]

An increasing number of evidence has demonstrated a relationship between the deregulation of these mechanisms with the emergence of diverse human complex diseases. Among them is cancer,[80] Alzheimer's disease (AD) pathogenesis,[81] autoimmune disease[82] and diabetes.[83] Thus, the code of histones is not only important for the maintenance of epigenetic information but also to allow the eukaryotic cells to function properly.

The last gene expression regulation cited here is ncRNAs, small molecules that do not code any protein. Although, the most common and well-known types are ribosomal RNA (rRNA) and transfer RNA (tRNA), other classes of ncRNAs have arisen, such as microRNAs (miRNAs),[84] circular RNAs (circRNAs),[85] and long noncoding RNAs (lncRNAs),[86] and play pivotal role in gene expression control.[47]

miRNAs can be described as small endogenous RNA molecules having ≈20–30 nucleotides (nt) in length.[87] They can regulate the gene expression by binding to the 3'UTRs of target messenger RNAs (mRNAs).[88] miRNAs are involved in several biological processes in different organisms, such as post-traumatic stress disorder,[89] synaptic plasticity,[90] in the regulation of tumor cell plasticity,[88] salt-stress responses, and tolerance in plants.[91]

circRNAs can be defined as an endogenous noncoding RNA molecule closed forming a loop. This kind of ncRNAs is very abundant in mammalian cells, being express from

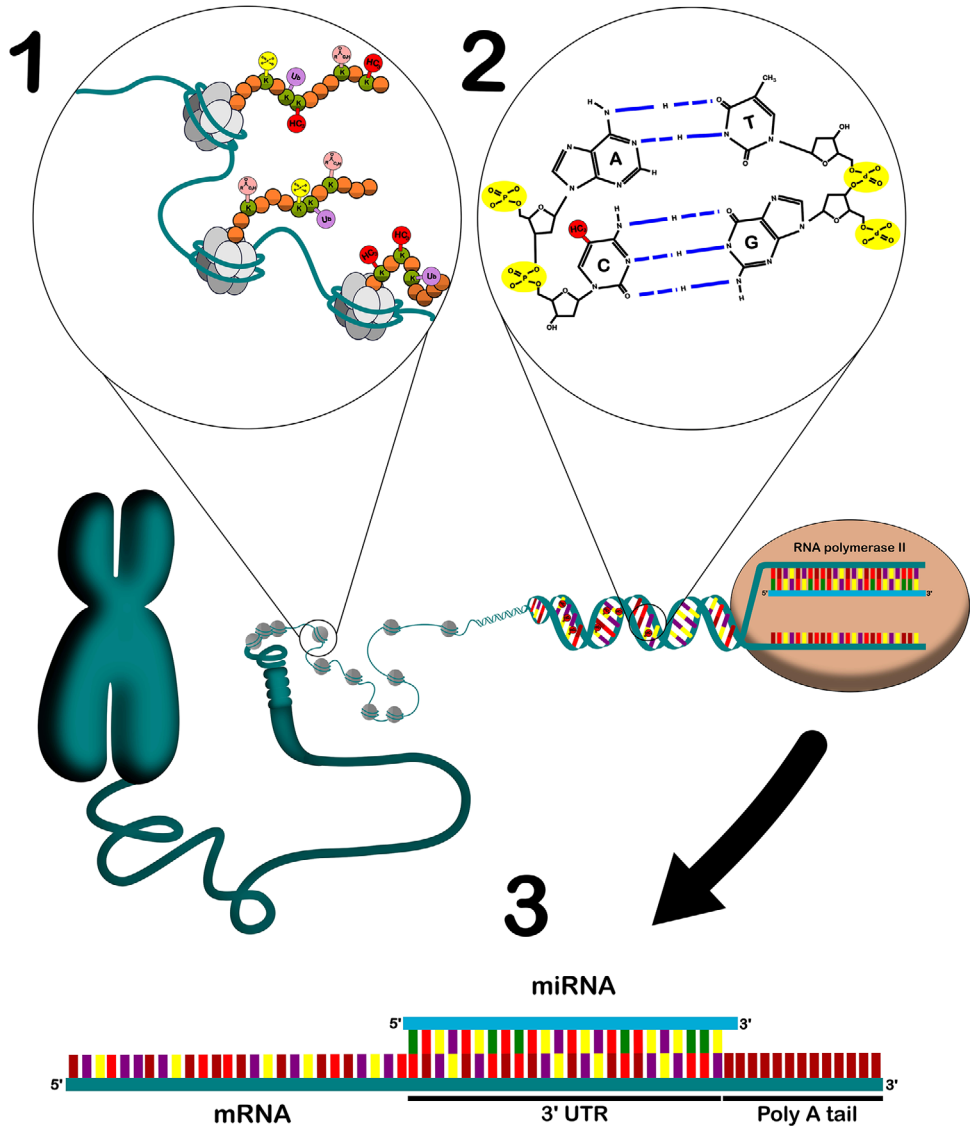

Figure 2. Epigenetic mechanisms responsible for the regulation of gene expression. 1-Chromatin modifications: The addition of chemical groups to the lysine residues present in the histidine tail results in the structural alteration of chromatin preventing RNA pol access to the gene. 2-DNA methylation: addition of methyl group to the cytosine carbon-5′ group in the CpG islands. 3-Noncoding RNA transcription (represented here by miRNA): miRNAs bind to the 3′UTR region preventing protein translation.

different locations of the animal genome and they also are resistant to degradation.[85,92–94] In the recent study, Yu and colleagues demonstrated that two circRNAs, *circBIRC6* and *circCORO1C*, are associated with human embryonic stem cells (hESCs) pluripotent state.[95]

LncRNAs are molecules that have more than 200 nt in length and do not have Open Reading Frames (ORFs), although they are transcribed by RNA Pol II, receive 5′ capping, undergo splicing, and 3′ polyadenylation.[96,97] A growing body of evidence demonstrated that lncRNAs play several roles in gene expression and biological processes.[98] They can be involved in the metastasis process,[99] hepatic function and liver diseases,[100] and they can act in the transcriptional and post-transcriptional regulation.[101]

4. Postsequencing Era: The Rise of Precision Medicine

Precision (or personalized) medicine is a biomedical strategy to individualize the treatment through the use of new diagnostics and therapeutics resources (**Table 1**). This approach is targeted to the needs of a class of patients based on their genetic, biomarker, phenotypic, or psychosocial characteristics.[102,103] Therefore, precision medicine integrates an investigation of mechanisms of disease with prevention, treatment, and cure.[104]

The Human Genome Project allowed the emergence of several molecular tools and techniques to process and generate a massive amount of genomic and proteomic data, such as NGS, mass spectrometry, microarrays, PCR-based methods, nanobiotech-

Table 1. Summary of selected articles showing different high-throughput technologies to obtain epigenetic biomarkers (DNA methylation, noncoding RNAs and histone modifications), which can be used in the precision medicine for the prognosis, diagnosis and treatment of complex diseases.

Biomarker	Analysis	Disease	Suggested Application	Ref.
Promoter methylation of TP53 gene by the overexpressed SETDB1	Western blot, ChIP-PCR, IHC, and qPCR	Colorectal cancer	Treatment by inhibitors targeting SETDB1	[105]
Di-methylation of p53K370 by the overexpressed SETDB1	Western blot and LC-MS/MS.	Hepatocellular carcinoma	Treatment by inhibitors targeting SETDB1	[106]
Hypermethylation of SFRP1 and SFRP2	MSP and bisulfite sequencing	Colorectal cancer	Treatment by monoclonal antibody blockade or reversal of *SFRP* silencing	[107]
Expression of the oncogenes H19 and IGF2 induced by the overexpressed lncRNA 91H	qRT-PCR, qPCR and chromatin-immunoprecipitation	Breast cancer	Treatment by inhibitors targeting lncRNA 91H	[108]
Methylation of CDO1, ZSCAN18 and ZNF331	qMSP and RT-qPCR	Gastrointestinal cancer (colorectal-, gastric-, pancreatic- and bile duct cancer)	Biomarker for cancer detection	[109]
Promoter hypermethylation of the BRCA1 gene with allelic imbalance	MSP and microsatellite analysis	Sporadic breast cancer	Diagnosis and prognosis	[110]
Differential expression of the lncRNAs FMO6P and PRR26	RNA-Seq and a multivariate Cox proportional hazards regression	LUSC	Potential outcome predictor for LUSC patients	[111]
Differential 5hmC loci in cell-freeDNA.	PCR and NGS	Colorectal and gastric cancer	Noninvasive diagnosis and prognosis	[112]
17 DMRs	MBDCap-Seq, Sequenom MassARRAY methylation and Cox proportional hazards model	Triple-negative breast cancer	Prognostic potential of DNA methylation	[113]
Histone H3 lysine 4 acetylation	ChIP-Seq	Breast cancer	Treatment by HAT inhibitors	[114]
DMRs in miRNAs and lncRNAs promoters	MBDCap-Seq and RNA-seq	Breast cancer	Diagnostic biomarkers for breast cancer	[115]
Upregulation of ELOVL5	HumanMethylation450K BeadChip, qRT-PCR and western blot	Type 2 diabetes mellitus	Noninvasive diagnosis and prognosis	[116]
Tri-Methylation of histones H3K4 and H3K27	Chromatin Immunoprecipitation and ChIP-Seq	Breast cancer	Therapeutic targets	[117]
Promoter methylation of ZNF331 gene	qMSP	Colorectal cancer	Diagnosis and prognosis of cancer	[118]
Differential expression of hsa-miR-125a-5p, hsa-miR-342-3p, and hsa-miR-365b-3p	qRT-PCR	Endothelial dysfunction	Diagnosis, prognosis and evaluate therapeutic efficacy	[119]
Up-regulation of lncRNA LINC00342 and differential expression of 6066 lncRNAs	RT-qPCR	Nonsmall cell lung cancer	Prognosis or treatment by silencing the LINC00342	[120]
DMRs of nine CpGs	HumanMethylation450K BeadChip and multiple Cox regression	All-cause and cardiovascular mortality-related smoking	cardiovascular risk prediction and disease prevention	[121]
High levels of circulating miR-10a, -10b, -21-5p, -30c, -155, and -212	RT-qPCR	PDAC	PDAC risk stratification	[122]
Low levels of circulating let-7c, miR-145, and miR-155	RT-qPCR	Coronary artery disease	Diagnosis and potential targets for therapeutic intervention	[123]
Overexpression of histone-modifying genes EZH2 and SUV39H2	RT-qPCR and TMA-based IHC	Hepatocellular carcinoma	Prognosis and predict patient survival	[124]
High proportion of histones H2AK5ac+, H3K9/K14ac+, and H3K27ac+ hepatocytes	TMA and IHC	Cirrhosis	Assessment of the liver function and prognosis	[125]
Decreased levels of histones H3K4me2 and H3K18ac	TMA, IHC, Western blot and ChIP	Lung and kidney cancer	Prognosis or treatment with HDACis	[126]

TMA tissue microarray; *IHC* immunohistochemical staining; *ChIP* chromatin immunoprecipitation combined with microarrays; *PCR* polymerase chain reaction; *RT-qPCR* reverse transcription polymerase chain reaction quantitative real time; *MSP* methylation-specific PCR; *qMSP* Quantitative methylation-specific PCR; *LC-MS/MS* Liquid chromatography with tandem mass spectrometry; *MBDCap-seq* methyl-CpG binding domain (MBD) protein column followed by next-generation sequencing; *DMRs* differentially methylated regions; *LUSC* lung squamous cell carcinoma; *HAT* histone acetyltransferase; *PDAC* pancreatic ductal adenocarcinoma; *HDACis* Histone deacetylase inhibitors.

nology, and various other direct analysis methods or assays. Also, these technologies brought huge information that allows the development of therapeutic interventions for precision medicine based on OMICS data (genomics, epigenomics, proteomics, metabolomics, etc.).[103,127–129]

For the development of customized diagnostics in precision medicine, it will be necessary to integrate OMICs data (big data) with clinical information, increasing the understanding of pathophysiology and treatment of diseases.[130] This approach allows us to understand the genetic and epigenetic basis of the disease and improve the treatment as per the genetic structure.[129] However, there are some challenges to overcome due to a large amount of information obtained from big data. Nowadays, generate the data is less expensive than is to store, secure and analyze it.[131]

Therefore, there is very limited knowledge regarding most of the genes and their functions in various cell types, tissues, physiological conditions, and disease phenotype.[129] The first challenge ahead is to improve the computational infrastructure to generate, maintain, transfer and analyze large-scale information securely in biomedicine and to integrate OMICS data with other data sets, allowing the combined analysis of multidimensional data which is referred to as "panomics."[130,131]

In addition to some big data concerns, life sciences professionals will need to work along with the advances in information technology (IT). Computer-aided medicine, web-based solutions, and big data analytics will need to be taken seriously by physicians in the treatments of diseases. Furthermore, these data-centric methods will enable researchers to transform information into predictive models, which is crucial for the application of precision medicine.[131]

Precision medicine will bring many benefits to human health with the development of targeted drugs and gene-oriented treatments but is necessary to identify and validate disease-specific, mechanism-based, or epigenetics-dependent biomarkers. The identification of disease biomarkers can improve and increase the accuracy of clinical diagnosis and patients' susceptibility, monitor disease progression, and therapeutic efficacy, and serve as an important tool in the pharmacogenomics, analyzing the benefits and adverse effects of particular drugs.[103,129]

5. Epigenetic Therapies to Treat Complex Diseases

The regulation of gene expression is one of the main topics addressed in genomic science currently. Understanding the mechanisms that involve the expression of genes in different cell types and how certain modifications can lead to the development of diseases is of great importance.[132,133] All processes related to epigenetic modifications such as DNA methylation and histone modifications, act on the remodeling of chromatin, resulting in a dynamic regulation of activities such as maintenance of intracellular homeostasis and regulation of the cell cycle, apoptosis, signal transduction, cell differentiation, DNA repair, tumor suppression, and transcription in response to environmental or biological changes.[134,135]

The combination of all epigenetic marks allows cells to determine and inherit distinct and stable patterns of gene expression by defining a single phenotype that may be normal or diseased.[136] Mutations in regions encoding miRNAs and DNMTs or environmental factors may lead to positive or negative regulation of enzymes, causing the emergence of complex diseases. Neurological disorders such as AD, autoimmune (rheumatoid arthritis) and vascular diseases may also be a result of these epigenetic changes.[137,138] Although epigenetic changes are directly associated with the emergence of complex diseases, they are also present in normal processes such as essential aspects of the immune system such as cytokine production, dendritic cell activation, $CD4^+$ T cell plasticity, regulatory T cell function, and plasma cell differentiation also depend on epigenetic regulation.[139]

Epigenetic changes are strongly associated with the risk of development of autoimmune and complex diseases, such as type 1 diabetes mellitus (T1D) and type 2 diabetes mellitus (T2D).[140] Almost 25% of the heritability in T1D and more than 80% in T2D cannot be explained by known genetic defects.[141] Besides, in monozygotic twins pairs, the concordance rate for T1D varies widely from 13–67.7%, suggesting that in addition to genetic factors, nongenetic mechanisms are responsible for the development of T1D.[142,143]

These data suggested that the environmental factors including diet, physical activity, circadian rhythms, stress, temperature, etc., can also lead to transient or persistent changes in the epigenomics, altering the gene expression and cellular phenotypes, resulting, thus, in the epigenetic pattern alteration.[144,145] The early exposure to intrauterine growth restriction (IUGR), for example, impacts the expression of genes responsible for controlling glucose homeostasis and pancreatic islet energy, as well as peripheral tissues.[143]

T1D is an autoimmune disease characterized by the destruction of insulin-secreting pancreatic β cells by activated T lymphocytes.[142,146] Epigenetics mechanisms are related to the decline of β cells with age. DNA methylation in β cells of mice has differences when compared between young and old individuals since the loci of pro-proliferative genes are de novo methylated, while enhancers near genes involved in glucose metabolism undergo demethylation.[147]

Several studies of islet autopsies of individuals with T2D have shown changes in epigenetic marks including DNA methylation, modification of histones and ncRNAs.[148] Nutritional status is strongly related to the development and progression of diabetes mellitus (DM) and diabetic kidney disease (KDB) because the diet can alter the epigenetics state.[140] Recent studies have suggested an association between environmental factors, T2D and cardiovascular diseases (CVD).[149] Understanding the epigenetic mechanisms is vitally important, as it will allow the development of specific epidrugs to treat classes of patients with some type of diabetes.

New therapies based on epigenetics can be developed to reverse the epigenetic anomalies associated with the disease.[150] The use of drugs for the prevention or treatment of diseases related to epigenetic factors is one of the fundamental topics of pharmacoepigenetic and pharmacoepigenomic fields. It is a new step in cancer therapy, and other diseases, which epigenetic regulation is present such as schizophrenia, bipolar disorder, depression, cardiac insufficiency, and hypertrophy, among neurological diseases as well.[137,151]

Table 2. Epigenetic Drug(s) & Gene Target(s).

Epigenetic modification	Epigenetic drug/	Function	Clinical status
Histone modification	Zolinza	Cell cycle arrest and apoptosis in several cancer types	Approved by FDA
	Istodax	Induction of apoptosis in different cancer cell lines	Approved by FDA
	Beleodaq	Decrease in cell proliferation and induction of apoptosis in several cancer types	Approved by FDA
DNA methylation	Vidaza	Reduction of malignant cells in leukemia and myelodysplastic syndrome	Approved by FDA
	Dacogen	Antitumor activity in different leukemic cell lines and solid tumors	Approved by FDA
MicroRNAs	miR-34a	Tumor suppressor in cancer cells	Preclinical trial
	miR-15a and miR-161	Tumor suppressor in colorectal cancer	Phase II clinical cancer trial
	MiR-29	Tumor suppressor and antiviral factor induced by IL-21 in CD4 T cells	Preclinical trial
LncRNAs	SPRY4-IT1	Decrease in cell proliferation and tumor suppressor	Preclinical trial
	MEG3	Tumor suppressor in cancer cells	Preclinical trial
	RP11-160H22.5, XLOC_014172, and LOC14908	Biomarkers for hepatocellular carcinoma	Preclinical trial
	MALAT-1	Reduce cell proliferation and induction of apoptosis in Several Different cancer types	PreclinPrical Preclinical trial

Drugs that provide structural chemical interactions with enzymes related to epigenetic changes increase the evidence that it is possible to induce changes in DNA patterns or histone conformation.[151] Molecules that act as modifiers of epigenetic mechanisms allow the reactivation of silenced genes. Therefore, targeting these modifications can be used as a promising strategy for the development of new therapies to treat different diseases. Technologies such as second and third-generation sequencing combined with epigenomic tools can aid in the search for better molecular targets and the identification of biomarkers.[132,152]

Most of the epigenetic drugs developed so far demonstrate antineoplastic effects for the treatment of cancer and myelodysplastic syndromes. One of the goals of cancer research has been the development of epigenetic drugs, such as inhibitors of DNMTs and histone deacetylases (HDACs). These drugs have received approval from the US Food and Drug Administration (FDA) for therapeutic purposes (Figure 1 and **Table 2**). Nevertheless, other epigenomic-related diseases are emerging and the demand for new epigenetic therapies for noncancerous conditions has increased.[151,153]

So far, the study of the epigenome has made possible the discovery of how the epigenetic modifications work and how they are linked to different diseases. Molecules that can alter these epigenetic marks can be an alternative pathway for the development of new drugs that can be used as a treatment of epigenetically regulated diseases.[154]

5.1. DNA Methylation: A Target to be Explored by Epigenetic Therapies

Known for regulating gene expression in the normal development of mammalian cells, DNA methylation is one of the most studied epigenetic changes.[155] Modifications related to this epigenetic alteration play important roles as diagnostic biomarkers and/or therapeutic responses in complex diseases such as cancer and autoimmune diseases.[156] In the last decades, the investigation of the DNA-methylation status, as well as its functional effects become one of the main focuses in cancer research.[157,158] At present, different methods to inhibit DNA methylation have been produced.

The FDA has already approved drugs of both classes for the treatment of patients, and inhibitors such as 5-azacytidine (Azacitidine) and 5-aza-2′-deoxycytidine (Decitabine) have been used in the treatment of leukemia and myelodysplastic syndrome.[136,138,152] Clinical trials for the treatment of other cancers such as prostate, colon, bladder, melanoma, lung have also been performed.[151] These drugs can intercalate between DNA or RNA molecules, in CpG dinucleotides and to trap DNMTs, leading to their degradation. This promotes hypomethylation and reactivation of silenced loci.[134]

5.1.1. Vidaza (5-Azacytidine)

Azacitidine, a DNMTs inhibitor, was first approved in 2004 in the US to treat chronic myelomonocytic leukemia and myelodysplastic syndrome. As the demethylating agent of the 10–13 promoter, it has been shown to induce the re-expression of silenced genes both in vitro and in vivo. Studies have shown that to induce and re-express the gene, the required drug concentration is much lower than that required to produce cytotoxicity.[159,160]

Laribi and colleagues demonstrated that in patients with myelodysplastic syndromes (MDS), subcutaneous treatment with azacitidine on a 5-day regimen of treatment, 2 days off and 2 days of treatment (5-2-2) was efficacious in global survival. The median overall survivors were 32 months for responders and 8 months for nonresponders, with the probability of 1 year to 2 years in overall survival of 91% versus 28% and 66% versus 6%, respectively.[161]

Plumb and colleagues performed an in vivo assay in which they confirmed the effect of treatment of the 2-deoxy-5-azacytidine demethylating agent on the re-expression of MLH1 (the gene responsible for the repair of DNA incompatibility) after three days of treatment using a dose of 5 mg kg^{-1} every three hours. They also proved that synergy between 2-deoxy-5-azacytidine and cytotoxic drugs (cisplatin, carboplatin, epirubicin, and temozolomide) causes a delay in ovarian carcinoma and human colon xenografts through the sensibilization of the tumor.[162]

In an in vitro study by Jilg and colleagues, the impact of a novel therapeutic approach in patients with myelodysplastic syndromes and secondary acute myeloid leukemia was evaluated. Combination therapy using azacitidine and venetoclax can reduce primary malignant cells even at low concentrations. A reduction of 65% in cell viability in 68.4% of patients was observed with a high dose of azacitidine, and in 62.5% of patients when using low doses.[163]

Different combinations of azacitidine and HDAC inhibitors were also evaluated to increase the response rate and duration, maintaining low rates of toxic effects.[164] In an in vivo assay conducted by Gopalakrishnapillai and colleagues, mice achieved complete remission in preclinical models of acute myelogenous leukemia (AML) when administered with the combination azacitidine-panobinostat (2.5 mg kg^{-1} each) in a four-cycle treatment lasting five days a week, with two days of rest. The animals remained healthy >519 days after starting treatment.[165]

5.1.2. Dacogen (Decitabine)

Decitabine (5-aza-2′deoxycytidine), a cytidine deoxynucleoside analog, was first described more than 40 years ago. By inhibiting DNMTs, it is capable of inducing hypomethylation of DNA including promoter regions of the gene, providing for the re-expression of tumor suppressor and other genes involved in cell proliferation and differentiation. Several in vitro and in vivo studies were done evaluating the antitumor activity of decitabine in different leukemic cell lines and solid tumors.[166,167]

In a Phase I clinical and pharmacodynamic study conducted by Appleton and colleagues, the feasibility of administration of decitabine combined with carboplatin to produce a reduction in DNA methylation equal to or greater than that observed in carboplatin-sensitized xenograft mice was analyzed. According to the results, a dose of 90 mg m^{-2} of decitabine on the first day of treatment (administered as a 6-hour intravenous infusion) can be safely combined with carboplatin under the concentration-time curve (AUC) 6 in the eighth day of treatment every 28.[168] This study may be the first step for further clinical studies targeting a potential epigenetic mechanism of drug resistance.

Similarly, decitabine may also be used in combination with low dose clofarabine and cytarabine for the treatment of acute myeloid leukemia (AML) in the elderly. In a phase II trial study conducted by Kadia and colleagues, treatment with clofarabine IV 20 mg m^{-2} on days 1–5 combined with 20 mg cytarabine at low doses subcutaneously twice daily on days 1–10 alternating with cycles of decitabine, provided a remission rate in 60% overall and median overall survival of 11.1 months among all patients. The synergistic combination of this treatment was effective, with low toxicity rates and with better remission rates when compared to intensive chemotherapy.[169]

In a study conducted by Jiang and colleagues, patients with atypical chronic myelogenous leukemia treated with four cycles of decitabine chemotherapy (20 mg m^{-2}, days 1–5) achieved remission. Before initiation of treatment, one of the patients had a range of 4.5% myeloblasts (normal range, 1–2%), 2% promyelocytes (normal range, 2–3%) and 68.5% granulocytes (normal range, 40–60%) detected in the total cell count of the nucleated medulla. After the end of the treatment, the patient was in good health with 2.5% of myeloblasts and 1.5% of promyelocytes, with 0.528% of residual leukemic cells, according to flow cytometry.[170]

Hypomethylating agents have been used for more than a decade, especially as maintenance or preventive treatment. They are clinically relevant in myelodysplastic syndromes (MDS), chronic myelomonocytic leukemia (MML) and acute myelogenous leukemia (AML), even at low concentrations. Scientific progress in the area of epigenomics is opening space for second-generation hypomethylating agents, studies are being optimized for the oral formulation, dose adaptation, and new combinations. Thus, hypomethylating agents remain important in the fight against myeloid neoplasias in the coming years.[171]

5.2. The Role of Histones Modification to Treat Complex Diseases

Among the epigenetic modifications involved in the regulation of gene expression, the most studied are histone modifications.[172] Histone acetyltransferases (HATs) are key enzymes in transcriptional activation whereas HDACs are responsible for transcriptional repression and gene silencing. Excessive histone deacetylation is directly related to different human cancers, promoting the repression of tumor regulatory genes. In this way, HDACs are seen as promising therapeutic targets for the treatment of cancer, developing a wide variety of inhibitors.[173,174]

Histone deacetylases inhibitors (HDACIs) are among the best described epigenetic drugs. They inhibit HDAC-mediated deacetylation by providing histone hyperacetylation and decondensation of the chromatin structure with consequent re-expression of silenced genes. They can lead to the death of tumor cells, inhibit angiogenesis and immunogenicity, among other functions. Currently, several HDACs are being evaluated for possible applications such as chemopreventive and chemotherapeutic, alone or in combination with other treatments.[175,176]

The following HDAC inhibitors have been approved by the FDA: Vorinostat (SAHA), Romidepsin (Istodax), and Belinostat (Beleodaq). These medications are usually used for progressive, persistent, or recurrent cutaneous T-cell lymphoma treatments; but are also being evaluated as a single agent or in combination with different drugs for other hematological malignancies and solid tumors, as well as for other diseases.[173]

5.2.1. Zolinza (Vorinostat)

The first FDA-approved HDAC inhibitor was Zolinza (Vorinostat), a linear hydroxamate compound, developed by Merck & Co. Inc., that inhibits class I, II, and IV HDACs, also known

as SAHA or Zolinza.[173,174] Capable of inducing cell cycle arrest and apoptosis in several cancer types, including cutaneous T-cell lymphomas. Vorinostat also showed antitumor activity in lymphoma, leukemia, and solid tumor models in vivo in preclinical studies.[177,178]

The approval of this drug was based on phase II clinical trials for the treatment of 74 patients who had an advanced stage of cutaneous T-cell lymphoma (CTLC). The observed objective response rate was 30% of the disease with a median response duration of 168 days and was considered to be a clinical benefit by the FDA reviewers. The recommended dose is 400 mg orally once daily with food for the treatment of cutaneous manifestations of CTLC in progressive, persistent or recurrent disease in or after two systemic therapies.[179]

Duvic and colleagues conducted a phase II clinical trial to evaluate the response of oral vorinostat in patients with cutaneous refractory T-cell lymphoma (CTCT). The first treatment group was started at 400 mg vorinostat every day, group 2 received 300 mg twice daily for 3 days, and group 3 received vorinostat 300 mg twice daily for 14 days. According to the data obtained, 19 (58%) of the 33 patients benefited from the treatment, achieving relief of symptoms and progression of the disease. The continuous treatment of groups 1 and 3 was more effective when compared to group 2.[180]

In another phase II clinical trial, Kwak and colleagues investigated a synergistic combination between Vorinostat and ixabepilone (antitumor activity drug in a variety of tumors) for metastatic breast cancer. In a 21-day protocol with the administration of 300 mg vorinostat per day (days 1–14) and 32 mg ixabepilone (day 2), the clinical benefit rate was 22%, the overall survival was 14.8 and the progression-free survival of 3.9 months. In a second 28-day protocol with the administration of 300 mg vorinostat per day (days 1–7, 15–21) and 16 mg ixabepilone (days 2, 9, 16); the clinical benefit rate was 35%, the overall survival was 17.1, and the progression-free survival was 3.7 months.[181]

5.2.2. Istodax (Romidepsin)

Romidepsin (Istodax, FK228, FR901228, depsipeptide), developed by Gloucester Pharmaceuticals (acquired by Celgene in 2009), predominantly inhibits class I HDACs and it was the second HDAC inhibitor approved by the FDA in 2009, also for the treatment of T-cell lymphoma. It is a cyclic tetrapeptide isolated from the fermentation product of Gram-negative bacteria *Chromobacterium violaceum* from a Japanese soil sample, and it has also been approved for peripheral T-cell lymphoma (PTCL) in patients who received at least one prior treatment in 2011.[182]

The second HDAC inhibitor approved for the treatment of CTCL was based on two large studies. After observing the response in patients with cutaneous and peripheral lymphoma of T-cells treated with romidopsin in a phase I study, Piekarz and colleagues evaluated the effect of romidepsin in patients with cutaneous T-cell lymphoma in a phase II study. The overall treatment response was 34%, with an average response duration of 13.7 months. Complete or partial response was obtained in 18 (29%) of 62 patients with stage IV disease.[183] The efficacy of randopsin may be increased when in combination with other agents and drugs.

It has been reported in the literature that the synergistic combination between HDAC and proteasome inhibitors may lead to the induction of apoptosis in different cancer cell lines. Hui and colleagues found that the combination of romidepsin and bortezomib is capable of inducing apoptosis and autophagy of gastric carcinoma cells in vitro and in vivo at concentrations of 2.5 nM and 7.5 nM respectively. The data indicate that this combination can be used as a potential therapeutic regimen for metastatic or recurrent gastric carcinoma.[184]

In addition to being used for the treatment of T-cell lymphoma, romidepsin is a candidate therapy for patients with idiopathic pulmonary fibrosis (IPF). When tested in human IPF fibroblasts in vitro, this anticancer agent demonstrated antiproliferative and antifibroblastic properties. In the murine model of pulmonary fibrosis was also able to decrease the number of fibroblasts. Its effects were corroborated by the increase in histone acetylation, consistent with the drug acting as an epigenetic modulator.[185]

5.2.3. Beleodaq (Belinostat)

Belinostat (also called PXD101 or Beleodaq) is a hydroxamic acid-based compound developed by Spectrum Pharmaceuticals, which inhibits class I and II HDACs, and was the third drug approved by the FDA for the treatment of peripheral T cell lymphoma recurrent or refractory in 2014.[174,182] It was based on a multicenter, single-arm trial in which 120 patients with peripheral T cell lymphoma who were refractory or relapsed after the first treatment. The overall response rate of this study was 25.8%.[186]

Belinostat has been shown to be effective against pancreatic ductal adenocarcinoma (PDAC), a highly aggressive gastrointestinal tumor. Dovzhanskiy and colleagues observed that treatment with belinostat causes a decrease in cell proliferation, as well as induce apoptosis in three pancreatic cancer cell lines (T3M4, Panc-1, and AsPC-1), both dose-dependent with a half-maximum inhibitory concentrations (IC50) concentration in the nanomolar range.[187]

In another study, Belinostat also demonstrated efficacy in squamous cell carcinoma (SCC) cells of the lung. Kong and colleagues evaluated the differential sensitivity in squamous cell carcinoma cells caused by the inhibitor in question. Six of the ten strains tested were highly sensitive to belinostat, with increasing doses inducing apoptosis, the same as in other studies analyzing the effects of other HDAC inhibitors.[188]

A study by Tumber and colleagues validated that the Belinostat and 5-fluorouracil (5-FU) in combination for 48 h produces synergy at different concentrations for colorectal cancer. Significant beneficial responses were observed in HCT116 nude rat xenograft models when administered a sub-therapeutic concentration of 5-FU (15 mg kg^{-1}) combined with PXD101, which suggests that the belinostat compound significantly increases the antitumor efficacy of 5-FU in vivo.[189]

Buckley and colleagues first demonstrated the lowest effective concentration of belinostat in human bladder cancer cells. There was inhibition of proliferation in all cell lines of urothelial carcinoma, with the most potent inhibitory effect in cells 5637 (IC50 of 1.0×10^{-6} м), in addition to cell cycle disruption. The data

suggest that belinostat is a potential agent for the treatment of superficial bladder cancer.[190]

Given the biological results that HDAC inhibitors have shown in several antitumor clinical trials, the search for new, more efficient and selective inhibitors has been intensified. Four HDAC inhibitors have been approved for use in the treatment of cutaneous and peripheral T cell lymphoma, and when combined with other agents, provide better results. Therefore, they have great potential as antitumor drugs and future studies on their inhibitory capacity would be of interest to provide a greater number of cure to patients with lymphoproliferative disease.[191]

5.3. Noncoding RNAs

Most transcripts produced in human cells do not encode any protein. ncRNAs account for almost 60%-70% of transcript production depending on the cell type and regulates different genes, including oncogenes and tumor suppressor genes. Hence ncRNAs represent new targets for different clinical applications, such as in the diagnosis and treatment of cancer.[192–194] Among the types of ncRNAs, microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) are the most relevant until now.[195]

5.3.1. miRNAs

miRNAs are the most studied among small ncRNAs and over 2500 human miRNAs were identified and annotated in the miRBASE database of miRNAs.[193] MicroRNAs are very promising for therapeutic applications because of certain favorable characteristics they possess, such as short and well-conserved sequences in several vertebrates, and the ability to target multiple genes simultaneously.[193,196,197] In addition to acting in several aspects of cancer, by controlling large target networks instead of some genes, disease-related miRNAs can be found circulating in the bloodstream, representing an opportunity for their use as a biomarker.[198,199] The presence of certain miRNAs related to complex diseases can be explored as biomarkers for the diagnosis of different types of cancers, such as miR-32, miR-182, and miR-143, which are associated with intestinal-type gastric cancer.[200]

These molecules are capable of inhibiting tumor suppressor genes (oncogenic miRNA—OncomiRs) contributing to the carcinogenesis process, or they can inhibit the expression of proto-oncogenes (tumor suppressor miRNAs) preventing cancer development. Different therapeutic strategies for cancer treatment are being based on miRNAs, such as using miRNA inhibitors or replacing suppressive miRNAs with synthetic miRNAs for silencing OncomiRs.[201]

miRNAs in therapy can be classified as miRNA mimics and miRNA inhibitors (also known as antimiRs). The mimetizers replace the function of a miRNA lost due to disease, whereas the antimiRs bind to the target miRNA through a complementary sequence and block their function. Through chemical modifications, great strides have been reached regarding stability, binding affinity, and target modulation effects of miRNA mimics and antimiRs. Thus, many miRNA-related therapies have reached clinical development.[194,196]

One of the most described tumor suppressor miRNAs is MiR-34a, which is expressed at reduced levels in tumor tissue or even lost. As an alternative to inhibit cancer cell development in vitro and in vivo, the use of miR-34 mimetics has been shown to be effective as a tumor suppressor in cancer cells, to cope metastasis, chemoresistance, and tumor recurrence.[201]

Cortez and colleagues have demonstrated in mice with lung cancer that MRX34 (liposomal formulation complexed with miR-34a mimics) alone or in combination with radiotherapy (XRT), is able to reduce the expression of PDL1 (programmed death 1 ligand 1, overexpressed in many human cancers, promoting T-cell tolerance and escape host immunity) in the tumor and did not cause T cell exhaustion. MRX34 associated with XRT also increased the production of interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα) indicating miR-34 can reactivate the immune system in immunocompetent mice.[202]

In another study, Adoro and colleagues suggest a relationship between interleukin-21 antiviral activity against HIV-1 with the highest miR-29 expression in primary lymphoid CD4 T cells. Exogenous IL-21 treatment in humanized mice was able to suppress early HIV-1 infection, and the presence of microRNA-29 (miR-29) was identified as an IL-21-induced antiviral factor. These results suggest a new antiviral axis IL-21-miR-29 that promotes intrinsic resistance of CD4 T cells to HIV-1 infection.[203]

miR-15A and miR-16-1 are also studied for the emergence of new therapies. Liu and colleagues investigated the relationship of these microRNAs to B-cell mediated immunosuppression by colorectal tumors. Mice that received miR-15A and miR-16-1 mimics showed progression in colorectal cancer and lower accumulation of IgA-positive immunosuppressive B cells (IgA+ B), while the absence of microRNAs promoted the accumulation of these cells.[204]

Another microRNA, MicroRNA-122 (miR-122), is required for efficient replication of the hepatitis C virus. So Ree and colleagues evaluated changes in miRNA plasma levels in patients with chronic hepatitis C treated with miravirsen (anti-oligonucleotide -miR-122), and a substantial decrease in miR-122 levels was observed in these patients.[205]

MiRNAs may also increase the angiogenesis process in pathological conditions such as myocardial infarction and limb ischemia.[206] Using a mini-circle DNA vector encoding the miR-210 precursor, it was possible to increase myocardial neovascularization in rats with myocardial infarction.[207]

It is noteworthy that microRNA-based therapies have achieved clinical trials for various diseases, especially cancer. However, obstacles still need to be overcomed, such as increasing specificity and effectiveness, reducing associated side effects and dose optimization.[208]

5.3.2. Long Noncoding RNAs (LncRNAs)

Since the characterization of the first lncRNAs, H19 and Xist, many others have been identified through next-generation sequencing and transcriptomic studies and inserted into databases such as NONCODE, GENCODE, and lncRNAdb.[209]

Long noncoding RNAs (lncRNAs) participate in basic physiological activities such as cell proliferation, differentiation and apoptosis, and are also associated with different diseases, such

as cancer initiation, tumor progression, and metastatic spread. They may also be related to other pathological situations such as nervous system diseases and cardiovascular diseases either. Besides, these molecules can act as biomarkers for the diagnosis and prognosis of human diseases.[210,211]

These molecules have remarkable cell and tissue specificity and can be used as biomarkers and therapeutic targets in different diseases. From the profile of lncRNAs transcribed in body fluids such as plasma, serum, urine or sputum, various diseases can be detected. The presence of PCA3 lncRNA in urine, for instance, is used as a clinical detection of prostate cancer.[209,211]

SPRY4-IT1 lncRNAs are related to the tumorigenesis of many types of cancer, such as esophageal squamous cell carcinoma and nonsmall cell lung cancer.[212] The long noncoding RNA SPRY4 intronic transcript 1 (SPRY4-IT1) is upregulated in hepatocellular carcinoma (HCC). However, in vitro HCC proliferation and tumor growth in vivo are significantly inhibited when SPRY4-IT1 is knockdown, exhibiting therapeutic potential in HCC progression.[213]

The lncRNA MEG3 exhibits a tumor-suppressive function. A study conducted by Lu and colleagues, overexpression of this lncRNA was able to slow cell proliferation in nonsmall cell lung cancer (NSCLC) tumor tissues, in addition to inducing apoptosis in vitro and preventing tumorigenesis in vivo.[214]

Sun and colleagues analyzed the expression of lncRNA GAS5 (Growth Stop Specific Transcript) in gastric cancer tissues. The results showed that the expression of this low-level noncoding RNA is associated with more invasive tumors, while ectopic expression decreases the proliferation of gastric cancer cells. Thus, GAS5 can act as a tumor suppressor through regulated cell growth and apoptosis.[215]

There are also lncRNAs, in body fluids that can be used as biomarkers for the diagnosis of different types of cancer. The lncRNAs RP11-160H22.5, XLOC_01 4172, and LOC14908 are found in plasma and works as biomarkers for hepatocellular carcinoma. The UCA1 is present in the urine and is a biomarker for bladder cancer. Others like AA174084, AA174084, and LINC00152 are present in tissue gastric juice and plasma, they are biomarkers for gastric cancer.[216]

MALAT1 (Metastasis-Associated Pulmonary Adenocarcinoma Transcription 1) is one of the most studied lncRNAs known to enable tumor development through cell cycle regulation.[216] Its in vitro down-regulation promotes reduced cell proliferation and apoptosis in different types of cancer such as breast,[217] colorectal,[218] esophageal squamous cell carcinoma,[219] renal cell carcinoma and others.[220]

Another study suggested that inhibition of MALAT1 might also interfere with the process of angiogenesis in the tumor's hypoxic environment, acquiring a therapeutic potential. According to the results, MALAT1 expression is increased by endothelial cell hypoxia and contributes to the proliferative response.[221]

In a preliminary phase 1/2a clinical trial, plasmid BC-819, which contains the diphtheria toxin gene under the control of lncRNAs (H19) regulatory sequences, is capable of tumor reduction in pancreatic cancer patients. It has additional therapeutic benefits when combined with chemotherapy. This is the only completed clinical trial related to the application of lncRNAs for cancer therapy. Further studies should be performed to evaluate higher doses, prolonged treatment periods and combination with other medications.[222]

The use of biomarkers for disease identification is critical for personalized treatments. Understanding biological processes such as epigenetic alterations by lncRNAs are necessary, several of these molecules could be of importance as diagnostic biomarkers and therapeutic targets in solid tumors.[223] The main epigenetic modifications reported here are summarized in Table 2.

Epigenomic medicine studies have been reporting numerous epigenetic diseases and developed some epigenetic drugs and biomarkers related to cancer and other diseases. However, many challenges and obstacles will need to be addressed, such as side effects and low specificity/selectivity.[224] As a result, lack of selectivity can cause undesirable effects in other parts of the genome such as up-regulation of prometastatic genes and disruption of cell pathways due to changes in proteins such as P53, nuclear receptors, heat shock protein-90, among others.[225]

In addition to epigenetics mechanisms, other approaches such as CRISPR (clustered, regularly interspaced, short palindromic repeats)-cas9 platform have been developed for site-specific genome editing.[144] Recently, modifications in the CRISPR/Cas9 system have made it possible not only to regulate gene expression but create the possibility of introducing epigenetic modifications, without causing breaks in the double strand of DNA.[226] In this approach, the nuclease activity of Cas9 can be inactivated (dCas9) by joining it to the catalytic domain of a DNMT or Tet enzyme, enabling hydroxyomethylation, as well as the demethylation of the CpG islands.[147]

In 2016, Klan and colleagues described a CRISPR/Cas9-based epigenomic regulatory element screening (CERES) for improved high-throughput screening of regulatory element activity in the native genomic context. Using CERES, they were able to find all regulatory elements of the well-characterized β-globin locus and known and unknown elements that regulate the expression levels of the HER2 oncogene.[227] Thakore and collaborators merged dCas9 with the Krüppel-associated box (KRAB) repressor, forming the dCas9-KRAB complex. The results suggest that dCas9-KRAB can be used as a highly specific epigenome editing tool to target the cell phenotype and reveal connections between regulatory elements and gene expression.[228] Although many more studies are needed, the CRISPR/dCas9 mechanism can be a potential alternative for the study and induced regulation of genes, without damaging the DNA.

6. Conclusion and Future Perspectives

Although the term epigenetics was first coined to describe the "causal mechanisms" that result in phenotype from genotype, it was only after the iconic publication by Watson and Crick that this term gained new meaning. The elucidation of the 3D structure of DNA allowed the emergence of molecular biology, an area in which we broadened our knowledge about the mechanisms related to heredity and gene expression regulation, among others.

Epigenetics is now defined as a branch of genetics that studies alterations caused by structural modifications in chromatin, inherited by mitosis or meiosis. These structural changes are

caused by the addition of several types of chemical groups to the lysines present in the histone tails. Moreover, DNA methylation and transcription of a multitude of ncRNA molecules are related to several biological processes essential for cell maintenance, as well as being responsible for many complex diseases such as cancer, neurological disorders, diabetes mellitus, etc.

The emergence of automatic sequencers has enabled the molecular understanding of many of these complex diseases. Over the years, state-of-the-art devices capable of sequencing the entire human genome in just one day have been launched, something unthinkable a few decades ago. These technological advances have enabled a paradigm shift in modern medicine. Today it is possible to glimpse a precision medicine, which is based on the genetic, phenotypic, biomarkers and psychosocial characteristics of patients.

Precision or personalized medicine comes with a multidisciplinary character and integrates a series of mechanisms to investigate, prevent, treat and cure diseases, including those considered very complex. From this perspective, epigenetic therapies can be used as true allies. These approaches consist of the use of drugs capable of interacting and suppressing the activity, of enzymes responsible for maintaining or altering the epigenetic pattern, as well as altering nc RNA transcription that may trigger the emergence of complex diseases.

Thus, the search for new biomarkers that may aid in diagnosis and treatment is essential, since the knowledge acquired from DNA sequencing and studies of epigenetic mechanisms enable the development of drugs that will act specifically on their target, increasing chances of favorable prognosis for patients. Therefore, we believe that studies like this can contribute to the advance of accurate medicine since a compendium of recent discoveries about molecules capable of being used in epigenetic therapies is emerging.

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N.B.d.C. and F.F.C. contributed equally to this work.

Conflict of Interest

The authors declare no conflict of interest.

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REVIEW

Strategies for recombinant production of antimicrobial peptides with pharmacological potential

Kamila Botelho Sampaio de Oliveira[a], Michel Lopes Leite[a], Gisele Regina Rodrigues[a], Harry Morales Duque[a], Rosiane Andrade da Costa[a], Victor Albuquerque Cunha[a], Lorena Sousa de Loiola Costa[a], Nicolau Brito da Cunha[a], Octavio Luiz Franco[a,b,c] and Simoni Campos Dias[a,d]

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ABSTRACT

Introduction: The need to develop new drugs for the control of pathogenic microorganisms has redoubled efforts to prospect for antimicrobial peptides (AMPs) from natural sources and to characterize its structure and function. These molecules present a broad spectrum of action against different microorganisms and frequently present promiscuous action, with anticancer and immunomodulatory activities. Furthermore, AMPs can be used as biopharmaceuticals in the treatment of hospital-acquired infections and other serious diseases with relevant social and economic impacts.

Areas covered: The low yield and the therefore difficult extraction and purification process in AMPs are problems that limit their industrial application and scientific research. Thus, optimized heterologous expression systems were developed to significantly boost AMP yields, allow high efficiency in purification and structural optimization for the increase of therapeutic activity.

Expert opinion: This review provides an update on recent developments in the recombinant production of ribosomal and non-ribosomal synthesis of AMPs and on strategies to increase the expression of genes encoding AMPs at the transcriptional and translational levels and regulation of the post-translational modifications. Moreover, there are detailed reports of AMPs that have already reached marketable status or are in the pipeline under advanced stages of preclinical testing.

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1. Introduction

Antimicrobial resistance is recognized as one of the most important global health challenges of our time [1]. About 700,000 people die each year from resistant bacterial infections, and if the current scenario does not change, the estimate is 10 million deaths by 2050, generating an overall cost of 100 USD trillion [2–4]. Resistance against antibiotics may be innate (intrinsic resistance), acting through the alteration or inactivation of the antibiotic, alteration of the target site and efflux of the antibiotic. The occurrence of spontaneous mutations in the bacterial genome (vertical evolution) and the transfer of resistant genes (horizontal evolution) between bacteria also confer resistance to these microorganisms [4,5].

In 1940, the first case of bacterial resistance to an antibiotic was mentioned: strains of the bacteria *Staphylococcus aureus* showed to be highly resistant to penicillin [6]. Currently, cases like this have become common. Strains of *S. aureus* resistant to methicillin (MRSA) and *Escherichia coli* resistant to β-lactamases (ESBL) are recurrent in a number of hospitals around the world [7–9], mainly harming patients whose immune systems have been compromised by other diseases or by invasive therapies [10,11]. The indiscriminate use of antibiotics and antifungals over the years is one of the main factors that contribute to the selection of resistant strains in hospitals [12]. Due to increasingly frequent reports of antibiotic-resistant pathogens, the search for alternative antimicrobial therapies with a broad spectrum of action, low toxicity, a novel mechanism of action and new antimicrobial targets has become the major challenge of scientific research groups and the pharmaceutical industry [13–15]. Under current circumstances, natural antimicrobial peptides (AMPs) emerge as a potential approach for the development of new therapies [16].

AMPs are small molecules capable of performing antimicrobial activities and are produced naturally by several species. They are found in microorganisms, insects, birds, marine invertebrates and vertebrates, and mammals, among others and act as defense components of the innate immune system [17–20]. The first AMP to be reported was gramicidin, isolated from the microorganism *Bacillus brevis* [21]. The therapeutic potential of this peptide was confirmed in vitro and in vivo assays, which demonstrated its antimicrobial ability to inhibit a wide range of Gram-positive bacteria. It has also been effective in infected wounds, enhancing its clinical use [3,20].

Like gramicidin, many of these natural molecules exert activity against bacteria and other pathogens, including

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Article highlights

- Increased microbial resistance against antibiotics due to natural processes such as intrinsic resistance, vertical or horizontal evolution, and frequent use associated with widespread and long-term applications.
- The challenge to develop alternative antimicrobial therapies that are more effective and less likely to select resistant strains.
- AMPs as a new strategy for the development of antimicrobial therapies.
- Heterologous production of ribosomal and non-ribosomal AMPs synthesized using different expression systems.
- The purification methods of recombinant AMPs and the importance of this step for the development of biopharmaceuticals.
- Different AMPs have already achieved clinical trials. However, difficulties in reaching market status are outlined here.

viruses, protozoans, and fungi. Therefore, they are suitable candidates not only for the development of alternative antibiotics but also for new antifungals, antivirals, and antiprotozoals [18]. They have certain advantages when compared to conventional antibiotics, such as a lower risk of resistance, broad-spectrum activity, less toxicity, synergy with other compounds and rapid death [20]. Natural AMPs also have immunomodulatory activity, resulting in inhibition of inflammatory response, stimulation of proliferation and recruitment of macrophages, neutrophils, eosinophils, and T lymphocytes [20]. Furthermore, they can present anticancer activities [22,23]. These qualities make them attractive candidates for the advancement of other drugs besides alternative commercial bioproducts.

These molecules differ from antibiotics precisely because of their mode of interaction with the microorganism. In general, the first interaction of an AMP with the bacterial cell membrane occurs through electrostatic interactions between the cationic residues of the AMP and anionic components of the microorganism's membrane, followed by the structural disorganization and formation of pores in the membrane, with the consequent rupture of the lipid bilayer [24,25]. The rupture of microbial membranes by AMPs consists of different mechanisms, such as barrel-stave, toroidal pore models, and carpet model. Peptides are also capable of inhibiting the essential intracellular targets such as DNA and RNA by crossing the membrane without disruption. This inhibition may occur through different methods, including direct penetration and endocytosis [18,25,26]. A few of these molecules can also act as antivirals [27,28] and antitumor agents [29].

AMPs may contain about 100 amino acid residues and have a molecular weight of up to 5,000 Da. However, there are exceptions, such as peptides with about 130–150 amino acid residues. Secondary and tertiary structures are common and may contain post-translational modifications, essential for the performance of their biological function [30,31]. Large-scale antimicrobial peptide production is a powerful tool that allows the general characteristics of these molecules to be studied, including their composition, structure, function, and mechanisms of action [10,32,33].

To evaluate the antimicrobial activity of the peptides of interest, the first investigations into AMPs were based on the purification of peptides from natural sources [32]. This technique allows the production of AMPs with high biological activity, such as lipotoxins extracted from the spider *Lycosa carolinensis* [33] and the strongylocin peptide of *Strongylocentrotus droebachiensis* [34]. However, this procedure is often not feasible; the concentrations of AMPs in organisms are low, usually produced in stress situations. It is also a slow and costly process that can cause environmental impacts [35]. Despite obtaining the highly active molecules of interest, the yield is generally low and the products have impurities [17].

Chemical synthesis has been used for decades for the production of natural and synthetic peptides with a high level of yield and purity. However, the costs of this method may be relatively high, especially for peptides that have post-translational modifications. The complexity of the different steps involving the addition of each amino acid residue also makes it difficult to synthesize long-chain peptides (> 35 residues) and increases the risk of errors in their sequence [32,36]. These observations highlight the demand for a cheaper and faster method that allows a large quantity of the natural peptide of interest to be obtained, with a good degree of purity for therapeutic use.

2. Heterologous production of ribosomal synthesized AMPs

Recombinant DNA technology enables the scalable, sustainable and cheaper production of peptides when compared to the other options described above. Genes with different characteristics and origins are cloned into certain vectors for expression in prokaryotic or eukaryotic hosts [36]. In recent decades, several expression systems such as bacteria, yeast, mammalian cells, insect cell cultures (baculovirus), and plants have been used for heterologous production of molecules with pharmacological and industrial interest. All these systems have advantages and limitations (see Figure 1), requiring the use of strategies to enhance the production of recombinant AMPs [37–40]. Such strategies are the use of codons, specific strains and organisms, strong promoters, tandem multimeric expression, as well as the use of fusion proteins to enable high production [17]. In this review, we will cover systems and approaches that are already being widely used for the production of recombinant molecules.

2.1. Bacteria

Bacteria are extensively used as a host for heterologous expression [17], as most bacterial systems can grow in low-cost media, have very short doubling time and some of them are highly efficient in receiving (incorporating) foreign DNA and expressing recombinant proteins at a very high rate. However, a few bacterial systems present a limitation in secretion systems for the release of peptides into the growth medium and have limited ability to form disulfide-bonds, post-translational modification, and glycosylation [17,34,41].

Many different bacteria can be used as a host for the heterologous system, including *Corynebacterium* [42], *Streptomyces* [43] and *Pseudomonas* [44,45]. These hosts can be associated with different promoters and different vectors to produce a peptide or

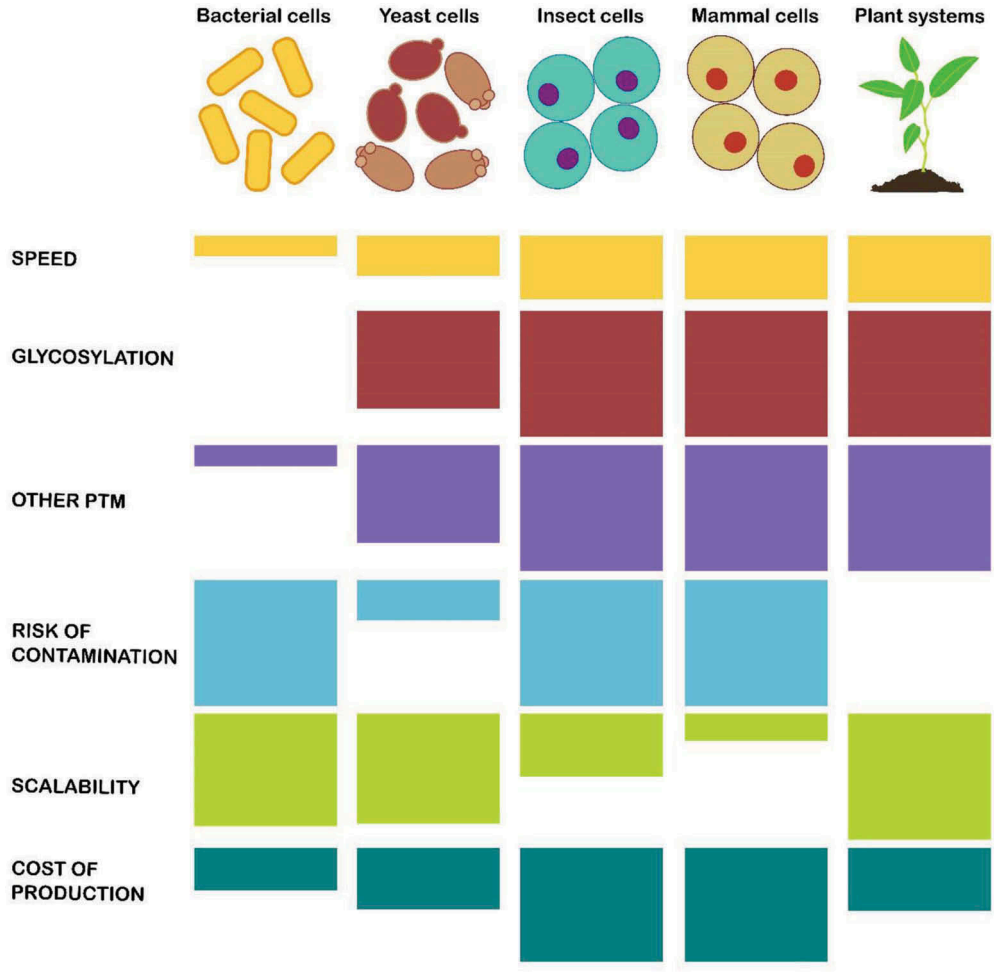

Figure 1. Comparison between different expression systems for production speed (yellow), ability to perform glycosylation (red) and other post-translational modifications – other ptm (purple), risk of contamination (blue), scalability (light green) and cost of production (dark green).

protein. In this section, we provide an overview of the most common bacterial systems – *E. coli, Bacillus subtilis*, and *Lactoccocus lactis* – as hosts.

2.1.1. *Escherichia coli*

Escherichia coli is a bacterium that has been extensively used for amplifying the expression of recombinant peptides, proteins and membrane proteins [34,41,46,47]. According to the Protein Data Bank (PDB), approximately 99% of peptides and proteins were expressed in an *E. coli* expression system. In addition, this system has a low cost and is relatively fast and simple, due to the genetic simplicity and well-established protocols [41,48]. Thus, numerous expression systems are available for different industrial applications [41,46,48,49].

Theoretically, the production of recombinant peptides in *E. coli* is relatively easy, but the diversity of peptide sequences, post-translational modification, and glycosylation increases the complexity of obtaining the active peptide [34,41,46–49]. In an attempt to solve these problems, the proper *E. coli* strains must be selected as the host, to allow efficiency in peptide expression [50] (Table 1).

L21 and BL21 DE3 are strains from *E. coli* and demonstrated a deficiency in the genes of Lon protease (cytoplasm) and OmpT protease (outer membrane). In addition, these hosts can be expressed using RNA polymerase promoters like lac, tac, trc, ParaBAD, PrhaBAD, T5 and T7 [51–54]. Another strain is BL21 tar, which has a mutation in the gene rne131 that allows higher stability and increases the efficiency of peptide expression for heterologous genes. However, this strain is not a good option to express peptides with toxicity for the host [55–58]. In addition, there are engineered strains of *E.coli* capable of correctly folding proteins that have multiple disulfide bonds in the cytoplasm. This occurs due to a mutation in the thioredoxin reductase (trxB) and glutathione reductase (gor) genes [59–63].

It is very important to know the characteristics of the target that will be expressed, as this enables the correct strain to be chosen and helps to define the best vector to compose the system. These vectors can control the basal expression associated with the promoters [41]. The commercial vectors pGEX, pMAL and pUC are used for lac, tac and lacUV5 promoters, respectively. To promote tetA, the use of the pASK vector is recommended; to promote T5, pQE is used and to promote T7 the vector is the pET system. In addition, some peptides need to add a fusion tag in the vector. These different tags help in the correct folding of protein, solubilization, purification and protecting the recombinant peptides against the degradation produced by intracellular

Table 1. *E. coli* strains frequently used for protein heterologous production.

Bacterial strain	Features	Benefit	Company
BL21(DE3)	*Has DE3 lysogen that expresses T7 RNA polymerase *Deficient in lon and ompT proteases	Appropriate for the expression of nontoxic genes	Novagen
BL21 trxB	trxB mutant	Promotes cytoplastic disulfide bond formation	Novagen
BL21(DE3) pLysS	*Has DE3 lysogen that expresses T7 RNA polymerase *Has T7 lysozyme to inhibit the enzyme T7 polymerases before induction	Prevents leaky expression Suitable for the expression of toxic genes	Novagen
Lemo21™ (DE3)	*Contains features of BL21(DE3) *Tunable expression of difficult clones by varying the level of lysozyme (lysY)	Suitable for the expression of challenging protein including: toxic proteins, membrane proteins, and low soluble ones	NEB
Tuner™ (DE3)	Has lac permease (lacY) mutation	Suitable for toxic and insoluble proteins	Novagen
Origami™	Has mutation in trxB and gor genes	Enhances disulfide bond formation in the cytoplasm	(Novagen, 2006–2007)
SHuffle®	*Expresses disulfide bond isomerase DsbC *Deficient in proteases Lon and Omp	Promotes the correct folding of mis-oxidized proteins Resistance to phage T1 (fhuA2)	NEB
Rosetta™	*BL21 lacZY (Tuner) derivatives *Has additional copies of genes encoding the tRNAs for rare codons AUA, AGG, AGA, CUA, CCC, GGA	Appropriate for the expression of heterogeneous proteins	Novagen
Overexpress™ C41(DE3) and C43(DE3)	Mutant strains of BL21(DE3) that prevent cell death associated with the expression of toxic recombinant proteins	Expression of toxic and/or membrane proteins from all classes of organisms	Lucigen

™: Unregistered mark, ®: Registered mark.

proteases [41,50,64]. In this context, various successful studies have been described, using different tags for AMP expression.

According to the information in Kim et al., 2019, these researchers expressed abaecin, an antimicrobial peptide isolated from honeybees. They used *E. coli* BL21 as a host for vector pKSEC1 with 6xHisSUMO-abaecin. The results demonstrated that a SUMO-tag is a relevant codon-optimization strategy for the production of antimicrobial peptides in *E. coli* without affecting the viability of the host cell [65].

Costa et al., 2018 expressed a recombinant hepcidin Hep25c fused with elastin-like recombinant (ELR) with 200 repetitions of the pentamer sequence VPAVG. The genetic construction of the recombinant peptide Hep-A200 was transformed in *E. coli* XL1-Blue and *E. coli* BL21(DE3). Hep-A200 was successful only when produced by *E. coli* BL21(DE3). According to the authors, the production using this system increased the final volume of Hep-A200 2–4 times after purification using inverse transition cycling (ITC). Hep-A200 demonstrated antimicrobial activity against Gram-positive and negative bacteria; besides, it presented no cytotoxicity [66].

2.1.2. *Bacillus subtilis*

Bacillus subtilis is a Gram-positive bacterium that can be found in soil and the gastrointestinal tract of ruminants and humans. Assigned as GRAS (generally regarded as safe), this is a nonpathogenic organism [67,68]. *B. subtilis* has the ability to secrete enzymes or antibiotics into medium and to produce spores; it does not produce LPS and can become competent for genetic transformation [46,67]. Due to these characteristics, *B. subtilis* is an alternative to the *E. coli* expression system. Thus, the highest expression level was obtained for the mutation in gene sacUh for strain WB30 [69].

For strain WB600, an expression cassette was constructed carrying sacY, a sacB-specific regulatory gene, and this gene was placed under the control of P43, a strong constitutive promoter. Therefore, *B. subtilis* has been widely used in industry to produce homologous and heterologous peptides and protein, reaching production yields of 20–25 $g.L^{-1}$ [70]. Nevertheless, to raise the secretion level of heterologous proteins, it is necessary to take care with important points like promoters [71], signal peptides [72–74], ribosome-binding sites [71], secretory pathways [75–77], proteolytic degradation of proteins [78–81] and genome reductions [82,83].

Thus, He et al., 2015, expressed cathelicidin from *Bungarus fasciatus* (CBF), in a *B. subtilis* expression system. They used *B. subtilis WB*800 N strain; the shuttle vector pHT43 was selected for the secretion of target proteins and the vector pTWIN1 was used for constructing fragments encoding CBD–intein–fused CBF. The authors reported recovering 0.5 mg peptide from 1 liter of culture medium. In addition, the CBF peptide maintained the antimicrobial activity, and the expression system used was a safe and efficient method by which to produce soluble peptides and proteins [84].

Another example was the expression of plectasin (first fungus defensin) in a *B. subtilis* sequence encoding plectasin, which was fused in the small ubiquitin-like modifier (SUMO) gene, the 6 × his gene and the signal peptide of sacB. The authors used WB800N *B. subtilis* as a host and the shuttle plasmid pGJ148. They reported that the fusion protein was successfully secreted in culture, and approximately 41 mg was purified per liter. After purification and digestion of SUMO protease, they obtained 5.5 mg of plectasin with a purity of 94% from 1 liter of fermentation culture. The recombinant plectasin demonstrated antimicrobial activity against *S. pneumoniae, S. aureus*, and *S. epidermidis*. The authors showed this expression system could be a safe and efficient way for the large-scale production of soluble peptides in *B. subtilis* [85]. *B. subtilis* is a promising system for industry, due to all the characteristics previously mentioned. Recently, several reviews and studies have been published to establish and improve this system [46,67,70,80,86,87].

2.1.3. *Lactoccocus lactis*

Lactococcus lactis is a Gram-positive bacterium which has been used for fermentation and is considered safe (GRAS), having gained this status from the Food and Drug Administration

(FDA) [88,89]. This bacterium has ideal aspects for industrial applications as it is an easy and low-cost culture, has a single membrane and relatively mild proteolytic activity [67,87,88,90]. In addition, *L. lactis* is broadly used in the biotechnology industry for large-scale production of antimicrobials, polyphenols, vaccines, and flavor-compounds [87]. The efficacy of *L. lactis* is related to the genetic knowledge available, and this knowledge has allowed researchers to develop novel expression systems by changing the constitutive and induction system [89]. Xu et al., 2019 expressed two plantaricins, J and K, for which they used the plasmids pNZ8124-plnJ and pNZ8124-plnK, and expression of these peptides was accomplished in *Lactococcus lactis* NZ9000, producing plnJ and plnK. The results demonstrated plnJ, plnK and combination plnJK, which showed antimicrobial activity against different Gram-positive bacteria [91]. Other antimicrobial peptides expressed in *L. lactis* and the other bacterial systems can be seen in Table 2.

2.2. Yeasts

The heterologous production of AMPs presents challenges such as the toxicity of some of them to *E. coli* and other host bacteria used for the recombinant production of these peptides. Other problems are instability, susceptibility to proteases and the requirement of post-translational modifications for the proper performance of the peptide function. Although it is possible to add post-translational modifications to peptides or proteins produced in *E. coli*, it is an even more expensive and time-consuming process to obtain the molecule of interest. These issues have led to a search for alternative expression hosts such as yeasts, filamentous fungi, insects, and plant and mammalian cell lines [30,36,107,108].

Yeasts are unicellular organisms and have a lower nutritional demand when compared to insect and mammalian cell lines [108]. An expression system based on yeast cells has many advantages when compared to other systems, including rapid growth, simple genetic manipulation, and the ability to perform adequate post-translational modifications, scalable fermentation, high cell density, and pathogen-free protein production [109–111].

Saccharomyces cerevisiae was the first eukaryotic organism to be used for the production of recombinant proteins in the 1980s. The vast knowledge of its genetics, physiology, fermentation, and the attribution of the GRAS status are some of the reasons for the use of this host [112–115]. In addition to its ability to produce and secrete biologically active eukaryotic proteins, this yeast easily adapts to severe industrial-scale conditions [108].

This system was successfully used for the production of the hepatitis B vaccine and the hantavirus vaccine [116,117]. Nonetheless, there is a limited variety of AMPs produced in this host [35,67], as can be seen in Table 3. Some disadvantages like fermentative metabolism, hyperglycosylation and the low protein concentrations of this system must be taken into account for the production of the protein of interest [110,112,118,119]. Thus, new expression systems have been developed using the so-called unconventional yeasts. Among these systems, the most established examples of unconventional yeasts are *Hansenula polymorpha, Pichia pastoris, Kluyveromyces lactis, Kluyveromyces marxianus, Yarrowia lipolytica, Arxula adeninivorans, Pichia methanolica, Schizosaccharomyces pombe* and *Zygosaccharomyces bailii* [119].

Unlike *S. cerevisiae, Pichia pastoris* (reclassified as *Komagatella phaffii* [136]) does not divert part of its carbon to ethanol production, allowing for high biomass as well as recombinant molecule production [108,110,137]. Besides, the presence of methanol-induced alcohol oxidase promoter (PAOX1) enables high cell density to be obtained, resulting in heterologous production over a specific period. *K. phaffii* is still capable of secreting recombinant proteins into the culture medium, decreasing purification steps and thereby increasing production efficiency [137–139]. Due to all the characteristics mentioned, these microorganisms have been widely used in recent years as heterologous systems for the

Table 2. Recombinant antimicrobial peptides produced in bacterial systems.

AMP	Source	System	Application	Yield	Ref.
β-Defensin 2	Human	*E. coli*	Bactericidal activity	57 $mg.L^{-1}$	[92]
PtDef	*Populus trichocarpa*	*E. coli*	Antibacterial and antifungal activities	N/D	[93]
Hybrid magainin–thanatin	Frog skin and hemolymph of *Podisus maculiventris*	*E. coli*	Antimicrobial activity	49.5 $mg.L^{-1}$	[94]
Pallidocin	*Aeribacillus pallidus* 8	*E. coli*	Antimicrobial activity	N/D	[95]
Tachyplesin I	*Tachypleus tridentatus*	*E. coli*	Antimicrobial activity	17 $mg.L^{-1}$	[293]
β -defensin-3	Human	*E. coli*	Antimicrobial activity	210 $mg.L^{-1}$	[96]
Lactoferricin 33	Bovine	*E. coli*	Antimicrobial activity	N/D	[97]
Cecropin ABP-CM4	*Bombyx mori*	*E. coli*	Antimicrobial and anticancer activity	48 $mg.L^{-1}$	[98]
Abaecin	*Apis mellifera*	*B. subtilis*	Bactericidal activities	up to about 1 $g.L^{-1}$	[99]
Cecropin A-melittin mutant	Insect	*B. subtilis*	Antimicrobial activity	159 $mg.L^{-1}$	[100]
Porcine β-defensin-2 (pBD-2) and cecropin P1 (CP1)	Epithelial cells in animal skin, gastrointestinal and respiratory tracts	*B. subtilis*	Antimicrobial activities	45 $mg.L^{-1}$	[101]
Cecropin AD	*Hyalophora cecropia*	*B. subtilis*	Antimicrobial activities	30.6 $mg.L^{-1}$	[102]
Enterocin A, enterocin P and hiracin JM79	Bacteria	*L. lactis*	Antimicrobial and preservative activities	N/D	[103]
Mabinlin II	*Capparis masaikai*	*L. lactis*	Sweetener	1.28 to 2.72 $mg.L^{-1}$	[104]
Pediocin PA-1	*Pediococcus acidilactici*	*L. lactis*	Dairy industry	N/D	[105]
Exendin-4	*Heloderma suspectum*	*L. lactis*	Treatment of type-2 diabetes	N/D	[106]

N/D: Not determined.

Table 3. Recombinant antimicrobial peptides produced in *S. cerevisae* and *P. pastoris*.

AMP	Source	System	Application	Yield	Ref.
β-defensin-1	Human	*S. cerevisiae*	Infectious and inflammatory diseases	55 μg.L^{-1}	[120]
Pediocin PA-1	*Pediococcus acidilactici*	*S. cerevisiae*	Food biopreservative	N/D	[121]
Plantaricin 423	*Lactobacillus plantarum* 423	*S. cerevisiae*	Food biopreservative	N/D	[122]
Enterocin L50A and Enterocin L50B	*Enterococcus faecium*	*S. cerevisiae*	Food biopreservative	19.30 ng.mL^{-1} and 52.50 ng.mL^{-1}	[147]
Thrombocidin-1	Human blood	*P. pastoris*	Antimicrobial and antioxidant	190 μg.mL^{-1}	[123]
PaDef	*Persea americana*	*P. pastoris*	Antibacterial	79.60 mg.mL^{-1}	[124]
Apidaecin	*Apis mellifera*	*P. pastoris*	Antibacterial	418 mg.L^{-1}	[125]
NZ17074	*Arenicola marina*	*P. pastoris*	Antibacterial	515.10 mg.L^{-1}	[126]
Mytichitin-A	*Mytilus coruscus*	*P. pastoris*	Antibacterial	45.50 μg.mL^{-1}	[127]
Plectasin	*Pseudoplectania nigrella*	*P. pastoris*	Antibacterial	748.63 μg.mL^{-1}	[286]
Perinerin	*Perinereis aibuhitensis Grube*	*P. pastoris*	Antibacterial	35 mg.mL^{-1}	[128]
Hispidalin	*Benincasa hispida*	*P. pastoris*	Antibacterial	98.60 μg.mL^{-1}	[129]
Pleurocidin	*Pleuronectes americanus*	*P. pastoris*	Antimicrobial	N/D	[130]
Lactoferrin	*Camelus*	*P. pastoris*	Antibacterial and antifungal	N/D	[131]
Hepcidin-25	Human	*P. pastoris*	Iron homeostasis and antimicrobial	1.90 mg.L^{-1}	[132]
Abaecin	*Apis mellifera*	*P. pastoris*	Antibacterial	N/D	[133]
Protegrin-1	*Susscrofa domesticus*	*P. pastoris*	Anticancer activity	40 mg.mL^{-1}	[134]
Cecropin P4	*Ascaris suum*	*P. pastoris*	Antimicrobial	N/D	[135]

N/D: Not determined.

expression of molecules with pharmacological and industrial interest. However, as with other available expression systems, *K. phaffii* has certain disadvantages such as proteinase production, which may make recombinant production unfeasible [112].

The mechanism of proteolysis usually occurs during the vesicular transport of the recombinant protein or when it is in the extracellular space, leading to reduced yield or even the activity of recombinant molecules [140]. As a solution to this problem, some protease deficient strains may be used for proteinase sensitive protein expression. The commonly used strains are SMD1163 (pep4 prb1 his4), SMD1165 (prb1 his4), and SMD1168 (his4 pep4). Due to the deletion of the specific genes, proteinase activity could be substantially decreased or even eliminated. However, these strains are not as robust as wild strains, showing slow growth, low transformation efficiency and short viability [141].

This yeast is widely used for the production of antimicrobial peptides, and many studies showed better results when compared to *E. coli* [17]. The AMP scygonadin achieved a yield of 70 mg.L^{-1} when expressed in *K. phaffii*, and its antimicrobial activity was better when compared to scygonadin expressed in *E. coli*, which may have occurred due to the ability of the yeast to make post-translational modifications [142]. The vpdef defensin isolated from the mollusk *Venerupis philippinarum* reached a concentration of 60 μg.mL^{-1} of culture medium when expressed in *K. phaffii*, and the purified molecule demonstrated a minimum inhibition concentration of 50 μg.mL^{-1} against the growth of *E. coli* (ATCC 35150). The same peptide expressed in *E. coli* showed no significant antimicrobial activity [143].

The AMP snakin-1 from plants contains six sulfide bridges and was successfully produced in *K. phaffii*. Clavanine, an α-helical AMP that is toxic to Gram-positive and negative bacteria, was also expressed in the same host [144,145]. The antimicrobial peptide ch-penaeidine was successfully expressed in *K. phaffii* and *S. cerevisiae*. However, secreted production was higher in *K. phaffii*, at around 100 mg.mL^{-1} [146]. EnterocinL50A and enterocinL50B were expressed in both systems, but the bioactivity of these peptides was higher when produced in *K. phaffii* [147,148].

The use of constitutive and inducible promoters for heterologous expression in yeast is common. Inducible promoters are generally the most used because they allow greater control of expression, providing higher product yield [17]. The expression of the antimicrobial peptide MP1102 in *K. phaffii* reached a final yield of 807.42 mg.L^{-1} using glyceraldehyde 3-phosphate dehydrogenase (PGAP) promoter [149]. A much higher level of expression could be observed by using the AOX1 promoter for NZ2114 production in the same system, achieving 2390 mg.L^{-1} yield [150]. Although inducible promoters are being widely used for basic and applied research, industry prefers the use of constitutive promoters for the production of recombinant molecules at high concentrations [17]. Other promoters are used for gene expression in *K. phaffii*, like the constitutive TEF, PGK and methanol-induced FLD1 [119].

In *S. cerevisiae* the constitutive promoters TEF1 and GPD are often used for heterologous gene expression. Others like ADH1, GAPDH, PGK1, TPI, ENO, PYK1 are also used. However, these promoters may cause the aggregation of folded proteins, making their secretion difficult. To avoid this complication it is possible to use inducible promoters, such as galactose-induced GAL1 and GAL10, and others such as CUP1 and ADH2 [108].

Plectasine showed a yield of 146 mg.L^{-1} when expressed in *K. phaffi* with 8 copies of gene fragments. Otherwise, with only one genetic copy of plectasine, the yield obtained was 50 mg.L^{-1} [151]. These results suggest that tandem multimeric expression increases gene transcription levels and consequently improves the protein expression yield [17]. Adenoregulin AMP, for instance, was tested for expression in *E. coli* BL21 (DE3) and *K. phaffii* GS115 with 2, 4 or 6 tandem repeats. The peptide was expressed and secreted in the culture medium by using the yeast, whereas in *E. coli* the expression level decreased

according to the tandem repeats number [152]. Other AMPs expressed in *K. phaffii* are described in Table 3.

High mannose N-glycosylation creates a clear limitation on using microorganisms as cell biopharmaceutical factories. Although not as remarkable as in *S. cerevisiae*, hypermanosylation can interfere with the modified protein half-life and trigger allergic reactions, which is a challenge that needs to be overcome. However, thanks to genetic engineering, these yeasts are already capable of performing human-like N-glycosylation patterns, which even include the terminal addition of sialic acid to glycosylation [111,153].

Among strains constructed to generate a human glycosylation pattern, we can mention YSH44, which eliminated the yeast endogenous glycosylation pathway. To establish the synthetic glycosylation pathway that generates the human oligosaccharide GlcNAc2Man3GlcNAc2, eukaryotic proteins (mannosidasidases I and II, N-acetylglucosaminyl transferases I and II and uridine 5′-diophosphate-N-acetyl glucosamine transporter) were used [154].

2.3. Plants

In recent years, plants have been used as hosts capable of producing biologically active recombinant proteins and antigens used as important vaccines [155], as well as producing recombinant peptides with antimicrobial potential [156]. When compared to other available heterologous expression systems (e.g., bacteria, yeast, mammalian cells or transgenic animals), plants are advantageous due to the absence of introgression risk [157,158]. These systems allow high yield and low production cost, as they do not present pathogens common to humans, oncogenes, and endotoxins in the final product, contributing to a low cost of producing heterologous biomolecules [157,159].

In addition to antibodies, antigens, molecular transporters, and enzymes [10], several recombinant AMPs have already been expressed in plant systems. Efforts to produce AMPs are aimed at obtaining these molecules at high concentrations [160] or increasing the resistance of transgenic plants [161,162]. Nowadays, a number of recombinant AMPs (natural or synthetic) have already been released on the market or are in advanced clinical trials [163].

Nearly all proteins and many peptides require post-translational modifications, which include the addition of hydroxyl, phosphoryl, acetyl and carboxyl groups, proteolytic cleavage and glycosylation, and plants are capable of performing almost all of these modifications [164]. Plant cells are also able to correct folding of complex glycoproteins, such as antibodies, due to the presence of chaperones homologous to those of animals [165,166]. Another advantage is that plants produce protein disulfide isomerase (PDI), which catalyzes the formation of disulfide bonds, essential for the activity of certain antimicrobial peptides [167].

Although plants have shown several advantages for AMP heterologous production, the glycosylation pattern that is slightly different from that found in mammalian cells is one of the major limitations that need to be overcome [168]. Unlike animals, plant *N*-glycans have mannose, α-1,3-fucose, and β-1,2-xylose, which have strong potential for triggering an immunogenic response [158,165]. Therefore, for the heterologous production of AMPs in plant systems, it is necessary to inhibit the addition of plant-specific glycans [169].

One strategy is to knock out genes from the enzymes (α-1,3-fucosyltransferase and β-1,2-xylosyltransferase) responsible for the addition of undesirable plant glycans or by co-expressing human β-1,4-galactosyltransferase [170]. In vitro modification of biosynthesized proteins in plant cells using purified enzymes, β-1,4-galactosyltransferase and sialyltransferase is another strategy that can be used [157]. Inactivation of *N*-glycosylation sites on asparagine (Asn), serine (Ser) or threonine (Thr) residues can also prevent an undesired pattern of glycosylation [171].

Currently, there are several approaches to the heterologous production of AMPs in plant systems. They are based on the plastidial nuclear transformation of whole plants or even plant cell suspensions, which can express transgenes in a stable or transient manner [168,169]. The stable (nuclear) transformation consists of the integration of exogenous genes into the nuclear genome, or circular plastidial DNA, of plant cells [164,172]. The transgene will be integrated into the genome (nuclear or plastidial) of the plant, which implies its transmission to future generations [173].

In 2018, Jin and colleagues stably produced recombinant β gallinacin-3, a cysteine-rich peptide isolated from chicken immune cells, in *Arabidopsis thaliana* seeds. The objective of the work was the use of transgenic seeds as feed additives for the prevention of poultry diseases [174]. Recently, (CBD) 2-DrsB1, a peptide dermasptin B-1 fusing tandem repeat to CBD, was stably and recombinantly produced in tobacco hairy roots (HRs). When compared to the non-fused peptide (DrsB1), (CBD)2-DrsB1 demonstrated an increase in antimicrobial activity against phytopathogens, especially *Alternaria alternata* with MIC of 11.25 $\mu g.L^{-1}$ [175].

Nuclear transformation is advantageous because it allows the accumulation of heterologous peptides in the seeds, allowing a reduction in costs since no special packaging is required. In addition, this approach is still widely used for the expression of transgenes in plant systems, accounting for most products available on the market. On the other hand, the nuclear transformation has as a disadvantage the need for transgene containment, since transgenic plants are able to cross with native species [168].

Transplastomic expression, which consists of heterologous peptide production within chloroplasts, is a viable alternative to stable transformation [176]. In 2004, Molina and colleagues demonstrated high-level production of 2L21, a linear antigenic peptide from the VP2 capsid protein of the canine parvovirus (CPV), in transgenic *Nicotiana tabacum* (var. Petit Havana) chloroplasts. Data suggest that stable expression in the plant chloroplasts can be used to produce vaccines due to the high yield of the recombinant molecule of interest [177].

The main advantages of transplastomic expression are the stability of the transgene over generations, the absence of gene silencing mechanisms, a high accumulation (3%-31%) of the recombinant protein, when compared to the amount of total soluble proteins (PST), the large number of plastids by cells, and the expression of polycistronic units [178]. In

addition, the fact that the offspring inherit their chloroplasts from the mother plant, which eliminates the risk of dispersion of the transgene during pollination, is another advantage [173]. On the other hand, chloroplasts have some limitations, such as the inability to correctly produce molecules that need to be glycosylated; however, they are capable of correctly folding and forming disulfide bridges, allowing the production of biologically active heterologous peptides [179].

At the beginning of Molecular Farming, biomolecule production through stable transformation was widely used. However, its use has been decreasing over recent years due to the need for a long period for the establishment of transgenic plants [169,180]. To circumvent this limitation of the technique, the transient heterologous expression has recently been employed for the production of recombinant peptides [181].

Transient expression is the production of antimicrobial proteins and peptides of pharmacological interest without the integration of the transgene into the plant genome [180,182–185]. Depending on the physicochemical properties of the proteins or the vector used for transformation, transient expression of transgenes occurs rapidly, starting within 24 hours of infiltration and lasting for days or even weeks [181].

Moreover, another advantage of heterologous peptide production through transient recombinant expression is that it can inhibit post-transcriptional gene silencing (PTGS) mechanisms by preventing the presence of abundant RNA species for a long period [178]. In addition, the transient expression of transgenes in plants allows the production of peptides that exhibit an irregular demand and unstable markets [167]. Another advantage of the heterologous transient expression system is the ability to co-express, depending on the case, multiple transgenes easily and at the same time [181].

Well-established plant cell transformation protocols also contribute to the popularization of this technique. Biolistics is a transfection technique consisting of bombarding plant cells with microprojectiles coated with the DNA of interest. These microparticles are accelerated at high speed in a vaccination chamber, allowing them to penetrate the cell wall [159,186]. Transformation by biolistics allows the production of transgenic plants from many species of reclining monocotyledons [187].

Another approach is the infiltration of plant tissues with a suspension of Gram-negative soil bacteria cells, *Agrobacterium tumefaciens*, carrying the recombinant vectors, a process known as agroinfiltration [172,188]. *A. tumefaciens* has a high molecular weight plasmid (Ti plasmid), which has a sequence (region T) delimited by the left and right borders that are transferred and randomly integrated into the genome of the host plant [189,190]. This type of transformation allows the efficient transfer of genes in both monocotyledons and dicotyledons [191]. Defensin Rs-AFP2, isolated from *Raphanus sativus*, was expressed in rice (*Oryza sativa* L. cv. Pusa basmati 1) by transformation into *A. tumefaciens*. This peptide confers resistance to fungal phytopathogens [192].

Plant-based heterologous systems are mainly used for the production of proteins and peptides that are capable of improving the harvest and nutritional quality of plants [34]. An example was barley transformation for heterologous production of metchnikowin, an antifungal peptide isolated from *Drosophila melanogaster*, which gave barley resistance to biotic stress [193]. Also, the hordothionin gene from *Hordeum vulgare* was introduced in apple through *A. tumefaciens* strain AGL0 for crop improvement against fungal infections [194].

Although there have not been many therapeutically important AMPs produced by plant-based platforms, there are some examples, such as the synthetic peptide C4V3, which was introduced into *N. tabacum* chloroplasts and induced mucosal and systemic antibody responses in mice. These results show that plant chloroplasts can be used as biofactories for HIV vaccine candidates [195].

2.4. Mammalian cells

Due to their capacity for proper protein folding, assembly and posttranslational modifications, such as glycosylation, this system allows the production of recombinant complex molecules that cannot be produced in microbial systems. The proteins produced by mammalian cells are similar to those naturally occurring in humans regarding molecular structures and biochemical properties. Although yeasts and plants can glycosylate proteins, the resultant glycans are not the structures normally found in human proteins. Thus, the quality and efficacy of a peptide or protein can be greater when expressed in mammalian cells than in other hosts, such as bacteria, plants, and yeast [196–198].

On the other hand, mammalian cell culture yield is much lower than that of microbial cells and their cultivation is a laborious, cost-intensive and complicated process [199]. Mammalian cells are fragile and sensitive to impurities because they are approximately 10 to 50 fold larger than microbial cells, without the tough cell walls of microbes [197]. Thus, the high costs required for mammalian expression systems represent a barrier for the industrial production of non-post-translationally modified peptide drugs [200].

Even so, this host is the one most used for the production of recombinant biopharmaceutical compounds, mainly antibodies and vaccines [196,197,201,202]. Chinese hamster ovary cells (rCHO) are the main mammalian cells used for the production of recombinant proteins because of their ease of use, established regulatory track record, and safety profile. The cell lines derived from murine myeloma lymphoblastoid-like (NS0 and Sp2/0-Ag14) cells, baby hamster kidney (BHK), human embryo kidney (HEK-293) and human retinal cells can also be used to produce recombinant protein for biomedical and pharmaceutical research [196,198,200,201,203,204]. However, there are only a few studies using this expression system for the production of AMPs.

In 1994, the antibacterial peptide magainin2 was produced using mouse erythrocytes as a bioreactor [205]. The authors prepared a red blood cell-specific expression construct that encodes a fusion peptide comprising alpha-globin and the antibacterial peptide. A transgenic mouse model produced the recombinant fusion protein at >25% of the total hemoglobin in the erythrocytes. A protein cleavage site was inserted between the alpha-globin chain and the desired peptide; therefore, after cleavage of the fusion protein with an enzyme that recognizes this cleavage signal as their substrate, the peptide magainin2 was recovered.

Magainin2 is a 23 aa antimicrobial peptide found in the african clawed frog (*Xenopus laevis*) that can inhibit the growth of numerous species of bacteria and fungi and induce osmotic lysis of protozoa at low concentrations [206]. In 2008, Fun and Li designed and synthesized a gene construct encoding a carrier protein and the antibacterial peptide, and tested this construct in the rCHO cell line. The designed gene construct was expressed in rCHO cells at a relatively high level and, in order to confirm that this gene is also expressed suitably in mammalian models in vivo, they engineered mice by a mammary gland-specific expression vector system, pBC1. The gene was expressed efficiently in the mouse mammary gland with a yield of 3–10 $g.L^{-1}$, demonstrating that this system provides a potentially efficient and affordable manner by which to produce an antibacterial peptide in a mammary gland bioreactor system [207].

In 1996, Yarus and his colleagues tried to produce the bovine tracheal antimicrobial peptide (bTAP) using a mouse mammary gland bioreactor in a non-fusion manner. The bTAP is a member of the b-defensin family of antibiotic peptides found in the tracheal mucosa of the cow. The researchers generated an expression vector driven by control sequences from the murine whey acidic protein (WAP) gene to produce this AMP in the mammary tissue of transgenic females. As a result, they obtained a bioactive bTAP in transgenic mouse milk, but the expression level was too low (5 $mg.L^{-1}$) to meet the need for large-scale production of TAP [208].

Guzmán-Rodríguez and colleagues tried to produce the antibacterial peptide PaDef in the bovine endothelial cell line BVE-E6E7. PaDef is a 78 aa peptide homologous with plant defensins, and it was identified in the avocado fruit (*Persea americana* var. *drymifolia*). The researchers obtained polyclonal and clonal populations of cells transfected with the coding sequence of the AMP. In spite of the total protein from the clones that did not display activity against *Candida albicans*, it inhibited *E. coli* and *S. aureus*. These results showed the potential use of this AMP for the control of pathogens [209].

HEK-293 cells were used in a study by Li and colleagues for the production of the antimicrobial peptide human β-defensin 3 (hBD3). They made the liposomal transfection of the cells using the vector pVAX1 in two approaches, the vector carrying only the coding sequence of hBD3 (pVAX1/hBD3) and the vector carrying the coding sequence of hBD3 fused with a carbohydrate-binding domain (pVAX1/hBD3-CBD). The carbohydrate-binding domains (CBDs) can interact with sugar chains, but do not modify them. Therefore, it is a potential method for the biological activity of AMPs to combine them with a CBD, which allows the peptide to interact with the peptidoglycan present in the bacterial cell wall, but which is non-existent in human cells. In this study, both peptides were successfully produced, but the recombinant pVAX1/hBD3-CBD showed better bactericidal activity and stability than the eukaryotic AMP pVAX1/hBD3 against *S. aureus* N315 [210].

The antimicrobial peptide thionin Thi2.1 cDNA from *A. thaliana* was expressed in the bovine endothelial cell line BVE-E6E7 and its activity was evaluated against bovine mastitis *S. aureus* isolates [211]. As a result, they obtained a dose-dependent inhibition of *S. aureus* isolates, showing total inhibition at concentrations higher than 3.12 $mg.L^{-1}$. In addition, a study by Liu and colleagues transfected goat mammary epithelial cells (GMECs) with an HBD3 mammary-specific expression vector by electroporation in order to produce transgenic clonal cells expressing the peptide. They studied the in vitro development of these cells and, as a conclusion, they suggested that genetic modification of GMECs might not influence the in vitro development of cloned embryos. These results are important for the future development of transgenic goats that express increased concentrations of b-defensins in their milk [212].

Over recent years, many research groups have developed different strategies to improve the production of recombinant biopharmaceuticals in mammalian cells. There are reviews that bring several approaches to improve the production by vectors and cell engineering technologies, improvement of the culture media components, codon optimization, gene amplification, transfection methods, and screening tools [198,199,213–216]. However, there are no reviews about the optimization of recombinant AMP production in mammalian cells. The most commonly used expression hosts are bacteria and yeasts, because AMPs are small molecules that generally do not require complex post-translational modifications, which makes their products in mammalian cells less attractive due to the high cost of this system.

2.5. Insect cells

According to Berger and Poterszman, the baculovirus expression system has been widely used for more than 30 years, for the production of heterologous proteins, AMPs, viral and parasitic antigens [217–220]. The first paper describing the potential of the baculovirus system for the production of heterologous proteins was published by Smith in 1983, who used the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) virus to express human INF-β [221].

Several advances have already been made in this expression system, which includes simpler methods of isolating and quantifying recombinant viruses, enhancing cell culture, and modifying glycoproteins [219,222]. Other advantages are the use of recombinant baculoviruses as gene delivery vectors for mammalian cells and as expression vectors for adeno-associated virus (AAV) – based gene therapy products [219].

Performing post-translational modifications (glycosylation) and processing mechanisms (folding, modifying, trafficking and assembling newly synthesized polypeptides to produce soluble end products) are some features that insect cells have in common with mammalian cells. Besides, insect cells typically use fewer resources, time, effort and maintenance, which makes this system advantageous for protein production with the correct folding [223].

This system is nonpathogenic to mammalian cells. The insect cells used are derived from Lepidoptera, which are simple to culture (a CO_2 incubator is not required). The recombinant occlusion bodies based on the properties of baculovirus were developed to improve protein production, facilitating purification and long-term storage capacity without refrigeration. Also, eukaryotic proteins fold better in insect cells than in the *E. coli* expression system [218,219,224]. However, when comparing to mammalian cells, post-translational modifications in the insect cells are still limited, and an example of this is glycosylation, which may be

detrimental to the functioning of some recombinant proteins if it is not done correctly [222,225,226].

Although this system has some limitations, such as cell lysis through baculovirus infection and decreased production of molecules due to excessive passage of recombinant baculovirus, it still has the advantage of high cell density, which allows an increased yield of the final product [219]. Some therapeutic proteins have already been produced using the insect cell-based baculovirus expression vector system. The prophylactic human papillomavirus (HPV)-16/18 vaccine, was the first FDA-approved insect cell product, and a vaccine based on the influenza virus hemagglutinin protein [227].

The baculovirus insect cell expression system can be used as an alternative for producing peptides that are toxic to bacteria and fungi [220,228]. This system still uses the nucleopolyhedrovirus of *Autographa californica* (AcMNPV) for the development of the baculovirus vector, and the insect cell lines commonly used are Sf9 and Sf21 (IPLB-Sf21AE) from *Spodoptera frugiperda* [229] and Hi5 cells (BTI-TN-5BN1-4) from tissues of the ovary of *Trichopusia ni* [230–232].

This system was used for the production of snakin-1 peptide and osmotin, respectively. The first is an antimicrobial peptide derived from *Solanum tuberosum*, which is rich in cysteine and thus harmful to prokaryotic cells, and the latter is a pathogenesis-related plant protein. Both authors reported high production of the product, indicating that, in fact, the system is suitable for heterologous expression of AMPs and protein for pharmacological configurations [220,228].

Zitzmann and colleagues obtained 25 $mg.L^{-1}$ of the AMP gloverin derived from the *Galleria mellonella* wax moth, using *Drosophila melanogaster* S2 cells as an expression system. Gloverins have shown antimicrobial activity against *E. coli* strains. Although gloverins antimicrobial peptides have already been expressed in a microbial system, the antimicrobial properties of the peptide can impede efficient production. This shows that insect cells can be used as an alternative expression system in cases like that [233].

3. Heterologous productions of nonribosomal peptides

Nonribosomal peptides (NRP) are molecules synthesized by huge enzymatic complexes called nonribosomal peptide synthetases (NRPSs). Enzymatic complexes of this 'mega-enzyme' type are able to synthesize short cyclic or linear peptides (usually up to 20 amino acid residues) using as building-blocks D and L amino acids, besides exotic amino acids, which can be conjugated to a glycidic, a lipidic or an acyl chain, contributing to a wide diversity of molecules [234]. A mega-enzyme usually synthesizes a family of nonribosomal peptides, that is, more than one peptide isoform which presents a core of amino acid residues, and this allows variations of amino acids at specific points on the chain [235,236].

This ribosome-independent mechanism of synthesis is mainly present in Gram-positive bacteria (such as *Bacillus* and *Actinomycetes*), filamentous fungi and marine organisms [235]. Those biomolecules may have exclusively peptidic structure, such as gramicidin and tyrocidin [237]; they could be a lipopeptide, such as a daptomycin [238–240] and polymixin [241,242]; or even a glycopeptide such as vancomycin [243].

NRPs are known to present activity against Gram-positive and Gram-negative bacteria [244–248], including multidrug-resistant strains [249–253] and fungi [254,255]. The heterologous expression of an NRP, similarly to ribosomal peptides, could be used as a strategy for the overproduction of a specific isoform, improving the yield of a native peptide or producing it in a host organism more suitable to large-scale growth conditions [256]. Another possibility is obtaining new molecules with specific changes in their structure through directed mutations [257] in order to reduce cytotoxicity or increase antimicrobial activity. The heterologous expression also permits access to new molecules from an uncultivable microorganism or directly from environmental DNA [258,259].

A few important elements should be considered between the native producer organism and the heterologous host, such as similar G + C content and codon utilization. It is expected that the use of a phylogenetically related host will produce more efficient expression. It is important that the host is able to synthesize necessary NRP precursors and have a slow background of secondary metabolites [256]. This can be achieved by mutagenesis in the host genome or/and exogenous feed over the fermentation process.

Due to factors such as fast growth, simple cultivation conditions, and well understood metabolic pathways, *E. coli* is frequently chosen as a host to produce ribosomal antimicrobial peptides [260]. However, NRPS heterologous expression in this bacterium is a particular challenge, mainly because of the mega-enzyme's size and intermediate metabolites required. However, after modifications to the host genome are made in order to guarantee the precursors required, the expression of an NRPS in *E. coli* could be successful [261,262].

Host engineering has been used to reduce the host's genome size in order to build a more efficient heterologous host. Thus, after a *Streptomyces avermitili* strain had about 15.5% of its genome deleted in strategic genes, two mutants of the host engineered were able to produce streptomycin and cephamycin C in a higher yield than the original producer strain. This seems to be related to the fact that the transformants no longer produced the main endogenous secondary metabolites, thus being able to direct their biosynthesis more efficiently to the production of the precursors of the exogenous secondary metabolites [263].

Heterologous expression of NRPS usually involves a cosmid or fosmid library approach, which allows genes up to 40kb to be cloned. That could be a limiting factor since some NRPS gene clusters sometimes achieve up to 100kb [264]. Other strategies such as BAC (bacterial artificial chromosome) derived libraries are able to capture and clone inserts that are twofold larger [265]. The TAR system could be used to capture larger gene clusters directly from eDNA and express them in *S. cerevisiae* [266,267].

A useful approach to developing new antimicrobial molecules is the rationally directed modification of NRPSs. The method consists of cloning an NRPS gene cluster, including one or more genes that encode structural modification reactions that are able to change the molecules expressed by the host organism [268]. This strategy was successful in obtaining

a daptomycin-like lipopeptide, a variation that is more potent than the wild type [269] (Table 4).

NRPs are good candidates as antimicrobial molecules, which is important since there is an increasing demand for new and more effective bioactive molecules. Nonetheless, bioprospecting new NRPSs directly from environmental DNA or improving well-known compounds via heterologous expression is still a challenge, as the yield achieved in a heterologous host is often lower than in the native producer (Table 4) [256,279]. However, overexpression could be achieved with additional gene editing in the host and optimization in culture conditions [280]. In recent years, the development of new tools in genetic manipulation and heterologous expression has contributed to the rising interest in this area, making it a promising technique by which to obtain more and more effective molecules.

4. Purification of recombinant production of antimicrobial peptides

One recombinant protein production challenge involves purification. Optimization of the process is critical for many reasons, among them the recovery of the target molecule [281]. The AMP purification method in question depends on the system that was used for the heterologous production of the molecule. AMPs produced in bacteria or plants are recovered usually from the host's intracellular environment, while those expressed into yeasts or mammalian cells can be recovered from supernatant due to secretion capability in the extracellular medium [30,32]. In bacterial systems, the use of peptide/fusion proteins and cleavage sites is common in the purification process [17,282], which increases the steps in the process [283].

AMPs recovered from the culture medium and without fusion proteins are routinely precipitated with high salt concentrations (i.e., ammonium sulfate) [284–286]. Otherwise, AMPs expressed without signal peptides have been localized in the host cell, and lysis induction is necessary for recovery [281]. Dialysis or chromatographic methodologies (e.i., reverse phase HPLC, molecular exclusion chromatography, etc.) can be used in these cases [224–226]. Proteins synthesized in such conditions can be expressed in soluble or insoluble forms [287], especially in bacterial systems. Frequently, the insoluble product's expression occurs due to cellular stress response [288]. However, most of the AMPs are recovered from soluble fractions due to the use of carrier proteins [35,36]. Buffer solutions such as Tris-HCl [289] or phosphate-buffered saline PBS are necessary in this case [290].

Another possibility is the formation of inclusion bodies (insoluble forms), in which denaturing conditions are necessary for protein recovery [282]. Only a few AMPs were extracted from insoluble fractions, such as pAMP1 [291], fowlicidin-2 [292] or tachyplesin I [293]. During protein extraction, additional chaotropic agents were used, such as guanidinium chloride [293], β-mercaptoethanol [294] or urea [291,292]. After that, target AMPs should be purified by dialysis followed by chromatographic methodologies [17,282].

4.1. Use of fusion proteins as a strategy for AMP purification

Fusion proteins have been seen to be important for AMP expression and later purifications, especially when they are produced in the bacterial system [17,35]. Several fusion proteins have been used as a strategy for AMP purification [17,282] and they can be used for facilitating target recombinant protein solubilization, purification, and expression [295]. Sometimes, the strategy for AMP expression involves a combination of both tag and carried protein to facilitate the target protein's extraction and purification [36].

However, some of them have dual properties because they can also be recovered by affinity chromatography [281]. Thioredoxin (Trx), a fusion protein from *E. coli*, plays an important role in the solubilization of expressed proteins [295] and further facilitates exogenous gene expression [296]. This has routinely been used for recombinant AMP expression [36]. AMPs such as plectasin [297], G13 [298] or plantaricin [299] are some examples. Other fusion proteins such as maltose-binding protein (MBP) and

Table 4. Nonribosomol peptides produced in different host organisms.

Molecule	Original producer	Host organism	Gene cluster size (kb)	Vector	Comparative yield	Ref.
A54145	*S. fradiae*	*S. ambofaciens* *S. roseosporus*	Appr. 60	BAC	NR	[270]
Amicetin	*S. vinaceusdrappus*	*S. lividans*	37.3	Cosmid	NR	[271]
Argyrin	*Cystobacter sp.*	*Myxococcus xanthus*	30	pSynBio3	↑4-fold	[272]
Bacitracin	*B. licheniformis*	*B. subtilis*	49	Plasmid	↑2-fold	[273]
Cephamycin	*S. clavuligereus*	*S. flavogriseus* *S. coelicor* *S. albus*	35.6	Cosmid	↓ than original producer	[274]
Congocidine	*S. ambofaciens*	*S. lividans*	43	Cosmid	NR	[275]
Daptomycin	*S. roseosporus*	*S. lividans*	128	BAC	↓50-fold	[279]
Daptomycin	*S. roseosporus*	*S. lividans*	128	BAC	↓1/3 of original producer	[280]
Eniatin	*Fusarium oxysporum*	*B. subtillis*	NR	Fosmid	NR	[276]
Echinomycin	*Streptomycetes sp.*	*E. coli*	38	Cosmid	NR	[261]
Pacidamyci	*S. coeruleorubidus*	*S. lividans*	32.2	Cosmid	↑ than original producer	[259]
Polymyxin	*P. polymyxa*	*B. subtilis*	40.6	Fosmid	NR	[277]
Valinomycin	*Streptomyces*	*E. coli*	Appr. 18	Plasmid	↑33-fold	[278]

NR: Not reported, ↑: Higher yield than original producer, ↓: lower yield than original producer.

glutathione-S-transferase (GST) facilitated both the solubilization and purification by immunoaffinity chromatography of AMPs [17]. Crotamine [300] and ORBK [282] were expressed in fusion with MBP, while dermaseptin S4 [301] and bldesin [302] were expressed in fusion with GST.

Small ubiquitin modifier (SUMO) fusion proteins cannot be used as a tag, but they play a role in both solubilization and signaling of cleavage proteins [303]. SUMO is recognized by SUMO protease, allowing the later release of target AMPs [36]. Another fusion protein, the green fluorescent protein (GFP), is currently used as an indicator of gene signal expression [304] and it was also used for AMP expression [30]. In addition to the fusion proteins mentioned, his6-tag is a polypeptide that has been widely used for AMP purification, and when recombinant fusion proteins with this tag are expressed, they are purified from extract compounds by affinity chromatography [17,35,281,282]. This six-histidine sequence can be fused to the N- or C-terminal from target proteins and is easily recognized by fixed metal resins such as Cu^{++}, Zn^{++} or Co^{++} [295,305].

Some AMPs, such as plectasin [85], lsGRP1 [306] or cecropin A-LL37 [307] were fused to his6- and SUMO tags to facilitate the solubilization and purification of these recombinant proteins. However, the use of any fusion peptide/protein is not the general rule for achieving successful AMP expression. Some carrier proteins currently used do not facilitate the expression process of AMPs. Generally, AMPs are molecules of a hydrophobic and positive nature [308]. So, these characteristics make them a challenge for heterologous expression. MIP-3α, for instance, was fused to Trx, MBP, SUMO, his6-tag, ketosteroid isomerase (KSI) and calmodulin (CaM) -tag for expression in *E. coli* [309]. Only CaM allowed optimal conditions of expression, while Trx, MBP, SUMO or KSI produced this AMP in inclusion bodies, and his6-tag was toxic for the host cell [309].

Alternatively, the AMPs IMPI(I38 V) and gloverin were fused to cry4AaCter tag and their expression was compared with current recombinant GST and GFP fusion proteins [310]. Cry4AaCter tag is a protein that induces the inclusion bodies' formation and facilitates re-solubilization by alkaline pH [311]. IMPI(I38 V) and gloverin were seen to be more easily expressed and purified using cry4AaCter tag than when conventional GST and GFP fusion proteins were used [310]. These findings show that each AMP has its intrinsic features suitable for any specific fusion tag. For example, the acidic nature of calmodulin (CaM)-tag counterbalanced the basic feature of the AMP, allowing its expression in the soluble form [309].

4.2. Digestive strategies for AMP release from fusion proteins

Fusion tag/carried proteins need additional in vitro cleavage and chromatographic steps [295]. To recover target AMPs from fusion proteins, three cleavage strategies have been used [36]. The most common strategy to recover AMPs has been using endopeptidases [35], and several of these have been tested [36]. Endopeptidases recognize a specific short sequence of amino acids that should be previously inserted into the gene construction designed. These short signal sites can be hydrolyzed by some endopeptidases at their final C-terminal, releasing an intact N-terminal of target proteins [295,312]. However, some endopeptidases can cleave into their signal sequence [313–315]. Endopeptidases can change the primary structure of the excised protein, caused by the addition of extra amino acid residues. This may influence the target AMP activity [303]. An example is PDC1, which showed the best antifungal activity when its his6-tag was removed [316]. Another challenge for some endopeptidases is the capability for recognition of specific signal sites. Some of them have shown unspecific cleavage sites [317]. Enterokinase is an enzyme that releases an intact N-terminal in target proteins (DDDDK↓) [313], but it has also been shown to cleave other nonspecific sites such as TLR↓ [318], EDK↓, DNDK↓, QNAR↓, and WEYR↓ amino acid sequences [319]. Nonetheless, AMPs such as hepcidins [320], plectasin [297] or plantaricin [299] have been successfully recovered using enterokinase.

Endopeptidases such as thrombin or tobacco etch virus protease (TEV) have been added to amino acid residues in recombinant AMPs [36]. Thrombin is a serine protease that catalyzes coagulation-related reactions through the (LVPR↓GS) recognition site [314], while TEV recognizes and cuts the (ENLYFQ↓G) amino acid sequence [315,321]. Other enzymes such as SUMO protease technology [303] and factor Xa, which allow specific, efficient and accurate cleavage [295], were used to release recombinant AMPs [36]. Factor Xa, like thrombin, is involved in coagulation-related reactions, but its recognition site (IEGR↓) allows the target protein to be released without extra amino acids [314]. Similarly, SUMO protease, previously mentioned, structurally recognizes its target amino acid sequence, allowing a specific cut [303,312,322].

In addition to self-cleavage, AMPs can also be removed from fusion protein by using specific reagents such as cyanogen bromide (CNBr) or formic acid [30,241]. CNBr cleavage is a strategy that allows the peptide to be released from fusion protein by cutting the C-terminal of methionine residues [323]. Some AMPs, such as magainin2 [206] or ecAMP1 [324] and others, have this amino acid residue as part of their structural component (see AMPs database [325]). Recombinant cecropin A, for instance, decreased its antimicrobial activity when compared with its natural counterpart because of an alteration at the C-terminal before cleavage with CNBr [326]. Otherwise, the formic acid cut between (D↓P) amino acids, leading to the release of peptides at low costs [327]. Cecropin CM-4 [282] or lactoferricin/AMP-HP [328] were recovered using formic acid. However, this cleavage system adds an amino acid to any extremity of AMPs treated.

4.3. Non-chromatographic strategy for AMP purification

Although chromatographic methods are currently required for biopharmaceutical products due to the level of purity achieved [281], a cheaper strategy is being developed [329]. Non-chromatographic methods adopt physicochemical features such as aggregation and solubilization of some polypeptides in the purification process [330]. However, the purity level of the products is lower when compared to chromatographic methods [281].

Recombinant AMPs aim to produce them on a large scale and at low cost [30], and the use of elastin-like polypeptides (ELP) is important for applications in the pharmaceutical and biotechnological industry [329]. While other expression strategies use enzymes or reagents to recover AMPs, ELP added to intein decreases the production costs, making this system the cheapest [30]. The ELP system is a polypeptide made of repeated sequences of (VPGXG), in which X is any amino acid (except proline) [331]. When ELP is fused to the target protein, this fusion protein could be expressed in soluble or insoluble forms, depending on the strategy utilized [330]. ABP-CM4, CM4 and hep AMPs were fused to ELPs, showing effective antimicrobial activity [66,332,333].

AMPs carrying fusion proteins use an ELP soluble expression [334]. During the purification process, ELPs aggregate with temperatures up to 30 degrees, or adding salt, while they are re-solubilized at low temperatures such as 4 degrees [329]. Nevertheless, these solubility/aggregate conditions would vary with the target protein fusion [335]. Subsequent to purification, it is necessary to release the target protein from ELP. For recombinant AMPs, intein has been used [30]. Intein is a self-cleavage system that allows the target proteins to be released [336]. AMPs such as CM4 and human β-defensin 4 were released from ELPs by self-cleavage with this system [337].

Another strategy recently developed, as cheap purification steps, was the use of surfactant proteins [338]. DAMP4 is a protein packed in four alpha-helices when solvated, which endures high temperatures (90°C) and relatively high salt concentrations [339]. These advantages allow it to be separated from other compounds of cellular extract even when submitted to this temperature and/or salt conditions [338]. Another advantage of DAMP4 is that it is expressed efficiently in *E. coli* and has been seen to facilitate the expression of AMPs in fusion [340,341], such as pexiganan and omniganan, which were successfully expressed using this system [338].

5. AMPs under clinical trials

To date, many AMPs have reached at least one of three clinical trial phases. In these circumstances, the contrast is evident with the relatively small number of molecules that have reached the market. Several challenges mark the path that AMPs go through from their initial assessment to their marketable status. Undoubtedly, the main barrier to the commercial development of AMP-based drugs is the poor therapeutic performance in vivo as compared to in vitro [342].

This finding is a consequence of preclinical tests being conducted under conditions different from those observed in human physiology, which overlooks the presence of ions at altered levels under physiological conditions, which may negatively influence the biological activity of AMPs; test conditions were under higher salt concentrations (which increases the ionic strength governing interactions between electrically charged AMPs). The presence of physiological incompatibilities from various natures, such as active proteases, considerably reduces the half-life of drug candidates and, finally the physiological heterogeneity present in the different animal models used in vivo tests is not reproducible in humans with the same level of performance [343,344].

In addition, another limitation to the complete development of AMPs for clinical use is the usually low oral bioavailability of such peptides under physiological conditions. In the gastrointestinal environment, many peptides are degraded by gastric proteases, lose their activity influenced by low gastric pH, and/or have little absorption in the intestinal epithelium [345,346]. Thus, several strategies are being pursued to increase the chances of success in preclinical tests and in the development of AMP-based drugs, not only in terms of improved bioavailability but also in increased pharmacokinetics, stability and pharmacodynamics [342]. The improvement of AMPs follows two complementary approaches: (i) chemical modifications to increase bioavailability and (ii) bioengineering for increased pharmacokinetics and pharmacodynamics.

As the vast majority of AMPs tested in vivo exhibit up to 1% bioavailability and poor distribution, structural chemical modifications are carried out to enhance their metabolic stability, lipophilicity, and permeability [24,347]. Among the desirable effects of structural modification of AMPs, the most popular are: increase in metabolic stability after cyclization; increase in structural stability and permeability in the intestinal epithelium or by substitution of amino acid residues genetically encoded by non-canonical amino acids or by N-/C-terminal modifications (C-terminal amidation and/or N-terminal acylation, eventually with peptide lipidation and alkylation of the ε-amine group of Lys side chains).

Bioengineering for the improvement of pharmacokinetics and pharmacodynamics can be carried out in different ways. Most notable is AMP encapsulation using nanoparticles (NPs) (hydrophobic, in gold or magnetic) for targeted site delivery [348]. The construction of multiple peptide sequences fused to a nucleus of branching – the highly stable and poorly hemolytic dendrimer peptides [349]; the method of biosynthesis and delivery of AMPs by genetically modified bacteriophages to increase the lysis of pathogenic host bacteria [350] and the covalent immobilization of AMPs in biomaterials for the production of active biofilms against adherent bacteria [351,352].

Among the most promising AMPs evaluated in clinical trials, many were synthesized according to structural enhancement or bioengineering strategies. Table 5 shows some of the AMPs currently in the pipeline for commercial development and emphasizes which phase of clinical trials each one falls into.

6. Market-approved AMPs

Although AMPs have highly desirable intrinsic characteristics for their development in commercial products, and the number of patent deposits describing new AMPs has increased considerably over the past 10 years, relatively few AMPs have reached the final stages of clinical trials prior to manufacturing [367]. The major issues raised by pharmaceutical companies regarding the low availability of commercial AMP-based drugs are the usually low absorption and half-life of these molecules, resulting in commonly poor pharmacokinetics [368].

Other limitations in the development of commercial AMPs from bench to batch involve the usually low oral bioavailability

Table 5. Antimicrobial peptides at clinical trials stage. Patents were obtained from Justia Patents (https://patents.justia.com/Accessed April, 2020).

Patent number	Peptide	Origin	Patent year	Evaluated condition	Clinical trial phase	Institute/Company	Ref.
US6514692	p113	Human saliva	4 February 2003	Treatment of HIV	II	Demegen, Inc	[353]
US7060677	hLF1-11	Derived from human lactoferrin	13 June 2006	Treatment of bacterial infections	I/II	AM-Pharma B.V	[354]
US7544814	nPI0052	Marine microrganisms *Salinispora tropica* and *Salinispora arenicola*	9 June 2009	Treatment of human colorectal carcinoma, human prostate carcinoma, human breast adenocarcinoma, human non-small cell lung carcinoma, human ovarian carcinoma and human multiple myelomas	I	Nereus Pharmaceuticals, Inc.	[355]
US7713927	c16G2	Synthetic peptide	11 May 2010	Prevention of dental caries caused by *Streptococcus mutans* and others Gram positive bacteria infections	II	C3 Jian Inc.	[356]
US7968588	wap-8294A2	Lysobactor spp.	28 June 2011	Gram positive bacteria VRE and MRSA	I/II	eRigen Pharmaceuticals, Inc.	[357]
US8283371	nVB-302	Lantibiotic	9 October 2012	Treatment of microbial infections, in particular *Clostridium* infections	I	Novacta Biosystems Limited	[358]
US8507647	surotomycin	Cyclic lipopeptide	13 August 2013	*Clostridium difficile* (diarrhea) and others bacterias, including resistant strains	III	Cubist Pharmaceuticals, Inc.	[359]
US8530409	pexiganan	Magainin-2 analogue	10 September 2013	Treatment of infected ulcers	III	Dipexium Pharmaceuticals LLC	[360]
US8828922	DiaPep277™	Human Heat Shock Protein 60 (Hsp60)	9 September 2014	Treatment of type I Diabetes mellitus	III	Andromeda Biotech Ltd.	[361]
US9181303	nP213	Cyclic cationic peptide	10 November 2015	Treatment or prevention of microbial infections especially fungal infection of the skin and nails.	IIb	NovaBiotics Limited	[362]
US9521846	pOL7080	Protegrin analog	20 December 2016	Treatment of *Pseudomonas aeruginosa* pneumonia	II	Polyphor Ltd.	[363]
US9567367	p2TA (AB 103)	Synthetic peptide	14 February 2017	Treatment of bacterial infections and associated inflammation, especially necrotizing soft tissue infections	III	Atox Bio Ltd.	[364]
US9850279	iMX942 (SGX942)	Indolicidin analog	26 December 2017	Treatment of oral mucositis	II	Soligenix, Inc.	[362]
US9963488	mU1140	*Streptococcus mutans* (lantibiotic)	8 May 2018	Gram positive bacteria (MRSA, *C. difficile*)	Preclinical	Oragenics, Inc.	[365]
US10226508	IL37	Cathelicidin analog	12 March 2019	Treatment of a chronic ulcers	IIb	Promore Pharma AB	[366]

HIV: Immunodeficiency virus infection, VRE: Vancomycin-Resistant *Enterococcus*, MRSA: Methicillin-resistant *S. aureus*, ™: Unregistered mark.

of AMP formulations, in addition to inadequacies in gene expression in vitro and in vivo and high production costs due to low scalability [367]. Thus, relatively few AMPs have received the required certification for market entry and clinical use, issued by regulatory agencies such as the US FDA and the European Medicines Agency (EMA). Notably, all certified AMPs show therapeutic potency equal to or greater than already available commercial analogs, low toxicity to humans, broad spectrum of action/targets, low residual tissue accumulation, and low potential for resistance induction [342]. Table 6 shows all AMPs approved for clinical use by the FDA. In common, they have the anti-polymicrobial activity of the skin and soft tissue.

7. Conclusion

The search for new antimicrobial drugs has increased in recent years due to the emergence and staying-power of resistant bacteria selected by the inappropriate use of antibiotics and other factors. AMPs present themselves as promising molecules that have a broad antimicrobial spectrum and a specific antimicrobial mechanism of action that decreases the possibility that bacterial strains will develop resistance. However, obtaining these molecules is not at all easy. The traditional method of extraction includes low yield and low purity, while chemical synthesis can be costly for the production of certain peptides, depending on molecular weight and folding. Genetic engineering presents different heterologous expression strategies for the production of pure and functional AMPs through the use of different hosts, such as bacteria and yeast. Many tools have been developed in this area to solve common problems in the production of recombinant AMPs, such as post-translational modifications, folding and quantity. Choosing the right host according to AMP-specific chemical and physical characteristics, as well as techniques such as codon optimization, multimeric gene copying, fusion proteins, and optimized promoters, can enable these molecules to achieve not only clinical trials but also to enter the pharmaceutical market as another option for antibiotics.

8. Expert opinion

Although antibiotics have revolutionized medicine and saved millions of lives, their misuse around the world since the end of World War II has made bacterial infections become a global problem once again due to the emergence of antibacterial resistance. As a result of overuse, inappropriate prescription, extensive agricultural use, low availability of new antibiotics and regulatory barriers, several bacteria are already classified as a threat by different organizations such as the Centers for Disease Control and Prevention (CDC), the World Health Organization, and the Society for Infectious Diseases of America. Among antibiotic-resistant bacterial infections, resistant species of *S. aureus* and *Enterococcus* represent the largest threat among Gram-positive pathogens. The most severe gram-negative ones are *Klebsiella pneumoniae, P. aeruginosa*, and *Acinetobacter*. These resistant bacteria generate considerable costs for healthcare systems because they require prolonged treatments that are often ineffective, leading to a higher number of deaths compared to nonresistant infections. Measures such as new policies for tight control of antibiotic use and

Table 6. Marked antimicrobial peptides approved by the FDA. The marked drugs were obtained from Drugs Data Base http://www.drugsdb.eu/index.php?l=c (Accessed July, 2019) n.d., and from THPdb: Database of FDA-approved peptide and protein therapeutics [369]).

AMP	Examples of developer companies	Clinical prescription	Administration
tyrothricin	Nutribon Inc. Pharmavite Laboratories Inc. Les Produits Gerbex Inc.	Isolated from *B. brevis*. Administered with other peptides and antibiotics Indicated for the treatment of skin and membrane of the pharynx infected by Gram-positive bacteria	Topical application
gramicidin S	Merck	Dibasic cyclic hexapeptide peptide isolated from soil bacilli in the early 1940 s Broad spectrum antibacterial and antifungal peptide utilized combined with polymyxin B or neomycin	Topical application Toxic for systemic use
gramicidin D	Pharmetics Inc. Glaxo Wellcome Novartis Pharmaceuticals Sandoz Canada Inc.	Linear pentadecapeptides from *B. brevis* Recommended for the treatment of topical lesions, superficial skin wounds and eye infections	External use
bacitracin	Akorn, Inc. Bausch & Lomb Inc. E. Fougera & Co. Physicians Total Care, Inc. Rebel Distributors Corp. E. Fougera And Co. Fera Pharmaceuticals Pharmacia And Upjohn Co. Pharmacia And Upjohn Co. Physicians Total Care, Inc. Sagent Pharmaceuticals	Mixture of cyclic peptides from *B. subtilis* Skin and eye infections	Topical and intramuscular application
daptomycin	Merck Sharp & Dohme Corp. OSO BioPharmaceuticals Manufacturing, LLC.	Cyclic lipopeptide from *Streptomyces roseosporus* Skin infections caused by Gram-positive bacteria	Intravenous
polymyxin E	Physicians Total Care, Inc. Endo Pharmaceuticals, Inc.	Mixture of cyclic lipopeptides from *Bacillus colistinus* Recommended for the treatment of gastrointestinal tract infections and for multidrug-resistant Gram-negative bacteria	Intramuscular, intravenous
polymyxin B	App Pharmaceuticals, LLC. X-gen Pharmaceuticals, Inc. Bedford Laboratories	Mixture of cyclic lipopeptides from *Bacillus polymyxa* Indicated for multidrug-resistant gram-negative bacterial infections	Intramuscular, intravenous, ophthalmic

population education regarding management are important to mitigate the global impact of this socioeconomic problem. However, when it comes to antimicrobial resistance, it cannot be overlooked that the development of research for the production of new antimicrobial drugs is of fundamental importance. New targets and mechanisms of action are desirable for new therapeutic applications. In this context, AMPs arise as a new antibacterial therapeutic strategy. These molecules are part of the innate immune system of many organisms and are induced in the presence of pathogens and cytokines, capable of eliminating infections caused by many microorganisms such as Gram-positive and negative bacteria, fungi and viruses. One of the favorable features of AMPs is their specificity, as they interact electrostatically with the bacterial wall, forming pores and causing cell disruption. They can also penetrate the cell and disrupt the synthesis of molecules essential for the functioning of cellular metabolisms such as DNA, RNA or proteins, which can inhibit cell wall synthesis or may alter the cytoplasmic membrane. Although extraction from natural sources allows for high biological activity from AMPs, the genes encoding these peptides are expressed in small amounts and under conditions of environmental stress, thus incurring high costs, running the risk of impurity and achieving low yields for the peptides. Chemical synthesis has emerged as a solution to increase the therapeutic application and scientific research on AMPs, overcoming the low yield problems and the complicated extraction process with high purity. However, this technique can be costly for the synthesis of large-scale peptides and peptides containing many amino acid residues and post-translational modifications. The heterologous expression of AMPs has certain advantages compared to these strategies. The low cost and short production period are some, as is high yield. Many systems are available to produce AMPs, and different strategies can be used to optimize the production of these molecules. The use of fusion tags, tandem multimeric expression, promoters and strains appropriate to the specific characteristics of each AMP and codon optimization can considerably improve the production of stable and functional AMPs in sufficient quantities for further activity, pharmacodynamics, pharmacokinetics, toxicity studies and half-life time.

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Research Article

Chemical Characterization of *Aspergillus flavus* α-Amylase Synthesized in Tobacco Plants Engineered by CRISPR/Cas 9

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ABSTRACT

The endo-type group digestive enzymes, such as α-amylases, play a crucial role in insect carbohydrate metabolism. They primarily catalyze the conversion of starch into structurally simpler carbohydrates by hydrolysis. Despite causing post-harvest damage to grains, such as peanuts and beans, *Apergillus sp*. α-amylases can be used for industrial purposes in food, chemical, and textile modification, adding value to the production chain by increasing the conversion of useful carbohydrates for manufacturing purposes. The amylolytic enzyme studied in this report was biosynthesized in tobacco plants transiently transformed by genome editing using CRISPR/Cas 9 system. It showed specific characteristics when compared to other α-amylases from different organisms, such as unique kinetics, an optimal temperature of 50°C, and an optimal pH (6.0). Starch hydrolysis catalyzed by *Apergillus flavus*. α-amylase was measured using Ionic enhancers and different inhibitors, revealing a decreased amylolytic activity when complexed with metallic ions and organic compounds.

Keywords: α-amylase; Genome edition; CRISPR/Cas9 System; *Apergillus flavus*

INTRODUCTION

Amylases (α-amylase, β-amylase, and glucoamylase) are among the most important products obtained from Biotechnology nowadays. Great amounts of these enzymes can be produced by many microorganisms such as bacteria, yeast, and filamentous fungi, especially from species of the genera *Aspergillus* such as *Aspergillus niger*, *Aspergillus awamori*, and *Aspergillus oryzae* [1,2].

The basic amylolytic property of fungal derived α-amylases (1,4 a-glucan glucanohydrolase, EC 3.2.1.1), is the catalytic conversion of starch into different soluble sugar sub-products, what confers the enzyme an increased interest in food, detergent, chemical, textile, and other industrial purposes [3].

Nevertheless, the starch degradation promoted by the seed crop infesting fungus *Aspergillus flavus* α-amylase (Af α-amylase) confers an important carbon source readily metabolized *via* glycolysis for and is intimately associated with aflatoxins production, having an important role in the induction of these potent liver toxins and carcinogens family of structurally related secondary metabolites biosynthesis [4].

This species has economic importance since it can infect many agronomically important host crops such as cottonseed, carrot, corn kernels, peanut, and tree nuts causing millions of dollars in losses a year worldwide [5-7].

Despite its importance, Af α-amylase is only synthesized in conditions of fungal infection of grains and at low concentrations, which makes it difficult to obtain from natural sources. In this way, recombinant systems of gene expression can become alternatives for the biosynthesis of α-amylases, in sufficient quantities for its use in scientific research and for industrial purposes [8].

In recent years, tobacco plants have proved to be interesting tools for the biosynthesis of recombinant proteins. In addition to being easy to handle, tobacco has the characteristics of a model organism for biotechnology. Its genome has already been sequenced, it has easy genetic transformation and hot spots of gene expression, preferred targets for genome editing, have already been properly mapped [9].

One of the challenges in the use of tobacco as a reactor for recombinant proteins is the randomness in the integration of

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the expression cassette in genomic sites of the plant, which often leads to low levels of transgenic expression [9]. In this context, the CRIPSR/Cas 9 system shows itself as a recommended technology to direct the integration of transgenes to genomic sites associated with high levels of gene expression that allow obtaining satisfactory amounts of protein [10].

The evaluation of the biochemical properties, and kinetic parameters of new related types of Af α-amylase synthesized by genome edited tobacco can provide an increase of efficiency in industrial applications and improve crop storage conditions to avoid degradation of seed storage tissues, loss of agronomical quality, and mycotoxin contamination.

The focus of this report is the evaluation of the biochemical properties of an Af α-amylase, such as optimal temperature and pH for starch degradation, determination of kinetic parameters and ionic and organic compound inhibition, elucidation of protein tertiary structure by a three-dimensional model construction, and analysis of possible amino acid residues directly involved in the catalytic activity of the enzyme.

MATERIALS AND METHODS

Construction of the gene expression vector

DNA amplification by PCR: The amplification of the DNA fragment encoding Af α-amylase (GenBank accession number: AF139925.1), was performed using the primers shown in Table 1 and cloned into the vector p-sgRNA34 (3954 bp), under the control of the cauliflower mosaic virus (CaMV) 35S promoter and octopine synthase (OCS) terminator, upstream of the coding sequence of the guide RNA for genome editing *via* CRISPR/Cas9.

Primer	Sequence	Size	Tm
Alpha-Forward	5'-AGGTATTTACCATGAAGACACGATGTTGAA-3'	30 bp	51°C
Alpha Reverse	5'-AAGCTGAGACCTTAATGATATGGAAGTGT-3'	29 bp	53°C

Table 1: Primer oligonucleotides used to amplify the Af α-amylase coding sequence.

The sgRNA coding sequence, complementary to the last 30 nucleotides of the 5` UTR of the *Nicotiana tabacum* ribulose bisphosphate carboxylase (RuBisCO), the most highly abundant transcript in tobacco [11]. Plasmid p-TruCas9 (6576 bp) was used as the expression vector for Cas9, containing the Cas 9 gene of Streptococcus pyogenes under the control of the folded 35S promoter CaMV and the Tobacco Mosaic Virus (TMV) replicase terminator.

Genetic transformation of *Agrobacterium tumefaciens*: The electroporator (MicroPulserTM Electroporation Appartus, Bio-Rad, USA) was adjusted to the appropriate conditions: voltage of 2.2 kV, capacitance of 25 μF and resistance to 200 Ω (for a cell with 0.1 cm of distance between the electrodes). Then, aliquots containing 45 μL of electrocompetent *Agrobacterium tumefaciens* (GV3101) cell suspension were thawed on ice. 100 ng of DNA related to the recombinant vectors were added to each aliquot. The aliquots containing the mixture of cells and DNA were transferred to the cuvettes, which were inserted into the electroporator and the electric pulse applied. Immediately, the cells were suspended in 1 ml of liquid LB medium; transferred to a 1.5 mL microcentrifuge tube and incubated at 28°C, under agitation of 180 rpm for 90 minutes. Then, 200 μL of the suspension was plated in Petri dishes containing solid LB medium plus the appropriate selection antibiotic (kanamycin (100 μg/mL).

Agroinfiltration of *N. tabacum* leaves: A colony of *A. tumefaciens* from each of the constructions was separately inoculated in 5 mL of liquid LB medium plus kanamycin (100 μg/mL), and then the inoculants were incubated at 28°C with 180 rpm shaking for 48 H. After the incubation period, the cells were collected individually by centrifugation at 7,000 rpm for 4 minutes. Then, the precipitate was re-suspended in 2 mL of saline (infiltration buffer; 10 mM MgCl2) and the OD was measured by means of a spectrophotometer under a 600 nm wavelength (OD 600 nm). Then, dilution calculations were made to produce a cell suspension containing the pro-vectors and recombinant vectors in a final OD of 0.3. Thirty *N. tabacum* plants were used with 15 days of emergence in the soil. Leaves were agroinfiltrated in the abaxial part, using a syringe without a needle.

Extraction of total soluble proteins: Four leaf discs of *N. tabacum* were highlighted for the extraction of total soluble proteins (TSPs), 5 days after agroinfiltration. These were immediately placed in microcentrifuge tubes (2 mL) and stored at -80°C for future analysis. TSPs were extracted using 4x (m/v) of the extraction buffer (50 μM Tris HCl, pH 7.5; 150 μM NaCl; 0.1% Tween-20; 0.1% β-mercaptoethanol) [12-14], plus 10% (v) of proteinase inhibitors (PIC; Sigma-Aldrich, Missouri, United States). The leaf discs were macerated with the aid of metal spheres, under agitation of 30 Hertz in a tissue processor (Tissuelyser II, Quiagen, Hilden, Germany) for 3 minutes. Then, the samples were centrifuged at 8,000x g for 15 minutes at 4°C and the supernatant was transferred to a new microcentrifuge tube (1.5 mL). The samples were clarified by means of a new centrifugation at 8,000x g, for 10 minutes at 4°C, and the supernatant transferred to a new tube.

α-amylase purification: Protein extracts (20 mg/ml) were applied into an affinity chromatography Sepharose-6B conjugated with β-cyclodextrin equilibrated with 0.1 M phosphate buffer, pH 7.2, containing 20 mM NaCl and 0.1 mM CaCl2. Positively charged proteins were collected from previously numbered tubes 1 to 17. After the addition of 20 ml of 1 M of NaCl into the column, proteins with negative charge were eluted and collected in tubes 18 to 44. Fractions (2.0 ml) were collected at a flow rate of 28 ml h^{-1} and used to measure a-

amylolytic activity. Retained fractions were pooled, concentrated with TCA (Trichloroacetic Acid) 12%. After having purified and selected the proteins for changes, it went measured to absorbance (280 nm) in the spectrophotometer, detected of the purified enzyme and was analyzed by SDS-PAGE mini-gels 15% at a standard concentration of 10 mg.ml^{-1} according to Laemmli (1970).

Enzymatic assays: We used Bernfeld protocol (1955), to measure Af α-amylase. Enzyme activity was evaluated under concentrations of 2 mg/ml, diluted in Tris-HCl buffer 0.05 M, pH 7.0. Starch (1% w/v) was added to the reaction as substrate. Each fraction was incubated at 37°C for 20 min. The enzymatic reaction was stopped by adding 1.0 ml of 3.5 DNS (1% dinitrosalicylic acid dissolved in 0.2 M NaOH and 30% sodium potassium tartrate) and was evaluated by optical density at 530 nm. Each assay was carried out in triplicate.

Biochemical characterization of *Aspergillus flavus* α-amylase: The determination of Af α-amylase biochemical properties was carried out under a standard concentration of 20 mg.ml^{-1} diluted in 0.05 M Tris-HCl buffer, pH 7.0.

The structural stability and activity of the enzyme were evaluated by catalytic assays under different pHs and temperatures. Triplicate samples conatining α-amylase (2 mg/ml-20 µl) were submitted to treatments with 0.05 M Tris-HCl buffer under acidic pHs (pHs 4,5 and 6), neutral (pH 7) and basics (pHs 8, 9 and 10), and different temperatures (25°C, 30°C, 40°C, 50°C, 70°C and 90°C/10 min). After incubation, 100 µl of starch 1% (w/v) was added to each sample, and incubated for 20 min at 37°C.

The modulation of the enzymatic activity was measured using 14 ions and organic compounds, such as: Acetone, AgCl2, AgNO3, CaCl2, CuSO4, FeCl3, Glicine, KCl, KNO3, MgCl2, MnCl2, NaCl, Proline and ZnSo4 with final concentrations of 0.05 mg.ml^{-1}. Samples were composed by enzyme (2 mg.ml^{-1}), the respective ion solution (0.05 mg.ml^{-1}), Tris-HCl buffer (50 mM), pH 7 and starch 1% (w/v), and incubated for 20 min at 37°C. All samples were carried out in triplicate.

Kinetic parameters of *Aspergillus sp.* α-amylase: Starch enzymatic hydrolyzation was carried out according to Tester. For evaluation of the kinetic parameters of Af α-amylase. Samples (20 µl) from reaction mixture containing Af α-amylase and also with substrate concentrations (0,33%; 0.30%; 0,26%; 0,23%; 0,20%;0,16%; 0,13%; 0,10%; 0,06%; 0,03% and 0,01%) were dissolved in 50 mM Tris-HCL buffer (pH 7.0) at 37°C/20 min. The reactions were immediately stopped by adding dinitrosalicylic acid (0.1% v/v) and incubation for 20 min at 95°C. The amount of reducing sugars produced from soluble starch was measured according to Lindsay (1973). Kinetic data were transformed to Lineweaver-Burk plots and Km values were calculated from the slopes of the curves.

RESULTS

Biochemical characterization of *Aspergillus sp.* α-amylase

For the evaluation of Af α-amylase biochemical properties, enzymatic samples were submitted for purification and loaded onto an Epoxi-Sepharose 6B affinity column. A chromatographic profile was obtained from protein elution by a single step of NaCl 1 M, (Figure 1).

Fractions with larger peaks observed in the graphic (tubes 8,9,21,22,23,24,25,26 and 39) were selected and stored at 5°C. Tube 39 showed the highest level of absorbance at 530 nm and its contents were resolved by SDS-PAGE mini-gel 15%, at concentrations of 2, 4, 6, 8, and 10 µg.ml^{-1} as shown in Figure 2.

Purified α-amylase sample presented molecular mass near 52 kDa as observed using SIGMA's low mass protein marker and related previously in the literature.

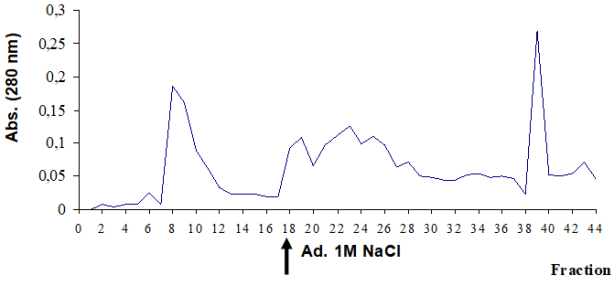

Figure 1: Chromatographic profile obtained during the purification of Af α-amylase using EpoxiSepharose 6B chromatography collumn equilibrated with 0.1 M phosphate buffer, pH 7,2, containing 20 mM NaCl and 0.1 mM CaCl2. The black arrow indicates the single-step application of 1 M NaCl.

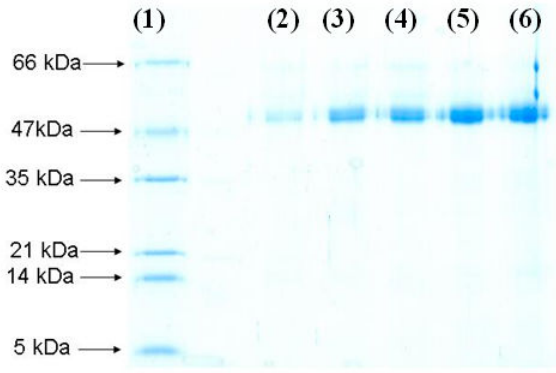

Figure 2: Electrophoretic profile of Af α-amylase showing an approximated mass of 52 kDa resolved purified fraction. 1 shows 7 µl of SIGMA's low mass marker. 2, 3, 4, 5, and 6 show respectively 2, 4, 6, 8, and 10 µg.ml of the purified fraction by affinity chromatography.

Kinetics parameters of amylolytic activity were determined by a reaction ratio vs. substrate concentration curve, which produced a Michaelis-Menten kinetic profile, indicating a hyperbolic curve represented by an increase in starch hydrolysis until a substrate saturation point remarked by enzymatic activity stabilization (Figure 3).

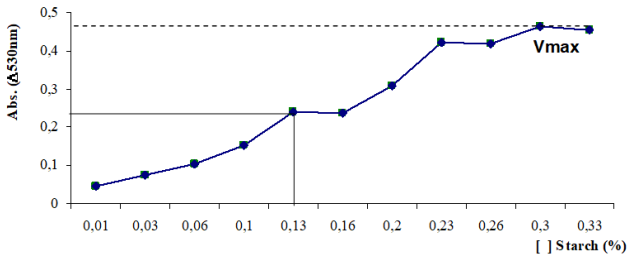

Figure 3: Enzymatic activity by a variation in starch concentration. An accentuated increase in amylolytic activity is observed until the starch concentration reaches 0,23%, followed by the curve`s stabilization. The calculated Km ([S]=1/2 Vmax) for starch was 0,13% (w/v).

The construction of a thermostability curve showed higher α-amylolytic activity under 25 to 50°C (Figure 4A). The enzyme structural stability was maintained until 45°C as suggested by Figure 4B, getting a pronounced decrease after 55°C. Around 70°C it is possible to observe both decreases of activity and structural stability, showing that under this temperature denaturation process is taking an advanced course.

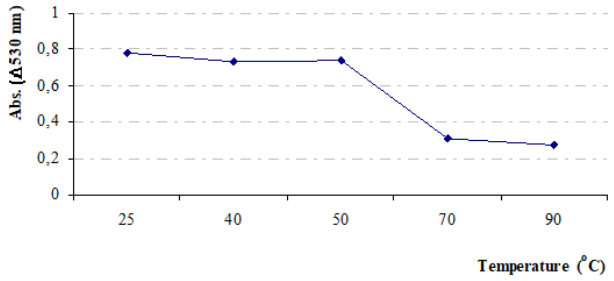

Figure 4: (A) Effects of temperature on Af α-amylase amylolytic activity.

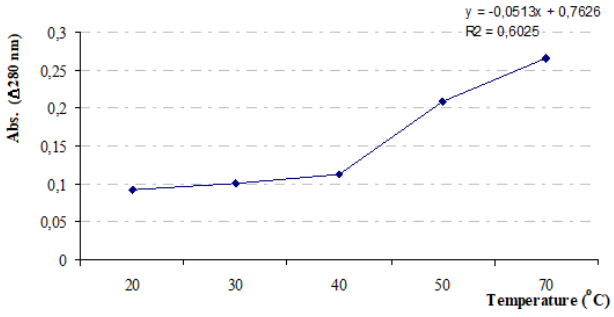

Figure 4: (B) Structure stability.

The denaturation process results in aromatic hydrophobic residues exposition, increasing the absorbance ratio greatly after 50°C, corroborating with the decrease of amylolytic activities. The highest Af α-amylase activity was observed under pH 6.0 (Figure 5A), decreasing greatly after pH 7.0. Lower activities occurred under acidic pH 4.0 and under alkaline pHs (9.0 and 10.0).

The decrease of enzymatic activity at pH 7.0 corroborates with the greater absorbance ratios under this condition, indicating a less-stable protein form under neutral pH like suggests in Figure 5B.

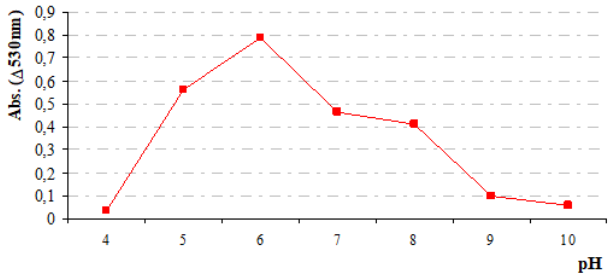

Figure 5: (A) Af α-amylase amylolytic activity.

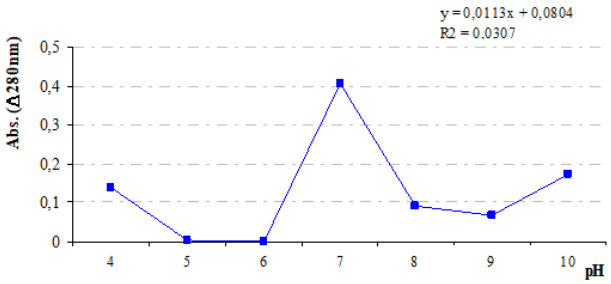

Figure 5: (B) Structural stability under different pH conditions.

Af α-amylase amylolytic activity was tested in the presence of ions and organic compounds Acetone, AgCl2, AgNO3, CaCl2, CuSO4, FeCl3, Glicine, KCl, KNO3, MgCl2, MnCl2, NaCl, Proline, ZnSO4. All substances decreased amylolytic activity, specially AgNO3 and Glicine. Acetone was responsible for the sweetest decrease, followed by CaCl2, NaCl, MnCl2, FeCl3, KNO3 and AgCl2 (Figure 6).

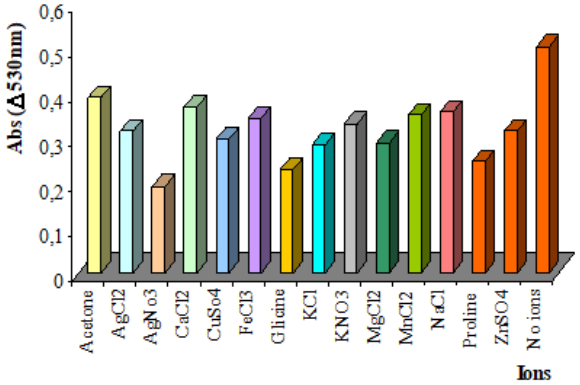

Figure 6: Ionic and organic compounds effects on Af α-amylase amylolytic activity.

DISCUSSION

This report determined several properties for Af α-amylase. According to our results, the enzyme showed higher activity between 25 to 50°C, and the lowest activity at 90°C, which suggests the effect of thermal denaturation and loss of specificity and catalytic efficiency by the enzyme.

Denaturation may cause the exposure of hydrophobic amino acids that were hidden inside the internal surface of the active site and were crucial for catalysis and protein stabilization as tryptophan and phenylalanine [15].

Thermal stability (between 20 and 40°C) and optimum temperature for catalytic activity (near 40°C) for this Af α-amylase reflects similarities between the enzyme and α-amylase isolated from bean plague *Acanthoscelides obtectus* [16].

Also, similar results for the activity of an α-amylase from Peruvian carrot (*Arracaia xanthorrhiza* Bancroft.) under optimum pH of 6.0 were found by Pires et al., 2002. Both enzymes, from *Aspergillus sp*. and Peruvian carrot has a pH range between 5.0 and 7.0, that still confers catalytic activity [17].

What makes this Af α-amylase interesting for industrial purposes is the maintenance of the catalytic efficiency under a wide range of pH without abrupt decrease, which is desirable for long-term carbohydrate hydrolysys [18].

The pH range observed for the enzymatic activity of *Rhizopus oligosporus* α-amylase varied from 3.0 to 5.5, significantly different from our observation in *Aspergillus sp* [19]. This indicates variation between the two fungi enzymes on the ionized ratio of catalytic functional groups from residues presented in the internal surface of the catalytic site.

In the presence of al ions and organic compounds tested Af α-amylase showed a lower activity ratio than in absence of these substances. Differently from what Dutta et al., 2005 have observed, since crustacean *Heliodiaptomus viduus* (Gurney) [20]. the α-amylase amylolytic activity showed a great enhance in the presence of metal ions like $Fe2^{+}$, $Ba2^{+}$, $CO2^{+}$, $Ag2^{+}$, and $Mn2^{+}$, up to 130%-200% of the original activity. Otherwise, in the presence of $Cu2^{+}$, $Mg2^{+}$, Amylase activity was completely inhibited like in Aspergillus sp., but in a more drastic way than ours, preserving only 5% of the original activity.

CONCLUSION

The structural variability between α-amylases found in nature can explain the variation in the activity profiles between these enzymes in a great range of organisms. This is particularly interesting when α-amylases from individuals from different species under the same kingdom show less kinetic similarity than that observed organisms of different kingdoms.

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CHAPTER FOUR

Screening for cysteine-stabilized scaffolds for developing proteolytic-resistant AMPs

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Contents

Abstract

Antimicrobial peptides (AMP) are present in all organisms and can present several activities and potential applications in human and animal health. Screening these molecules scaffolds represents a key point for discovering and developing novel biotechnological products, including antimicrobial, antiviral and anticancer drugs candidates and

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insecticidal molecules with potential applications in agriculture. Therefore, considering the amount of biological data currently deposited on public databases, computational approaches have been commonly used to predicted and identify novel cysteine-rich peptides scaffolds with known or unknown biological properties. Here, we describe a step-by-step *in silico* screening for cysteine-rich peptides employing molecular modeling (with a core focus on comparative modeling) and atomistic molecular dynamics simulations. Moreover, we also present the concept of additional tools aiming at the computer-aided screening of new Cs-AMPs based drug candidates. After the computational screening and peptide chemical synthesis, we also provide the reader with a step-by-step *in vitro* activity evaluation of these candidates, including antibacterial, antifungal, and antiviral assays.

1. Introduction

Antimicrobial peptide (AMP) are molecules that present up to 10 kDa and has been found in all organisms (Huan, Kong, Mou, & Yi, 2020; Nijnik & Hancock, 2009). Cysteine-stabilized peptides (Cs-AMPs) correspond to a group that presents conserved cysteine motifs (Table 1) and a broad variation in sequence and numerous structural profiles (β-sheets, α-helical, or a mixture of these two secondary structures) (Porto, Pires, & Franco, 2017). These peptides can present various activities (de Oliveira Dias & Franco, 2015; Srivastava et al., 2021), acting as promising model molecules for the generation of future peptide based-drug candidates with and potential applications in medicine, veterinary medicine, and agriculture, including the development of novel antimicrobial and antiviral properties

Table 1 Conserved cysteine motifs present in antimicrobial peptides families.

Peptide family	Conserved cysteine motifs	Reference
Lipid transporter proteins (LTPs)	$Cx_{(7–9)}Cx_{(12–14)}Cx_{(8–19)}Cx_{(1)}Cx_{(19–23)}Cx_{(13–15)}C$	Shelenkov, Slavokhotova, & Odintsova, 2020
Snakins	$CX_{(3)}CX_{(3)}CX_{(8)}CX_{(3)}CX_{(2)}CCX_{(2)}CX_{(1)}CX_{(11)}CX_{(1)}CX_{(12)}C$	
Heveins	$CX_{(3,8)}CX_{(4)}CCX_{(5)}\ CX_{(6)}CX_{(3,5)}CX_{(1,3)}C$	
Thionin	$CCX_{(11)}CX_{(9,15)}CX_{(5)}CX_{(6,11)}C$	
Cyclotides	$CX_{(3)}CX_{(4,5)}CX_{(4,6)}CX_{(1)}CX_{(4,5)}C$	
Cis-defensins	$CX_{(10)}CX_{(5)}CX_{(3)}CX_{(9)}CX_{(6)}CX_{(1)}\ CX_{(3)}C$	

(Boas, Campos, Berlanda, de Carvalho Neves, & Franco, 2019; Fleitas Martínez, Cardoso, Ribeiro, & Franco, 2019; Maximiano & Franco, 2021).

In this context, computational approaches are increasingly used to study peptide/protein functions from their primary sequence to their tridimensional (3D) structural arrangement, which can be investigated through *in vitro* and *in silico* techniques. *In silico* methodologies have assisted researchers to predict peptide/protein atomic coordinates in the absence of experimentally elucidated structures, which are commonly characterized by X-ray crystallography, solution nuclear magnetic resonance (NMR) or solid-state NMR, and cryo-electron microscopy (Cardoso, Oshiro, Rezende, Candido, & Franco, 2018).

Thus, comparative molecular modeling comprises a useful computational tool for 3D peptides' structure prediction (Fiser & Šali, 2003). Specifically, comparative molecular modeling uses one or more peptide/proteins of known structure (templates) to predict the 3D structure of a given peptide/protein sequence (target) based on sequence alignments (Webb & Sali, 2016). Currently, there are numerous web servers for automating comparative peptide/protein modeling (Table 2).

Table 2 Web servers are used to predict the 3D structures for peptides and proteins.
Web servers—comparative modeling

Name	Link	References
3D-JIGSAW server	https://bmm.crick.ac.uk/~svc-bmm-djigsaw/help_crick.htmL	Bates, Kelley, MacCallum, and Sternberg (2001)
HHpred	https://toolkit.tuebingen.mpg.de/tools/hhpred	Söding, Biegert, and Lupas (2005)
IntFOLD	http://www.reading.ac.uk/bioinf/IntFOLD/IntFOLD6_form.html	McGuffin et al. (2019)
I-TASSER	https://zhanggroup.org/I-TASSER/	Roy, Kucukural, and Zhang (2010)
RaptorX	http://raptorx.uchicago.edu/	Xu, Mcpartlon, and Li (2021)
Robetta	http://robetta.bakerlab.org/	Song et al. (2013)
SWISS-MODEL	https://www.expasy.org/resources/swiss-model	Schwede, Kopp, Guex, and Peitsch (2003)

In this scenario, this work highlights the strategies to screen for Cs-AMPs structure, aiming at developing peptide-based drug candidates, with potential biotechnological applications in several areas, as medicine, veterinary medicine, and agriculture.

2. Materials and equipment

2.1 *In silico*

Minimal recommended configuration:

- Core I5 4GB RAM 500GB HD. Linux Ubuntu 20.04 LTS
- GROMACS 5.1.4
- MODELLER 10.1

2.2 *In vitro*

2.2.1 Antimicrobial (antibacterial and antifungal) assay

- Mueller–Hinton broth;
- Mueller–Hinton agar;
- Potato dextrose agar;
- RPMI 1640 medium;
- Spectrophotometer/microplate reader;
- Sterile 96-well microplates;
- Microcentrifuge tubes, 1.5 mL;
- Falcon tubes;
- Shaker;
- Sterile Petri plates.

2.2.2 Antiviral assay

- CO_2 incubator at 37 °C for cell growth
- Flask for culturing cells (e.g., glass flat bottom 60 mL);
- Inverted microscope
- 6, 24 and 96 wells plates
- DMEM—Dulbecco's Modified Eagle Medium
- Penicillin/Streptomycin solution for cell culture
- Fetal Bovine Serum for cell culture
- Sodium bicarbonate powder
- Carboxymethyl cellulose (CMC);
- Formaldehyde
- Crystal violet powder

3. Step-by-step method details

3.1 *In silico*

This topic presents how regular expressions (RegEx) can be applied for Cs-AMPs screening in public databases and repositories, including proteomes, genomes, and transcriptomes. Additionally, we present the concept of molecular modeling and how comparative modeling, with a core focus on MODELLER, has been used to predict the 3D structure of known and unknown Cs-AMPs sequences. Finally, we highlight how atomistic molecular dynamics simulations have profoundly contributed to calculating the trajectory of Cs-AMPs in molecular systems mimicking a desired biological condition.

3.1.1 Searching for Cs-AMPs in public databases

3.1.1.1 Preparing the computational environment and databases

(a) Perl or Python is necessary to perform the searches proposed here. An updated repository for installation on the Linux environment is available in the link: (https://www.cpan.org/src/5.0/perl-5.28.1.tar.gz, for Perl; and https://www.python.org/downloads/ for Python)

(b) A target database can be chosen based on specific preferences. For demonstrative purposes, here we will use the *Arabidopsis thaliana* proteome. The database is available on the Uniprot Consortium (https://www.uniprot.org/proteomes/UP000006548)

(c) There are several Cs-AMPs from plants (e.g., defensins, lipid transfer proteins (LPTs) and snakins), and the pipeline could be performed for most of them. However, this methodology can not be performed for families with a poor amount of sequences described such as β-barrelin. A RegEx that represents the peptide family is required to screen for Cs-AMP sequences within a target proteome/genome/transcriptome. Many of these families already have one or more RegEx described in the literature (Costa et al., 2020; Silverstein et al., 2007; Tomczak et al., 2012; Zhu, 2008). For this pipeline, the CSαβ-defensin RegEx: $CX_{2-18}CX_3CX_{2-10}$[GAPSIDERYW]XCX_{4-17}CXC (each amino acid is represented by one-letter code; "X" represents any proteinogenic amino acid, and brackets indicate positions that allows one of those amino acids between brackets), described by Zhu (2008), will be used as an example. A commented Python script is available for data mining (Supplementary File 1 in the online version

at https://doi.org/10.1016/bs.mie.2021.11.001). The packages *re* (Van Rossum, 2020) and Bio (Cock et al., 2009) are necessary for the proper performance of the script

3.1.2 Comparative modeling of Cs-AMPs by using MODELLER

3.1.2.1 Installing MODELLER

(a) MODELLER should be installed to proceed with the analyses. The software is available for Mac operating system (OS), Windows and Unix/Linux (https://salilab.org/modeller/download_installation.html).

(b) The files required to complete the basic protocol can be found on http://salilab.org/modeller/tutorial/basic-example.tar.gz (Unix/Linux) or http://salilab.org/modeller/tutorial/basic-example.zip (Windows).

(c) Here, the most recent version of MODELLER (v10.1) was used. The algorithms applied were tested for MODELLER v9.17 and superior.

3.1.2.2 Searching for suitable template structures

(a) Here, we will use as an example the following target Cs-AMP sequence:
>PDB ID: 1ti5
R T **C** M I R R E G W G R **C** L I D T T **C** A H S **C** K N K G Y I G G N **C** K G M T K T **C** Y **C** L V N **C**

Where cysteine residues are in bold type

(b) The template structures can be searched using different algorithms, including BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM= blastp) and HHpred (https://toolkit.tuebingen.mpg.de/tools/hhpred). BLAST (Basic Local Alignment Tool) uses local sequence alignments to find proteins with high sequence similarity. By contrast, HHpred applies a secondary structure alignment to find similar proteins. In both cases, a target sequence is necessary as input

(c) In this process, alignment statistics such as sequences' identity, e-value, and sequence coverage are essential to select a template structure. Therefore, the identity values should be ~30% or higher for closely related Cs-AMP sequences. Moreover, the e-value defines the statistical measure of the alignment, representing the chances of random alignments

(d) All these parameters detect similarities between the target sequence and database template structures

(e) Once the template is selected, the .pdb file (the atomic coordinates of a structurally determined peptide) can be obtained from Protein Data Bank (https://www.rcsb.org/) (Fig. 1).

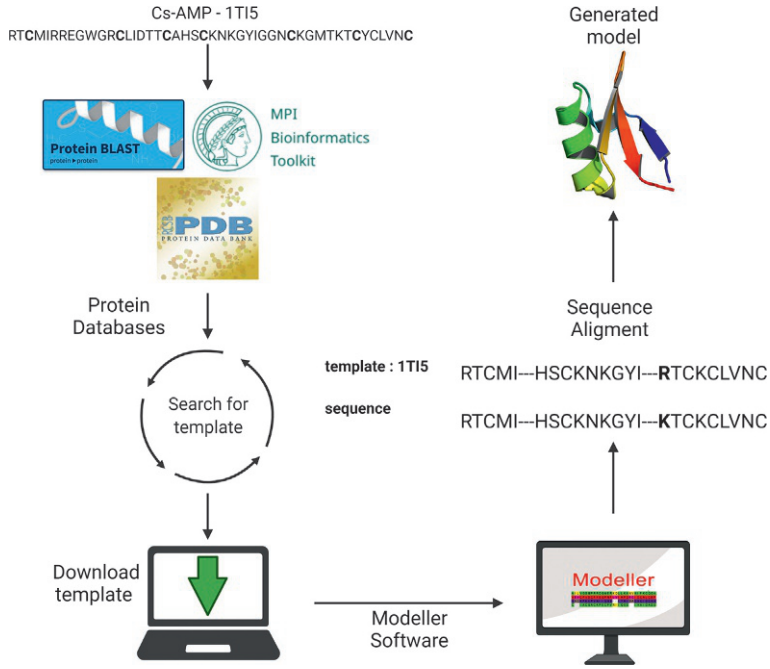

Fig. 1 Scheme demonstrating the steps to download .pdb files for a given peptide/protein structure from databases (Protein Data Bank Server, Blastp and HHpred). These experimentally determined structures are then used for comparative modeling. After downloading the template .pdb structure using a four-letter code (e.g., 1ti5), it is submitted to software MODELLER, based on sequence alignment simulations the model would be generated. Created with the support of Biorender (BioRender.com).

(f) In addition, MODELLER also provides a script for template searching. The script is named build_profile.py (https://salilab.org/modeller/tutorial/basic.html).

3.1.2.3 Checking necessary scripts to perform sequence alignment and generating 3D theoretical models

(a) TvLDH.ali—this PIR format file is necessary for MODELLER to proceed and write alignments. In this script, the target sequence is inserted in FASTA format. The first line consists of (>P1;code), followed by the sequence's identifier. After populating the sequence target, insert (*), which marks its end (Fig. 2)

(b) Align2d.py—this file is a script used to align the template (.pdb) sequence with the target sequence described in the TvLDH.ali script

```
>P1;TvLDH
sequence:target sequence:::::::0.00: 0.00
RTCMIRREGWGRCLIDTTCAHSCKNKGYIGGNCKGMTKTCYCLVNC*
```

Fig. 2 Representation of the TvLDH.ali script, which contains the target sequence in PIR format.

(c) model-single.py—this script generates the theoretical models based on the template atomic coordinates and spatial orientation. However, for Cs-AMPs, if the cysteines are not aligned in align2d.py output, another script should be used to specify each disulfide bond, as explained below

(d) model-disulfide.py—this script is a punctual script to generate models that contain disulfide bonds not covered by the template. The appropriate disulfide bond restraints are generated for the output model/models

3.1.2.4 Aligning TvLDH.ali with the selected template

(a) Insert the target sequence according to the instructions above in the TvLDH.ali file

(b) With the template (e.g., 1ti5.pdb) structure in the same folder, open the file align2d.py. This script will align the target sequence in the TvLDH.ali file with the template structure described in the .pdb file

(c) Once the align2d.py script is opened, the first line will indicate the template structure (FILE) 1ti5.pdb. It is necessary to change the parameters in (ALIGN_CODES) to "1ti5", followed by (ALN.APPEND), where the variable (FILE) receives "TvLDH.ali", which contains the target sequence. Next, the sixth line will execute the command ALIGN2D to perform the alignment (Fig. 3).

```
from modeller import *

env = environ()
aln = alignment(env)
mdl = model(env, file='1ti5', model_segment=('FIRST:A','LAST:A'))
aln.append_model(mdl, align_codes='1ti5', atom_files='1ti5.pdb')
aln.append(file='TvLDH.ali', align_codes='TvLDH')
aln.align2d()
aln.write(file='TvLDH-1ti5.ali', alignment_format='PIR')
aln.write(file='TvLDH-1ti5.pap', alignment_format='PAP')
```

Fig. 3 Script align2d.py is used to align the target sequence with its selected template.

3.1.2.5 Command lines to execute the alignment

(a) Execute through the command line in Linux (Ubuntu), using the following command: mod10.1 (the number 10.1 depends on the installed version).

(b) To do the alignment, use the command mod10.1 align2d.py

Two output files will be produced: a PIR (TvLDH-1ti5.ali) and a PAP (TvLDH-1ti5.pap) format file. MODELLER uses the PIR format for the model-building stage, whereas the PAP alignment format is more intuitive to check the alignment results. Identical amino acid residues are underscored with an asterisk (Fig. 4A and B).

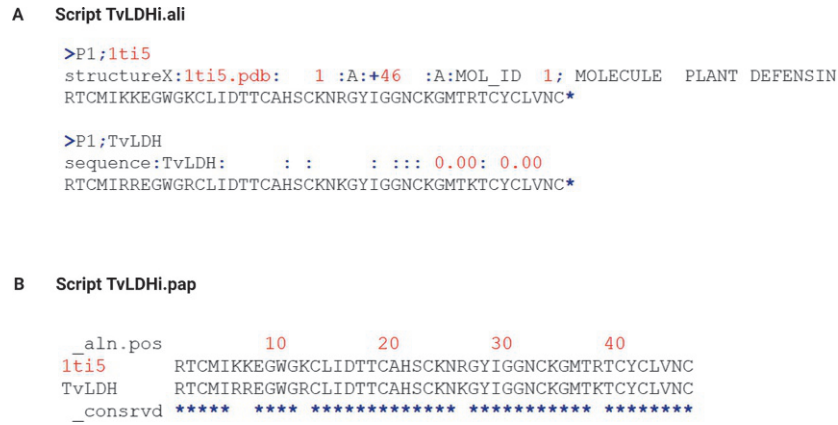

Fig. 4 (A) TvLDH-1ti5.ali, illustrating the alignment between the template and target sequences. (B) TvLDH-1ti5.pap, highlighting by "*" the conserved residues between the two sequences. Created with the support of Biorender (BioRender.com).

3.1.2.6 Generating theoretical models using model-single.py

(a) After the alignment has been executed by the script align2d.py, open the file model-single.py, which calculates 3D models for the target sequence based on the template structure. The first line of this script sets the environment. The following lines comprise parameter values for the "model" routine. The variable (ALNFILE) sets the name of the alignment file "TvLDH-1ti5.al," the parameter (KNOWNS) defines the known template structure and (SEQUENCE) needs to be assigned as TvLDH, which has the target sequence. At last, the parameters (A.STARTING_MODEL) and (A.ENDING_MODEL) define the number of models that will be calculated. The last line refers to the command to run the "model" routine (Fig. 5A).

A **Script model-single.py**

```
from modeller import *
from modeller.automodel import *

env = environ()
a = automodel(env, alnfile='TvLDH-1ti5.ali',
              knowns='1ti5', sequence='TvLDH',
              assess_methods=(assess.DOPE, assess.GA341))
a.starting_model = 1
a.ending_model = 100
a.make()
```

B **Script model-disulfide.py**

```
# Comparative modeling by the automodel class
from modeller import *              # Load standard Modeller classes
from modeller.automodel import *    # Load the automodel class

# Redefine the special_patches routine to include the additional disulfides
# (this routine is empty by default):
class MyModel(automodel):
    def special_patches(self, aln):
        # A disulfide between residues 3 and 46:
        self.patch(residue_type='DISU', residues=(self.residues['3'],
                                                  self.residues['46']))
        # A disulfide between residues 12 and 32:
        self.patch(residue_type='DISU', residues=(self.residues['12'],
                                                  self.residues['32']))

log.verbose()
env = environ()


env.io.atom_files_directory = ['.', '../atom_files']

a = MyModel(env,
            alnfile='TvLDH-1ti5.ali',
            knowns='1ti5', sequence = 'TvLDH',
            assess_methods=(assess.DOPE))
a.starting_model= 1
a.ending_model  = 100

a.make()
```

Fig. 5 Representation of scripts used to run molecular modeling simulations (A) representing model-single.py script used to generate 3D theoretical models for the target sequence based on the template structure. (B) model-disulfide.py script, used to generate models that contain disulfide bonds in their structure. Created with the support of Biorender (BioRender.com).

(b) When modeling a sequence with disulfide bonds, it may be necessary to use a modified version of model-single.py, named model-disulfide.py (https://salilab.org/modeller/manual/node24.html#SECTION:model-disulfide). The restraints to disulfide bonds are added in the script. To generate CHARMM topology it is used the file DISU in the parameter (RESIDUE_TYPE), the residues with the disulfide bonds are inserted in (SELF.RESIDUES[“]) parameter. The instruction should be repeated according to the number of disulfide bonds with each specific pair of cysteines (i.e., cys3-cys46 and cys12-cys32) (Fig. 5B).

3.1.2.7 Using model-disulfide.py used to generate 3D theoretical

(a) Command lines to generate 3D theoretical models with unpaired disulfide bonds

- **i.** To generate models without disulfide bonds restrains, run mod10.1 model-single.py
- **ii.** To generate models containing disulfide bonds without coverage in template structure, run mod10.1 model-disulfide.py
- **iii.** Choose the lowest free-energy generated model based on the DOPE score. The DOPE (Discrete Optimized Protein Energy) is added into MODELLER, based on a refined reference state that coincides with non-interacting atoms in a uniform sphere with a sample native structure radius dependent. It was tested with several non-redundant sets of crystallographic structures. Therefore, it is among the best parameters to calculate peptides/proteins free-energy (Shen & Sali, 2006).

(b) The report of the output files can be found in "model-single.log" and "model-disulfide.log," depending on the scripts used. Helpful information about generated models, including report warnings, errors, and input restraints, can be found in .log files. The model can be visualized using the PyMOL software (https://pymol.org/2/).

3.1.2.8 Structural statistics for a given 3D theoretical model

MODELLER evaluates final models using the scoring function DOPE (Shen & Sali, 2006), developed independently based on a conformation analysis of well-folded peptides and proteins. After the selection of the lowest free-energy model, an additional evaluation step is recommended. For that, many servers can be used. General examples include the ProSA-web server (https://prosa.services.came.sbg.ac.at/prosa.php) and QMEAN (https://swissmodel.expasy.org/qmean/) to evaluate the 3D theoretical model fold in comparison with other peptides of similar size and structurally determined by X-ray crystallography or NMR. Additionally, PROCHECK (https://saves.mbi.ucla.edu/) can be used to check the stereochemistry quality of models. High-quality models are expected to present an NMR or X-ray similar z-score on ProSA, a modular z-score below to 2 on QMEAN and, at least, 90% of the residues in most favored and additionally allowed regions on the Ramachandran Plot.

3.1.3 Molecular dynamics

3.1.3.1 Installing GROMACS

(a) The first step is to download and install GROMACS software at (http://www.gromacs.org/Downloads), distributed for Mac OS, Windows and Unix/Linux system but works natively on a Unix-type system (such as Linux, or Mac OS X).

(b) The installation can be done according to the GROMACS version that the user chose. There are two ways to build GROMACS. We will use a user-friendly installation. For this, the configuration described below is necessary:

(i) Get the latest version of C and C++ compilers;

(ii) Check if the computer has CMake installed (it varies according to GROMACS version);

(iii) Download and unpack the latest version of GROMACS;

(iv) Select a separate build directory and change to it;

(v) Run the command cmake with a path to the source as an argument;

(vi) Run commands make, make check and make install (Fig. 6).

(c) Here, an MD simulation demonstration will be performed using GROMACS version 5.0.4 on Ubuntu 16.04 LTS. For more information about system configuration and performance, please see: (https://manual.gromacs.org/current/user-guide/mdrun-performance.html).

```
tar xfz gromacs-5.0.7.tar.gz
cd gromacs-5.0.7
mkdir build
cd build
cmake .. -DGMX_BUILD_OWN_FFTW=ON -DREGRESSIONTEST_DOWNLOAD=ON
make
make check
sudo make install
source /usr/local/gromacs/bin/GMXRC
```

Fig. 6 Commands to run when executing the quick and dirty installation of GROMACS.

3.1.3.2 Programing the MD simulation

PDB structure

(a) Go to Protein Data Bank and download (https://www.rcsb.org/) a target structure or use your theoretical molecular model generated by comparative modeling. Here cysteine-rich defensin VrD1 (pdb: 1tl5) was used as an example

Checking scripts

(a) Check the necessary scripts that will be used throughout the MD parametrization. The scripts are:
ions.mdp—to generate ions counting to parametrize the system;
minim.mdp—system energy minimization;
nvt.mdp—temperature parametrization;
npt.mdp—pressure parametrization;
md.mdp—to generate the file that will execute md.tpr.

(b) Open the terminal command on Linux/Ubuntu, then source GROMACS using the following command line:
source/usr/local/gromacs/bin/GMXRC.

3.1.3.3 Start programming your MD simulation

(a) First, it is necessary to generate GROMACS coordinate files (.gro) and topology using as input the .pdb file downloaded from PDB or obtained from molecular modeling. The pdb2gmx tool generates a GROMACS atomic coordinate (.gro) from the input .pdb file. To map a disulfide bond, use the flag: -ss (they will be automatically identified). The other parameters refer to the solvent type (e.g., water: flag -water spce), net-charge (neutral; protonation: flag: -inter), N- and C-terminus modifications (flag: -ter), and polar hydrogen addition or removal (flag: -ignh). For more information, access (https://manual.gromacs.org/archive/5.0.4/programs/gmx-pdb2gmx.html).

(b) Type the following command:
gmx pdb2gmx -f 1ti5.pdb -o 1ti5.gro -ignh -inter -ter -water spce -ss

(c) Next, a list of force fields will pop up (Fig. 7). Select the force field that suits best the kind of macromolecules and system you want to simulate. The GROMOS96 43a force field is commonly used for linear and cyclic peptides and, therefore, it was selected for the methodology described here. Select option 9 for GROMOS96 43a

(d) Next, it will be necessary to protonate the peptide/protein. Register how many residues will be protonated. This data will be used in the following steps

(e) The following step includes the simulation box generation. The command (flag: -editconf) converts generic structure from .gro, (flag: -o) to .pdb. To set the geometric center of the system, use (flag: -c), to center the system in the box, use (flag: -d) followed by the measure of the

```
Select the Force Field:
From '/usr/local/gromacs/share/gromacs/top':
 1: AMBER03 protein, nucleic AMBER94 (Duan et al., J. Comp. Chem. 24, 1999-2012, 2003)
 2: AMBER94 force field (Cornell et al., JACS 117, 5179-5197, 1995)
 3: AMBER96 protein, nucleic AMBER94 (Kollman et al., Acc. Chem. Res. 29, 461-469, 1996)
 4: AMBER99 protein, nucleic AMBER94 (Wang et al., J. Comp. Chem. 21, 1049-1074, 2000)
 5: AMBER99SB protein, nucleic AMBER94 (Hornak et al., Proteins 65, 712-725, 2006)
 6: AMBER99SB-ILDN protein, nucleic AMBER94 (Lindorff-Larsen et al., Proteins 78, 1950-58, 2010)
 7: AMBERGS force field (Garcia & Sanbonmatsu, PNAS 99, 2782-2787, 2002)
 8: CHARMM27 all-atom force field (CHARM22 plus CMAP for proteins)
 9: GROMOS96 43a1 force field
10: GROMOS96 43a2 force field (improved alkane dihedrals)
11: GROMOS96 45a3 force field (Schuler JCC 2001 22 1205)
12: GROMOS96 53a5 force field (JCC 2004 vol 25 pag 1656)
13: GROMOS96 53a6 force field (JCC 2004 vol 25 pag 1656)
14: GROMOS96 54a7 force field (Eur. Biophys. J. (2011), 40,, 843-856, DOI: 10.1007/s00249-011-0700-9)
15: OPLS-AA/L all-atom force field (2001 aminoacid dihedrals)
```

Fig. 7 List of force filed that can be chosen depending on the user's needs.

distance from solute and the box (e.g., 1.0). To define the box shape (e.g., cubic, dodecahedral, octahedral, among others), use the command (flag: -bt). For more information, access (https://manual.gromacs.org/archive/5.0.4/programs/gmx-editconf.html).

(f) The final command should be something as described below:

gmx editiconf -f 1ti5.gro -o box.gro -c -d 1.0 -bt cubic

(g) After preparing the simulation box, proceed to the system's solvation. For this, use the flag: -solvate, followed by the flag: -cp to specify the output files from the previous step (editconf). The flag: -cs is used to specify the solvent, which will be the Simple Point Charge water (SPC) using the flag: -spc216.gro. Nevertheless, other co-solvents can also be added to the system, including 2,2,2-trifluoroethanol and methanol

(h) The final command should be something as described below:

gmx solvate -cp box.gro -cs spc216.gro -o solv.gro -p topol.top

(i) The topology file (topol.top) will be updated constantly from this step, as this file contains information about molecule types and the number of molecules

(j) An additional input file with the molecular dynamics parameter file extension (.mdp) will be used to produce a .tpr file with grompp. Grompp assembles specified parameters from the .mdp file with the coordinates and topology information to generate a .tpr file. Type the following command:

gmx grompp -f ions.mdp -c solv.gro -p topol.top -o ions.tpr

(k) With the atomic-level description of the system in the binary file ions.tpr, use the genion command. To add the necessary ions to neutralize the system's net charge by adding the correct number of negative ions (Na+) or positive ions (Cl-), use the flag: -neutral. Moreover, if a given ionic strength is desired, for instance, 0.15 M NaCl, use the flag: -conc 0.15

(l) For more information, please visit (https://manual.gromacs.org/archive/5.0.4/programs/gmx-genion.html).

The final command should be something as described below:

gmx genion -s ions ions.tpr -o solv_ions.gro -p topol.top -neutral or

gmx genion -s ions.tpr -o solv_ions.gro -p topol.top -neutral -conc 0.15

For each ion that is added to the system, one solvent molecule will be removed.

3.1.3.4 MD energy minimization

(a) Energy minimization steps are used to ensure no steric clashes or inappropriate geometry in the system. A process called energy minimization

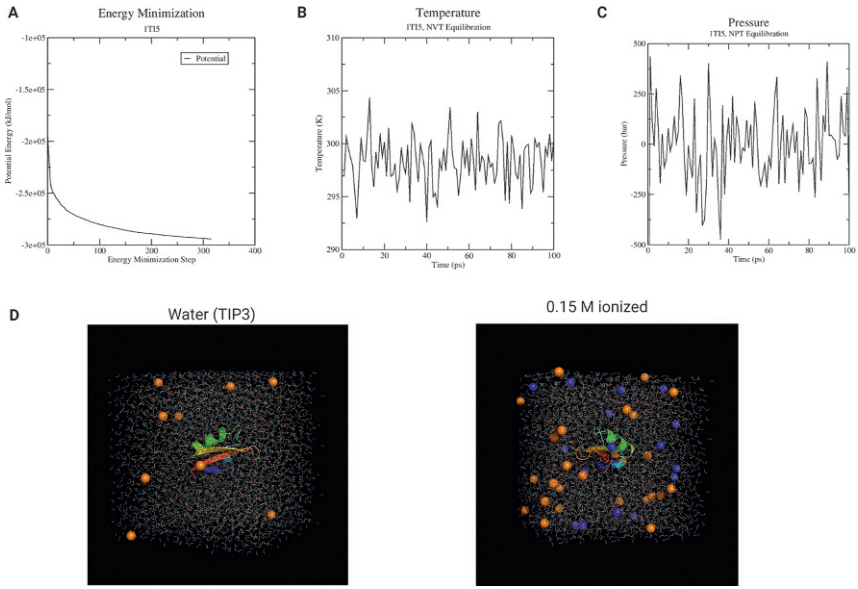

Fig. 8 (A) The plot is represented by an energy potential that results from energy minimization, demonstrating that energy is converging. (B) Plot generated showing the temperature progression of the system according to the number of steps set in the nvt.mdp script. The graph was built using xmgrace. (C) Plot generated using xmgrace shows the system's pressure equilibration, which is usually set at 1 bar. And (D) cubic boxes containing the target cysteine-rich peptide, the TIP3 water molecules and one system only with the necessary ions to neutralize the system's charge (left); whereas the other system (right) shows the same system, however at 0.15 M ionic strength. Both systems had their energies minimized, as described above. Created with the support of Biorender (BioRender.com).

is applied for structure relaxing (Fig. 8A and D). For more information visit: (https://manual.gromacs.org/archive/5.0.4/programs/gmx-grompp.html) and (https://manual.gromacs.org/archive/5.0.4/programs/gmx-mdrun.html).

(b) Run the following command:

gmx grompp -f minim.mdp -c solv_ions.gro -p topol.top -o em.tpr.
gmx mdrun -v -deffnm em

(c) The potential energy graph can be plotted by the following command:

gmx energy -f em.edr -o potential.xvg.

Select the option: Potential.

(d) Then open the plot using xmgrace software. For more information: (https://plasma-gate.weizmann.ac.il/Grace/).

3.1.3.5 MD equilibration

(a) Equilibration is commonly conducted in two steps. The first is conducted under an NVT ensemble (constant Number of particles, Volume, and Temperature). This ensemble can also be referred as "isothermal-isochoric" or "canonical" (Fig. 8B). The system's temperature should reach a plateau at the desired value. For more information, see: (https://manual.gromacs.org/documentation/2018/user-guide/mdp-options.html).

(b) To run temperature equilibration using the nvt.md script type the following commands:

```
gmx grompp -f nvt.mdp -c em.gro -p topol.top -o nvt.tpr.
gmx mdrun -v -deffnm nvt
```

(c) To plot the temperature graph, run the following command:

```
gmx energy -f nvt.edr -o temperature.xvg
```

Select the Temperature option and open the. xvg output file using xmgrace

(d) Pressure equilibration is the second step conducted under an NPT ensemble, as the number of particles, pressure, and temperature are constant. The ensemble is also called the "isothermal-isobaric" ensemble and closely resembles experimental conditions (Fig. 8C). For more information, please visit (https://manual.gromacs.org/documentation/2018/user-guide/mdp-options.html).

(e) To generate NPT files run the following commands:

```
gmx grompp -f npt.mdp -c nvt.gro -t nvt.cpt -p topol.top -o npt.tpr
gmx mdrun -v -deffnm npt
```

(f) To plot the pressure graph, run the following command:

```
gmx energy -f npt.edr -o pressure.xvg
```

Select the Pressure option and open the. Xvg output file using xmgrace.

3.1.3.6 MD simulation

(a) After minimization and equilibration steps, the next step is to release the position restraints and run the MD simulation. It is a similar process to the commands described above for NVT and NPT. For this representative simulation, we will run 10 ns of MD simulation. Therefore, it is necessary to edit script md.mdp and alter the value in nsteps parameter (Fig. 9).

(b) Then execute the following commands:

```
gmx grompp -f md.mdp -c npt.gro -t npt.cpt -p topol.top -o md.tpr.
gmx mdrun -v -deffnm md.tpr
```

Current Trends and Prospects in Antimicrobial Peptide Bioprocessing

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Abstract The increase in resistance to conventional antimicrobials in recent years has boosted the search for new antibiotics to treat serious infectious diseases, especially those generated by multi-resistant bacteria. In this context, antimicrobial peptides (AMPs) are alternative molecules for use as new therapeutic agents. AMPs are small bioactive proteins commonly produced by all living organisms, and they can be part of innate immunity. Due to their broad-spectrum antibacterial potential and other activities, including immunomodulatory and antitumor, they are of great interest to the pharmaceutical industry's production of biopharmaceuticals. Among the technological platforms applied in the process of development and manufacturing of AMPs, recombinant DNA technology has enabled the production of such molecules using bacterial and yeast cells as expression host systems on a laboratory scale and in large-scale environments. Furthermore, different bioprocessing strategies can be used for peptide industrial production, aiming to optimize the yield, make cultures more robust and significantly increase cell density. In this chapter, we will address recent developments and future directions in AMPs bioprocessing, including microbial expression systems, as well as bioprocessing and purification technologies. Here we also describe successful cases in this field and emphasize the prospects and challenges related to AMPs bioengineering.

1 Introduction

The increase in microbial resistance to antibiotics is a major public health problem around the world. Methicillin-resistant *Staphylococcus aureus* (MRSA) and β-lactamase-resistant *Escherichia coli* (ESBL) are bacterial strains that recur in many hospitals (Assis et al. 2017; Wang et al. 2019), mainly infecting patients whose immune system is compromised by other diseases or invasive therapies (Leite et al. 2019). Bacteria such as *Pseudomonas aeruginosa, Enterococcus faecium*, *Acinetobacter baumannii*, *Klebsiella pneumoniae,* and *Enterobacter* species are also commonly responsible for nosocomial infections exhibiting high microbial resistance to currently available drugs (Laws et al. 2019).

The emergence of multi-resistant microorganisms such as those mentioned above may occur due to the excessive and/or incorrect use of antimicrobial drugs by humans and other animals (Lombardi et al. 2019; Bhopale 2020; Dijksteel et al. 2021). As a result, many drugs commonly used to fight those pathogens are no longer effective (Leite et al. 2019). About 700,000 people die annually from resistant

bacterial infections. If measures are not taken to change the current scenario, it is estimated that by 2050 the number of deaths will be ten million per year (Mishra et al. 2017; Laws et al. 2019). The development of new, broad-spectrum, and low-toxicity drugs as an alternative therapy to conventional antibiotics is highly desirable. Both academia and industry are working on prospecting candidate molecules and producing new therapies to combat infectious diseases (Andersson et al. 2016; Leite et al. 2019).

In this context, antimicrobial peptides (AMPs) have emerged as a new approach for replacing or complementing traditional therapeutic compounds in combating resistant microorganisms (da Costa et al. 2015; Chen and Lu 2020). They are naturally produced by various living organisms, such as microorganisms, plants, and animals, acting as important immune system components against exogenous pathogens (Wang et al. 2019; Leite et al. 2019). Generally, they present activity against several microorganisms and can perform more than one function in some cases, including acting as an immunomodulator or antitumor, so they are considered promiscuous molecules (Franco 2011; Haney et al. 2017; Leite et al. 2019).

Due to the different functions that they can perform simultaneously, AMPs are molecules that seem to have great potential as candidates for new drugs, especially antibiotics (Tornesello et al. 2020; Liscano et al. 2020). The development of new AMP-based drugs needs these molecules to be obtained in large quantities for biotechnological studies and pharmaceutical evaluation (Parachin et al. 2012). There have been notable improvements in producing AMPs on an industrial scale during the last few years, due to several new technologies (Gupta and Shukla 2017). Innovations in the upstream bioprocessing steps, such as selection and development of the cell line, optimization of cell culture parameters, and the use of feeding strategies, have the ultimate goal of large-scale production (Gronemeyer et al. 2014; Tripathi and Shrivastava 2019). The application of recombinant systems in cell line construction is opportune in terms of yields and costs for AMPs production (Parachin et al. 2012).

Among these recombinant systems, microorganism hosts are generally the most used, because they have characteristics that enable high levels of synthesis, modification, and secretion of heterologous AMPs. Various recombinant proteins are already available on the market, including AMPs produced by these hosts (Gupta and Shukla 2017). *E. coli* bacteria is the most widely used microbial cell factory, since its genetic and biological processes are well known, exhibiting fast growth rate, high yield and simple upstream process (Khow and Suntrarachun 2012; Briand et al. 2016; Kaur et al. 2018). Otherwise, among the yeast systems, *Saccharomyces cerevisiae* is the most established. It is a robust, stress-tolerant yeast and uses simple nutrients (Öztürk et al. 2017).

After developing the expression system, the next step consists of producing the small-scale recombinant protein to screen and select transforming clones. At this point, it is essential to monitor the cultivation conditions, as they directly affect the expression of the recombinant AMP. Optimizing the temperature, pH, aeration, agitation, media composition, the concentration of inducers, induction time and the feeding strategies make the development of bioprocesses more effective (Kaur

et al. 2018). Continuing the process, bioreactor systems and bioprocess strategies such as batch, fed-batch, and continuous culture are employed for the production of large-scale AMPs as biopharmaceuticals (Tripathi and Shrivastava 2019). Subsequently to large-scale production, a key purpose is the recovery of the target biomolecule and removal of the impurities present in the culture medium (Gupta and Shukla 2017).

Known as the downstream process, this phase has innovative technologies for handling large volumes in production processes, as well as for the recovery of the recombinant biomolecule of interest (Gupta and Shukla 2017). Filtration removes cell biomass, providing culture medium clarification, and affinity chromatography captures the recombinant protein using specific separation resins, promoting a high degree of purity for the proteins of interest (Singh et al. 2013; Kimple et al. 2013; Arora et al. 2017). In summary, this chapter addresses innovative approaches to upstream and downstream processes for AMPs yielding biopharmaceuticals. Selection of the proper expression hosts, development of bioprocesses, recent strategies in bioprocessing, techniques related to purification, and ways to achieve lower production costs, while boosting manufacturing flexibility and final product quality, are here described.

2 Antimicrobial Peptides

2.1 Properties

AMPs are small bioactive proteins that are effective against several species of Gram-positive and -negative bacteria, fungi, parasites and viruses (enveloped and non-enveloped) (Bhopale 2020; Moretta et al. 2021). These molecules are also known a host defense peptides (Boto et al. 2018; Liang and Diana 2020), and they are essential elements of the innate immune system of living organisms, acting as a first defense line against microbial actions displaying microbicidal, bacteriostatic, and cytolytic properties (Sinha and Shukla 2019; Moretta et al. 2021). AMPs are the primary defense line against pathogenic microorganisms for plants and insects, as these do not present an adaptive immune system. In bacteria and other microorganisms, AMPs act in defense of their environmental niche (Browne et al. 2020). The bacteria *Paenibacillus polymyxa*, for instance, which develops in plant roots, produces the antibiotic polymyxin, capable of breaking down *P. aeruginosa* or *S. aureus* biofilms (Quinn et al. 2012).

AMPs are evolutionarily conserved molecules that are extremely diverse in composition and length, with different sequences, structures, and sources, but they have some typical features (Bhopale 2020). Most AMPs are generally characterized as short molecules containing fewer than 100 amino acid residues and have molecular masses ranging from 1 to 10 kDa. These peptides commonly possess a positive net charge ranging from +2 to +11, attributed to the presence of positively charged residues such as lysine and arginine residues, thus being characterized as cationic

molecules (Mahlapuu et al. 2020). These molecules usually have a considerable amount of hydrophobic residues (typically 50%) such as valine, leucine, isoleucine, alanine, methionine, phenylalanine, tyrosine, and tryptophan in the peptide sequence (Lee et al. 2017; Thapa et al. 2020; Dijksteel et al. 2021).

2.2 Mechanisms of Action and Targets

Generally, the first AMP interaction with the bacterial cell membrane occurs through hydrophobic and electrostatic interactions between cationic residues and anionic components of the microorganism's membrane (Moravej et al. 2018). The negative microbial surface charge may be provided by phospholipid head groups such as phosphatidylglycerol, cardiolipin, or phosphatidylserine (Boto et al. 2018). Eukaryotic membranes have zwitterionic phospholipids such as phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin. These phospholipids contribute to membrane net charge at physiological pH. In addition to zwitterionic phospholipids, the presence of cholesterol molecules along the membrane may also contribute to a reduction in membrane fluidity and flexibility, which can generally reduce AMP activity (Boto et al. 2018; Dijksteel et al. 2021). Therefore, AMPs are generally non-toxic to mammalian cells, which is an attractive feature for their therapeutic use (Silva et al. 2011; Browne et al. 2020). Although AMPs' mechanism of action is still largely unknown, the electrostatic attraction between negatively charged cells components and positively charged AMPs is understood to be important as a first step, resulting in a strong interaction and further target cell membranes disruption (Tornesello et al. 2020).

After initial electrostatic interactions, self-assembly peptide accumulation on the bacterial membrane surface may cause membrane integrity loss and intracellular component leakage after reaching certain concentrations (Mirski et al. 2017; Dijksteel et al. 2021). Furthermore, there are a several widely accepted models of action that usually involve bacterial cytoplasmic membrane integrity being disrupted in many ways. Among them are the barrel-stave pore, detergent micellization, toroidal pore, disordered toroidal pore, membrane thinning/thickening charged lipid clustering, non-bilayer intermediate formation, oxidized phospholipid targeting, anion carrier, and non-lytic membrane depolarization, among others (Nguyen et al. 2011; Mahlapuu et al. 2020).

In the barrel-stave model, AMPs can self-organize into cylindrical bundles, which insert themselves in a perpendicular way into the membranes. Aqueous pore lumen formation may occur with the orientation of hydrophobic portions towards the hydrophobic bilayer interior. The pore may cause cytomembrane permeabilization, osmotic imbalance and further cell death (Seyfi et al. 2020). In the toroidal model, the pore formation occurs when the AMPs insert themselves perpendicularly into the bacterial plasmatic membranes, a mechanism similar to the barrel-stave model. However, in this model, the nonpolar AMPs amino acid residues interact with lipid head groups, causing membrane deflection and, further, generating torus

pores (Lee et al. 2017; Moretta et al. 2021). Another possibility is the carpet model, in which the AMPs are accumulated and arranged parallel to the cell membrane, covering it completely. At the same time, micelles are formed with the initial ruptured membranes, through the hydrophilic amino acids interactions of polar phospholipid heads, causing cytomembrane disruption (Deng et al. 2017; Lee et al. 2017; Zandsalimi et al. 2020). Moreover, peptides are capable of interacting with several targets, including proteins and carbohydrates, acting not only on the surface, but also inside the cell (Silva et al. 2011; Kumar et al. 2018). In addition to destabilizing bacterial membranes and causing their rupture (membrane-acting peptides), AMPs can also cross the membrane, destabilizing normal cellular processes, such as cell division, protein, nucleic acid and cell wall synthesis, being classified as "non-membrane acting peptides." AMPs can act against pathogens by causing different stresses at the same time until the combined action causes cell death (Boto et al. 2018). AMPs that act through membrane destruction are also able to act through non-destructive membrane, in addition to acting independently or synergistically with other AMPs (Dijksteel et al. 2021).

Nonetheless, AMPs have a therapeutic potential that goes beyond their antimicrobial activity. Some AMPs can also exhibit cytotoxic activity against tumor cells (Wang et al. 2019). Most tumor cells also have a surface negative charge on the membrane due to anionic overexpression of molecules such as phosphatidylserine. This characteristic allows the interaction with cationic AMPs (Wang et al. 2019). AMPs are also capable of inhibiting inflammatory responses and stimulating the proliferation of immune system cells, acting as immune modulators (Drayton et al. 2020). They are capable of recruiting and stimulating the proliferation of macrophages, neutrophils, eosinophils, activation of T lymphocytes and differentiation of dendritic cells, in addition to inducing or modulating pro-inflammatory cytokines and producing chemokines, chemotaxis, causing apoptosis and inhibiting the inflammatory response. This supports their use as potential therapeutic molecules against immune-related diseases (Liang and Diana 2020).

AMPs are thus able to act on several cell targets and are considered as promiscuous molecules in certain cases (Franco 2011). Multiple functions can be related to a single peptide structure, contrary to what was supposed years ago, which held that peptides have an unconditional structure directly associated with a particular function. Knowledge about AMPs promiscuity has been gaining ground in several fields of research, such as in antibiotic development (Silva et al. 2011; Franco 2011). Therefore, natural AMPs have enormous potential as an alternative approach for the development of new therapies, acting alone or in synergy with conventional drugs (de Oliveira et al. 2020; León-Buitimea et al. 2020).

Several studies have already demonstrated AMPs' therapeutic efficacy (Vilas Boas et al. 2017; Lima et al. 2017; Fensterseifer et al. 2019; León-Buitimea et al. 2020; Almeida et al. 2020). Another important feature is that AMPs are less susceptible to microbial resistance since they act on evolutionarily conserved cell membrane components. Therefore, bacterial cells would need different mutations over an extended period to completely redesign the structure of their cell membranes (Mercer et al. 2020; Mahlapuu et al. 2020). Thus, AMPs seem to have great potential

for antibiotic adjuvants, making it possible to reduce or circumvent the occurrence of antibiotic resistance. The synergy between AMPs and antibiotics, at lower dosages, can provide a reduction in the toxicity or adverse side effects of a drug (Browne et al. 2020).

Despite being less common, some anionic AMPs have been reported, such as the anionic AMP maximin-H5, isolated from amphibians, and dermcidin, secreted by the human eccrine sweat glands (Rios et al. 2016; Boparai and Sharma 2019). They are made up of negatively charged glutamic and aspartic acid residues, with a net negative charge ranging from −1 to −7. Precisely because of its negative charge, its mode of action differs from cationic AMPs. However, some anionic AMPs can be disruptive to bacterial cell membranes. These peptides are capable of using metal ions to form cationic salt bridges with the negative microbial membrane constituents, which enables cell penetration. When they reach the cytoplasm, they can bind to intracellular components such as ribosomes or inhibit ribonuclease activity, inducing cell death (Wang et al. 2019; Boparai and Sharma 2019; Moretta et al. 2021).

2.3 Classification

AMPs can be classified according to their structural aspects into three major subgroups: α-helical, β-sheet, and extended peptides (Tornesello et al. 2020; Moretta et al. 2021). The α-helical peptides, when in interaction with bacterial membranes, can be organized into a flexible amphipathic structure (Koo and Seo 2019; Cardoso et al. 2021). Examples of α-helical AMPs include magainin, temporins and melittin (Ge et al. 1999; Raja et al. 2017; Ramirez et al. 2019). Peptides in β-sheets may have greater structural stability in solution, due to the cysteine residues that are conserved and form disulfide bonds, which minimize proteinases degradation. This class includes AMPs such as protegrins from the cathelicidin family, defensins and tachyplesins (Kumar et al. 2018; Seyfi et al. 2020). Extended peptides are composed of a large proportion of proline and glycine residues and have no specific secondary structure, but when in contact with membranes, they generally form an amphipathic helical structure (Koo and Seo 2019). Proline-rich short-chain extended peptides, such as indolicidin and tritrpticin, can be isolated from mammals and from insects, such as apidaecin. Glycine-rich extended peptides can be isolated from insects and have sizes ranging from 8 to 30 kDa (Wang et al. 2019).

3 Upstream Process Development

Large-scale AMPs production is definitively a challenging task. The direct isolation and purification of these molecules from natural sources is normally extremely labor-intensive, in addition to resulting in low yields (Wibowo and Zhao 2019). Chemical synthesis, including Fmoc and other methodologies, is one of the main methods

currently used to obtain purified AMPs with high biological activity. Despite providing a high yield and level of purity, the high manufacturing cost of this technique is a limiting factor for the development of AMPs as biopharmaceuticals, particularly for peptides with more than 35 residues and that have post-translational modifications (Deng et al. 2017; Wibowo and Zhao 2019). Peptide synthesis by SPPS (Solid Phase Peptide Synthesis) is a complex and expensive production technique, despite being efficient. AMPs manufacture by SDDS is estimated to cost around US$50–400 per gram of amino acid produced (Moretta et al. 2020). Alternative approaches that increase production and make the AMPs development process cheaper are necessary (Sinha and Shukla 2019).

Upstream bioprocessing consists of several stages to achieve high yield and final product quality. Through recombinant DNA technology, scalable, economical, and sustainable AMPs production is possible. This strategy allows the cloning of foreign genes into specific vectors for expression in host systems, such as bacteria and yeast (Jozala et al. 2016; Wibowo and Zhao 2019; de Oliveira et al. 2020). The positive screening and selection of clones is performed, as well as small-scale assays to evaluate cell growth and protein product levels. Lastly, bioprocesses are conducted in bioreactor systems for large-scale production, and batch, fed-batch, and continuous strategies are applied for the mass production of the recombinant AMPs of biopharmaceutical interest (Gronemeyer et al. 2014; Jozala et al. 2016; Tripathi and Shrivastava 2019). Figure 1 exemplifies the upstream bioprocess of AMPs biopharmaceutical manufacturing.

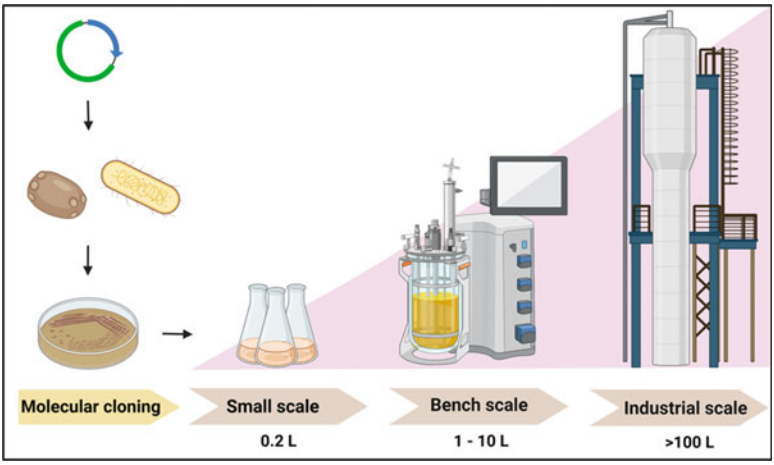

Fig. 1 Upstream bioprocess of AMPs biopharmaceutical manufacturing: molecular cloning, small-scale assays and scale-up of bioprocess. The figure was created by BioRender

3.1 Recombinant AMP Production in Microbial Cells

Bacterial expression systems are the type most used for heterologous gene expression since they are capable of producing a high level of recombinant molecules, show rapid multiplication, and have a simple media requirement (Gomes et al. 2016). Among them, the Gram-negative bacterium *E. coli* is the most extensively used for amplifying recombinant peptides' gene expression (Terpe 2006; Ahmad et al. 2018). In this regard, ~99% of proteins and peptides deposited in the Protein Data Bank (PDB) were produced in the *E. coli* expression system (de Oliveira et al. 2020). Under ideal culture conditions, bacterial cell number practically doubles every 20 min (Sezonov et al. 2007). Additionally, this host is also attractive due to its well-established genetic and expression protocols, wide availability of commercial expression vectors and cost-effectiveness (Sinha and Shukla 2019). There are several reports in the literature about the use of *E. coli* cells for recombinant AMPs expression. Recently, the proline-arginine-rich cationic peptide PR-39 was expressed fused to the SUMO or intein-chitin binding domain (CBD) in *E. coli* BL21 (DE3) pLysS, aiming to compare the expression level. Comparing the fusion protein concentration and single-step purification aspects, the intein system was better, even though similar amounts of pure PR-39 were recovered (250 μg L^{-1} of SUMO and 280 μg L^{-1} of intein) (Azari et al. 2020).

The *E. coli* (Rosetta DE3) system was also chosen to express more than 250 μg mL^{-1} of the human β-defensin 118 (DEFB118). This defensin is related to epididymal innate immunity, protecting the sperm against microorganism attacks in both male and female reproductive tracts. Antimicrobial assays demonstrated that DEFB118 activity occurred against Gram-negative and -positive bacteria at a minimum inhibitory concentration (MIC) of 4 μg mL^{-1} (Lin et al. 2020). Also using *E. coli* BL21 (DE3), a group of scientists created a modular system to express the lanthipeptide mersacidin, produced by *Bacillus amyloliquefaciens*. Mersacidin has bactericidal activity against several Gram-bacteria species, including methicillin-resistant *S. aureus* (Viel et al. 2021).

In recent years, *Bacillus subtilis* has been used as an alternative to the *E. coli* expression system for recombinant AMP production. *B. subtilis* is an endospore-forming Gram-negative soil bacterium and is generally recognized as safe (GRAS) because it lacks endotoxins, so it has been exploited as a production host for aquaculture industries (Pan et al. 2016; Cui et al. 2018). One of the greatest advantages of this bacterium is the possibility of naturally secreting the recombinant peptide (simplifying the downstream process), reducing hydrolysis of cell-associated proteins. Moreover, it simplifies the detection and purification processes of the target molecule (Zhang et al. 2020). Besides, *B. subtilis* does not produce lipopolysaccharides (LPS), preventing some degenerative disorders in humans and animals (Gomes et al. 2016).

Although bacterial cell-based expression systems are mostly used for the production of recombinant molecules, they are more prone to the degradation of cationic peptides (Li 2011). In addition, *E. coli* cells can produce inclusion bodies formed by

insoluble protein aggregates that hinder the recombinant AMP extraction and purification process (Gomes et al. 2016). Although engineered *E. coli* expresses periplasmic disulfide bond isomerase (DsbC) in the cytoplasm or through exporting the periplasm recombinant peptide (Abbas et al. 2013), this bacterium does not carry out other complex post-translational modifications (Parachin et al. 2012). Thus, for cases where there is a need for post-translational modifications, other systems should be chosen, such as yeasts or bacterial strains engineered for this purpose.

In addition to post-translational modifications, yeasts can carry out the correct protein folding. However, they have as a disadvantage the hyperglycosylation mechanisms and the need for aerobic fermentation, which reduces the growth rate, resulting in a lower recombinant protein yield (Juturu and Wu 2018). *S. cerevisiae* (baker's yeast) is a model organism for heterologous expression because its cell biology, genetics and biochemistry are well described (Gomes et al. 2016). This yeast is a suitable expression system for recombinant peptide production, as demonstrated by a group of scientists (Jiang et al. 2021). They expressed the cecropin P1, a positively charged α-helical peptide, isolated from the nematode *Ascaris suum*, which shows activity against *E. coli*, *Salmonella* sp., *Shigella* sp., and *Pasteurella* sp. In addition, this peptide also has antiviral activity against the PRRSV NADC30-like strain (Jiang et al. 2021). Defensins have also been successfully expressed in *S. cerevisiae*. By using the *MET17* promoter, Møller and colleagues expressed β-defensin-2 in *S. cerevisiae* cells (Møller et al. 2017).

In recent decades, the yeast *Pichia pastoris* (reclassified as *Komagataella phaffii)* (Naumov et al. 2018) has been extensively used for heterologous production of peptides and proteins, as it is capable of performing disulfide bridges, O- and N-glycosylation, and the correct processing of signal sequences (Wibowo and Zhao 2019). Several peptides have been expressed in a *K. phaffii* system (Wang et al. 2009; Basanta et al. 2010; Zhao et al. 2015; Zhang et al. 2018). Tachyplesin I (TP-I), a cationic peptide isolated from the Japanese horse crab (*Tachypleus tridentatus*) hemocytes, which inhibits the bacterial lipopolysaccharide (Li et al. 2019), was expressed in this system. Another example is the expression of the immunomodulatory and anti-inflammatory hybrid peptide (IAHP) LL-37Tα1. This peptide was efficiently produced in *K. phaffii* cells, demonstrating their ability to produce recombinant bioactive peptides (Ahmad et al. 2019).

Even though the choice of expression system must be made considering recombinant molecule properties, systems based on prokaryotic and eukaryotic cells have both strengths and weaknesses. The disadvantages can be overcome with multiple strategies, aimed at increasing recombinant molecule production. Codon optimization for specific organisms or engineered strains, the use of strong promoters and multimeric AMP expression in tandem or fused to a higher molecular mass protein are strategies that allow for a stable and high level of recombinant production (Fig. 2) (Deng et al. 2017). Below, we will address all these strategies to improve recombinant AMPs production.

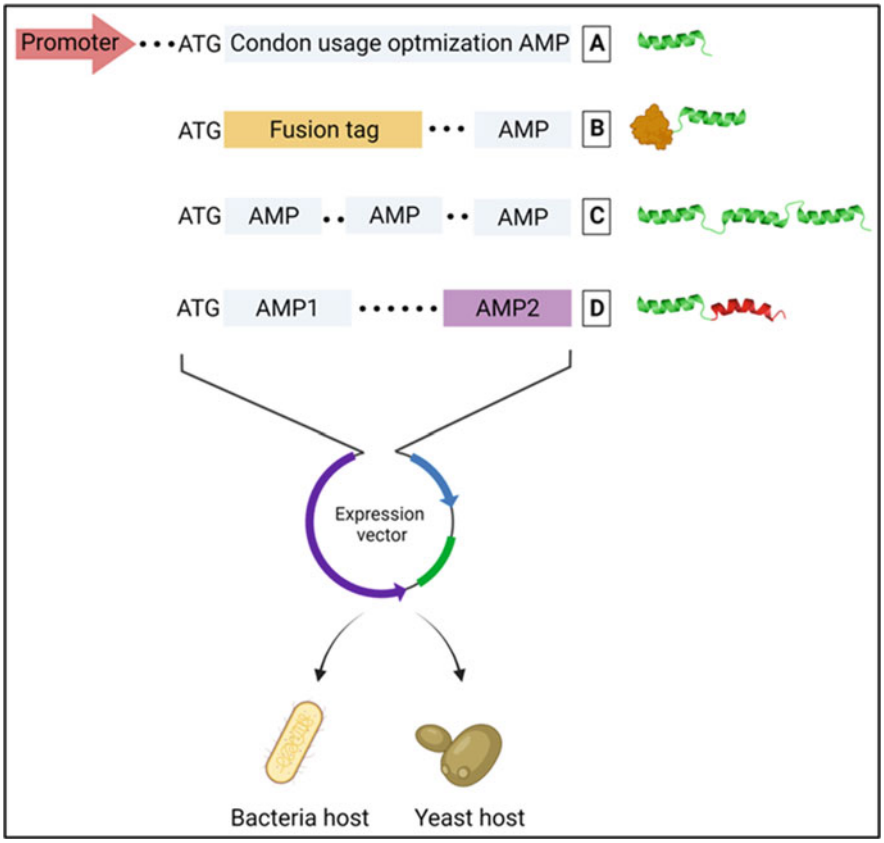

Fig. 2 Multiple strategies to increase the production of recombinant AMPs. (A) The use of strong promoters and codon optimization, (B) fusion to a higher molecular mass protein, (C) multimeric AMP expression in tandem and (D) hybridization of different AMPs. The figure was created by BioRender

3.2 Strategies to Enhance the Heterologous Expression Level

3.2.1 Cell Line Engineering and Host Strain

The choice of strain affects recombinant production success. At the end of the twentieth century, various potentially advantageous characteristics for heterologous production were tested in *E. coli* strains, generating line B, of which BL21(DE3) is the favorite host (Rosano et al. 2019). *E. coli* BL21 (DE3), and other parental B strains, have a deficiency in Lon protease, preventing exogenous protein degradation, an advantage to this system. In addition, the gene encoding the outer membrane protease OmpT is absent from the *E. coli* BL21 (DE3) genome, preventing the degradation of extracellular proteins (Rosano and Ceccarelli 2014). Another

advantage of the BL21 strain is that it expresses genes under the RNA polymerase promoters' control, such as lac, tac, trc, ParaBAD, PrhaBAD, T5, and T7 (de Oliveira et al. 2020). BL21(DE3) is able to provide a high AMP expression level, as observed in a study in which the yield of peptide P-113, derived from human saliva protein histatin 5, was 4 mg L^{-1} of bacterial suspension (Cheng et al. 2018).

Moreover, by transforming the *B. subtilis* (WB800) strain with a recombinant plasmid pHT-CI-CBF, scientists expressed cathelicidin-BF (CBF), a peptide isolated from snake venom (*Bungarus fasciatus*), fused with intein (Vogt et al. 2016). Data demonstrated that the intein expression system is a safe and efficient method by which to produce recombinant proteins in *B. subtilis,* since the yield of fusion CBF secreted reached ~0.5 mg L^{-1} (He et al. 2015). In addition to secretory capacity, this system can be exploited to produce recombinant molecules exposed on biofilm surface. Vogt and colleagues selected peptidic regions from tropomyosin and paramyosin, from the parasite *Echinococcus granulosus*, producing the peptides EgTrp and EgA31, respectively. They fused these peptides directly to the TsaA C-terminus, an important matrix protein (Vogt et al. 2016).

B. subtilis cells were genetically modified by the addition of a small tobacco etch virus (TEV) protease and the two-cistron expression vector gene of abaecin, previously isolated from *Apis mellifera* and further fused to TEV (Li et al. 2017). It has also been demonstrated that an expression system based on *B. subtilis* cells is capable of expressing a eukaryotic non-ribosomal peptide synthetase (esyn) gene that codes for the biosynthesis of the enniatin molecule (Zobel et al. 2015). Such examples reinforce the versatility of this system to produce recombinant peptides. In *K. phaffii*, recombinant peptides and proteins can be degraded through proteolysis mechanisms during transport or even in the extracellular space. To circumvent this limitation, protease-deficient strains such as SMD1163 (pep4 prb1 his4), SMD1165 (prb1 his4), and SMD1168 (his4 pep4) can be used. Although they have reduced proteolytic activity, due to gene silencing, these strains grow more slowly than the wild type, in addition to having low transformation efficiency and short cell viability (Daly and Hearn 2005; Ahmad et al. 2014). Even so, *K. phaffii* recombinant peptides' yield can be higher, as in the case of defensin VpDef, isolated from the mollusk *Venerupis philippinarum*, which was produced at a concentration of 60 μg mL^{-1} of culture medium (Meng et al. 2018). Another strategy to improve recombinant AMP yield is the use of both strong constitutive and inducible promoters.

3.2.2 Promoters and Codon Usage Optimization Strategies

The DNA region responsible for given gene transcription, or promoter, is another important component for recombinant AMPs production. Certain hosts have compatibility with different types of promoters. There is currently a plethora of commercially available promoters. Promoter T7, commonly found in pET series expression vectors, is a strong promoter most used for AMPs expression in *E. coli* cells (Deng et al. 2017). Promoters lac, tac, lacUV5, and T5 are also widely used

today, and research for the development of new and more efficient promoters continues to be developed. Inducible promoters were developed and derived from the constitutive phage promoters T5 (T5N25) and A1 (T7A1), capable of being recognized by RNAP of *E. coli* σ^{70}. Promoters not only decreased the basal expression but also increased the production of the recombinant protein (Schuller et al. 2020).

Another strategy to increase gene expression levels in bacterial systems is the use of dual promoters. Unlike eukaryotic systems, which have two separate expression cassettes, the dual promoters used in prokaryotic organisms are chimeric and are located, in tandem, upstream from the gene to be expressed (Öztürk et al. 2017). Using an optimized dual-promoter (P_{HpaII}–$P_{amyQ'}$) system, a group of scientists has demonstrated the increasing extracellular expression of β-CGTase, pullulanase, and α-CGTase in the *B. subtilis* (CCTCC M 2016536) strain. This strain was genetically manipulated to delete the *srfC*, *spoIIAC*, *nprE*, *aprE,* and *amyE* genes (Zhang et al. 2017).

In yeasts, the use of constitutive and inducible promoters is common. When compared to constitutive promoters, the inducible promoters are more used, because they allow a certain control over gene expression, which ends up resulting in a higher yield of recombinant peptides. Constitutive TEF1 and GPD promoters are the ones most used for *S. cerevisiae* expression. Although they can cause aggregation of folded proteins, making their secretion difficult, the promoters ADH1, GAPDH, PGK1, TPI, ENO, PYK1 also present an alternative (de Oliveira et al. 2020). Another factor that can influence the efficiency of promoters is polymorphism, which is already being evaluated (de Paiva et al. 2018).

Although the use of strong promoters could increase the heterologous expression levels, alternative strategies such as codon usage optimization can be used to boost the recombinant peptide expression. The codon usage bias refers to the availability of certain codons over others in the genome of organisms (Hanson and Coller 2018), and it can be a challenge for heterologous expression. Different species have a frequency of codons in a DNA sequence that is positively related to the corresponding tRNA, and the tRNA concentration in a cell is decisive for the number of amino acids accessible for protein translation extension (Fu et al. 2020).

The codon optimization approach had been used, in synthetic biology and the metabolic and cellular engineering fields, as an alternative by which to enhance heterologous gene expression levels (Lanza et al. 2014). In other words, codon optimization is suggested as a crucial factor in gene expression, since it consists of modifying synonymous codons through genetic engineering techniques, resulting in increased protein up-regulation and RNA levels (Mauro and Chappell 2014; Zhoua et al. 2016). In addition to contributing to mRNA stability, codon optimization also can impact ribosome translocation, connecting the processes of translation, elongation, and decay (Presnyak et al. 2015). Due to its importance to heterologous gene expression, scientists have developed, in recent years, new tools to facilitate codon optimization of synthetic genes. Such approaches explore the self-learning capacity of artificial intelligence (Tian et al. 2017; Fu et al. 2020), computational procedures (Chung and Lee 2012), and mathematical algorithms (Taneda and Asai 2020; Sen

et al. 2020). Organisms such as *B. subtilis* have another advantage, since the bias in codon usage is not a determining factor for heterologous expression. Additionally, transcription, translation, folding, and protein secretion processes, as well as methods of genetic manipulation and large-scale bioprocesses of this organism, are well described in the literature (Gomes et al. 2016).

Just changing a single codon synonym is enough to increase protein expression. The introduction of single codon synonym mutation (TCT → AGT) in the gene encoding the mrTNF-PADRE recombinant vaccine resulted in their enhanced production (~30% of total *E. coli* proteins) (Chu et al. 2018). It is also possible to improve translation efficiency in *S. cerevisiae* using the "condition-specific codon optimization" approach, as demonstrated by Lanza and colleagues (Lanza et al. 2014).

3.2.3 Tandem Multimeric Expression and Fusion Proteins

Another strategy commonly used for enhancing recombinant AMPs production is the tandem multimeric expression (Deng et al. 2017). Tandem peptide expression is an approach used for both prokaryotic and eukaryotic (yeasts) systems (Zhou et al. 2005; Fida et al. 2009; Wang et al. 2012). The expression of peptide LfcinB15-W4,10, a bovine lactoferricin, in four tandem repeats is more effective than monomers and the other repeats (2–8). At the end of the purification process, 10 mg of the tetramer with 99% purity was achieved (Tian et al. 2007). As demonstrated in the previous work, although it is possible to improve AMPs expression through multimeric tandem expression, the synthesis efficiency is not, unfortunately, proportional to copy number (Lee et al. 2002).

However, another strategy to overcome the low yield of recombinant AMPs is by expressing them fused to other proteins called fusion proteins which have been widely used in the expression of heterologous AMPs (Beaulieu et al. 2007; Liu et al. 2011; Mulder et al. 2015; Sousa et al. 2016; Xiao et al. 2017; Kaur et al. 2020). In addition to increasing the production level, these proteins facilitate the solubilization and purification of recombinant molecules (Costa et al. 2014). The small ubiquitin modifying (SUMO) fusion protein has an important function in AMPs' solubilization (Butt et al. 2005). Like SUMO, thioredoxin (Trx), a fusion protein from *E. coli*, also facilitates the solubilization of recombinant AMPs. However, unlike the former, Trx allows an increased expression rate (LaVallie et al. 1993; Costa et al. 2014). Another strategy is the use of polyhistidine ($_6$His) tag in the C- or N-terminal regions of a recombinant peptide. Although this tag is not a fusion protein, it facilitates the purification and detection by the western blot technique (Li 2011; Tavares et al. 2012; Belguesmia et al. 2020; Costa Ramos et al. 2021; Zhan et al. 2021).

3.2.4 AMP Hybridization

Hybridization, a combination of two native peptides, or even derivatives of hybrid peptides, is a method that has been used to produce new hybrid AMPs, in order to increase their antibacterial action, with reduced cytotoxicity (Wu et al. 2014; Klubthawee et al. 2020). A new hybrid AMP combined the α-helical fragments from peptides BMAP-27 and OP-145, producing the peptide H4, with the aim of maintaining its potent antimicrobial activity and reducing the cytotoxic profile against mammalian cells. The peptide H4 demonstrated activity against a broad spectrum of both Gram-negative and -positive bacteria, including the multidrug-resistant bacterial strains, in the range of 2.5–25 μM (Almaaytah et al. 2018).

Furthermore, the combination of two peptides can increase the plasma membrane permeabilization of the "parent" peptide. In order to evaluate the potential increase in activity of the hybrid peptides, hybrids were developed by joining membrane permeabilizing peptides (parasin or magainin 2) with membrane translocating peptides (DesHDAP1 or BF2). The results suggest that the permeabilizing activity is increased when the parent permeabilizing peptide is placed at the N-terminus, and through the addition of an alanine spacer between the sequences of the two parent peptides (Wade et al. 2019). Hybrid AMPs can also increase both the selectivity and stability of the molecule when compared to naturally occurring peptides (Yang et al. 2020). Through bioinformatic analysis, the 3.35 kDa hybrid magainin-thaumatin (MT) peptide was designed and expressed in *E. coli* BL21 (DE3) cells. Recombinant MT showed an inhibitory effect against *S. aureus*, *E. coli* DH5α, and *B. subtilis* at the MIC of 6.5, 20, and 9 μM, respectively (Tian et al. 2019).

Some hybrid peptides have strong antimicrobial activity against bacterial hosts, requiring the use of other expression systems. In this context, from hybridized plantaricin E (PlnE) and plantaricin (PlnF), type IIb bacteriocins, a ~5 kDa EF-1 hybrid peptide was developed (Li et al. 2020). This peptide can directly induce cell membrane permeabilization of *E. coli* cells. Expressing the recombinant EF-1 in the *K. phaffii* host, they recovered a yield of 32.65 mg L^{-1} with a purity of 94.9%. In addition to the bactericidal activity against enterohemorrhagic *E. coli* (EHEC) (MIC = 6.25 μM) and *E. coli* K88 (MIC = 3.125 μM) cells, recombinant EF-1 has no hemolytic activity (Li et al. 2020). The hybridization approach has the great potential to overcome some drawbacks, and it has been widely used (Jin et al. 2006; Xu et al. 2007; Arbulu et al. 2019; Agbale et al. 2019). Even though these strategies may have limitations, their use, alone or in combination, can increase the yield of recombinant AMPs, revolutionizing the production of biomolecules of medical and pharmaceutical interest.

4 Scale-Up from Small- to Large-Scale Fermentation

Bioprocess engineering has made considerable progress due to the high market demand for new biopharmaceuticals. New technologies related to bioprocessing techniques have been acquired for the large-scale production of proteins and peptides with biopharmaceutical potential for the treatment of various diseases. Economically viable production systems, which allow high yields to be achieved while maintaining the desired product quality, are now of great interest to bioprocesses industries (Potvin et al. 2012; Love et al. 2018; Tripathi and Shrivastava 2019).

An important step in the development process is the selection of a proper cell clone for the final production, which needs to fit the product quality requirements, processability and volumetric productivity. Clones can be selected for cell-specific and volumetric productivity, glycosylation profiles, aggregate formation, protein sequence heterogeneity and clone stability, among others. Cell culture conditions are decisive in productivity and product quality (Gronemeyer et al. 2014). AMPs biomanufacturing initially occurs on a small-scale, using shake flasks for expression system development. After this step, bioreactors are used to increase cell density and, consequently, the yield of the recombinant molecule of interest (Wibowo and Zhao 2019).

A defensin-like-peptide-P2 was successfully produced in *K. phaffii* using shake flasks. The induction of the recombinant peptide was performed with 0.5% methanol (v/v) every 24 h during the 120 h induction period, obtaining an expression level of 108.05 mg L^{-1}. Then, the process was scaled up, cultivation was carried out in a 5 L bioreactor, and a total of 1.69 g L^{-1} of peptide P2 was achieved. P2 exhibited bacterial reduction activity of 80–97% against multi-resistant *S. aureus* in RAW264.7 macrophages, among other activities (Yang et al. 2019).

Bacterial-based cell systems have also been used to express recombinant AMPs in shake flasks. One example is fowlicidin-2 expressed in *E. coli* BL21 (DE3). The induced expression of the peptide occurred by the addition of IPTG at a final concentration of 0.3 mM for 4 h in Luria-Bertani (LB) medium, at a temperature of 37 °C, under agitation. The results indicate a yield of 202 mg L^{-1} of the peptide of interest. The recombinant peptide demonstrated significant antimicrobial activity against a wide range of Gram-negative and -positive bacteria (Feng et al. 2015). Production of several other peptides was achieved on a small-scale using shake flasks (Sang et al. 2017; Meng et al. 2019).

Controlling certain parameters during production processes can make a big difference in AMPs yield. Monitoring critical operating parameters, including agitation, aeration, dissolved oxygen (DO), temperature, pH, and feed, is important and can be controlled in bioreactors. By monitoring the available oxygen rate, for example, it is possible to increase the availability of oxygen when it is low due to high cell density (Wibowo and Zhao 2019; Tripathi and Shrivastava 2019). The stability of these parameters allows high cell density and greater specific yield with a quality product to be achieved. In view of this, the physiological characterization of

production strains is essential for the proper development of a bioprocess (Gupta and Shukla 2017; Tripathi and Shrivastava 2019).

The composition of the culture medium also significantly influences cell growth and protein yield. The optimal selection of sources of carbon, nitrogen, salts, minerals and some growth factors and their proper concentrations are essential to achieve higher cell density and a higher level of recombinant proteins. The different carbon sources that are used as the main components of the cultivation medium in bioproduction, for instance, significantly affect cell metabolism, protein production and quality (García-Ortega et al. 2019). Complex, chemically defined or even semi-defined culture media can provide a nutrient-rich environment (Wibowo and Zhao 2019).

The parameters for obtaining a greater expression level of the hybrid magainin-thanatin (MT) recombinant AMP, produced in *E. coli*, were evaluated in shake flasks. Induction time (0, 1, 2, 3, 4, 5, and 6 h), temperature (32, 35, 37, 39 and 42 °C), IPTG concentration (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mM) and the culture medium were evaluated separately in different cultures. Maximum production of the MT was observed by cultivation in TB medium at 37 °C and induction with 0.8 mM IPTG for 5 h (Tian et al. 2019). In another work, the recombinant AMP UBI18-35, derived from the natural human AMP ubiquicidin, was also expressed in *E. coli* Rosetta (DE3) pLysS. SDS-PAGE electropherogram processing by densitometry demonstrated that the ideal conditions for the best production of AMP UBI18-35 include expression at 28 °C for 4 h after induction with 0.5 mM IPTG (Ashcheulova et al. 2018).

After carrying out the cultivation in shake flasks, cultures on larger scales are desired to obtain a superior recombinant AMPs yield. In this context, different bioprocess techniques can be employed. One example is a simple batch mode of cultivation in which the supply of essential nutrients only occurs at the beginning of bioprocess. Otherwise, in a fed-batch mode, nutrients are added at specific rates throughout the process. Commercial production of some antibiotics, such as penicillin, occurs in fed-batch. In continuous cultivations, the provision of nutrients to microorganisms constantly occurs through the addition of a fresh culture medium, and part of the culture is removed to collect the product simultaneously (Li et al. 2014b; Tripathi and Shrivastava 2019).

4.1 Batch Processes

Simple discontinuous batches can be useful in initial bioprocessing applications, for studies of the physiology and kinetic parameters of the microorganism. During this phase, which lasts between 24 and 30 h, the addition of the substrate occurs only at the beginning of the cultivation, which extends until it reaches a certain concentration of cells, or until the initial substrate is completely consumed during the process. As there is no other substrate addition during the procedure, cell density and yield are limited. Another disadvantage is that during this phase the accumulation of

secondary metabolites can occur, which can be toxic to the host microorganism, and may cause a limitation in obtaining the product of interest (Ÿztürk et al. 2016; Mears et al. 2017; Blunt et al. 2018).

Large-scale production of the recombinant AMP plantaricin (PlnE) expressed in *E. coli* BL21 (DE3) was performed in a bioreactor with a volume of 12 L of LB. Cultivation was carried out in a simple batch mode at 30 and 25 °C. The expression induction was done with IPTG (0.5 mM) for 3 h when OD600 reached 0.4. According to data presented, the yield of purified plantaricin E was 140 and 180 mg at 30 and 25 °C, respectively, in 12 L of LB after 3 h of induction (Pal and Srivastava 2015). To improve the expression level of the ABP-dHC-cecropin A in *E. coli*, large-scale bioprocess was performed. The production was carried out in a culture medium with 10 g L^{-1} of glucose in a 5 L bioreactor, at a stirring rate of 300 rpm, temperature at 37 °C, dissolved oxygen at 50% air saturation by cascade agitation between 300 and 800 rpm and pH 7.0. After 4 h of cultivation, the recombinant AMP production was performed using 0.5 mM IPTG and maintained for another 3 h. According to the results, 47.3 g L^{-1} of biomass was achieved in the batch, which is 14× more than the biomass obtained in culture flasks with LB medium. The amount of soluble protein recovered was 8643 mg L^{-1} (Zhang et al. 2016).

4.2 Fed-Batch Processes

The discontinuous fed-batch process allows a high biomass concentration to be obtained, and the product of interest is due to the possibility of extending process time, as well as the gradual feeding of the selected substrate in precise quantities (García-Ortega et al. 2019). Generally, it starts with a low concentration of substrate and, when its complete consumption occurs, more substrate is added to keep the fermentation process going without exceeding the ideal level. Product collection only happens at the end of the process, allowing for good sterilization conditions for the procedure (Li 2011). This cultivation strategy is advantageous for many microorganisms such as *E. coli*, *S. cerevisiae*, *B. subtilis*, and *K. phaffii* (Philip et al. 2017). In fed-batches, a simple batch phase is performed before feeding the system, in which the consumption of available substrate and accumulation of biomass will occur (Looser et al. 2015). When the initial carbon source is finished, the batch type is changed to fed-batch, in which the production of the recombinant protein will take place. Usually, the substrates used are the carbon source, together with minerals and trace elements needed (Ÿztürk et al. 2016). The feeding methods commonly used are continuous or constant, exponential and pulsed (García-Ortega et al. 2019).

During continuous and exponential feeding techniques, substrate supply is carried out constantly after simple batch bioprocess. Continuous feeding is based on nutrient addition at a continuous rate throughout the entire process. Exponential feeding allows the specific growth rate maintenance at a pre-defined level. The substrate amount needed for cells to reach the target concentration can be calculated

beforehand, and substrates are constantly supplied throughout the process (Ÿztürk et al. 2016). Due to nutrient addition during the procedure, there is a volume increase inside the bioreactor, causing a dilution of cell concentration. Thus, feeding is exponential due to exponential cell growth (D'Anjou and Daugulis 2000). Feedback feeding happens by monitoring indirect variables, such as oxygen or carbon dioxide dissolved in the system, or by the concentration of the carbon source, such as methanol or ethanol (Ÿztürk et al. 2016). Therefore, feeding can occur through pulses, according to dissolved oxygen increase rate, which indicates a metabolism reduction due to substrate depletion (Zhao et al. 2008; Looser et al. 2015).

The peptide NZX, a plectasin derivative, was expressed in *K. phaffii* (X-33) in a 5 L bioreactor, aiming to boost NZX production. After cell growth on glucose, the induction of NZW expression was performed in a fed-batch with methanol for 120 h. According to the results, the total biomass concentration obtained was 268 g $L^{-1,}$ and secreted protein was 2820 mg L^{-1}. The recombinant peptide showed high stability and low cytotoxicity, in addition to antimicrobial activity against *Staphylococcus hyicus,* both in in vitro and in in vivo experiments (Liu et al. 2020). Another group of scientists also used the yeast *K. phaffii* as a host for AMPs production. According to the data, the AMP clavanin MO was successfully expressed in *K. phaffii*. Therefore, the cultivation of the recombinant strain was then carried out in a bioreactor using BMGY medium with 40 g L^{-1} of glycerol in the discontinuous batch phase. After the total consumption of glycerol, the fed-batch carried out with 0.5% methanol added every 12 h during 72 h was initiated. At the end of the bioprocess, 5 mg mL^{-1} of the recombinant clavanin MO was recovered (Mulder et al. 2015).

Piscidin 4 (TP4), derived from tilapia *Oreochromis niloticus*, was produced in a 500 L bioreactor with a culture medium of basal salts with trace elements. In the fed-batch method, recombinant yeast *K. phaffii* growth was stimulated by adding glycerol as a carbon source until it ran out. Then, the discontinuous phase, also fed with glycerol, was conducted to obtain a higher cell density. Finally, the induction phase with 100% methanol was carried out for 65 h to induce the production of the recombinant TP4. Fed-batch bioprocess in recombinant strain *K. phaffii* (KM71) XS10 was also performed for the production of the recombinant bovine lactoferrampin–lactoferricin (LFA–LFC). The batch was kept for 12 h in a 10 L bioreactor with basal salts medium containing trace elements. After this step, a glycerol feed (50% (v/w)) was started at 10 mL/h for 12 h. Six hours later, peptide induction was initiated by the addition of 0.5% methanol (v/v) every 24 h. According to the results, it was possible to obtain about 1.025 $\pm$ 169 mg L^{-1} of LFA-LFC in the culture supernatant and about 53 $\pm$ 4 mg L^{-1} of the purified LFA-LFC (Tang et al. 2012).

4.3 Continuous Processes

Although relevant studies have already been published about discontinuous fed-batches, they can be less robust, more laborious and time-consuming. While the continuous mode allows for stable culture conditions, it provides a similar physiological state for all cells in the culture medium and, for these reasons, it has been one of the most widely used strategies for obtaining physiological data (García-Ortega et al. 2019). In the continuous mode, the target compound can be produced in large quantities due to the system maintenance at stationary phase. Fresh culture medium is added to the bioreactor, and part of the culture is continuously removed at a constant value: the dilution rate (D). This bioprocess is also known as chemostat (chemical and static environment), since the conditions inside the reactor (substrate, cell and product concentrations) can be stable (Mears et al. 2017; Blunt et al. 2018). Other parameters such as pH value, oxygen rate, working volume and nutrient supply are also kept constant (Koller 2018).

Continuous processes can keep the growth rate moderate and constant for long periods, which avoids the non-productive time spent on harvesting, cleaning, preparing a new medium, sterilizing and cooling the culture in a discontinuous process, generating higher average yield (Blunt et al. 2018). Among other advantages, it allows knowledge about bioprocess physiology, making it less laborious for the operation and maintenance of the process when it reaches a steady state (Blunt et al. 2018). Furthermore, the inhibition risk by substrate, or by-products, is lower as the final product is continuously collected throughout the process (Li et al. 2014a).

The interest in continuous cultures is not restricted to studies of microbial physiology and process development. It is also of great interest for the manufacture of recombinant proteins, as it allows cells to be kept in production states for a longer time, and consequently, the yield of the process significantly increases, while costs fall (Peebo and Neubauer 2018; Khanal and Lenhoff 2021). Several examples of continuous processes to produce recombinant proteins in different microorganisms have been reported in the scientific literature. However, some shortcomings associated with continuous processes make their commercialization limited (Rathore et al. 2015; Peebo and Neubauer 2018).

Both the stability and sterility during a long period of cultivation, as well as the lack of flexibility in the short term (caused by the need for long periods of execution), associated with the genetic inability of the cells can be cited as some limitations of this method of bioprocess (Rathore et al. 2015; Peebo and Neubauer 2018). Mainly due to these issues, continuous production of recombinant proteins is used particularly for the manufacturing of high-demand biopharmaceuticals. Recombinant insulin, produced in *S. cerevisiae* in the 1990s, is the only known example of a continuous industrial recombination process using microorganisms (Diers et al. 1991).

5 Purification of AMPs: Downstream Process Development

The downstream process consists of the recovery and purification of recombinant peptides, aiming to reduce costs (Clarke 2013). In this regard, innovative approaches have been applied in purification techniques of the downstream pharmaceutical industry (Gupta and Shukla 2017). These techniques are divided into three different stages: (a) an initial recovery (extraction or isolation), (b) purification (removal of most contaminants), and (c) polishing for removal of specified contaminants (Zydney 2016; Gupta and Shukla 2017; Tripathi and Shrivastava 2019). Taken together, all these steps improved the search, production and application of therapeutic peptides (Agyei et al. 2017). The purification process for recombinant AMPs is demonstrated in Fig. 3.

5.1 Recovery

At the recovery stage, the most common techniques applied in the industries are centrifugation, tangential flow microfiltration (MF-TFF), or depth filtration. The objective is to remove cells, fine particulates, colloids and soluble impurities prior to the initial purification steps (Pieracci et al. 2018). Generally, organisms such as yeasts export the recombinant peptides to the extracellular space, while in bacterial systems, the heterologous molecules are sent to the periplasmic space. To recover the extracellular recombinant peptide, it is necessary to concentrate it by a centrifugation

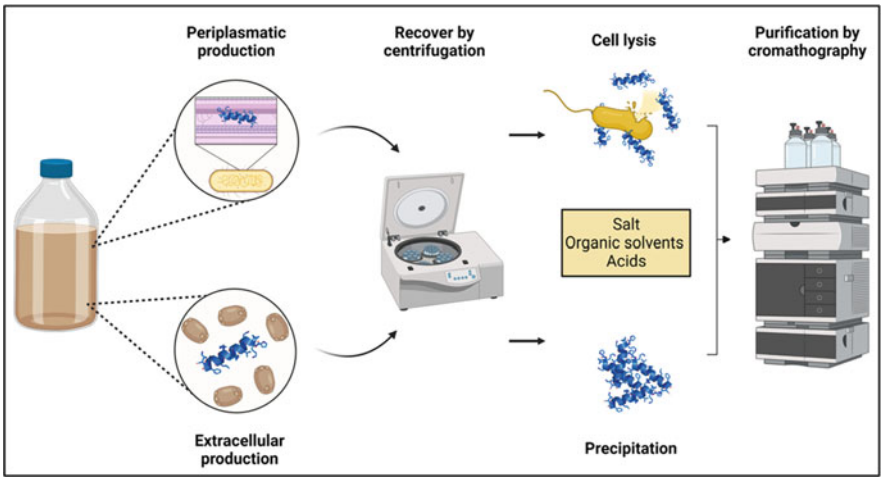

Fig. 3 Purification process of recombinant AMPs. The process addressed to both the periplasmic and extracellular spaces, through a signal peptide, can initially be recovered by centrifugation and, after this, from the cell lysis process (periplasm) or by precipitation (extracellular). The final step consists of the purification by chromatographic techniques. The figure was created by BioRender

or ultracentrifugation process. In addition to chromatography, the precipitation of the samples can be done to improve their concentration (Jozala et al. 2016).

The presence of a signal peptide located in the C or N-terminus region allows the export of the recombinant peptide to the extracellular (in *K. phaffii*) or periplasmic (in *E. coli*) space (Weinacker et al. 2013). For purified peptides in periplasmic space, the cells are submitted to lysis (sonication, high-pressure homogenizer, passing through mills, etc.) and clarification can be used to remove the cell debris. Therefore, the clarified product is purified using precipitation and/or chromatography. In some cases, recombinant peptides produced in periplasmic space can be accumulated as inclusion bodies (IBs), which are inactive, making an extra step necessary to refold the peptides to their native conformation (Ehgartner et al. 2017; Tripathi and Shrivastava 2019).

5.2 Purification

After the recombinant peptide recovery, the next step is the purification process by chromatography techniques (Tripathi and Shrivastava 2019). There are several types of chromatography, such as size exclusion chromatography, ion-exchange chromatography, low-pressure hydrophobic interaction chromatography, countercurrent distribution, partition chromatography, and reverse-phase high-performance liquid chromatography (RP-HPLC) (Tripathi and Shrivastava 2019). Although the chromatography techniques are well-established processes, they are more expensive, when compared to other purification approaches, as well as presenting limitations in throughput and scalability (Tripathi and Shrivastava 2019). To overcome some of those limitations, it is important that the purification strategy must be evaluated for every single case, and it can change according to the particular application (Carta and Jungbauer 2010). In this context, several AMPs have already been purified using chromatography techniques.

The host defense peptides (HDPs) IDR1, MX226, LL37, CRAMP, HHC-10, E5, and E6 were purified in a simplified 2-step purification method. After recombinant production in high yields, using *E. coli* strain BL21 with SUMO fusion, the peptides were first purified using a Ni-NTA Sepharose column. The recombinant peptide IDR-1 was successfully purified. From a yield of about 1.5 kg of wet biomass, 6% of the total protein produced is equivalent to the SUMO-IDR-1, with an estimated yield of 0.48 g/L of fermentation. Subsequently, the SUMO-specific protease sumoase was used to release the peptide from the fusion. Following the cleavage, reversed-phase chromatography was used to separate SUMO and sumoase from the peptide and recover the homogenously pure peptide (Bommarius et al. 2010).

6 Optimization of the Industrial Processes

Recently, different methods have been used in order to optimize the industrial process, such as high-throughput devices (HTD), design of experiments (DoE), and process analytical technology (PAT). The HTD method is divided into screening (HTS), process development and experimentation (HTPD), and experimentation (HTE). These processes together seek to obtain data quickly and reliably, doing tests on a small-scale, producing multiple data points to be generated with low usage of laboratory space, in addition to saving time (Silva et al. 2021).

HTS is responsible for producing hundreds of thousands of data points per day, using microtiter plates with 384 and 1536 wells (Mayr and Bojanic 2009). Another option is microfluidics, which use very small amounts of liquids, with quick analysis generating multiple data points and thus reducing time and laboratory space (Whitesides 2006). The goal of HTS is optimization of the process and delivering the final product actively and quickly (Shukla et al. 2017; Silva et al. 2021). HTPD makes use of complex computational tools to seek to establish a better design and understanding of the process (Bhambure et al. 2011). HTE is the union of HTS and HTPD to provide an overview of the miniaturization and automation process, enabling applicability in the biopharmaceutical industry (Silva et al. 2021; São Pedro et al. 2021).

In this regard, a group of scientists reported the combination of two different methods (high-throughput (HTP) processes and flow-mediated synthesis). They rapidly obtained a large amount of information about new peptides. They tested a library of potential AMPs against *P. aeruginosa*, and the results allowed them to identify the peptide with the highest antimicrobial activity. The workflow contributes to the discovery and optimization of peptide structures as new antimicrobial agents for biomedical applications (Judzewitsch et al. 2020). The suitability of both the diffusion self-interaction parameters (kDa) and osmotic second virial coefficients (B22) was investigated by using high-throughput screening, aiming to seek the peptides' formulation aggregation risk. In this study, thermal stress effects on colloidal stability were evaluated, as well as six buffering systems at two-selected pH values, four tonicity agents and a common preservative. Acetate and succinate buffer at pH 4.5, combined with glycerol or mannitol, demonstrated greater stability of peptides. Data suggest that HT methods display important information about the optimization of colloidal stability during the early development of peptide-based liquid formulations (Dauer et al. 2021).

In addition to the HTS method, DoE also allows bioprocesses to be optimized through monitoring and control of the experiment. DoE enables simultaneous evaluation of a large number of variables. Thus, this methodology obtains maximum information about the process, saving time and financial resources to obtain a high-quality product (Kasemiire et al. 2021). This methodology was used to optimize the AMP human β-defensin 2 (HBD2) expression yields in *E. coli* BL21 (DE3). The authors used 24-factorial design of experiments (DoE) to evaluate the following variables, namely cell density, temperature, induction period, and inducer

concentration. They got 19 different combinations, and the best condition was pre-induction temperature of 37 °C, a cell density of 1.0 U (600 nm), an induction temperature of 20 °C and a 0.1 mM of gene expression inducer (IPTG) over 4 h. Researchers concluded that these conditions produced the HBD2 peptide in a higher proportion than previously tested (Corrales-García et al. 2020).

Process Analytical Technology (PAT), initially from the US Food and Drug Administration (FDA), is responsible for the regulation of biopharmaceutical production, dealing with measuring, analyzing, monitoring, and ultimately controlling all substantial characteristics of a bioprocess (Scott and Wilcock 2006; Kornecki and Strube 2018). It is important to remember that the transition from up- to downstream in a bioprocess is always challenging. Any small change can affect final product quality. PAT was used for monitoring and controlling specific reactions during the manufacturing process of the recombinant lethal toxin-neutralizing factor (rLTNF), which acts against rattlesnake venom. Monitoring was performed in three critical production stages (solubilization of the IBs, enzymatic cleavage with α-chymotrypsin and quenching of the reaction at the optimal time). The authors reported that the process with PAT tools through various batches of rLTNF production would allow real-time analysis of quality and production control (Hebbi et al. 2020). Therefore, the methods described above are of great importance, as they seek to find the best operating conditions, in addition to determining the operating windows. These features are extremely useful in minimizing the impact of batch-to-batch variations and human error during the bioprocess.

7 Conclusions and Future Directions

Bioprocessing technology is a great opportunity for developing recombinant therapeutic AMPs on a large-scale. The promiscuity of these molecules makes them promising alternatives in the development of new biopharmaceuticals against a number of infectious diseases. The use of microorganisms and different fermentation processes allows large-scale high-quality AMPs production. Therefore, optimization of process conditions seems to be extremely important to generate biologically active and stable molecules. Different strategies can be applied for cell line engineering, such as promoters and codon usage optimization, tandem multimeric expression and fusion protein production, and AMP hybridization.

General optimization of AMPs bioprocessing seeks the best expression system and conditions for production and recovery of compounds. However, it must be emphasized that there is no ideal production system and that they all have advantages and disadvantages. Analysis of AMPs physicochemical characteristics is essential to find the most useful conditions to produce stable and active molecules, as well as in large quantity. The same occurs during the recombinant strain cultivation on a small or large-scale. Cultivation conditions and bioprocess strategies must be carefully analyzed, as they directly influence the recombinant AMPs expression.

The choice of an appropriate recovery method for an AMP after its production is also very important, as it can allow AMPs with a high degree of purity to be obtained.

In summary, optimization methods for industrial process are important developments for this field. Through best operating conditions, these optimized methods increase the production rate and reduce costs in both upstream and downstream process development. Such improvements are important because they can allow us to produce AMPs in high quantities and purity, and these will then be applied in the pharmaceutical industry, research, and pre-clinical and clinical trials. Furthermore, bioprocessing automation can reduce the impact related to variations during bioprocesses, as well as human error. All approaches related to upstream and downstream processes in the recombinant production of AMPs mentioned here generate lower production costs, manufacturing diversification and recombinant AMPs quality.

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