FACULDADE DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS FARMACÊUTICAS

DILEESH PRAKASAN

O DESREGULADOR ENDÓCRINO, DIBUTILESTANHO, É UM ANTAGONISTA DE PPAR-ALFA EM ENSAIOS DE GENE REPÓRTER

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Tese de Doutorado apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Ciências da Saúde, Universidade de Brasília, como requisito parcial á obtenção do titulo de doutor em Ciências Farmacêuticas.

Orientador: Prof. Dr. Francisco de Assis Rocha Neves

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Preface

Obesity is the direct result of an imbalance between energy intake and energy expenditure. The excess energy is primarily stored in adipose tissue in the form of triglycerides. Obesity also provokes structural and metabolic alterations in other organs, including skeletal muscle and liver. The excessive fat accumulation in adipose tissue, liver and other organs induce the developmental change in the metabolism and lead to metabolic syndrome. Obesity alters the signaling mechanism of the cell and induces an unbalanced amount of proteins such as adipokines and cytokines, which finally perturb the cell signaling mechanism and leads to diseased conditions such as diabetes. Synthetic agonist of Peroxisome proliferator activated receptor (PPARs) (thiazolidinedione) are considered as the best known efficient antidiabetic drug for the treatment of insulin resistance, but are not enough to solve the issues such as insulin resistance and type 2 diabetes. They over activate a set of genes, lead to serious side effects such as cardiac failure, cancer, liver toxicity and osteoporosis etc. It has been recently discovered that selective expression of genes are necessary for solving this issue. Recent discoveries have proven that edible plant parts contain diverse structured compound, which could activate the PPARs and could selectively modulate the expression of various genes with reduced side effects. Hence my Phd work was started to identify novels ligands for PPARα, β and γ from the extracts of cerrado related plants.

During my first 2 years, I had worked with various plant extracts for screening novel natural ligands for PPARα, β and γ. Various plants extracts of *Bauhinia variegata, Eugenia dysenterica, Erythroxylum suberosum, Erythroxylum daphnites, Erythroxylum subrotundum* have been used for the study. Among these various extracts tested, Bauhinia variegata (Hexanic) demonstrated weak activation for PPARα, while PPARβ exhibited a dose dependent activation in the transactivation. The results are not yet published.

In my 3rd year, another work was started with GQ-02, GQ19, which are modified ligands of TZD having anti-inflammatory properties. GQ-2 and GQ-19 has been screened for analyzing the agonistic effect on PPARα, β, γ and RXRα. These studies

exhibited no effect in the transactivation assay. Hence, another study was conducted to explore the antagonistic activity of these compounds on PPARα, β and γ. Unfortunately, compounds did not demonstrated any antagonistic activity to any of these receptors.

Later another work was assigned to me in order to test the agonistic effect of dibutyltin class of organotin compounds on RXRα. Various compounds of butyltin compounds such as dibutyltin dilaurate, dibutyltin dichloride, dibutyltin diacetate, dibutyltin maleate were studied using the transactivation assay. Studies concluded that dibutyltin dichloride,dibutyltin dilaurate acts as weak agonist for RXRα. The work has been presented in an International conference (*The Endocrine Society's 97th Annual Meeting*) held at San Diego, 2015.

The present work emerged from our previous laboratory studies. Dr. Flora Milton, Post doc was studying about the agonistic effect of dibutyltin compounds on various nuclear receptors. Found that dibutyltin compounds showed no agonistic activity on PPARα and hence we were interested to study the antagonistic effect of DBTs on PPARα. Various dibutyltin compounds have been investigated while dibutyltin dilaurate and dichloride showed the antagonistic effect on PPARα.

Resumo

PRAKASAN, dileesh. **O desregulador endócrino, dibutilestanho, é um antagonista de PPAR-alfa em ensaios de gene repórter**. Brasília, 2015. Tese (Doutorado em Ciências Farmacêuticas) Faculdade de Ciências da Saúde, Universidade de Brasília, Brasília,2015.

A exposição a poluentes ambientais está se tornando uma séria ameaça para os seres humanos, bem como para os animais. Estes poluentes apresentam perfis toxicológicos elevados, estruturas diversas e bloqueiam vários mecanismos de sinalização. Compostos organoestânicos são categorizados como desreguladores endócrinos devido à sua capacidade de perturbar a função endócrina. Estes compostos têm como alvo a membrana plasmática, enzimas, mitocôndrias, vários receptores nucleares ou induzem modificações epigenéticas. Compostos de diorganoestanho são a das classes mais importantes dos compostos organoestânicos. Dilaurato de dibutilestanho e dicloreto de dibutilestanho são derivados de compostos de dibutilestanho, que são utilizados como estabilizadores de PVC, catalizadores e agentes de desparasitação. Compostos de dibutilestanho têm sido detectados no sangue humano e no fígado. Diversas evidências indicam que os compostos de butilestanho induzem esteatose hepática, obesidade, *imposex*, adipogênese e inibem a osteogênese entre outras. A atividade agonista dos compostos organoestânicos nos PPARs tem sido extensivamente estudada, no entanto ainda não há evidências suficientes documentando a atividade antagonista em PPAR. Nesse estudo, pela primeira vez, demonstramos que o dilaurato de dibutilestanho, dicloreto de dibutilestanho atuam como antagonistas de PPARα no ensaio de transativação de gene repórter. Os resultados indicam que o dilaurato de dibutilestanho atua como um antagonista fraco, com um valor de IC_{50} de 4.1μM, enquanto que o dicloreto de dibutilestanho atua como um antagonista potente do PPARα, com um valor de IC_{50} de 0.26μM.

Palavras-chave: Desregulador endócrino, dibutilestanho, Receptores ativados por proliferadores peroxissomais (PPAR).

Abstract

PRAKASAN, dileesh. **Endocrine disruptor, dibutyltin is an antagonist for PPAR-alpha in reporter gene assay.** Brasília, 2015. Tese (Doutorado em Ciências Farmacêuticas) Faculdade de Ciências da Saúde, Universidade de Brasília, Brasília, 2015.

Environmental pollutant exposure is becoming a serious threat to humans as well as animals. These pollutants are having high toxicological profiles, diverse structures and they disrupt various signaling mechanisms. Organotin compounds are categorized as endocrine disrupting chemicals due to their capability to perturb the endocrine function. These compounds target plasma membrane, enzymes, mitochondria, various nuclear receptors or induce epigenetic modifications. Diorganotin compounds are one of the most important class of organotin compounds. Dibutyltin dilaurate and dilaurate dichloride are the derivatives of dibutyltin compounds, which are used as PVC stabilizers, catalytic and as deworming agents. Dibutyltin compounds have been detected in human blood and liver. Accumulating and emerging evidence indicate that butyltin compounds induce hepatic steatosis, obesity, imposex, adipogenesis and inhibit oesteogenesis etc. The agonistic activity of organotin compounds on PPARs has been studied extensively, while there are not enough evidences documented about its antagonistic activity. In the present study, for the first time, we demonstrated that dibutyltin dilaurate and dibutyltin dichloride act as PPARα antagonist in the transactivation assay. The results indicate that dibutyltin dilaurate acts as a weak antagonist with an IC_{50} value of 4.1μM, while dibutyltin dichloride acts as a potent PPARα antagonist with an IC_{50} value of 0.26μM.

Keywords: Endocrine disruptor, Dibutyltin, PPAR-Peroxisome proliferator activated receptor.

List of Abbreviations

ANT	Adenine nucleotide translocator
ATGL	Adipose triglyceride lipase
Abca1	ATP binding cassette, subfamily A member-1
AST	Aspartate amino transferase
ADIPOR1	Adiponectin recepor1
ALT	Alanine amino transferase
ATPase	Adenosine 5'-triphosphatase
ADIPOR2	Adiponectin receptor 2
ACS	Acetyl-CoA synthetase
AF2	Activation function 2
AF1	Activation function 1
AAMs	Activated adipose tissue macrophage
BADGE	Bisphenol A diglycidyl ether
BDNF	Brain Derived Neurotrophic factor
BMP7	Bone morphogenetic protein
BAT	Brown adipose tissue
BMP8	Bone morphogenetic protein 8
CNS	Central Nervous system
CAMs	Classically activated macrophages
COX2	Cyclooxygenase-2
CPT1α	Carnitine palmitoyl transferase1α
CEBPα	CCAAT/enhancer-binding protein alpha
DBTC	Dibutyltin chloride
DEHP	Diethylhexyl phthalate
DBT	Dibuyltin
Dmp1	Dentin matrix phospho protein 1
DNMT1	DNA methyl transferase
DR1	Direct repeats
EDC	Endocrine disrupting chemical
ER	Endoplasmic reticulam
FABP4	Fatty acid binding protein

FXR	Farnesoid X receptor
FAS	Fatty acid synthase
FAT	Fatty acid translocase
FATP	Fatty acid transporter protein
GQ16	(5-(5-bromo-2-methoxy-benzylidene)-3-(4-methyl-benzyl)-thiazolidine-2,4-dione
GR	Glucocorticoid Receptor
HAT	Histone acetyl transferase
HSL	Hormone sensitive lipase
HDAC	Histone deacetylase
IL-6	Interleukin -6
IL- 1β	Interleukin 1β
IL-10	Interleukin 10
IMMAC	Inner mitochondrial membrane anion channel
INFγ	Interferon γ
IkBα	Inhibitor of kB kinase complex
JNK	Jun N-terminal kinase
LBD	Ligand binding domain
LPS	Lipopolysacharide
MBT	Mono butyltin
MEHP	Monoethylhexyl phthalate
MAPK	Mitogen-activated protein kinases
miRNA	microRNA
MCP1	Monocyte Chemo attractant protein 1
MAPK	Mitogen-activated protein kinases
mRNA	messenger RNA
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NCOR	Nuclear receptor corepressor
Osx	Osterix
OCT	Ornithine Carbamyl Transferase
PVC	Poly vinyl chloride
PPAR	Peroxisome proliferator activated receptor
PFA	Penis Forming Area
PFOA	Perfluorooctanoic acid
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PAI-1	Plasminogen activator inhibitor
PRDM16	PR domain containing 16
PI-3 kinase	Phosphatidylinositide 3-kinase
PKA	Protein kinase A
PDK4	Pyruvate dehydrogenase kinase
Runx2	Runt-related transcription factor 2
RXR	Retinoid X Receptor
RAR	Retinoid Acid Receptor
RDX	Research Department Formula X
SAM	S - Adenosyl methionine
SAH	S- Adenosylhomocysteine
SIRT1	Silent mating type information regulation 2 homolog
SFRP5	Secreted frizzled-related protein 5
SOCS	Suppressor of cytokine signaling
TBTC	Tributyltin chloride
TCA	Tricarboxylic acid cycle
TPT	Triphenyltin
Tgm2	Transglutaminase
TNFα	Tumour necrosis factor α
TMT	Trimethyltin
TGF-β	Transforming growth factor – β
TRE	Thyroid responsive element
TR	Thyroid Receptor
UCP1	Uncoupling protein 1
VDR	Vitamin D Receptor
WAT	White adipose tissue
3βHSD	3β-hydroxysteroid dehydrogenase type

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1.Introduction

The safety of chemical compounds, which pollute our environment, is under wide concern due to its high toxicological profile. These compounds are having diverse chemical structures, high affinity towards various receptors and enzymes. Some of these chemicals mimic like endogenous ligands, can activate [1] or inhibit the expression of various genes or can make the epigenetic modification in the genes[2], which may further disrupt the signaling mechanism and leads to various diseased conditions. One of the example is obesity. Obesity is characterized with the dysregulation of various lipogenic as well as inflammatory genes[3], [4].

This work is focused towards the effect of dibutyltin class of organotin compounds on PPARα. Endocrine disrupting chemical(EDCs) can be any chemical, whether its synthetic or natural product[5], which alters the endocrine signaling mechanism. Most of the organotin derivatives alter the endocrine mechanism and hence it can be considered as the endocrine disrupting chemical.

Organotin compounds.

The First systemic study about organotin compounds was started by Edward Frankland. In 1853, diethyltin diiodide and in 1859, tetraethyltin were synthesized. Later on, other investigation followed and currently more than 800 organotin compounds are available[6], while few of them are having therapeutic potential against cancer. For example: Dibutyltin derivatives has been shown as antitumor agents[7]. The high toxic nature of the organotin compounds makes it intricate, hence the complete understanding of the inflammatory and the metabolic pathways become difficult. For example: Studies illustrates that PPARγ agonist repress the proinflammatory cytokines (i.e tnf-α, IL-6, IL-1, etc)[8]. Tributyltin chloride (TBTC) has been reported as an agonist for PPARγ[9], while others have demonstrated that PPARγ agonist, TBTC, activates the proinflammatory genes such as tnf-α in a dose dependent manner, when exposed to murine macrophage cell line (J774.1)[10]. In addition, TBTC exposure to murine keratinocyte cell line (HEL30) induced IL-1α, IL-6[11]. These evidences make it clear that TBTC trigger inflammatory genes through diverse mechanism. Another study demonstrated that TBTC reduces hepatic adiponectin in a dose dependent manner, which leads to hepatic steatosis

and obesity when exposed to male KM mice for 45 days[12]. Organotin compounds acts as obesogenic agent through the interaction with nuclear receptors such as PPARγ[1]. Various studies have been showed that organotin compounds induce adipogenesis in vitro as wells as in vivo[1], [9], [13]–[15]. Organotin compounds are diverse group of widely distributed environmental pollutants. The inorganic form of tin is considered as nontoxic, while the organotin compounds are having high toxicological profile. Triethyltin compounds are considered as the most potent toxic compound, entry of triethyltin in mammals show muscle weakness within hours, after a short period, tremor develops, ultimately leads to death after 2 to 5 days[16]. The biological effects of these compounds depend on the nature and the number of organic group bound to the tin (Sn) cation. On the basis of the presence of organic moieties, butyltin compounds are classified as mono, di, tri and tetra butyltin. Tributyltin compounds are the organic derivatives of tetravalent tin, which belongs to the tributyltin class of organotin compounds. TBT compounds include tributyltin hydride, tributyltin oxide, tributyltin benzoate, tributyltin chloride, tributyltin fluoride etc. While dibutyltin compounds are of various types which include, dibutyltin diacetate, dibutyltin dichloride, dibutyltin maleate, and dibutyltin dilaurate.

Dibutyltin compounds are mainly used as PVC stabilizer, catalytic agents, to control poultry tape worms and fungicides[17]. The PVC polymer becomes unstable under the influence of heat, light and it becomes more brittle. Later on it was found that addition of certain organotin compounds could prevent it. Organotin compounds have been detected in drinking water due to the leaching property from the PVC pipes[18] and in plastic covered food materials due to its leaching property from plastic materials[19]. Various studies suggest that organotin compounds have been detected in fish. For eg: Recent evidence suggests that TBT oxide acts as a potent antagonist for plaice PPARα and PPARβ at nM concentrations[20]. In Brasil, the Brasilian Navy restricted the use of organotin based compounds in antifouling paints in 2007 by means of the regulation (NORMMAM-23/DPC)[21]. For seawater and estuarine water quality parameters, the basal legal reference in Brazil, resolution 357 of the National Environment Council (CONAMA 2005) suggests that concentration limits for preservation of species in natural reserves, marine aquaculture in saltwater, protection of aquatic communities in estuarine waters were of 0, 10, 63 and 370ng/L respectively[21].An investigation of tin was carried out in 67 cetacean in the Rio de

Janeiro and Espiríto Santo states. The study reveals that the hepatic tin concentration in the marine tucuxi in the beaches of Guanabara bay varied between 1703 to 9638ng/g wet weight[22]. This evidence makes it clear that consuming sea food is a source for the organotin entry into the humans.

The entry of organotin compounds occurs mainly through the gastrointestinal tract. Studies found that severe hemorrhages in the mucus membrane, inner layer of gastric and intestinal wall of organotin treated white mice[23]. The major source of organotin intake for humans is dietary sources such as sea food and drinking water. Significantly high concentration of DBT(higher than 300ng/g) on dry weight has been detected in human blood samples collected from central Michigan[24], [25]. The concentration detected in the liver was < 360ng/g wet weight [26], [27]. TBTO treated wistar rats have showed higher concentration of tin in kidney and liver while the tin residues in the brain and adipose tissue were 5 to 10 times lower and has been found that the bile duct was inflamed[28].

Another study reveals that TBTC and DBTC in mice induced liver injury. The concentration of TBTC and DBTC that induced hepatotoxicity in mice at 24hrs after oral administration were 180 and 60μmol/kg, while guinea pig did not produce any liver injury even after 24 hrs[29]. Few reports suggest that TBT compound undergoes cellular metabolism by means of cytochrome P450 enzymes, produces di, mono and inorganotin compounds[30]. There are reports suggesting that TBT treatment in rats caused an initial increase of TBT content in the liver and is followed by a rapid decrease in hepatic TBT and showed an increase in DBT, MBT and inorganic tin content. Another interesting finding regarding the behavior of organotin compounds is about the accumulation of monobutyltin and inorganic tin in the brain. This clearly suggest that TBT passed through the blood brain barrier and is converted to di, monobutyl and further to inorganic tin in the brain[31].

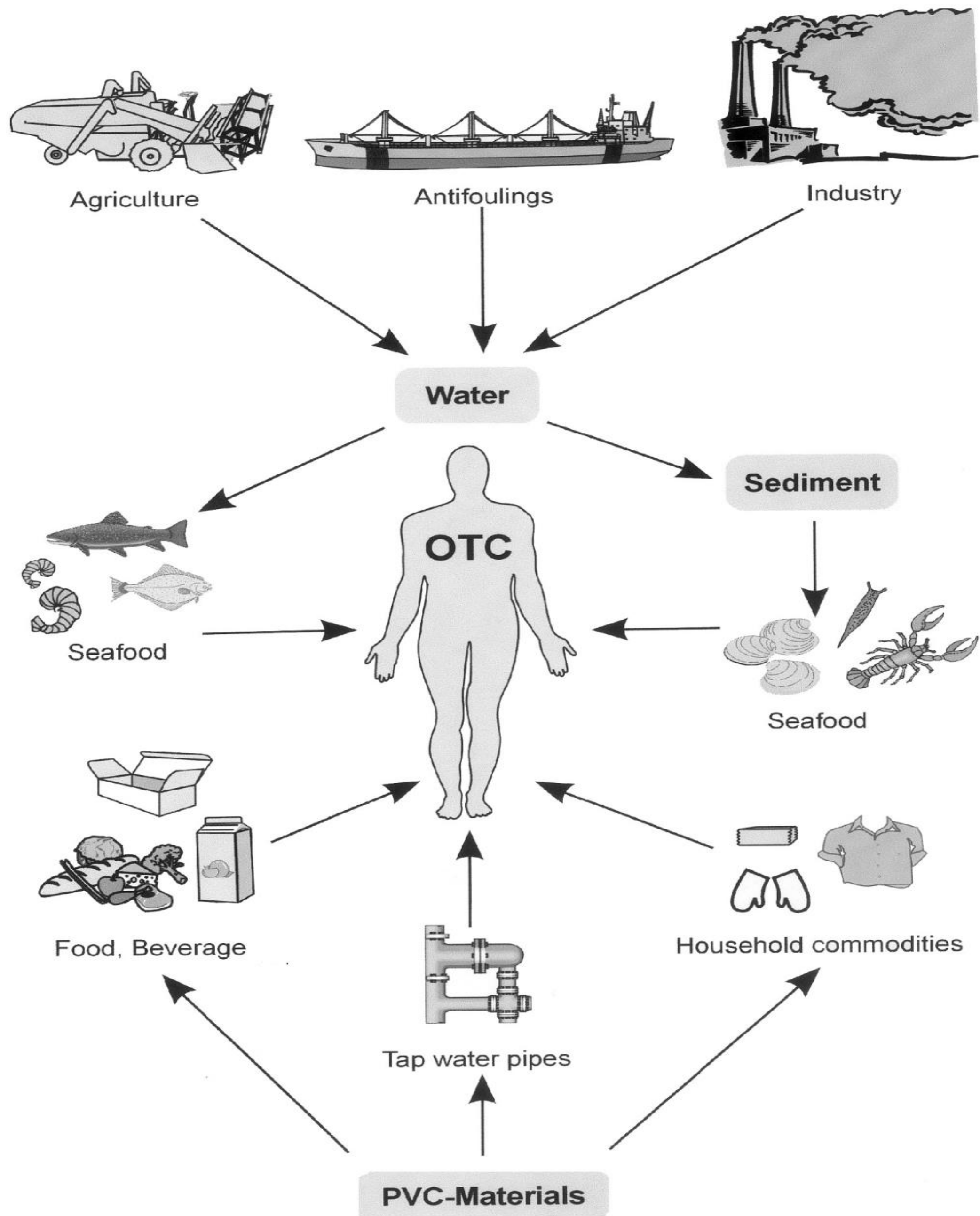

Figure-1: Different sources of organotin compounds exposure to humans.

Figure adapted from[6].

Imposex

The formation of male sex characteristic in females is called imposex. Organotin compounds such as TBT, TPT cause masculinization in female mollusk and induce imposex. Various studies suggest that organotin compounds especially TBT, TPT inhibit cytochrome 450 aromatase which converts androgen to estrogen. It is hypothesized that the induction of masculine characteristic in females is due to the increase of androgen levels compared with estrogen (a decreased conversion of androgen to estrogen). Studies conducted in steroidogenic human ovarian granulosa-like tumor cell lines, having a functional follicle stimulating hormone receptor (FSH) and a high aromatase activity in mammalian system suggests that tributyltin (TBT, TPT) showed a decrease in aromatase and it results in the decrease in the aromatase activity (Figure-2)[32], [33].

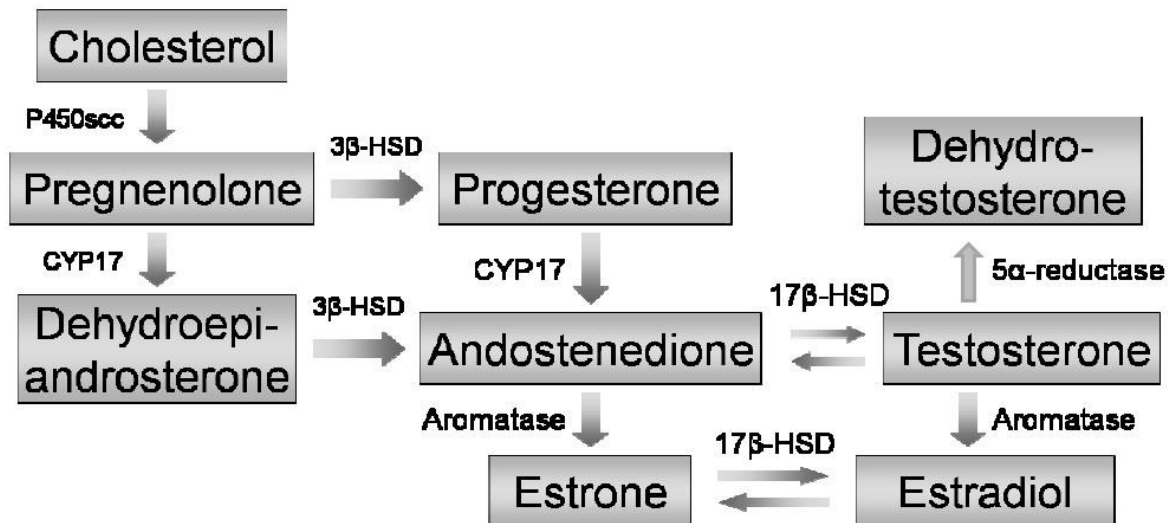

Figure - 2: The enzymatic action of steroid hormone biosynthetic pathways can be altered by organotin compounds. Figure modified from[34].

2. Organotin Compounds as antitumor agents.

Organotin compounds have wide range of industrial applications, few of them are having therapeutic property against tumors. Marcel Glien characterized few diorganotin derivatives of carboxylate which acts as antitumor agents. The studies were conducted in seven tumor cell lines of human origin and found that di-n-butyltin camphorates showed high inhibitory effects than Cisplatin, 5-Flourouracil and Etoposide but less inhibitory effect than methotrexate and doxorobicin. Dimethyltin camphorate were inactive[7].The values are noted in Table:-1[7].

Compounds	MCF-7	EVSA-T	WiDr	IGROV	M19 MEL	A498	H226
1	49	28	100	45	66	49	178
2	1342	903	3504	1006	1111	1548	764
Cisplatin	699	422	967	169	558	2253	3269
5-Fluorouracil	750	475	225	297	442	143	340
Etoposide	2594	317	150	580	505	1314	3934
Methotrexate	18	5	<3	7	23	37	2287
Doxorubicin	10	8	11	60	16	90	199

Table- 1. Inhibition dose ID_{50} of compounds with reference drugs such as Cisplatin, 5-Fluouracil, Etoposide, Methotrexate and Doxorubicin (Compound 1 =Di-n-butyl camphorate, Compound -2=Dimethyltin camphorate)

Another study reveals that triorganotin carboxylates showed antibacterial and antitumoral activity. The compounds showed high invitro antitumoral activity than clinically used Cisplatin[35]. Among the various organotin compounds dibutyltin showed higher antitumor activity and less toxicity. The studies suggest that dibutyltin reacts with Cantharidine, an effective ingredient of *Canthatis vesicatoria*, a chinese medicine used for malignancy treatment. The compounds showed high cytotoxicity for tumor cell lines of P38 and HL-60[7].

3. Mechanisms of action of Endocrine disrupting chemicals.

Endocrine disrupting chemicals which act like endocrine hormones and disrupt the normal endocrine signaling pathways. These chemicals have been detected in wildlife and in humans. The human blood collected from central Michigan, United States has been detected with dibutyltin compounds at a concentration of 300ng/g on dry weight and unfortunately these concentrations are significantly high[25]. A wide range of evidences have been reported that endocrine disrupting chemicals alter the normal signaling mechanism, induce apoptosis, inflammation, hepatotoxicity, etc[10], [36]–[38]. Hence priority has to be given to understand the endocrine disrupting chemical mediated mechanism of action.

The mechanisms of action by means of endocrine disrupting chemicals are mainly classified as two types: Receptor mediated mechanisms of action and non receptor mediated mechanisms of action. The receptor mediated effect occurs in different receptors and one of them is the nuclear receptor superfamily.

3.1 Nuclear receptor and endocrine disruptors

This thesis describes mainly about the action of organotin compounds on nuclear receptors and its consequences. The organotin compounds have high affinity towards nuclear receptors. Tributyltin is the most well characterized organotin compound. Studies suggest that TBT activate retinoid X receptor (RXR) and PPARγ. They induce adipogenesis in 3T3 cells, this effect seems to be mediated by PPARγ [9]. Another report suggest that organotin such as TBT is an agonist for vertebrate RXRα and PPARγ[14]. TBT has been identified as an endocrine disruptor, which targets adipogeneiss through the modulation of various adipogenic regulators. The exposure of TBT could lead to obesity and chronic lifetime exposure could leads to obesity related disorders[14].

Another study in male mice suggests that low dose of TBT resulted in the significant elevation of body weight gain, plasma insulin and plasma leptin. The hepatic adiponectin was reduced in a dose dependent manner compared to that of control while the resistin increased. TBT treatment resulted in severe hepatocyte cytoplasmic

degeneration, appeared as a special structure in cytoplasm which were not observed in control, have been detected with the presence of lipid droplets in the dose dependent manner[12].

Studies conducted in bone marrow stem cells suggest that tributyltin (TBT), triphenyltin (TPT) and dibutyltin (DBT) stimulated lipid accumulation, while DBT did not stimulated much lipid accumulation. The compounds induced the adipogenic marker, perilipin expression, while dibutyltin showed a very less expression. TBT induced a high expression of adipogenic genes such as PPARγ, FABP4 and perilipin. Dibutyltin also induced the adipocyte differentiation at a concentration of 50 -100nM and it was concluded to be less efficient compared to that of TBT or TPT. This confirms that organotin have the potency to induce adipogenesis in bone marrow mesenchymal cells even at low concentrations in cultured models and hence the environmental exposure could induce lipid accumulation[1].

Another study suggest that in bone marrow multipotent stromal cells, TBT induced the expression of FABP4, abca1 and Tgm2 genes in a RXR dependent manner, while TBT suppressed osteogenesis. Treatment with TBT in the primary bone marrow multipotent stromal cells showed a reduced alkaline phosphatase activity, mineralization, expression of osteoblast related genes. Runx2, (a master regulator of oesteogenesis), Osterix, (osteogenic transcription factor), Osteocalcin (another protein involved in mineralization), Dentin matrix phosphoprotein 1, which is expressed mainly in mineralizing oesteocytes and regulates mineralization. Rosiglitazone treatment reduced the Runx2 expression and suppressed the upregulation of Osx, oesteocalcin and Dmp1 expression, while Bexarotene and TBT suppressed the upregulation of Osx, Bglap and Dmp1 expression. This evidence suggests that TBT potently suppresses oesteogenesis, RXR ligands could suppress oesteogenesis[39].

Diethylhexyl phthalate (DEHP), class of phthalates, induce endocrine disruption, and found that they interact with nuclear receptors. They are used as a plasticizer, mainly used in cosmetics, industrial paints. Another report suggests that diethylhexyl phthalate is metabolized to monoethylhexyl phthalate (MEHP) through the action of intestinal lipases and are absorbed in the body[40]. Studies suggest that DEHP exposure to mice were protected from weight gain under both high fat diet and

regular diet. In liver, DEHP exposure induced peroxisomal beta oxidation (acyl coA oxidase), intracellular fatty acid shuttling protein 1 (FABP1), CPT1a were significantly increased. The oxidative functions of PPARα and PPARβ prompted to conduct the study in feeding high fat diet in PPARα, PPARβ null mice and concluded that DEHP activates PPARα. Studies conducted in humanized mouse model (mouse receptor replaced by human PPARα) suggest that DEHP reduces the weight gain, epidedymal mass and the fatty acid oxidation genes in mouse, while it did not showed any effect in humanized mouse having PPARα genes. The MEHP induced a stronger interaction of coactivators with humans PPARα than the mouse receptor. This concludes that DEHP regulate the PPARα mediated fatty acid oxidation in a species specific manner[41].

Studies conducted in various cell lines conclude that MEHP activates the PPARs. MEHP binds similar to that of rosiglitazone in the PPARγ ligand binding domain. The adipogenesis experiments with the 3T3 cells demonstrated that they induced adipogenesis almost similar to that of rosiglitazone, while DEHP showed a very less differentiation with increased triglycerides and were shown to possess a low level of PPARγ/CoR interaction upon MEHP binding[42].

Another study demonstrate that TBT induce imposex in gastropods species, while the exact mechanism is not fully known. The gastropod (*Nucella lapillus)* were treated with environmentally relevant concentration of TBT, investigated the expression of RXR gene transcription at different timings and tissues in both genders, before and after the intiation of imposex. The TBT exposures were investigated for the RXR gene expression in various tissues and sex specific manner. The expression of RXR elevated in different tissues such as CNS, Penis forming area (PFA) and gonads. In the CNS, a significant downregulation of RXR has been detected in females before and after imposex intiation. The male penis showed higher expression of RXR gene than that of PFA females. In the advanced stage of imposex, females displayed the RXR gene expression pattern similar to that of males. This concludes that TBT exposure induce imposex by modulating the RXR signaling in the CNS and male genitalia develop in both sexes [43].

Another study suggests that organotin compounds increase the production of progesterone. The stucture dependent effect of various organotin compounds have

been tested for the expression of 3β hydroxysteroid dehydrogenase type 1 and progesterone production in human choriocarcinoma jar cells. TBT, TPT, DBT, Monophenyltin, Tripropyltin and tricyclohexyltin enhanced progesterone production in a dose dependant manner. The compounds also induced the expression of 3βHSD, similar to that of rosiglitazone and the RXR ligands after the treatment with various organotin compounds. All the compounds have been tested for the activation of PPARγ, and showed a dose dependent activation, while the DBTC did not showed any effect on PPARγ. These evidences clearly confirm that the expression of 3βHSD1 mRNA is dependent on the PPARγ. The regulation of progesterone in human placenta is necessary for the human pregnancy[44].

Bisphenol is considered as an endocrine disrupting chemical, has been detected in urine, serum, breast milk and in fat. It is used for the synthesis of polycarbonate plastic and in the lining of food containers. Bisphenol has been shown as an obesogen, they induce adipogenic differentiation in 3T3L1 adipocyte. Bisphenol A diglycidyl ether (BADGE) has been identified as the antagonist of PPARγ, inhibits the differentiation of 3T3L1 preadipocytes and in 3T3442A preadipocytes[45]. BADGE administration orally decreased the triglyceride content in white adipose tissue and it increase fatty acid oxidation. Studies suggest that BADGE treatment enhances the differentiation in mesenchymal stromal stem cells into adipocytes, enhanced the gene expression profile related to adipogenesis, while it did not show any expression in PPARγ. The inhibitory potential of PPARγ antagonist (BADGE)[46] failed to inhibit the adipogenesis in mesenchymal stromal stem cells. These evidences suggest that BADGE is not an agonist for PPARγ but it induces adipogenic conversion in mesenchymal stromal stem cells[47].

Another report suggests that perflourooctanoic acid (PFOA) treatment caused a dramatic decrease in adipose tissue weight, the DNA content of adipose tissue were analyzed and did not found any change in total DNA content of epididymal fat pads. The administration of diet induced mice with PFOA treatment induced acyl CoA oxidase and it decreased the body weight. Fatty acid oxidation genes were prominently upregulated by PFOA treatment, which suggest that the hepatic mitochondria and the peroxisome have the ability to consume the fatty acid released from adipocytes, while the PPARγ production in adipose tissue was unchanged but the serum level of tnf-α have been increased[48].

3.2 Non Receptor mediated mechanisms of action

Non receptor mediated mechanisms of organotin action are mentioned bellows:

1) Epigenetic mechanisms of action.
2) Effect of organotin compounds on proteasome.
3) Interference of organotin compounds with enzymes.
4) Organotin compounds and its effects on mitochondria.
5) Organotin compounds and apoptosis modulation.
6) Organotin compounds and its action on plasma membrane.

3.2.1 Epigenetic mechanisms of actions

Epigenetic modification plays a major role in the gene regulation and the aberrant expression can alter the entire function of the gene. Environmental pollutants can alter the epigenetic regulatory mechanisms such as acetylation, methylation. Here we discuss about the various epigenetic mechanisms and how they alter the gene expression.

There are three common epigenetic mechanisms.

1) Histone acetylation
2) DNA Methylation
3) Regulation of gene expression by non-coding RNA.

1) Histone Acetylation

Histone acetylation is the transcription activating modification through the addition of acetyl group to the lysine residue at the amino group through the action of histone acetyl transferase. Acetylation of histone reduces the overall positive charge by neutralizing the positive charge of the lysine located in the histones, therefore it decreases its affinity towards the negatively charged DNA. This leads to the uncoiling of the condensed DNA, transcriptional co-activators binds and triggers the transcription.

Studies suggest that exposure of metals cause the epigenetic modification and can lead to the aberrant signaling. Another report indicate that $Cu2^{+}$ treatment with human hepatoma Hep3B cells reduced the H3 and H4 acetylation through the inhibition of histone acetyl transferase activity. In-vitro and in-vivo HAT assays showed a decrease in HAT activity after the treatment with Cu^{2+}. These evidences suggest that HAT is the target of Cu^{2+}[49].

Another study suggests that methyl mercury, considered as a neurotoxic, its exposure to humans mainly occur through the consumption of contaminated sea food. Exposure of methyl mercury to male mice induced depression like behavior and caused epigenetic changes in the BDNF gene in the hypothalamus region of mice. The expression level of brain derived neurotrophic factor (BDNF) has been studied and showed a significant decrease when compared to the control, while the treatment with fluoxetine (antidepressant drug) restored the expression level of BDNF expression. The acetylation of H3 (marker of active chromatin) has been analyzed, found a decrease in histone acetylation at the BDNF promoter IV after 12 week exposure with methyl mercury. The decreased acetylation of H3 is correlated with the reduced BDNF mRNA expression in hippocampus. The fluoxetine treatment increased H3 acetylation at BDNF promoter IV in perinatal methyl mercury treated mice. From these evidences it can be concluded that perinatal methyl mercury exposure downregulate the expression of BDNF in hippocampus and decreased acetylation of histone at the BDNF promoter region, while the fluoxetine treatment induced the H3 acetylation at the BDNF promoter IV[50]. The expression levels of BDNF is downregulated in various neurodegenerative disease including Alzheimers and Parkinsons disease[51]. This evidence shed light whether organotin compounds and heavy metals could induce neurological disorders, such as Alzheimers or Parkinson disease.

Organotin compounds have been detected as agents to enhance the HAT activity of core histone in a dose dependent manner. Studies suggest that TBT and triphenytin enhance the HAT activity. Purified HAT complex from rat liver interact together with histone or nucleosome, further incubation with organotin such as TBT, TPT or derivatives (10μM) showed an enhanced HAT activity, the derivatives such as dibutylin, diphenyltin, triethyltin and tripropyltin also increased HAT activity. This

concludes that organotin compounds can upregulate the HAT activity and increase the expression of histone acetyl transferase[52].

2) DNA Methylation

In multicellular organisms the DNA methylation occurs through the covalent modification of cytosine base at the fifth carbon position of CG rich dinucleotide through the action of the enzyme DNA methyltransferase, while the methyl donar of the reaction is supplied by S- Adenosyl methionine. DNA methylation is the mechanism involving the histone deacetylation, chromatin condensation and the gene silencing. During the unmethylated condition the CpG dinucleotide located in the gene promoter recruits the transcription factors and RNA polymerase to their specific nucleotides and the transcription of exon occurs. Methylation of CpG dinucleotide takes place by the action of DNA methyltransferase, which recruits the methyl-CpG binding protein 2, later the histone deacetylase and histone methyl transferase binds together and generates a complex in the promoter region. This complex deacetylase the histone and catalyze methylation of specific lysine residues. This modification makes the chromatin to become a compressed state, which prevent the transcription factors and RNA polymerase to bind the specific promoter region and results in the silencing of the transcription (Figure: 3).

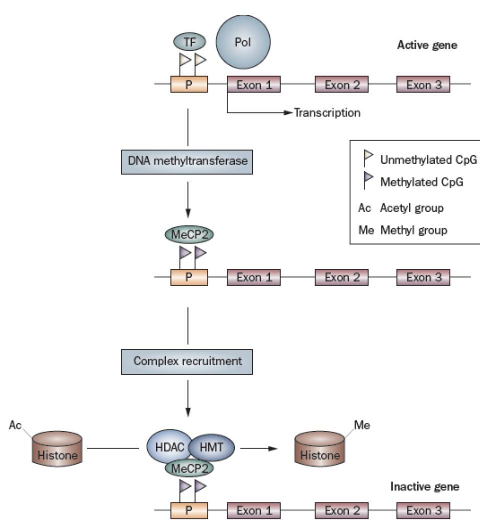

Figure-3. Mechanism of DNA methylation and gene silencing. Figure adapted from[53].

Adiponectin has been found to be downregulated in obese conditions, plays a major role in controlling the insulin resistance. Recent studies dissected the mechanism of adiponectin downregulation during obese conditions. Obesity increases the DNA methyltansferase, which results in the methylation of adiponectin promoter and suppresses the adiponectin production [54]. In addition, higher methylation at RXRα promoter in humans induced fat mass at 9 years of age. These evidences proves that epigenetic changes could perturb the metabolism of lipids[55].

Another study suggest that the maternal Bisphenol A exposure can change the offspring phenotype through the hypomethylation in the CpG islands and can normalize the methylation through the supplementation of diet[56].

Various studies have been demonstrated that organotin compounds have been detected in fresh water. The study provides evidences that TBT and TPT affect the DNA methylation. The organotin compounds have been detected in the fish liver and induced DNA hypomethylation in a dose dependent manner after the exposure of organotin. The levels of 5 methylcytosine content in the liver have been decreased significantly in a dose dependent manner after the exposure of organotin compounds for 48 days. During the methylation reaction SAM will be converted to SAH in the active site of methyl transferase enzyme. The expression level of SAM and SAH has been decreased after the treatment with toxic chemicals. The study concludes that TBT and TPT exposure caused hypomethylation by altering the SAM, SAH concentration while the activity of DNA methyl transferase have no effect in the hypomethylation[57].

3) Regulation of gene expression by non-coding RNA

Small non coding RNA play important role in the epigenetic regulation of a gene and genome expression through various mechanism, such as, translation repression of mRNA, mRNA degradation, chromatin modification and DNA methylation. miRNA are short nucleic acids having an average of 22 nucleotides in length. miRNA sometimes directly bind to the mRNA, inhibit the translation and mRNA stability.

miRNA can bind perfectly or non-perfectly to the coding sequence of mRNA and can induce RNA interference or cleave the mRNA.

Obesity and microRNA

miRNA143 is strongly induced during preadipocyte 3T3L1 differentiation. Ectopic expression of miRNA143 during the differentiation of 3T3L1 preadipocyte resulted in increased triglyceride accumulation[58]. The studies illustrates that the introduction of antisense oligonucleotide against miRNA143 in the 3T3L1 preadipocytes inhibited the diffentiation, this suggest that miRNA143 plays a major role in differentiation. The miRNA143 has been found significantly upregulated in the mesenrtic fat of high fat diet induced mice[59]. Another study suggest that miRNA519d is highly expressed in subcutaneous adipose tissue of obese and has been found that miRNA519d dose dependently suppress the translation of PPARα protein and increased lipid accumulation[60].

Effect of environmental pollutants on miRNA

Another report suggests that RDX also known as (Hexogen or Cyclonite) are environmental pollutant, mainly used in military and civil activities. It is known that exposure causes neurotoxiciy, immunotoxocity and some cancer. The influence of RDX on miRNA expression has been studied using B6C3F1 mice. The mice were exposed to RDX and found the upregulation of oncogenic miRNA and significant downregulation the tumor supressing miRNA. Significant changes in the miRNA expression in brains of RDX treated mice has been found, the miRNA206 has showed significant upregulation of 26 fold, compared to the control[2]. The overexpression of miRNA206 has been demonstrated early that they regulate the expression of BDNF[61]. The overexpression of miRNA206 has been detected in Alzheimer’s disease. The neurodegenerative disorders such as Alzheimer’s and Parkinson has been characterized with the reduced expression of BDNF gene[51].

3.2.2 Effect of organotin compounds on Proteasome.

The proteosome is a multisubunit enzyme complex that plays a major role in the regulation of proteins, they degrades unneeded or damaged proteins by means of proteolysis. Before the degradation, the proteins will be flagged for destruction by ubiquitin conjugation system, which results in the attachment of polyubiquitin chain in the target protein. Recent findings suggest that one of the main target of organotin compound is proteasome, the electrophilic tin atom in organotin could interact with the N terminal threonine of proteosomal β5 subunit and causes irreversible inhibition. The study demonstrated that TPT as the most potent inhibitor caused 63% against proteosomal chymotrypsin like (CT-L) activity and detected the accumulation of ubiquitilated proteins after the treatment with organotin compounds, of which TBT and TPT showed higher effect, while others have a very less effect. Bax protein was also increased dose dependently after the treatment. This concludes that TBT can inhibit the proteasome and can activate the cell death associated proteins, while the DNA strand break has not observed with TBT or TPT[62].

3.2.3 Interference of organotin compounds with Enzymes

It is known that organotin compounds can combine with enzymes, activate or inactivate the action of enzymes. The metal atom forms a strong bond with the active site of the enzyme prevent the enzyme from the reaction with its substrates, e.g TPTA interacts with lipoic acid thiols and inhibit the lipoic acid acetyl transferase and lipoamide dehydrogenase[63]. Studies suggest that organotin compounds can directly bind to enzymes. The in vitro studies suggest that the TBT or TPT inhibit the activity of Mg-ATPase in the mussel digestive gland mitochondria [64]. Another study suggest that TBT or TPT compounds inhibited aromatase activity and decreased P450 mRNA levels in the human granulosa like tumor cell line KGN[32]. Studies suggest that dithiocarbonates and organotin compounds inhibit the enzyme called human 11β-hydroxysteroid dehydrogenase 2[65]. TBT and TPT has been shown to induce the activity of aromatase and 11βHSD1[66]. Another report indicate that TBT and TPT treatment enhances the HAT activity of core histones in a dose dependent manner[52]. TBT and TPT has been identified as the potent inhibitors of mitochondrial ATPase and directly bind to the ATP synthase[64]. TBT decrease the

mitochondrial membrane potential, while it induce cytochrome c release from mitochondria in a dose dependent manner[67]. Subunit of ion channel is the target of ATPase inhibition by means of organotin compounds[68]. Another report suggest that DBT treatment with natural killer cells increased the activation of MAPK, could decrease the function of natural killer cells, while it did not showed any effect to protein tyrosine kinase[69]. TBT treatment to immature male mice has reduced the serum testosterone concentration , downregulated the expression of various enzymes for cholesterol side chain cleavage enzyme P450, 17α-hydoxylase, 3βhydroxysteroid dehydrogenase and 17β hydroxysteroid dehydrogenase. This provides evidences that TBT treatment reduce steroidogenic enzymes in the interstitial laydig cells, causes serious defects in the development in the testis[70]. Recent report indicate that butyltin compounds such as TBT, DBT treatment with natural killer cells activates the protein kinase C and protein kinase D[71].

3.2.4 Organotin compounds and its effect on Mitochondria

Mitochondria play a major role in regulating the metabolism of lipids. The mitochondrial dysfunction leads to the accumulation of lipid intermediates in the cell, downregulates mitochondrial respiratory genes[72]–[74]. Studies illustrates that organotin compounds inhibits oxidative phosphorylation in rat liver mitochondria[75], induce mitochondrial swelling[76]. It has been reported that the entry of DBT into the mitochondria is more toxic than TBT, inhibits the key energy metabolizing enzymes directly on the catalytic site of Mg ATPase. DBT prevents ATP hydrolysis by directly attacking catalytic subunit F1 and displaces the cofactor Mg2+[64].

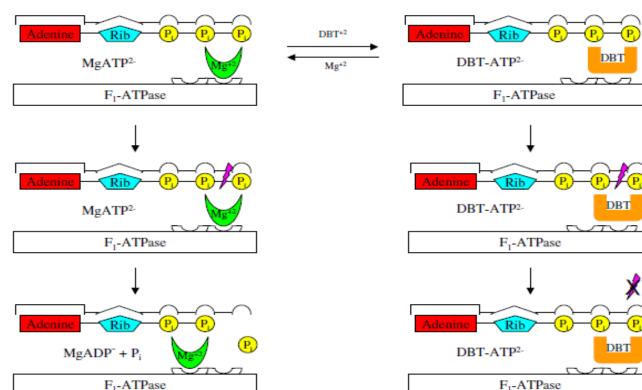

Figure-4: Action of DBT with ATPase. Figure adapted from[64].

Another study suggest that liver and heart have a specification conducting channel known as Inner mitochondrial membrane anion channel(IMMAC), Tributyltin inhibits the inner mitochondrial membrane anion channel and inhibits oxidative phosphorylation[77]. The studies conducted in mice suggest that DBT & TBT increased the serum enzymes activities of aspartic acid amino transferase(AST), alanine amino transferase (ALT) and ornithine carbamyl transferase (OCT), inhibited the succinate state 3 respiration due to the high affinity of butyltin compounds towards hepatic mitochondria[78]. In addition, Bisphenol A, a widely used endocrine disrupting chemical, induced mitochondrial dysfunction in the spleen and liver of mice when exposed with a low dose of bisphenol[79], [80].

3.2.5 Organotin compounds and apoptosis modulation.

Studies have been shown that organotin compounds can induce apoptosis or they can block the action of caspases. TMT increases the apoptotic cells after the treatment in primary neuronal cell cultures isolated from rat hippocampus and cortex[37]. Another study suggest that 3μM of DBTC or TBTC significantly increased DNA fragmentation in fresh isolated rat thymocyte after the incubation for 10 minutes with organotin compounds[81]. High dose of TBT were able to inhibit the caspase activity, TBT bind to the essential thiol groups and block the procaspase 8 activation. Another study suggest that TMT exposure to cerebral granule cells induce apoptosis while the higher concentration induced necrosis[82]. TBT treatment with human peripheral blood lymphocytes induced the release of cytochrome C, which results in caspase activation[83].

3.2.6 Organotin compounds and its action on plasma membrane.

Plasma membrane is the primary target of the organotin compounds. Due to the high lipophilic nature of the organotin, they bind to the plasma membrane, disturb the membrane structure, which can leads to disorganization of the cellular functions and can lead to necrosis or apoptosis. They can permeate the membrane without altering its structure and cellular function, can act as an agonist or an antagonist for various

receptors. Study suggests that organotin compounds transport organic anions across the phospholipid bilayer, caused hemolysis in the human erythrocytes in a dose dependent manner, hence is considered as a membrane poison[84]. Another reports suggest that organotin compounds are located in the upper part of the phospholipid bilayer close to the lipid water interphase (Figure-5)[85]. TPT inhibits the enzymatic activity of the membrane bound pyrophosphatase of *Rhodospirillum rubrum*[86].

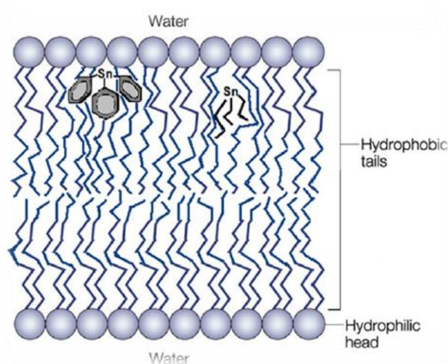

Figure -5. Localization of the organotin compounds in the membrane lipid bilayer, Figure adapted from[87].

4. Dibutyltin compounds (DBT).

Dibuyltin compounds are having two buytl groups, which are covalently bonded to a tin atom. There are different derivatives of diorganotin compounds, which include dibuyltin dilaurate, dibutyltin dichloride, dibutyltin diacetate, dibutyltin oxide and dibutyltin maleate, etc. Refer (Figure - 6) for the structures of dibutyltin compounds.

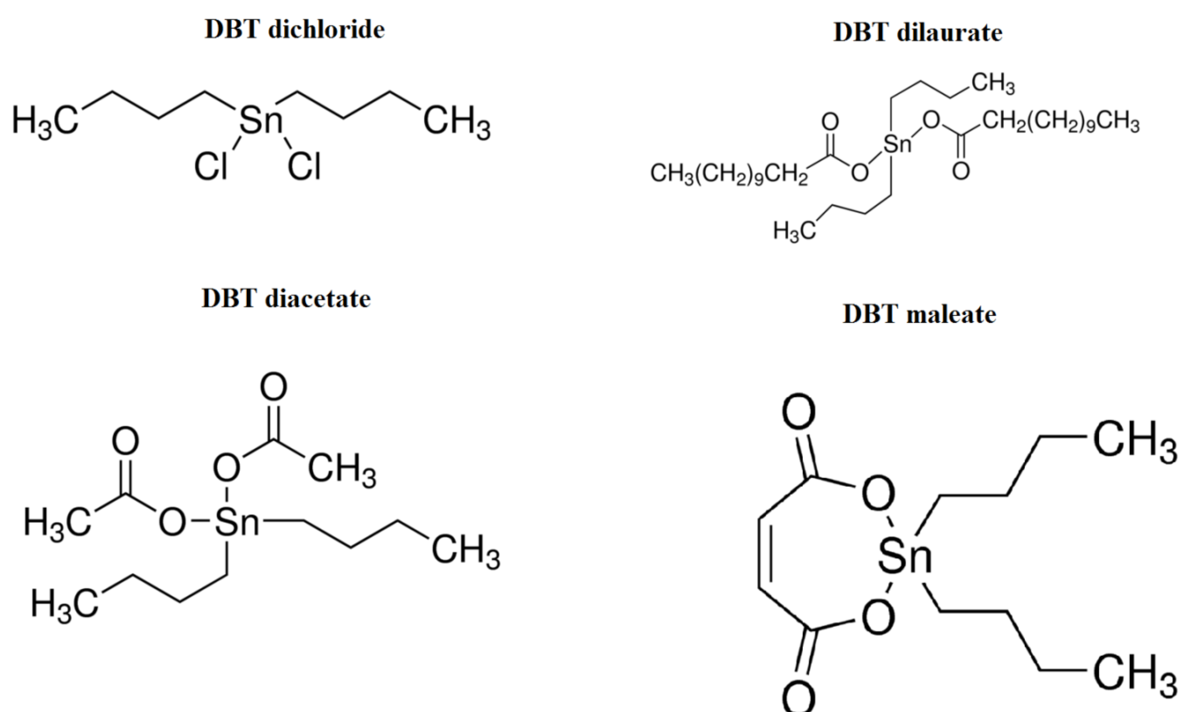

Figure: 6 Structure of various dibutyltin compounds.

Dibutyltin has been detected in drinking water due to the leaching of PC plastic pipes [18] and in food due to the migration from plastics to the food materials[19]. Studies with human natural killer cells demonstrated that the higher concentration of DBT showed decreased tnf-α secretion, while the low concentration showed an increase in secretion. This evidence clearly suggest that DBTs could alter the function of natural killer cells[88]. Another study dissected the DBT mediated immunotoxic mechanism in GR. DBT exposure, dose dependently inhibit the transcription activity of GR, inhibit the ligand binding to GR, which altered the glucocorticoid mediated suppression of cytokine production (TNF-α, IL-6) in macrophage[89]. The proinflammatory evidences is further supported by the study conducted in the BV-2

microglial cells. DBT treatment in BV-2 microglial cells increases the TNF-α and IL-6 mRNA expression, showed time and concentration dependent decease in ATP levels[90]. Furthermore intravenous treatment with DBT dichloride (8mg/kg) to rats induced pancreatitis, the expression of IL-1β, IL-10 and TGFβ has been elevated compared to normal, suggesting that pancreatitis induce macrophage infiltration and induces the cytokine, while IL-2, IL-2 receptor, interferon-γ and tnf-α has been detected in few samples, hence is not considered as significant[91].

Another study suggest that in vitro DBT exposure to natural killer cells activated the MAPK, while it did not showed any effect to protein tyrosine kinase[69]. In addition, another group have studied the effect of DBT dichloride related to adipogenesis and suggested that DBT dichloride stimulated the lipid accumulation, increased the perilipin in the bone marrow stromal cells[1],while the DBT dichloride did not caused any activation for PPARγ at a concentration of 0.1μM[44].

In addition, studies conducted in our laboratory conclude that dibutyltin compounds have unique effects in inducing adipocyte differentiation similar to that of tributyltin compounds. In reporter gene assays, DBTs has been shown as a partial agonist for PPARγ. (Unpublished data).

DBT dilaurate treated cerebrum of rats showed a reduction in diglyceride and phosphotydylinositide. This suggest that decrease in phosphoinositide leads to altered phosphoinositide signaling mechanism, which could leads to the altered behavior and neurotoxicity[92]. In addition another study suggest that dibutyltin dilaurate exposure caused DNA damage in the rat cerebral cortex, glial cells, crosses the blood brain barrier, caused swelling of mitochondria, reduced the cell organelles and induced apoptosis[93].

Dibutyltin dichloride and TBT treatment in mice showed an increase in level of liver enzymes such as aspartate transaminase (AST) and alanine amino transferase (ALT), swelling and collapse of mitochondria has been observed in mice liver through electron microscopy. The level of DBT dichloride has been increased significantly in the mice liver through the metabolization of TBT. This evidence suggest that DBT

dichloride impairs the liver function, induce hepatotoxicity and impair mitochondrial oxidative phosphorylation[78].

Mode of action of organotin compounds

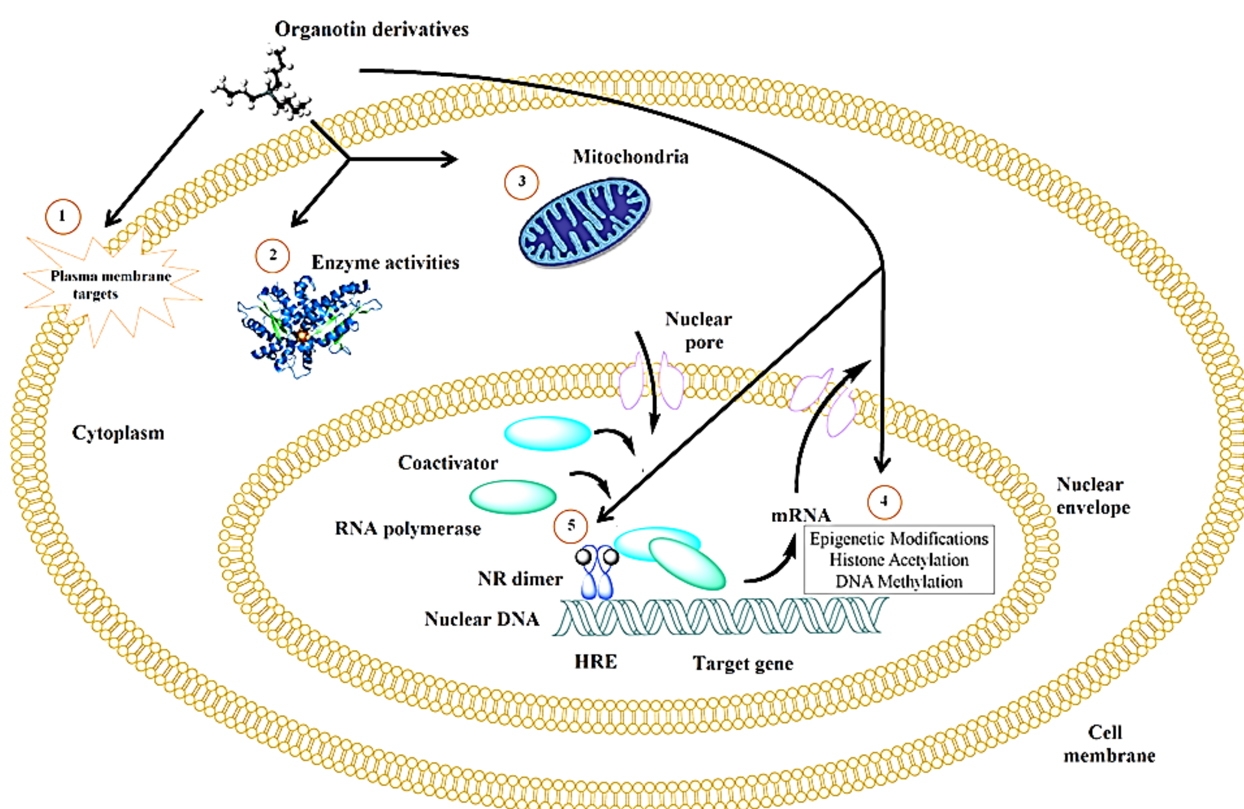

Figure: 7. The main target sites of organotin compounds. 1) Plasma membrane 2) Intracellular targets such as enzymes, Proteasome 3) Direct action on mitochondria, which further leads to inhibition of ATP synthesis 4) Epigenetic modification such as DNA methylation, Histone acetylation 5) Nuclear receptor mediated mechanism of action (activation or inhibition of various nuclear receptors).

5. Obesogen and obesity

Obesogen are chemicals which intiates or augment the obesity through the alteration of the precisely controlled pathways related to adipogenesis, lipid metabolism or energy balance. Various studies have been demonstrated that environmental pollutants perturb the critical pathways involved in the lipogenesis. The most well characterized environmental pollutant is Tributyltin chloride (TBT), which has been shown that they activated the PPARγ, stimulate adipogenic differentiation, adipogenesis in in-vivo studies[9],[12],[42]. The obesogen mechanism of action in the cell is mainly through receptor mediated and non-receptor mediated.

The number of obese individuals is increasing worldwide, obesity induces various metabolic health problems. Obesity occurs due to the excessive expansion of adipose tissue due to high nutrient intake and insufficient energy expenditure. In other way, it can be suggested that obesity occurs when the synchrony between lipolysis and lipogenesis alters, the overexpression of lipogenic genes leads to obesity. Studies have been demonstrated that during obese condition, the lipolytic genes such as adipose triglyceride lipase (ATGL) and hormone sensitive lipase(HSL) are downregulated[94]. Dysfunctional lipolysis is also an element which contributes to the pathogenesis of obesity. Obesity is associated with low grade chronic inflammation in the adipocytes, which ultimately leads of the abnormal production of cytokines. The high level of cytokine leads to a condition were the cells do not respond to the available insulin, referred as insulin resistance[95]. It has been identified that obesity leads to various pathological condition such as diabetes type 2, dyslipidemia, hypertension, cardiovascular dysfunction, etc.

Obesity is characterized with the impaired cytokine signaling and accumulation of lipids or its derivatives etc. One of the prominent factor which trigger obesity is the impaired signaling mechanism of cytokine. Obesity occurs due to the overexpression of inflammatory proteins and the reduction of anti-inflammatory proteins. Adipose tissue is the major site for the production of cytokines. Adipocytes and macrophages produce inflammatory proteins such as TNF-α, IL-6. The studies suggest that obesity induces local inflammation, chemokine production such as monocyte chemo

attractant protein (MCP1), which promotes the recruitment of circulating proinflammatory monocytes. Recruited monocytes further differentiate to M1 macrophage phenotype and create and imbalance between M1 and M2. The macrophage shift towards classically activated macrophages (CAMs also referred as M1 macrophage) from the M2 macrophage, referred as alternately activated adipose tissue macrophage (AAMs) (Figure:8)[4], [96]. The M1 macrophage produce proinflammatory proteins such as TNF-α, IL-6, IL-18, MCP1, TGF-β, leptin, resistin, plasminogen activator inhibitor(PAI-1), while the M2 is involved in the production of anti-inflammatory proteins such as secreted frizzled-related protein 5(SFRP5) and adiponectin. Adiponectin has been identified as a down regulated gene during obese conditions. Studies suggest that M1 macrophages promote adipose tissue insulin resistance and accumulate in obese WAT (Figure-8).

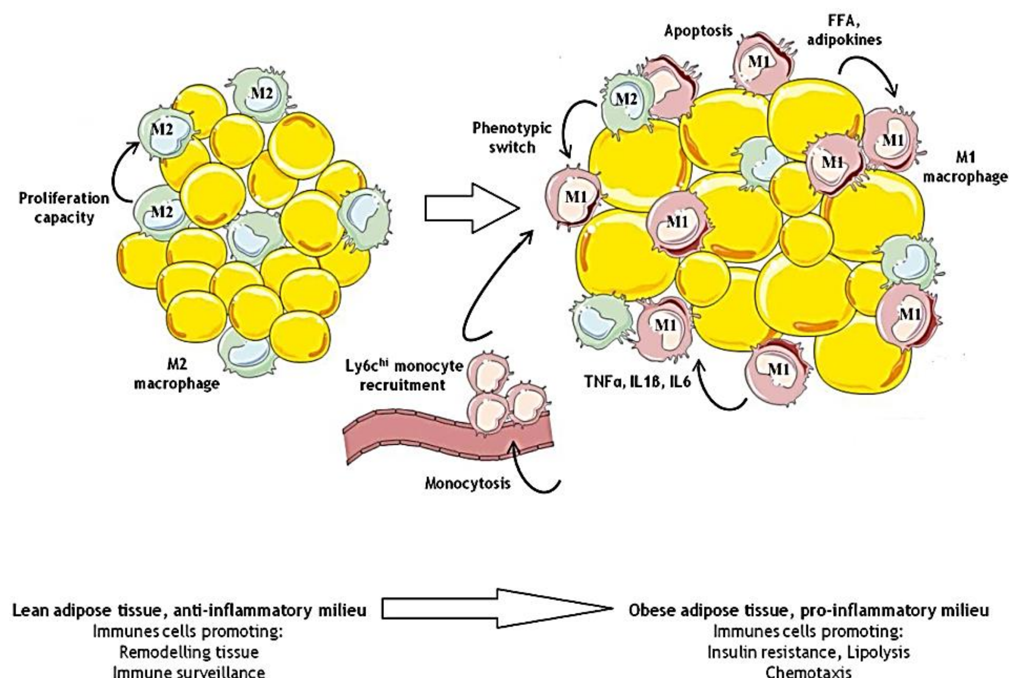

Figure: 8. Macrophage polarization from M2 to M1 during Obesity. Figure adapted from[96].

6. Adipose Tissue

Adipose tissue was considered as an inert tissue, while the recent evidences suggest that adipocyte have the large capacity to store the excess energy in the form of triglycerides. The accumulated triglycerides can be released during the period of fasting through the activation of various lipases. Another major function of adipocyte is the production of various cytokines and are termed as adipokines. Adipose tissue consists of preadipocytes (undifferentiated adipocytes), endothelial cells, fibroblast, leukocytes and macrophages. Adipose tissues are of mainly two types, which include brown adipose tissue (BAT), white adipose tissue (WAT) and the recent evidences identified beige adipocytes as the third one. Refer – (Figure: 9) for different types of adipocytes.

Brown adipose tissue is a specialized tissue whose main role is to produce heat, is different from the WAT cells. BAT cells are characterized with small multiple lipid vesicle and increased amount of mitochondria. The presence of huge amount of cytochrome located in the mitochondria provides brownish colour. The thermogenic potential of brown adipose tissue is due to the presence of UCP1, which can be found in the inner membrane of the mitochondria [97]. The white adipose tissue cells role is to store energy in the form of triglycerides. These triglycerides are stored in the large lipid droplet so that other cell organelles are protected from lipotoxicity caused by fatty acid. The lipid droplet is surrounded by a phospholipid monolayer and various proteins which are embedded on the phospholipid monolayer. The lipid droplet associated proteins regulates the lipolysis[98]. WAT cells are characterized with large lipid droplet and less mitochondria.

White adipocytes, in response to certain stimuli show the characteristics of BAT and the process is called browning. The browning recruit various coregulators, increases the amount of mitochondria and hence it acquires the ability to induce thermogenesis. Upon physiological stimuli such as chronic cold exposure, hormonal stimuli such as irisin, activation of few receptors such as PPARγ, PPARα , FXR and β-Adrenergic receptors induce the expression of thermogenic genes[99]–[104].

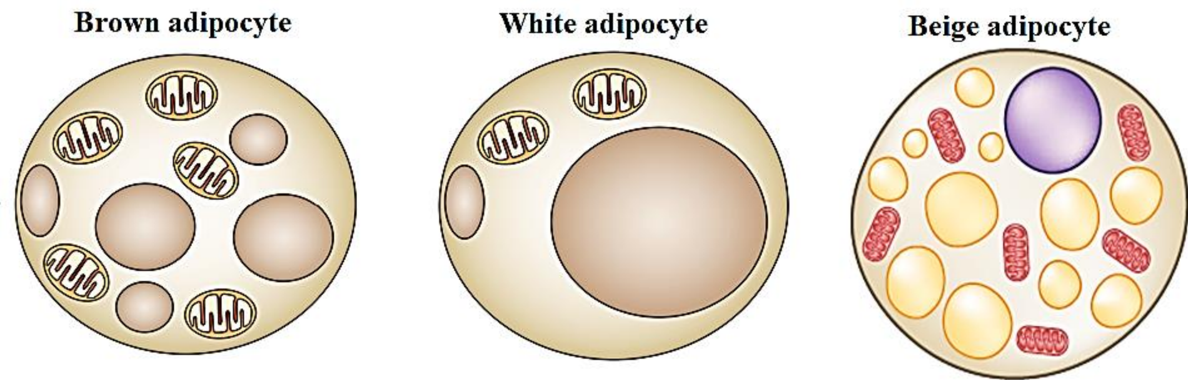

Figure: 9. Different types of adipocytes. Brown adipocyte, White adipocyte and Beige adipocyte.

7. Adipokines and its role in insulin resistance

Adipose tissue secretes an array of proteins, which are necessary to maintain the normal metabolic function, while the dysfunction of these proteins leads to activation of various metabolic genes. Obesity occurs due to the generation of an imbalance between proinflammatory and the anti-inflammatory cytokines. Most of the adipokines are secreted at high levels during the obese conditions, which include MCP1, TNF-α, resistin, leptin and TGF-β, while adiponectin has been found to be downregulated during obesity. The actual mechanism of action for the production of insulin resistance and inflammation has been described below.

Monocyte chemoatrant protein-1 is a chemokine, which regulate the migration and macrophage polarization. When adipocyte is overloaded with triglycerides, it induces the secretion of MCP1, which induce macrophage recruitment and stimulate the inflammation. Studies suggest that MCP1 induce the macrophages accumulation and leads to insulin resistance in the skeletal muscles of mice and humans[105]. During the chronic, inflammatory obese conditions, adipose tissue induces the overproduction of another proinflammatory protein called TNF-α[106]. Various studies have been demonstrated that TNF-α inhibit the insulin signaling[107]. It has been reported that TNF-α can inhibit the transcription of PPARγ as well as translation of PPARγ. TNFα has been shown to activate the NF-kB and stimulate the

expression of various genes [108]. In addition, the TNF-α decrease the expression of various adipogenic genes as well as few adipogenic transcription factors such as PPARγ and CEBPα[109]. TNF-α has been characterized as a major proinflammatory cytokine involved in various metabolic and inflammatory[110], [111]. Resistin, an adipokine which has been shown as an inducer of insulin resistance, the level of resistin is high during obese conditions[112]. Resistin inhibits the insulin signaling mechanism through the activation of suppressor of cytokine signaling protein 3(SOCS3)[113]. SOCS3 inhibit the insulin signaling by direct interaction with the insulin receptor substrate (IRS– 1/ 2) tyrosine phosphorylation or by targeting the IRS1/2 and induce proteosomal degradation, this ultimately blocks the action of insulin signaling (Figure:-10a)[114]. Recent studies suggest that suppression of resistin results in the decrease in lipid content without showing any effect on PPARγ and CEBPα during the adipocyte maturation of 3T3L1, while fatty acid oxidation increased[115]. The production of resistin induces the production of other cytokines such as TNFα, IL-6, IL-1β and IL-12 (Figure:10b)[116].

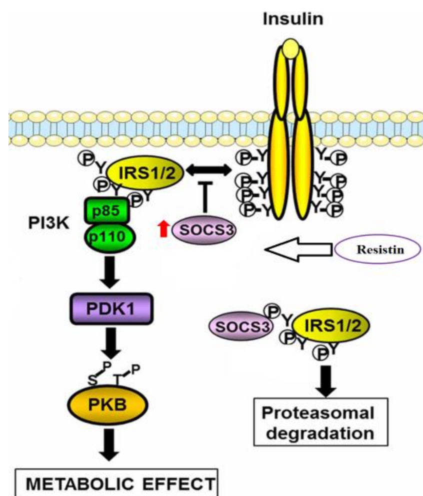

Figure:10 (a).

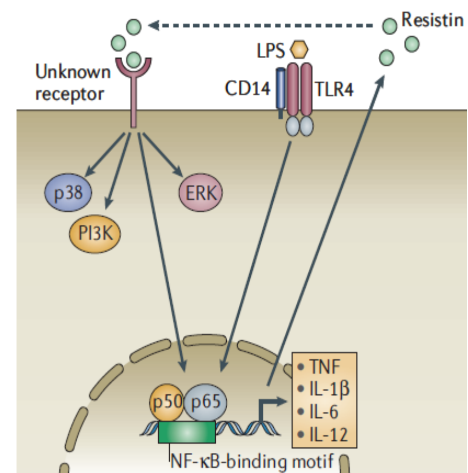

Figure: 10 (b).

Figure:10(a) Resistin induce the expression of SOCS3, which binds to the IRS-1 and inhibits the action of insulin. **(b)** The resistin induces the activation of NFkB, stimulates tnfα, IL1β, IL-6 and IL-12. Figure adapted from[114], [116].

Leptin is considered as a proinflammatory cytokine, produced by the adipocyte. The main function of leptin is to contol appetite, leptin circulates in the blood, acts on the brain to regulate the food intake. Leptin acts on the receptor present in the hypothalamus for regulating energy balance[117]. Studies suggest that leptin also

induces the production of proinflammatory cytokines such as tnf-α, IL-6, IL-12[116]. The ciculating leptin levels and the mRNA expression in the adipose tissue are high and induce leptin resistance at the blood brain barrier during obese conditions[118]. TGFβ, another cytokine has been charectorized as a proinflammatory protien, the expression of TGF-β is high in the adipose tissue of obese mouse [119]. Studies suggest that TGFβ inhibit the adipocyte differentiation[120].

Adiponectin, characterized as an anti-inflammatory protein synthesized mainly by adipocytes. The adiponectin action takes place through binding to its receptors such as ADIPOR1 and ADIPOR2[121]. The activation of adiponectin receptors stimulates fatty acid oxidation through the sequential activation of AMP-activated protein kinase, p38MAPK and PPARα, while the activation of PPARα suppresses the transcription of proinflammatory proteins[122]. Another role of adiponectin is to suppress TNFα and INFγ, while it induce the expression of anti-inflammatory proteins such as IL-10 and IL-1 receptor antagonist (Figure:12)[116]. Adiponectin has been reported as a downregulated gene during obesity[123]. Studies suggest that adiponectin reduces the insulin resistance and increases insulin sensitivity[124], [125].

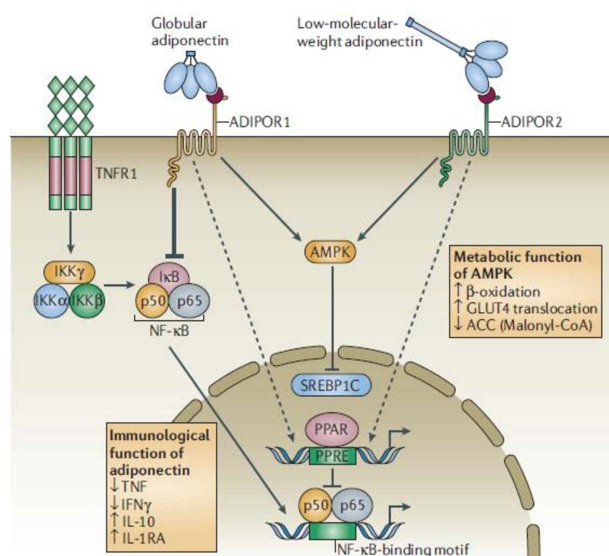

Figure: 11. Adiponectin binds to its receptor ADIPOR1 and 2 and induce β-oxidation of fatty acid through the activation of AMPK, PPARα. The adiponectin induces the IL-10 and IL-1RA and suppresses the TNFα and interferon γ. Figure adapted from[116].

7.1 Regulation of Adiponectin during obesity

Adiponectin has been found to be downregulated in obese conditions[123]. Recent studies reports that regulation of adiponectin occurs by means of epigenetic mechanism such as DNA methylation. The proinflammatory cytokines produced during obesity increase the expression as well as activity of DNA methyltransferase 1(DNMT1), which methylates at the promoter region of adiponectin, while the methylation levels at the promoter region of PPARγ and tnf-α were not affected (Figure-12). The increased methylation at the promoter region of adiponectin silences the expression of adiponectin. The treatment with DNA methyl transferase 1 inhibitor (RG108) elevated the adiponectin and improved the insulin resistance through the adiponectin expression[54].

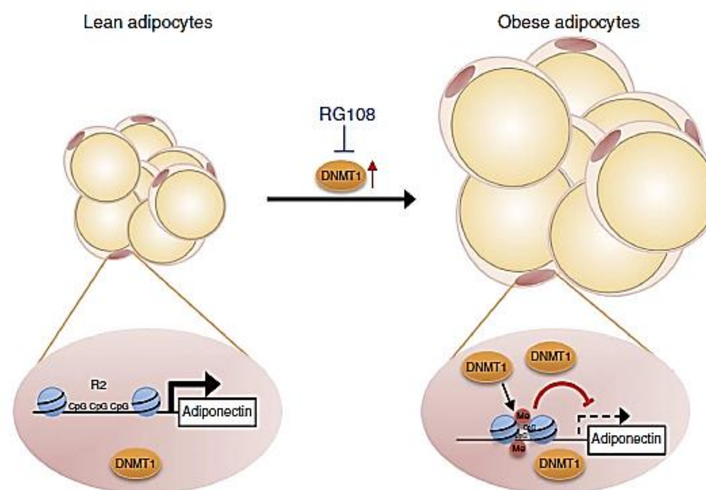

Figure: 12. Obesity induces DNA hypermethylation at the adiponectin promoter region (R2), which results in the suppression of adiponectin gene expression in the adipocytes. RG108 is DNA methyl transferase 1 inhibitor. Figure adapted from[54].

8. Nuclear Receptors

Nuclear receptors are multi-domain transcription factors that bind to DNA and regulate the expression of various genes. Nuclear receptors can be seen in cytosol or in nucleus. Multi cellular organisms require a specific intracellular signaling

mechanism for maintaining the complete signaling mechanism during embryogenesis and maintain the physiological function though out the life. Nuclear receptors play a major role in the intracellular signaling mechanism in animals because they converge different intra and extra cellular signals on the regulation of genetic program. Many of the natural ligands of nuclear receptor are lipophilic hormones which enter into the cell in a passive manner or by means of active transport mechanism.

All nuclear receptors are modular proteins which have a variable NH_2 -terminal region (A/B), one DNA binding domain, a linker region, one ligand binding domain (LBD) and a COOH- terminal region. The receptors also contain regions required for the transcription activation. The hyper variable A/F region of many receptors contains an autonomous transcription activation function which is referred to as AF1, which contributes to ligand independent activation by the receptor. A second transcription activation domain, termed AF-2, is located in COOH terminus of the LBD, but unlike the AF-1 domain, the AF-2 is strictly ligand dependent domain (Figure-13)[126], [127].

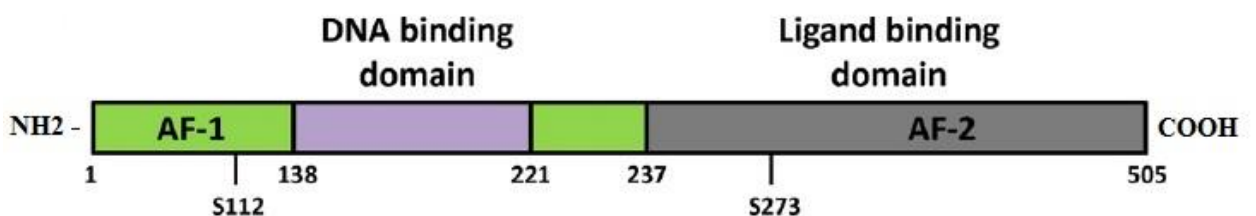

Figure: 13. Structural and functional organization of PPARγ receptor. Modified figure adapted from[127].

The Nuclear receptors are ligand controlled transcription regulators that functions as transcription activators or repressors of transcription. Nuclear receptors are DNA binding proteins which bind specifically to specific cognate sequences called hormone responsive elements (HRE), which is located in the control region of the target genes. Different nuclear receptor binds to different hormone responsive elements For eg: PPREs contain one or more copies of the hexameric DNA consensus sequence AGGTCA arranged as a direct repeat spaced by one nucleotide (hence termed DR1) and sequences recognized by the PPAR–RXR

heterodimer situated within six nucleotides of the 5´ flanking region of the DR1 motif (Figure-15)[128], thyroid receptor binds to the thyroid hormone responsive elements (TREs) in target genes as homodimers as well as heterodimers with RXR. TRE generally contains atleast two hexameric half sites, consisting of the consensus sequence AGGTCA. Thyroid receptor binds to the TRE in which half sites are arranged as direct repeats with 4 nucleotide spacer, hence its termed as DR4[129], The GR binds to a palindromic consensus sequences with 15 bp motif GGTCAnnnTGTTCT (where n is any nucleotide)[130], Estrogen receptor binds to the consensus sequence of 5-GGTCAnnnTGACC-3.(where n is any nucleotide) [131], [132].

Generally upon ligand binding, the nuclear receptor LBD undergoes a conformational change which results in the movement of helix 12, as a result the corepressor molecules are released and creates a new hydrophobic cleft on the surface of the receptor, which provides a docking site for the recruitment of various coactivators proteins[133]. These coactivators have intrinsic acetyl transferase activity, which decondense the chromatin through histone remodeling and promotes the creation of pre initiation complex[126]. In the absence of ligand, the unliganded nuclear receptor remains bound to the nuclear receptor corepressor protein (NCoR). The nuclear repressor proteins functions as a platform for the recruitment of various histone deacetylases (HDAC) to the specific DNA promoter region and deacetylation of histones takes place which results in chromatin compactation and silencing of various target genes takes place[126].

Nuclear receptors are grouped into a large superfamily and are taught to be evolutionary derived from a common ancestor. They are sub divided into six different subfamilies, which includes thyroid hormone receptors (TRs), Retinoic acid receptor (RARs), Estrogen receptors (ER), Vitamin D receptors (VDRs) and peroxisome proliferated activated receptors (PPARs) and different orphan receptors.

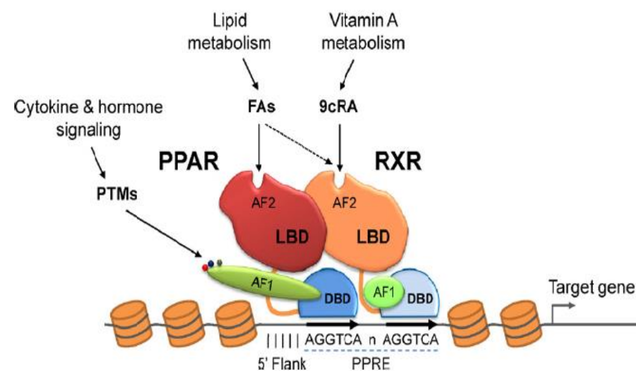

Figure: 14. Basic structure of PPAR-RXR heterodimer. PPAR can be activated by fatty acid and its derivatives. Figure adapted from[134].

8.1 Peroxisome Proliferator-Activated Receptor (PPAR)

PPAR are ligand activated transcription factors similar to other nuclear hormone receptor superfamily. PPAR can be seen in the nucleus, where they form a heterodimerisation with the 9 cis retinoic receptor upon ligand binding. The presence of Zn finger motif, which is located in the DNA binding domain, recognizes the promoter region of various genes which are involved in adipogenesis, metabolism and inflammation[135]–[140]. Upon ligand binding, PPAR forms dimerization and undergoes conformational change in the ligand binding domain, results in the displacement of H12, which create new surface for the coactivator recruitment. The coactivators include the family of p160 and p300/CBP. Most of the coactivators have intrinsic acetyl transferase activity, which forms a complex and decondense the chromatin through histone acetylation and favors the transcription (Figure: 15). PPAR suppresses the inflammatory gene transcription through the ligand mediated sumoylation of PPARγ. The sumoylated PPARγ stabilizes the corepressor NCoR-HDAC3 complex and blocks the recruitment of ubiquitinization machinery, as a result the coactivator complex cannot be able to promote transcription of inflammatory genes (Figure:16)[8].

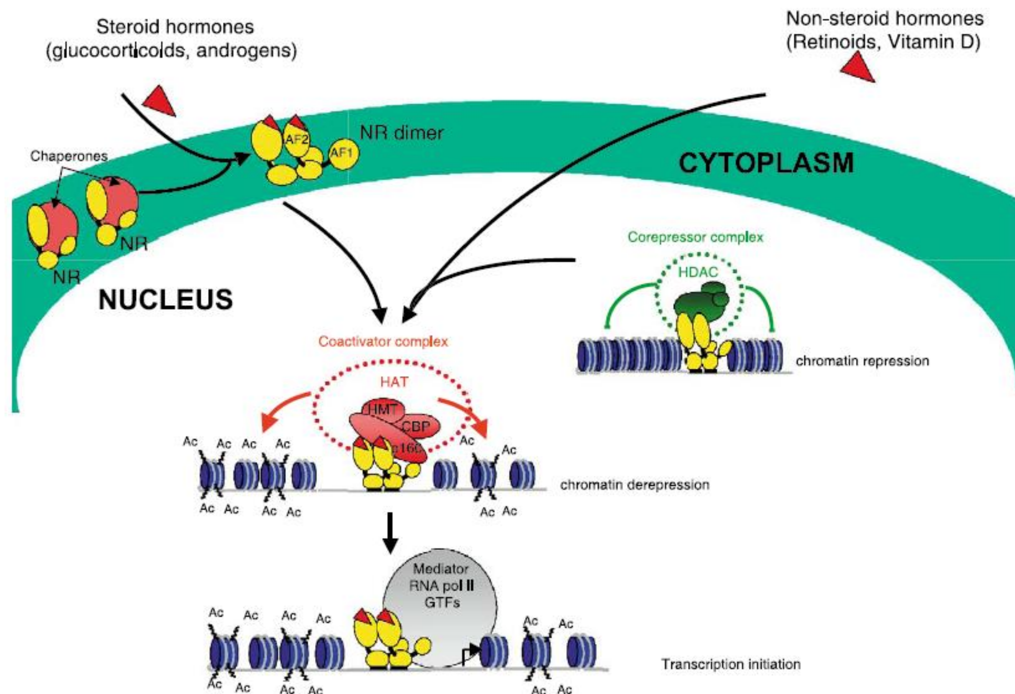

Figure: 15. Ligand mediated action of PPAR. Ligand binding forms a dimer of PPARγ and RXRα, translocates to nucleus, binds to DR1 sequences, recruits coactivators and transcription initiation occurs. Figure adapted from [141].

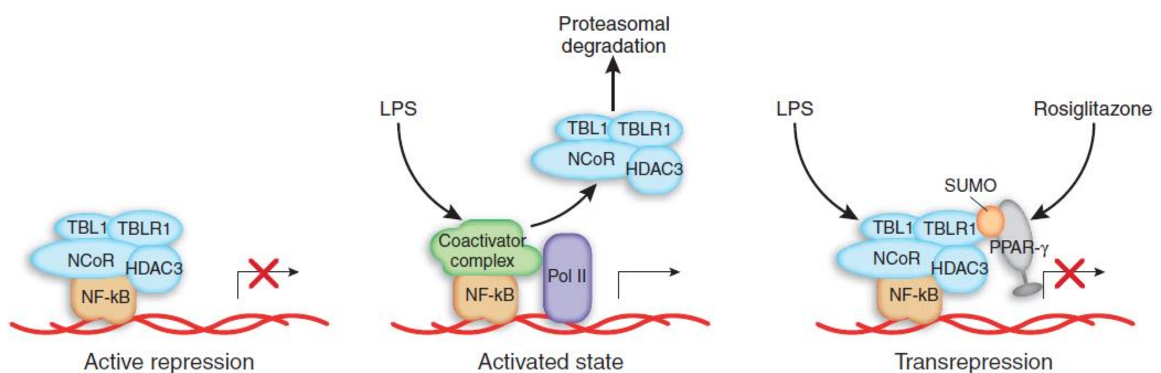

Figure: 16. PPARγ mediated sumoylation and suppression of inflammatory genes. Sumoylated PPARγ stabilizes with the corepessor complex, as a result the proinflammatory genes remains in repressed state even with the presence of LPS. Figure adapted from [142].

PPAR are of three types – PPARα, PPARβ and PPARγ. PPARγ is expressed in almost all tissues but is highly expressed in adipose tissue, heart muscle, colon, kidney, pancreas, spleen and macrophage. The main role of PPARγ is to induce adipogenesis and lipid storage through the regulation of various genes[135]. Various studies have been shown that activation of PPARγ can induce insulin sensitivity, reduce inflammation. Example: GQ16, a partial PPARγ agonist, which inhibited the serine mediated phosphorylation of PPARγ at S273, increased the insulin sensitivity without weight gain[143]. PPARγ activation can recruit beige through SIRT1 mediated deacetylation of PPARγ at lys268 and lys293, which is located in the LBD of PPARγ. Acetylation of PPARγ promotes triglyceride accumulation, increases the WAT and promotes insulin resistance upon high fat diet while, deacetylation of PPARγ promote s energy expenditure and insulin sensitivity[144].

PPARβ/δ express mainly in brain, adipose tissue and skin. PPARβ activation regulate the genes related to lipid catabolism in skeletal muscle cells[145].The PPARβ activation stimulates differentiation and lipid accumulation in keratinocytes[146]. Activation of PPARβ induces fatty acid oxidation in skeletal muscle cells, GW501516 administration to mice fed with high fat diet reduced diet induced obesity and insulin resistance, increased mitochondriogenesis, fatty acid oxidation and showed a significant reduction in the lipid droplet in skeletal muscles[147]. In addition, another group suggest that PPARβ activate fat mobilization and it protects from obesity, treatment with ligands showed a brown fat like feature in white fat, while did not showed any upregulation of PGC1α in white fat or brown fat isolated from the transgenic mice[136].

PPARα, transcription factor, regulate the levels of glucose, lipid and cholesterol. PPARα is highly expressed in oxidative tissues such as BAT, liver, heart and kidney. Similar to other PPARs, transcriptional regulation of PPARα takes place through direct binding to the AGGTCA elements, which is present in the promoter region of the target genes, while the function of PPARα is the combustion of lipids. PPARα activation induces fatty acid oxidation, differentiation in adipocytes without triglyceride accumulation[148]. PPARα null mouse, upon short term fasting, led to altered

expression of genes related of fatty acid oxidation, induce hepato steatosis, myocardial lipid accumulation and hypoglycemia[149]. Similar to PPARγ ligands, PPARα agonist also inhibit genes induced by NF-kB, such as IL-6, COX-2 and provide anti-inflammatory effects. PPARα agonist induces the expression of inhibitory protein IkBα in human aortic smooth muscle, this results in a decrease in the NFkB DNA binding activity, which ultimately results in repression of inflammatory proteins[150]. In addition to support the antiinflammatory property of PPARα ligands, another study suggest that activation of PPARα coupled with GRα, dose dependently enhance the transrepression of NFkB expression, which leads to repression of cytokine production[151]. Activation of PPARα also induces hepatic oxidation of fatty acid and showed reduced synthesis and secretion of triglycerides , improves insulin sensitivity[152]. Activation of PPARα induce beige cells in the subcutaneous white adipose tissue through the induction of beige signature genes such as PGC1α, BMP8, UCP1, PRDM16 and irisin. This clearly states that PPARα activation induce the combustion of triglycerides present in the white adipose tissue through the thermogenic mechanism[103], [153].

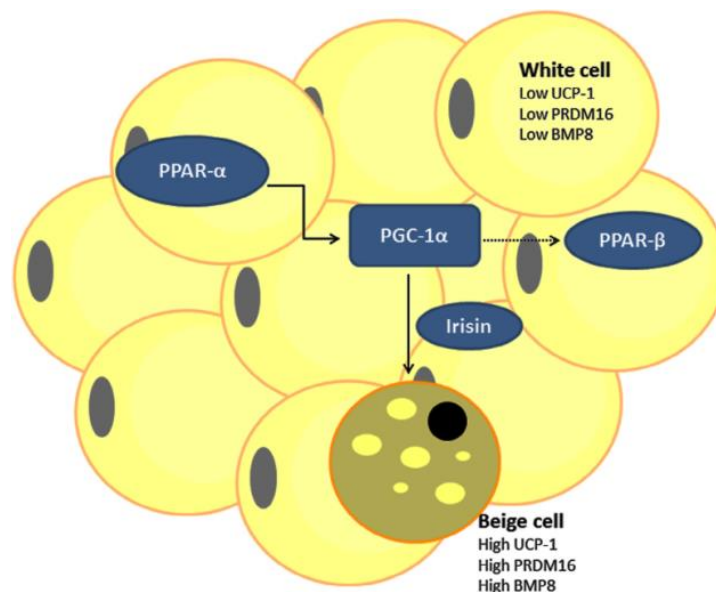

Figure: 17.PPARα activation induces beige adipose tissue through the upregulation of UCP1, PRDM16, BMP8 and irisin. Figure adapted from[103].

9. PPARα and fatty acid oxidation

The link between fatty acid oxidation and the PPARα was identified by Christine Dreyer in 1992. The studies demonstrated that the transcription factor, PPARα, activated the promoter of acylCoA oxidase gene, an enzyme involved in the activation of β-oxidation of fatty acid[154]. The free fatty acids liberated from the adipose tissue are taken up from the blood plasma and are activated by fatty acylCoA. The fatty acylCoA derivatives are later transported to the mitochondria or peroxisome for the degradation of acetylCoA via β-oxidation.

The first step for the fatty acid oxidation is the transport of fatty acid across the plasma membrane through the fatty acid transporter protein. The fatty acid transporter proteins are integral membrane proteins, the promoter of the FAT/CD36 has been identified with the presence of DR1 repeats and is regulated by PPARα[155]. Fatty acids bind to the lipid binding proteins and shuttles fatty acids from plasma membrane to the nucleus, is regulated by PPARα[156]. Later on the fatty acid are activated by esterification with CoA by means of fatty acyl CoA synthase enzyme and are shuttled into the mitochondria for the further oxidation. The transport across the mitochondria is controlled by two enzymes, which includes carnitine palmitoyl transferase 1 and 2. CPT1 is localized in the outer mitochondrial membrane, while CPT2 is restricted mainly in inner mitochondrial membrane, which catalyses the conversion of acyl carnitine to acetyl CoA. Once the fatty acylCoA reaches inside the mitochondria, it undergoes sequential action with various enzymes such as acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-L-hydroxyacyl-CoA dehydrogenase, and 3-ketoacyl-CoA thiolase and lead to the liberation of acetyl CoA, which will be further metabolized in the TCA cycle and ultimately liberates ATP after the oxidative phosphorylation reaction (Figure: 18)[157].

Studies have been reported that CPT1a protects the adipocyte against the insulin resistance and it reduced the proinflammatory genes such as TNFα and IL-6. The pharmacological inhibition of fatty acid oxidation by etomoxir increased the JNK activity[158] . Another studies conducted in macrophage suggest that inhibition of CPT1a increase macrophage inflammation, while the over expression of CPT1a reduced palmitate induced endoplasmic reticulum stress and inflammation. From

these aforementioned evidences, it is clear that increasing fatty acid oxidation can be used as a therapeutic strategy against obesity mediated metabolic disorders[159].

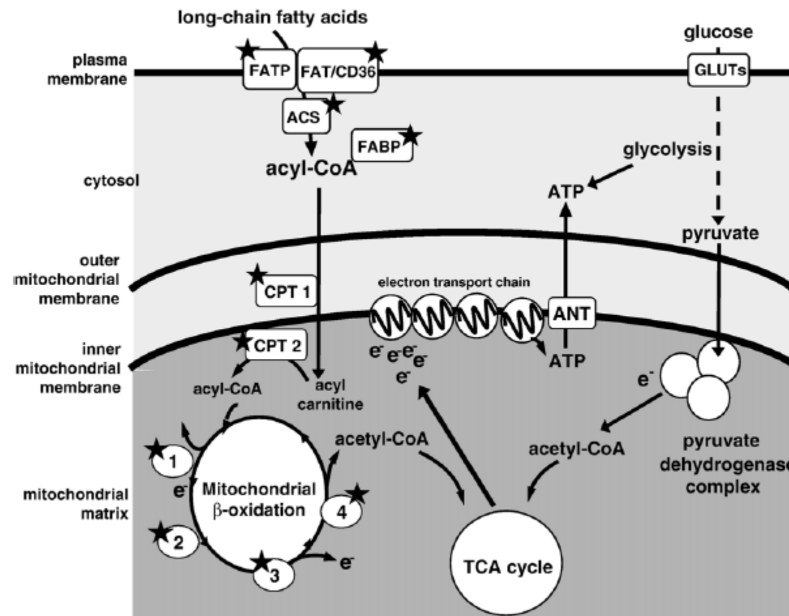

Figure: 18. Diagram shows the pathway of fatty acid oxidation by means of PPARα target genes, which are marked with a black star. Abbreviations (FATP, fatty acid transport protein; FAT/CD36, fatty acid translocase; FABP, fatty acid binding protein; ACS, acyl-CoA synthetase; GLUTs, glucose transporters; CPT, carnitine palmitoyltransferase; TCA, tricarboxylic acid; ANT, adenine nucleotide translocator; (1) acyl-CoA dehydrogenases; (2) enoyl-CoA hydratase; (3) 3-hydroxyacyl-CoA dehydrogenase; (4) 3-ketoacyl-CoA thiolase. Figure adapted from[160].

In addition, in-vivo and in-vitro studies suggest that stimulation of CPT1a activity is efficient to reduce the hepatic triglyceride accumulation. This evidence suggest that increase in fatty acid oxidation could reduce the triglyceride content in the hepatocytes[161].

10. Fatty acid induced insulin resistance

The level of fatty acid is high during obesity, insulin resistance and diabetes[162]. The increase in fatty acid, fatty acid metabolites such as diacylglecerol, fatty acylCoA, ceramides activate protein kinase C, induce the phosphorylation of insulin receptor substrate and hence the serine phosphorylated protein fail to transfer the signal to PI-3 kinase, resulting in decreased activation of glucose transporter, this

ultimately induce insulin resistance (Figure:20)[163]–[166]. The fatty acid liberated from the adipose tissue has to be removed from the blood plasma. The in-vitro study suggest that free fatty acid accumulation induce lipotoxicity, induces apoptosis and necrosis in HepG2 cells in a dose dependent manner[167]. Another study demonstrated that free fatty acid induced hepatic insulin resistance occur through the increase in diacylglyecerol content, increased activity of protein kinase C, IkB kinase and increased proinflammatory cytokines through NFkB signaling[163]. In addition to the above mentioned evidence, another report suggests that acetyl CoA carboxylase 2 knockout mice increases fatty acid oxidation, decreases triglycerides and the diacylglycerides. The reduction of diacylglyeceride reduced the activity of protein kinase C in liver and skeletal muscle, improved insulin sensitivity[168].

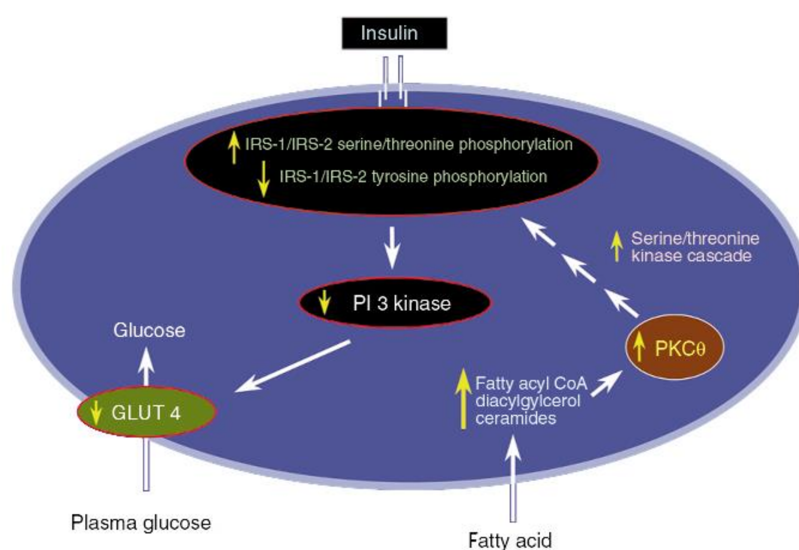

Figure: 19. Fatty acid mediated insulin resistance. An increase in free fatty acid leads to increase in fatty acyl CoA, diacylglyecerol and ceramides which leads to phosphorylation of Insulin receptor substrate 1 and 2, reduces the ability to activate phosphatidylinositol 3-kinase(PI-3)which ultimately results in diminished insulin receptor signaling and glucose transport activity. Figure adapted from[169].

11. Lipolysis and PPARα signaling

Adipocytes always maintain a balance between the triacylglyeceride (TAG) storage and lipolysis. Metabolic diseases such as obesity, type 2 diabetes arises when the triglyceride synthesis and the catabolism loses its synchrony. TAGs are stored in the lipid droplets, which is made up of phospholipid monolayer and various proteins such

as perilipin, ATGL etc. Various evidences suggest that during fasting, lipolysis is initiated when the β-AR gets the stimulus from the neuron, which activates the PKA and later the perilipin and HSL undergoes phosphorylation. The phosphorylated HSL migrates from cytosol to the lipid droplet, where it is enzymatically active and the lipid surface undergoes major rearrangement due to the phosphorylation of perilipin. The perilipin phosphorylation release the attached coactivator ABHD5, which interact with ATGL and initiate triacylglyeceride breakdown, generates a diacylglyeceride and a fatty acid. The action of the hormone sensitive lipase generates a monoacylglyeceride and a fatty acid, the monoacylgleceride lipase generate a fatty acid and a glyceride (Figure-21)[170], [171]. Studies suggest that in obese insulin resistant mice showed a reduced mRNA and protein expression levels for ATGL and HSL[94].

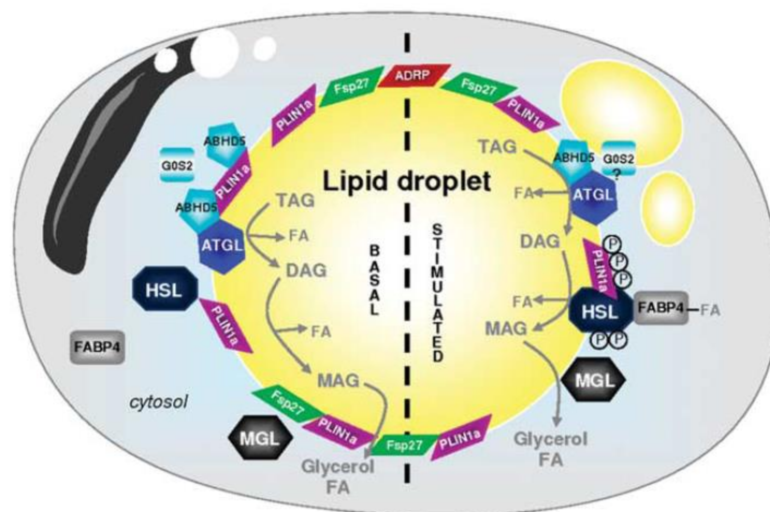

Figure: 20. White adipose tissue with lipid droplet. Upon stimulation, the adipose triacylglyceride undergoes degradation and liberates free fatty acid and diacylglecerol. (Refer text for details) Figure adapted from[172].

Studies suggest that the endogenous ligands for PPARα can be produced by means of lipolysis as well as lipogenesis. There are various evidences suggesting that lipolysis liberates various ligands for PPARs, especially for PPARα, while very few evidences are available for PPARβ (Figure:21)[173]. The liberated lipolytic products activate the PPARα, δ receptor and also induce the thermogenic gene expression such as PGC1α, PDK4, UCP1 in lipolysis simulated brown adipocytes[174]. Another evidence suggest that ATGL mediated lipolysis regulate the expression of PPARα

target genes, PGC1α and PGC1β in the heart muscle, the down regulated expression of PGC1α contributed a reduced mitochondrial biogenesis and impaired oxidative phosphorylation in the ATGL knockout mice. PPARα agonist treatment reversed the altered metabolic gene expression and the impaired oxidative phosphorylation, while the PPARβ agonist failed to reverse the altered gene expression[175].

Lipogenesis mediated by fatty acid synthase generates ligands for PPARα in the liver. It has been showed that FAS knockout induces hypoglycemia, alters the cholesterol and lipid metabolism, treatment with PPARα agonist reverted this effect and became normal. This suggests that during de-novo lipogenesis, FAS generates endogenous PPARα specific ligands, which regulates the normal metabolism through PPARα[176]. Another report suggest that a phosphatydyl choline species(1-palmioyl-2-oleoyl-sn-glyecerol-3-phosphocholine)(16:0/18:1-GPC) has been identified and found that they interact with ligand binding domain of PPARα[177]. From the above mentioned evidences, it is clear that lipogenesis, lipolysis mediated PPARα signaling is inevitable for the maintenance of lipid levels.

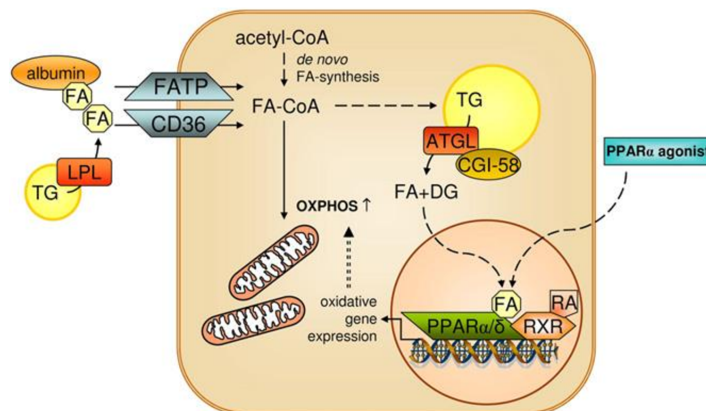

Figure: 21. Lipolysis is required for PPARα/β signaling. ATGL mediated lipolysis regulate fatty acid oxidation and oxidative phosphorylation through PPARα. Figure adapted from[170].

12. Objective:

1) To study the agonistic effect dibutyltin compounds on PPARα and PPARβ.

2) To investigate the antagonistic effect of dibutyltin compounds on PPARα.

Specific objectives:

- Evaluation of cytotoxicity of dibutyltin compounds by MTT assay.
- To study the agonistic effect of dibutyltin dilaurate on PPARβ.
- To study the agonistic effect of dibutyltin dilaurate and dibutyltin dichloride on PPARα.
- To study the antagonistic effect of dibutyltin diacetate on PPARα.
- To study the antagonistic effect of dibutyltin maleate on PPARα.
- To study the antagonistic effect of dibutyltin dilaurate on PPARα.
- To study the antagonistic effect of dibutyltin dichloride on PPARα.
- Comparison of the antagonistic effect of dibutyltin dilaurate and GW6471 on PPARα.
- Comparison of the antagonistic effect of dibutyltin dichloride and GW6471 on PPARα.

Materials and methods

Chemicals

Bezafibrate, DMSO, DBT dilaurate, DBT diacetate, DBT dichloride, DBT maleate and MTT were obtained from Sigma Aldrich. GW6471 was purchased from Cayman chemicals. DMEM was from Gibco.

All the DBTs, Bezafibrate, GW6471 were prepared by dissolving in 100% DMSO.

DMEM was supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 Units/mL penicillin and 50 mg/mL streptomycin).

Electroporation buffer was prepared by dissolving 0.1% of dextrose, 0.1% of $Cacl_2$ in 1X PBS with pH - 7 and membrane filtered using 0.22µm.

Transformation

E.coli (DH5α) competent cells were thawed on ice for 10 minutes and 50µl of competent cells were added to the eppendorf containing 1µg of plasmid and the mixture was incubated in ice for 30 minutes and the cells were given a heat shock at 42ºC for 1.5 minutes and immediately placed in ice for 2 minutes. 900µl of LB medium was added to the cells and incubated at 37ºC for 1 hour with shaking for phenotypic expression of antibiotic resistance genes. 50µl of transformed cells were plated on the LB ampicillin plates and the plates were inverted and incubated at 37ºC for 12 to 14 hours.

Plasmid isolation and quantification of plasmids

Plasmid having the human cDNA for PPARαLBD or PPARβLBD fused to a Gal4 DNA binding domain in pcDNA3 and a plasmid having the luciferase reporter gene under the regulation of five Gal4 DNA binding elements (UASG × 5TK-Luciferase) driven with a promoter of adenovirus E1b has been used for the experiments. All the plasmids used for the experiments were purified using QIAGEN Purification kit as per standard protocol prescribed by QIAGEN. The isolated plasmids were dissolved in

200µl of TE buffer with pH 8 and stored at -8 ºC. 1µl of plasmids were used for the quantification of plasmid DNA and all the plasmid isolated showed a high purity with A260/A280 - 1.8

MTT Assay

Principle of the assay

This is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria, where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells are then solubilized with an organic solvent (eg: acidic isopropanol) and the released, solubilized formazan reagent is measured using spectrophotometer. Since the reduction of MTT occurs only in metabolically active cells, the level of activity provides the measure of cell viability.

Hela cells were used for the MTT assay, 50µl of 30.000 cells were plated in 96 well plate. The cells were treated with DMSO (0.1%) or various concentrations of DBTs ranging from 10^{-10} µM to 10^{-4} µM for 24 hours, the cells were treated with MTT solution with a concentration of (5mg/ml), the plates were incubated in dark for at least 4 hours in Co2 incubator at 37ºC. After 4 hours the medium with MTT has been aspirated, added 100µl of acidified Isopropanol, then vortexed for 15 minutes and finally the reduced MTT was assayed using spectrophotometer Beckman coulter DTX800. The untreated cells with DBT were considered as negative control. The experiment was conducted as hexaplicates, 2 times before concluding the results. The DBTs were prepared by dissolving it in DMSO. The percentage of the cell viability of dibutyltin compounds has been calculated with respect to the vehicle (DMSO).

Transient transfection and luciferase reporter assay

The most common and successful approach for the search of new ligands is the cell based co- transfection assay. In this assay plasmid DNA encoding gene of interest, along with another plasmid encoding the responsive element tagged with a

reporter gene, capable of easily measurable product should be introduced into the cultured cells. The transfection was carried out by means of electroporation. The reporter gene contains a promoter with multiple responsive elements, which are highly specific for the nuclear receptor. This promoter regulates the expression of the reporter gene (for example a luciferase cDNA) by the activation or inhibition of the nuclear receptor by a potential ligand.

Hela cells were grown in Dulbecco Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) at 37ºC in a humidified atmosphere of 5% CO_2 with a confluence of 80%. 2.5ml of 0.2% of trypsin was added for removing the adherent HeLa cells from the plates and kept in Co2 incubator for 2 minutes, later on 8ml of DMEM was added for blocking the action of trypsin. The whole medium with trypsin was then aspirated and transferred to a sterile falcon tube aseptically and centrifuged at 2000rpm for 5 minutes. The supernatant suspension was discarded and the cells pellets were added with electroporation buffer and counted. 8.5 million cells with 500µl of electroporation buffer were added to a cuvette containing plasmids. 8.5 million cells were transiently co-transfected with a 1.5µg of plasmid having the PPARα LBD fused to a Gal4 DNA binding domain and a 3µg of plasmid having the luciferase reporter gene under the regulation of 5XGal4 DNA binding elements. The transfections were performed by electroporation at 240 mVolt and 950µF capacitance. After electroporation, 1 million transfected cells were plated to each well as triplicates. The cells were treated with vehicle (DMSO, 0.1%), DBTs (DBT dilaurate, DBT diacetate, DBT maleate and DBT dichloride) or GW6471 in a dose dependent manner. After one hour, gave another treatment with Bezafibrate (PPARα agonist), an equal volume of vehicle has been added to balance the percentage of DMSO and the final concentration of DMSO would become 0.2%. The cells were incubated for 24 hours at 37ºC. After 24 hours, the cells were lysed and reporter luciferase assay kit (Promega) was used to measure the luciferase activity according to the manufactures instruction using a luminometer (Promega).

Statistical Analysis

Data presented as mean +/- SEM of three independent experiments conducted in triplicate for DBTL and DBTC, while others were performed as single experiment conducted in triplicate. **, $p < 0.01$; ***, $p < 0.001$ by one way analysis of variance

(ANOVA) followed by Newman-Keuls multiple comparison test. **, *** or # indicate significant difference compared to the control or to the bezafibrate (10μM). MTT assay was performed as two independent experiments conducted as hexaplicates and the graph was plotted using nonlinear regression analysis. The activation or inhibition curves were constructed and IC_{50} values were determined by nonlinear regression analysis by using GraphPad Prism program version 5.0.

14. Results

Evaluation of cytotoxicity with DBT compounds.

To evaluate the cytotoxic effect of dibuyltin compounds, MTT assay has been performed using HeLa cells. The DBT dilaurate, DBT dichloride, DBT diacetate and DBT maleate were treated in a dose dependent manner to see its effect on the cell viability. The percentage of cell viability was calculated with respect to vehicle. DBT dilaurate 10^{-5}M (10μM) displayed 63.12% of cell viability with respect to the control (76.69%), higher concentration induced severe cell death, while DBT dichloride 10^{-6}M (1μM) displayed 65.76% and its control exhibited cell viability of 80.32%, higher concentrations demonstrated cell death. DBT diacetate 10^{-6}M showed 59% of cell viability with respect to its control (79.8%), while dibutyltin maleate 10^{-7}M displayed 64.8% and its control showed 75% (Figure-22). DBT dilaurate 10^{-5}M, DBT dichloride 10^{-6}M, DBT diacetate 10^{-6}M and DBT Maleate 10^{-7}M were selected as the maximum concentration for the transactivation assays.

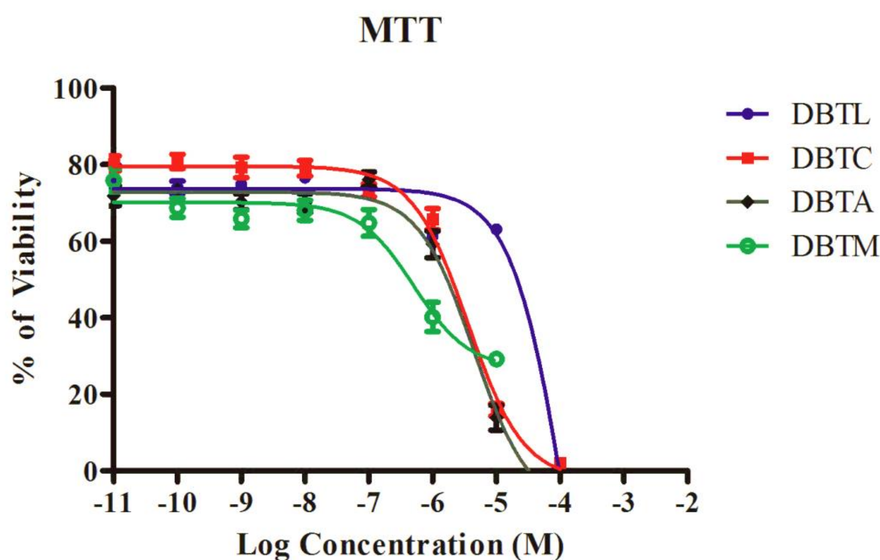

Figure: 22. Effect of dibutyltin compounds on viability of HeLa cells. HeLa cells were treated with increasing concentrations of DBT dilaurate, dichloride, diacetate and maleate for 24 hours, assessed by the MTT assay. Results indicated as mean +/- SEM of two independent experiments conducted in hexaplicates. Dose-response curve constructed using nonlinear regression analysis.

Dibutyltin dilaurate is not an agonist for PPARβ

Transactivation assay was performed to identify the agonistic effect of dibutyltin dilaurate on PPARβ. Bezafibrate was used as the positive control. Bezafibrate dose dependently increased the transcription activation of PPARβ and at 10^{-4} M exhibited a 3 fold activation, while the dibutyltin dilaurate did not showed any activation for PPARβ even with the maximal concentration. Thus we concluded that dibutyltin dilaurate is not an agonist for PPARβ (Figure-23).

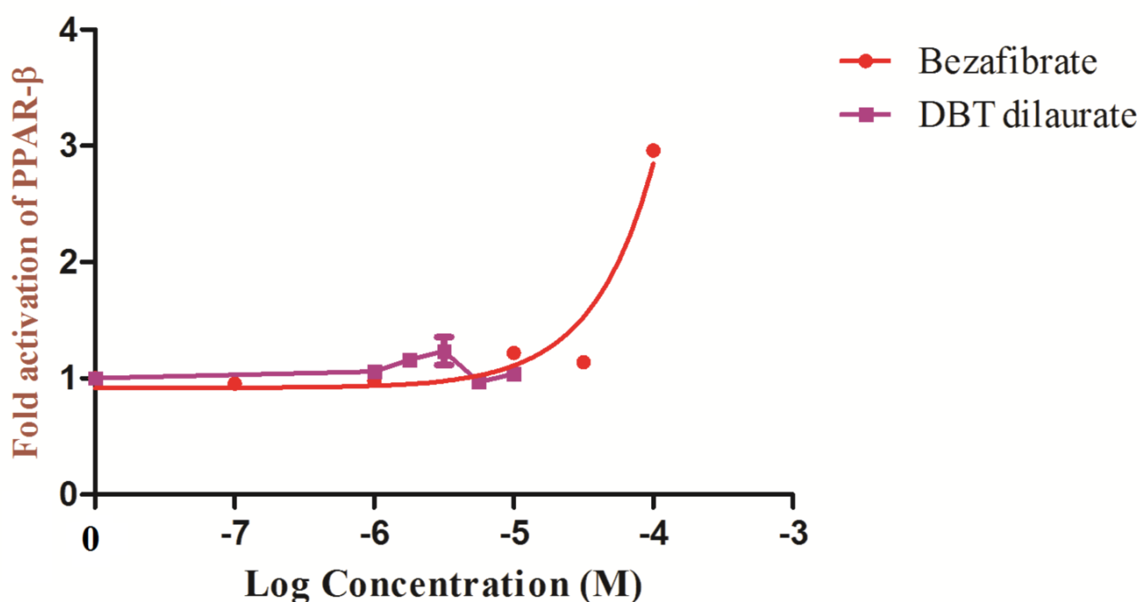

Figure:23. DBT dilaurate is not an agonist for PPARβ

HeLa cells were co-transfected with PPARβ LBD fused to a GAL4DNA binding domain and a luciferase reporter construct under the regulation of a 5xGAL4 DNA binding element by electroporation. Cells were treated with increasing concentrations of DBTL or Bezafibrate. Data presented as mean +/- SEM of single experiment conducted in triplicate. The activation curves were constructed by nonlinear regression analysis by using GraphPad Prism program version 5.0.

Dibutyltin dilaurate is not a PPARα agonist.

Next we investigated whether DBT can display an agonist effect on PPARα. Therefore a cotransfection assay has been performed. As observed in Figure-24, bezafibrate, showed a dose dependent fold activation of PPARα. The maximal concentration 10^{-4}M of bezafibrate exhibited 9 fold activation (Figure-24). Dibutyltin dilaurate dose dependently reduced the basal transcriptional activity of PPARα. The reduction in the basal transcriptional activity prompted us to investigate whether the dibutyltin dilaurate has an antagonistic effect on PPARα.

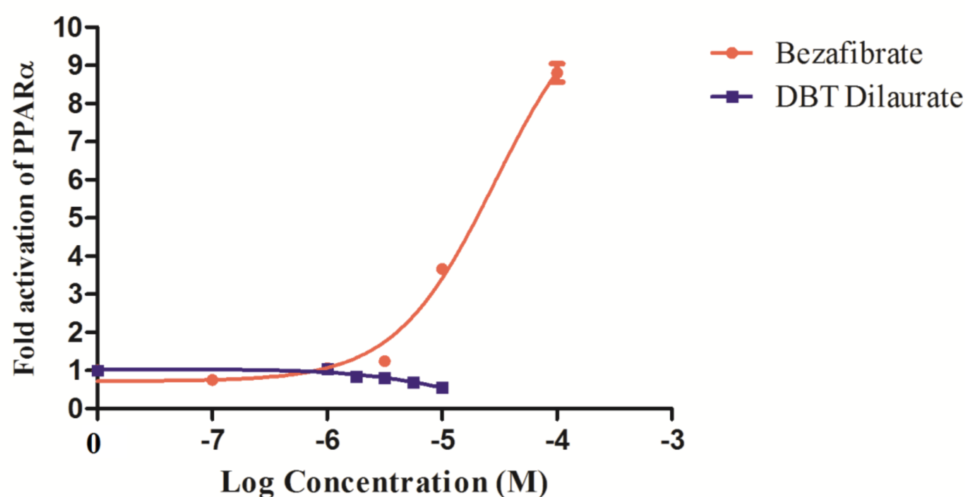

Figure:24. Dibutyltin dilaurate is not a PPARα agonist.

HeLa cells were co-transfected with PPARα LBD fused to a GAL4DNA binding domain and a luciferase reporter construct under the regulation of a 5xGAL4 DNA binding element by electroporation. Cells were treated with increasing concentrations of DBTL or Bezafibrate. Data presented as mean +/- SEM of single experiment conducted in triplicate. The activation curves were constructed by nonlinear regression analysis by using GraphPad Prism program version 5.0.

Dibutyltin dichloride is not a PPARα agonist.

The cell based cotrasfection assay has been utilized to investigate the effect of dibutyltin dichloride on PPARα. Bezafibrate has been used as a positive control. Bezafibrate induced a dose dependent increase in fold activation of PPARα upto 7. The dibutyltin dichloride exhibited a reduction in the basal transcriptional activity of PPARα. The reduction in the basal transcriptional activity prompted us to study the antagonistic activity of dibutyltin dichloride on PPARα (Figure: 25).

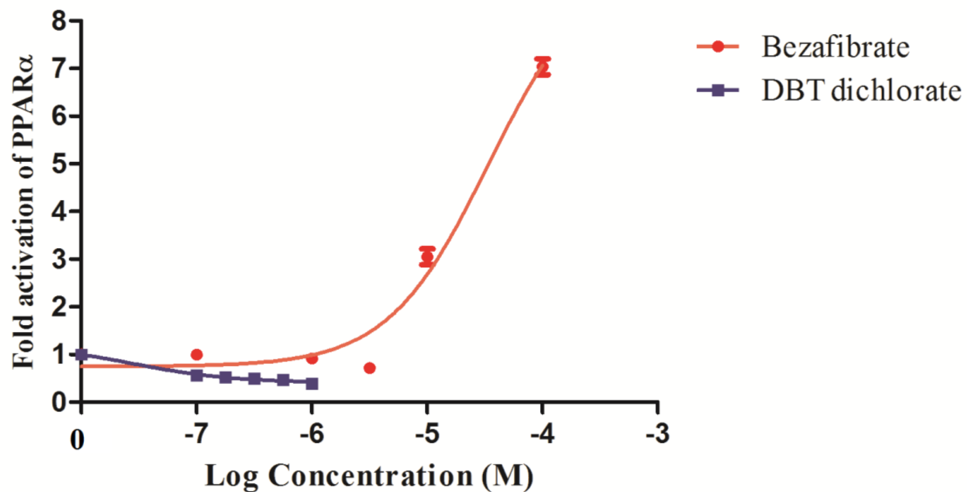

Figure:25. Dibutyltin dichloride is not a PPARα agonist.

HeLa cells were co-transfected with PPARα LBD fused to a GAL4DNA binding domain and a luciferase reporter construct under the regulation of a 5xGAL4 DNA binding element by electroporation. Cells were treated with increasing concentrations of DBTC or Bezafibrate. Data presented as mean +/- SEM of single experiment conducted in triplicate. The activation curves were constructed by nonlinear regression analysis by using GraphPad Prism program version 5.0.

Dibutyltin diacetate is not an antagonist for PPARα

To further clarify whether dibutyltin diacetate has an antagonist effect on PPARα we performed a cell based transactivation assay in HeLa cells. The Hela cells were treated with the PPARα agonist (Bezafibrate – 10μM) in absence (zero) and in the presence of increased concentrations of dibutyltin diacetate. Bezafibrate induced a 3.5 fold activation of PPARα reporter gene and the addition of dibutyltin diacetate (DBTA) did not significantly changed this transcription activity even at the higher concentration of 1μM (Figure - 26). Hence, dibutyltin diacetate is not considered a PPARα antagonist.

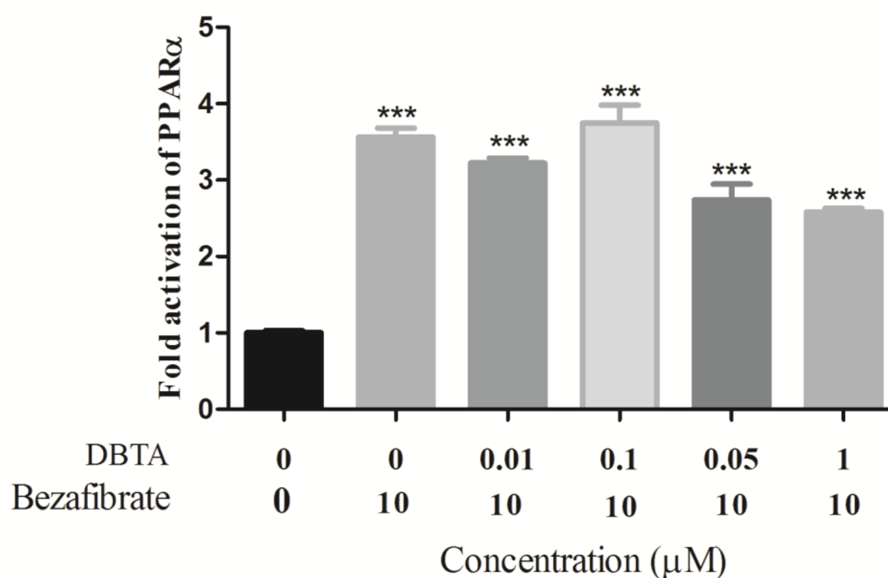

Figure: 26. DBT diacetate is not an antagonist for PPARα. HeLa cells were co-transfected with PPARα LBD fused to a GAL4DNA binding domain and a luciferase reporter construct under the regulation of a 5xGAL4 DNA binding element by electroporation. Cells were treated with increasing concentrations of DBTA in the absence or presence of 10 μM bezafibrate. Data presented as mean +/- SEM of single experiment conducted in triplicate. ***, $p < 0.001$ by one way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. ***, indicate significant difference compared to the control.

Dibutyltin maleate is not an antagonist for PPARα

To extend our knowledge of the DBTs on PPARα, we also evaluated whether dibutyltin maleate display an antagonist effect on PPARα. To investigate this question, we performed a cell based transactivation assay in HeLa cells. The HeLa were treated with the PPARα agonist (Bezafibrate – 10µM) in absence (zero) and in the presence of increased concentrations of dibutyltin maleate. In the co-transfection assay, bezafibrate (PPARα agonist) induced a fold activation of 2.6 with respect to the control, while the addition of dibutyltin maleate did not exhibited any inhibitory effect in the bezafibrate mediated PPARα activation (Figure-27). Hence, dibutyltin maleate is not an antagonist for PPARα.

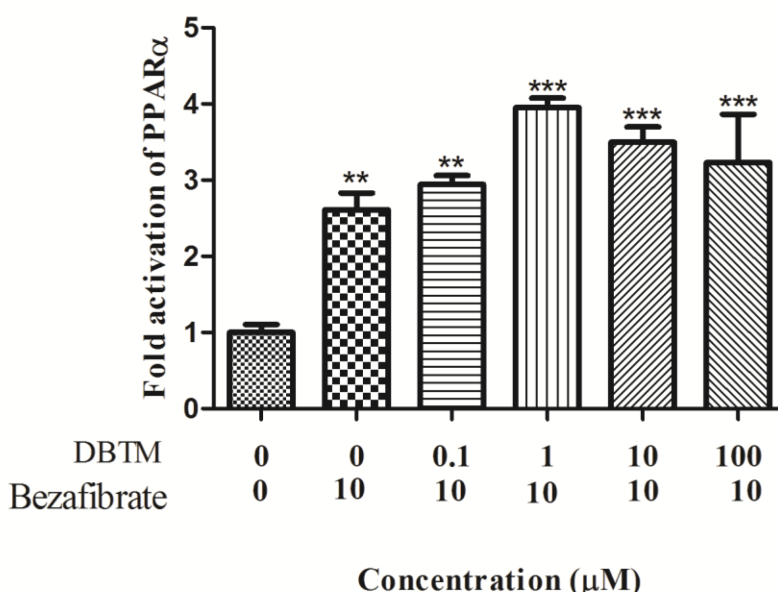

Figure:27. DBT maleate is not an antagonist for PPARα. HeLa cells were co-transfected with PPARα LBD fused to a GAL4 DNA binding domain and a luciferase reporter construct under the regulation of a 5xGAL4 DNA binding element by electroporation. Cells were treated with increasing concentrations of DBTM in the absence or presence of 10 µM bezafibrate. Data presented as mean +/- SEM of single experiment conducted in triplicate. **, $p<0.01$; ***, $p < 0.001$ by one way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. **; *** indicate significant difference compared to the control.

DBT dilaurate is a PPARα antagonist

Since dibutyltin dilaurate showed no agonistic effects on PPARα transcriptional activity and in fact reduced the basal transcriptional activity PPARα, we explored the antagonistic effects of this compound on PPARα. In the cell based reporter gene assay, treatment with PPARα agonist, (Bezafibrate), displayed a PPARα mediated fold activation of 3.3, while the DBT dilaurate treatment inhibited the transactivation induced by Bezafibrate in a dose response manner. The maximal reduction occurs at the concentration of 7.5 μM, a concentration much lower than the maximal non cytotoxic concentration (10^{-5}M). Therefore, this repression was not due to the cytotoxicity. Hence, DBT dilaurate is considered as an antagonist for PPARα (Figure:28).

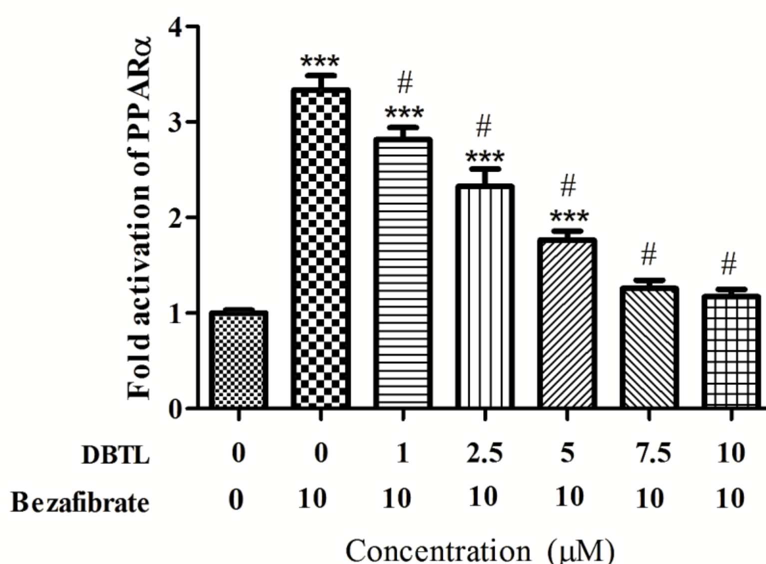

Figure:28. DBT dilaurate is an antagonist for PPARα. Effect of DBT dilaurate (DBTL) on bezafibrate induced PPARα activity. HeLa cells were co-transfected with PPARα LBD fused to a GAL4DNA binding domain and a luciferase reporter construct under the regulation of a 5xGAL4 DNA binding element by electroporation. Cells were treated with increasing concentrations of DBTL in the absence or presence of 10 μM bezafibrate. Data presented as mean +/- SEM of three independent experiments conducted in triplicate. ***, $p < 0.001$ by one way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. *** or # indicate significant difference compared to the control or to the bezafibrate (10μM).

DBT dichloride is an antagonist for PPARα

Also, considering that dibutyltin dichloride impairs basal transcriptional activity PPARα we examined if this compound has antagonistic effects on PPARα. In the transactivation assay, bezafibrate induced PPARα activation of 3 fold with respect to vehicle. The treatment with DBT dichloride impaired in a dose dependently way the bezafibrate-induced transcription activation of PPARα (Figure-29). This suppression was not due to the cytotoxicity of DBTs Refer to figure-22 for MTT cytotoxicity.

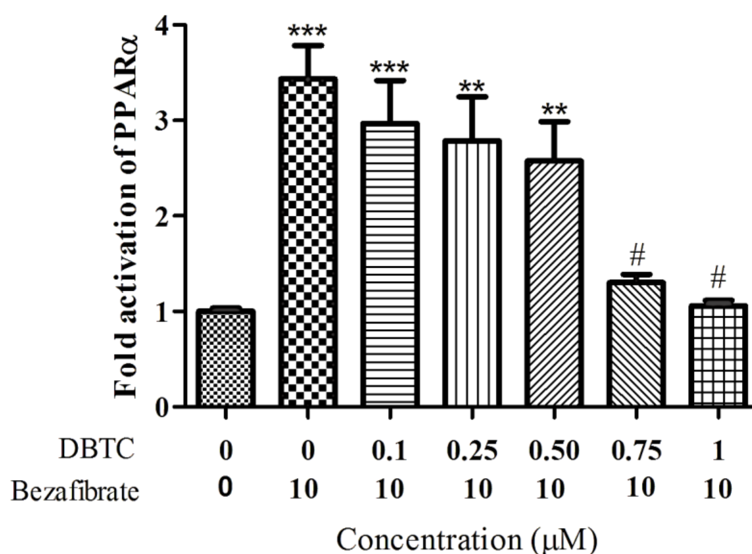

Figure: 29. Dibuyltin dichloride is an antagonist for PPARα. DBT chloride is an antagonist for PPARα. Effect of DBT chloride (DBTC) on bezafibrate induced PPARα activity. HeLa cells were co-transfected with PPARα LBD fused to a GAL4DNA binding domain and a luciferase reporter construct under the regulation of a 5xGAL4 DNA binding element by electroporation. Cells were treated with increasing concentrations of DBTC in the absence or presence of 10 μM bezafibrate. Data presented as mean +/- SEM of three independent experiments conducted in triplicate. **, $p < 0.01$; ***, $p < 0.001$ by one way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. **, *** or # indicate significant difference compared to the control or to the bezafibrate (10μM).

Comparison of the antagonistic effects of DBT dilaurate, DBT dichloride and GW6471 on PPARα.

To better characterize the antagonistic effect of DBT dilaurate and DBT dichloride, we compared their effect with GW6471, a very strong and specific PPARα antagonist. Transfected HeLa cells were treated with an increasing concentration of DBT dilaurate, DBT dichloride or GW6471 in the presence of 10μM of bezafibrate (Figures 30 and 31). Bezafibrate activation in the absence of antagonist was considered as 100%. The percentage of GW6471 (PPARα antagonist), DBT dilaurate and dichloride were calculated with respect to bezafibrate.

DBT dilaurate showed a dose dependent inhibition of bezafibrate induced PPARα transcriptional activity with a median inhibitory concentration (IC50) of 4.1μM, whereas GW647 displayed an IC50 value of 0.13μM (Figure-30).

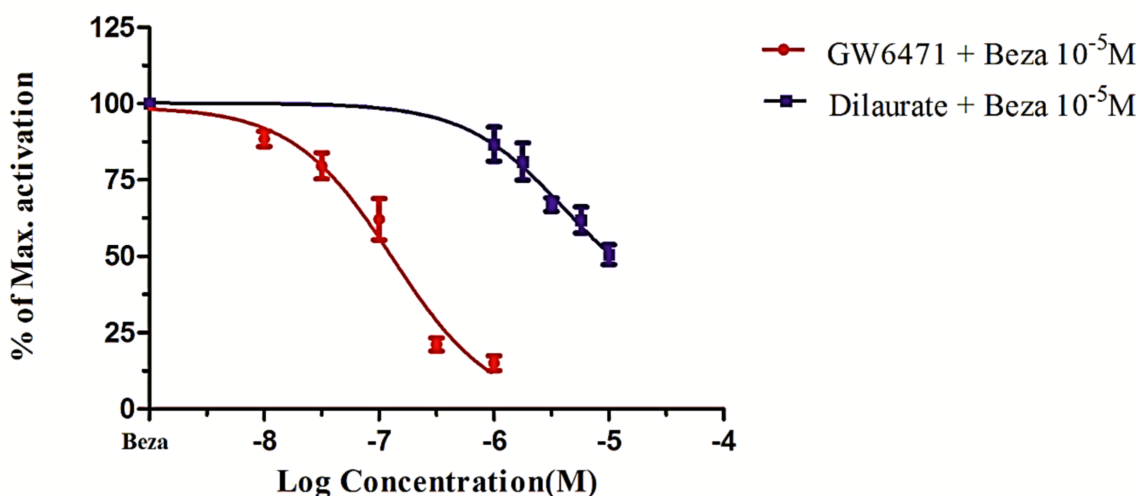

Figure: 30. Comparison of the antagonistic effects of DBT dilaurate, DBT dichloride and GW6471 on PPARα. HeLa cells were co-transfected with PPARα LBD fused to a GAL4DNA binding domain and a luciferase reporter construct under the regulation of a 5xGAL4 DNA binding element by electroporation. Cells were treated with increasing concentrations of DBTL in the absence or presence of 10μM bezafibrate or GW6471. Data presented as mean +/- SEM of at least three independent experiments conducted in triplicate. The inhibition curves were constructed and IC_{50} values were determined by nonlinear regression analysis by using GraphPad Prism program version 5.0.

As observed in Figure 31, GW6471 repressed bezafibrate mediated activation response, with a median inhibitory concentration (IC50) of 0.17μM, while DBT dichloride diminished bezafibrate mediated activation with a median inhibitory concentration of 0.26μM, very similar to GW6471 (Figure-31)

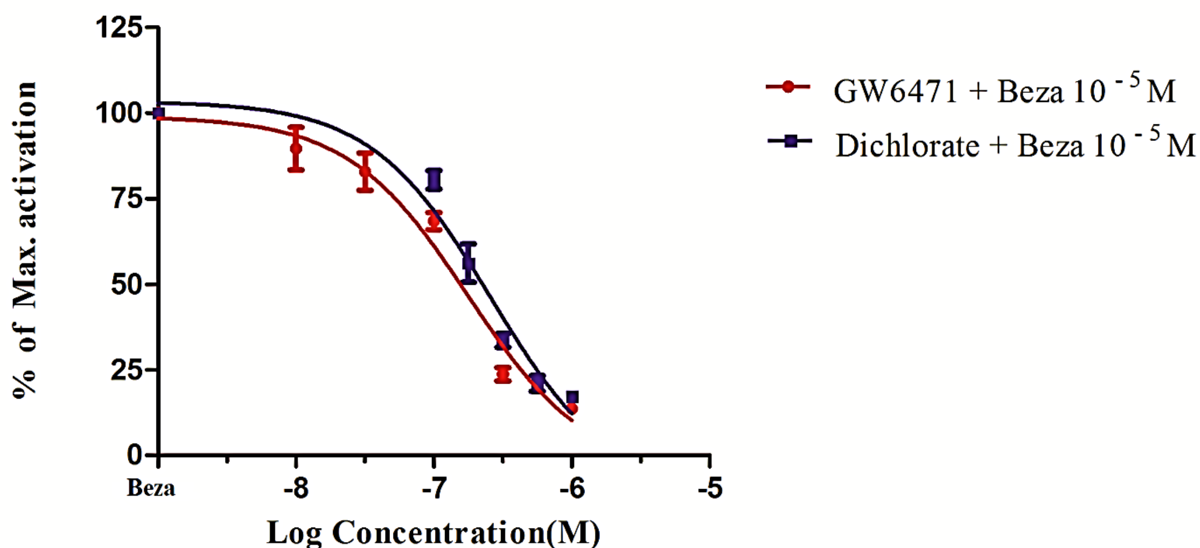

Figure:31. Comparison of the antagonistic effects of DBT dichloride and GW6471 on PPARα. HeLa cells were co-transfected with PPARα LBD fused to a GAL4DNA binding domain and a luciferase reporter construct under the regulation of a 5xGAL4 DNA binding element by electroporation. Cells were treated with increasing concentrations of DBTC in the absence or presence of 10μM bezafibrate or GW6471. Data presented as mean +/- SEM of at least three independent experiments conducted in triplicate. The inhibition curves were constructed and IC_{50} values were determined by nonlinear regression analysis by using GraphPad Prism program version 5.0.

15. Discussion

Organotin compounds bind to nuclear receptors, modulate the expression of various genes[1], [9], [89], [178]. Various organotin compounds are identified as agonist of PPARγ[1], [15], [179], while very few findings have been documented about its antagonizing property. Recent evidence suggests that TBT oxide act as a potent antagonist for plaice PPARα and PPARβ at nM concentrations [20]. In the present study, we demonstrated that dibutyltin dilaurate and dichloride acts as an antagonist for PPARα in transactivation assays.

Various reports suggest that PPARα regulates the fatty acid uptake, transport and oxidation of fatty acid for the production of energy[138], [180]. The deregulation could cause serious effects on the cells, which leads to lipid accumulation in non adipogenic tissues like liver, skeletal muscles[181]. Evidences suggest that activation of PPARα in adipocytes could increase the fatty acid oxidation and induce beige adipose tissue[103], [182]. These aforementioned evidences suggest that PPARα activation could burn the accumulated triglycerides.

The agonistic effects of dibutyltin dilaurate and dibutyltin dichloride on PPARα and PPARβ have been investigated using transactivation assay. The dibutyltin dilaurate did not showed any agonistic activity on PPARβ. The dibutyltin dilaurate and dibutyltin dichloride displayed a reduction in the basal transcriptional activity of PPARα, this prompted us to investigate about the antagonistic activity of these compounds on PPARα.

To determine whether dibutyltin compounds acts as an antagonist, we examined the cotansfection-mediated assay in HeLa cells. Dibutyltin dilaurate and dibutyltin dichloride reduced in a dose dependently manner the bezafibrate mediated activation in the transactivation assay, while the dibutyltin diacetate and dibutyltin maleate did not exhibited any inhibition in the bezafibrate mediated PPARα activation. The reduction in the bezafibrate-mediated activation was not due to the cytotoxicity of the compounds.

The median inhibitory concentration (IC_{50}) values of GW6471, a known PPARα antagonist, and dilaurate were 0.13μM and 4.1μM, while GW6471 and DBT dichloride displayed IC_{50} values of 0.17μM and 0.26μM respectively. Based on

the IC_{50} value, dibutyltin dilaurate was considered as weak antagonist, while the dibutyltin dichloride displayed a median inhibitory concentration close to that of the known PPARα antagonist (GW6471), hence it is considered as a potent antagonist for PPARα. This evidence cannot be completely acceptable, further studies are necessary to characterize the antagonistic activity.

Studies have been reported that PPARα antagonist recruit corepressors and hence the genes related to fatty acid oxidation could be inhibited[182], [183]. Based on the cotransfection assay results, we assume that these compounds could inhibit fatty acid oxidative genes. Prolonged inhibition of CPT1α in rats results in the accumulation of intramyocellular lipid and increase insulin resistance[184]. We assume that low fatty acid oxidation rates could lead to the decreased function of CPT1, associate with the insulin resistance, possibly through the accumulation of lipid intermediates and their interference with the insulin signaling, while our results do not support evidence.

In order to further strengthen the lipid mediated insulin resistance, reports suggest that saturated free fatty acid induced the accumulation of ceramide and diacylglecerol, which inhibited the insulin signaling pathway[164], [185], [186]. Moreover free fatty acid accumulation could induce the hepatic steatosis, inflammation through the increased activation of NfkB and stimulated the production of inflammatory proteins such as TNFα, IL1β and MCP1[163]. Our results do not provide any information in relation to the production of diacylglererol and its relation to the occurrence of inflammation, further experiments are necessary to dissect the mechanism.

Recent evidence suggest that DBT dichloride exposure to natural killer cells activates the protein kinase C and protein kinase D[71]. It is well characterized that activation of protein kinase C impairs the insulin signaling, induce reduction in the uptake of glucose into the cell and this ultimately leads to insulin resistance[187], [188]. Our data do not have any supporting evidence related to the DBTC mediated protein kinase C activation and the inhibition of insulin signaling. Further studies are required to explore this signaling mechanism.

DBT binds to the GR, inhibited the GR mediated transrepression of proinflammatory genes such as TNFα, IL-6[89]. Furthermore, DBT chloride induced the inflammatory proteins such as tnfα and IL-6, activated NFkB in microglial cells[90], moreover another study suggest that DBT dichloride treatment induced pancreatitis, unregulated the expression of TGFβ, IL-10, IL-1β, while there was no significant increase in the expression of IL-2, IL-4, TNFα[91]. Murine macrophage cell line, J774.1 studies suggest that dibutyltin dichloride was cyotoxic, inhibit tnfα and IL-12, while they induce IL-10. This evidence makes it clear that DBTC inhibit some inflammatory cytokines, while they do not repress all the cytokines[189]. The increased production of the proinflammatory proteins when treated with dibutyltin dichloride could inhibit the insulin signaling and induce the insulin resistance. These aforementioned evidences cannot be completely ruled out, while our data do not contain any information in relation to the increased production of inflammatory proteins.

Another study suggests that PPARα plays a major role during fasting, PPARα knockout mice induce hepatic steatosis, myocardial lipid accumulation, hypoglycemia[149]. This makes it clear that PPARα plays a major role during the period of fasting. Fatty acid function as endogenous ligand, modulate the PPARα activity. These ligands (Fatty acids), originate from two main sources. De novo lipogenesis and lipolysis. De novo lipogenesis is mediated by fatty acid synthase, which generate ligands and regulate hepatic PPARα activity[190]. Lipolyisis is initiated when the β-AR gets the stimulus, which activates the PKA and later the perilipin undergoes phosphorylation, this finally activates the lipases such as (ATGL, HSL) and releases the FFA[191]. The liberated lipolytic products activate the PPARα, δ receptor and also induce the thermogenic signature genes in lipolysis simulated brown adipocytes[174]. This clearly suggests that ATGL mediated lipolysis liberates PPARα specific ligands, which regulates the normal metabolism. Since dibutyltin compound has been detected in the blood and liver, they could easily interfere with the endogenous ligand mediated PPARα activity. It is not clear how efficiently the environmentally available concentration antagonize the PPARα receptor, while the possibilities cannot be excluded. Our transactivation assay results are not enough to provide clear information about the DBT mediated inhibition of the PPARα receptor.

Mitochondria play a major role in lipid metabolism[192]. Mitochondrial dysfunction is closely related to obesity, insulin resistance and diabetes[74], [193]–[195]. The impaired fatty acid oxidation could leads to hepatic steatosis, organ failure[196]. WAT from diabetes mice showed low level expression of mitochondrial proteins such as ATP synthase α, β, OXPHOS II and III, mitochondrial loss has been analyzed and confirmed in diabetic mice compared with wild type. Diabetic mice exhibited mitochondrial dysfunction, confirmed through reduced fatty acid oxidation and electron transport chain enzymatic activity compared with wild type[197]. Studies suggest that dibutyltin dichloride is associated with inhibition of mitochondrial respiration, induced mitochondrial damages[78]. Another evidence indicate that ATP levels are significantly reduced when natural killer cells were treated with dibutyltin compounds[198]. Another report suggest that DBT dilaurate induced lipid peroxidation, brain tissue injury and mitochondrial damage, when the wistar rats were treated[93]. From these aforementioned evidences, it can be concluded that these compounds induce mitochondrial dysfunction, hence the chances for the occurrence of insulin resistance, diabetes, accumulation of lipids in the non adipogenic tissues and lipotoxicity cannot be eliminated completely, while our results do not provide any evidence about the above mentioned evidences.

Tributyltin chloride undergoes metabolization by means of cytochrome P450, generates metabolites of dibutyltin, mainly dibutlytin chloride[30]. In addition another study suggests that when DBTC was exposed intraperitoneally to male rats, various dibutyltin metabolites were detected in kidney, liver and brain[199]. This evidence makes clear that dibutyltin compounds undergo metabolism, generate various metabolites and this makes it difficult to compare the results of in vitro and in vivo studies.

Further studies are necessary to characterize the antagonistic property of these compounds and how they alter the metabolism of lipids.

16. Conclusion

The current study was set out to explore the antagonistic activity of dibutyltin compounds on PPARα. MTT assay was performed with dibutyltin dilaurate, dibutyltin dichloride, dibutyltin diacetate and dibutyltin maleate in HeLa cells for studying the cell viability. Based on transactivation assay, dibutyltin dilaurate and dibutyltin dichloride were identified as an antagonist for PPARα. IC_{50} value for dibutyltin dilaurate was 4.1μM, while the dibutyltin dichloride was 0.26μM. For the first time, we demonstrated that dibutyltin dilaurate, dibutyltin dichloride acts as PPARα antagonist in the transactivation assay. These findings could be added as new information to the growing list of PPARα antagonist. It has been reported that dibutyltin compounds undergoes metabolization and generates butyltin metabolites. Hence, comparison between the results of in vitro and in vivo studies has limitations. Further studies are necessary to characterize the transcriptional activity of these compounds.

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