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# Discovery of Small Molecule Inhibitors of the Interaction of the Thyroid Hormone Receptor with Transcriptional Coregulators*

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**Thyroid hormone (3,5,3′-triiodo-L-thyronine, T3) is an endocrine hormone that exerts homeostatic regulation of basal metabolic rate, heart rate and contractility, fat deposition, and other phenomena (1, 2). T3 binds to the thyroid hormone receptors (TRs) and controls their regulation of transcription of target genes. The binding of TRs to thyroid hormone induces a conformational change in TRs that regulates the composition of the transcriptional regulatory complex. Recruitment of the correct coregulators (CoR) is important for successful gene regulation. In principle, inhibition of the TR-CoR interaction can have a direct influence on gene transcription in the presence of thyroid hormones. Herein we report a high throughput screen for small molecules capable of inhibiting TR coactivator interactions. One class of inhibitors identified in this screen was aromatic β-aminoketones, which exhibited $IC_{50}$ values of ~2 μM. These compounds can undergo a deamination, generating unsaturated ketones capable of reacting with nucleophilic amino acids. Several experiments confirm the hypothesis that these inhibitors are covalently bound to TR. Optimization of these compounds produced leads that inhibited the TR-CoR interaction *in vitro* with potency of ~0.6 μM and thyroid signaling in cellular systems. These are the first small molecules irreversibly inhibiting the coactivator binding of a nuclear receptor and suppressing its transcriptional activity.**

Thyroid hormone receptors (TRs)[3] regulate development, growth, and metabolism (1, 2). The TRs are nuclear receptors (NR), part of a superfamily whose members function as hormone-activated transcription factors (3). The majority of thyroid hormone responses are induced by regulation of transcription by the thyroid hormone T3 (4). Two genes, THRA and THRB encode the two protein isoforms TRα and TRβ, which yield four distinct subtypes by alternative splicing (5). Several functional domains of TRs have been identified: a ligand-independent transactivation domain (AF-1) on the amino terminus, a central DNA binding domain, a ligand binding domain (LBD), and a carboxyl-terminal ligand dependent activation function (AF-2) (6). TR binds specific sequences of DNA in the 5′-flanking regions of T3-responsive genes, known as thyroid response elements, most often as a heterodimer with the retinoid X receptor (7). Both unliganded and liganded TRs can bind thyroid response elements and regulate genes under their control. The unliganded TR complex can recruit a nuclear receptor corepressor (NCoR) or a silencing mediator of retinoic acid to silence basal transcription (8). In the presence of T3, TRs undergo a conformational change with the result that the composition of the coregulator complex can change with strong effects on transcriptional regulation. Several coactivator proteins have been identified (9). The best studied group of coactivators is the p160 or steroid receptor coactivator (SRC) proteins (7) including SRC1 (10), SRC2 (11, 12), and SRC3 (13). Another group of ligand-dependent-interacting proteins include the thyroid hormone receptor activating protein (TRAP) (14), peroxisome proliferate-activated receptor-γ coactivator-1 (PGC-1) (15), and the thyroid hormone receptor binding protein (TRBP) (16). Additionally, quantitative *in vitro* binding assays (17) have shown strong interactions between TR and the coregulators p300 (18), androgen receptor activator (ARA70) (19), receptor interacting protein 140 (RIP140) (20), dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region of the X chromosome gene (DAX1) (21), and the small heterodimer partner (SHP) (22).

The coregulators mentioned have in common that they have variable numbers of highly conserved L*XX*LL motifs; termed NR-boxes, in their nuclear receptor interacting domain (NID). The NR boxes are both necessary and sufficient for the interaction between CoR and TR. The coactivator binding site of TR LBD is formed by 16 residues from four helices (H3, H4, H5, and H12) (23). Scanning surface mutagenesis revealed that only six residues ($Val^{284}$, $Lys^{288}$, $Ile^{302}$, $Lys^{306}$, $Leu^{454}$, and $Glu^{457}$) are crucial for coactivator binding (24). This feature makes the AF-2 domain an ideal target for inhibitor development.

Several inhibitors of this interaction have been reported. The first reported inhibitors were macrolactam-constrained SRC2 NR box peptides (25). A combinatorial approach discovered novel α-helical proteomimetics that could selectively inhibit the interaction between coactivators and TR or the estrogen receptor (ER), with selectivity between ER isoforms ERα and ERβ (26). A similar approach, using disulfide bridges to constrain peptides, resulted in selective ERα coactivator inhibitor with a $K_d$ of 25 nM (27, 28). A report identifying a small molecule capable of inhibiting the interaction of a NR and its coactivator was published recently (29). These pyrimidine-based scaffolds showed affinities between 30 and 50 μM but did not inhibit NR signaling in cell culture or

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[3] The abbreviations used are: TR, thyroid hormone receptor; NR, nuclear receptors; T3, 3,5,3′-triiodo-L-thyronine, thyroid hormone; CoA, coactivator; CoR, coregulator; LBD, ligand binding domain; SRC, steroid receptor coactivator; NID, nuclear receptor interacting domain; ER, estrogen receptor; HTS, high throughput screening.

*in vivo* models. To date, none of these inhibitors may be used to regulate NR signaling in cellular systems.

All functional TR modulators known today are analogs of the T3 itself (30–33). These small molecule derivatives show selectivity toward different isoforms of TR resulting in tissue specific activities (34). GC-1, a TR$\beta$ selective agonist shows interesting properties *in vivo* and could be crystallized with TR LBD (35–40). The first functional T3 antagonist was NH-3, which inhibits thyroid hormone function in both cell culture and whole animal-based assays (41).

High throughput screening (HTS) together with computational screening and fragment discovery are current methods for discovering lead compounds for manipulation of protein function. Although such methods have been applied to discovery of small molecule inhibitors of protein-protein interactions (42), only a limited number of successes have been reported (43, 44). One of the most robust and sensitive HTS methods for studying protein-protein interactions is the competitive fluorescence polarization assay (45). Herein, we present the first HTS using an *in vitro* fluorescence polarization assay to measure the ability of small molecules to inhibit the interaction between the TR$\beta$ LBD and its coactivator, SRC2. This screen revealed a number of hits for inhibitors of the TR-CoR interaction. One particular class of compounds has been examined carefully, and its mechanism of inhibition has been investigated. The resulting lead compounds are potent and selective inhibitors of both the TR-CoR interaction *in vitro* and thyroid hormone signaling in cellular systems. They have potential both as drug candidates and useful biochemical tools for study of the role of the interaction of TR and its coregulators.

## EXPERIMENTAL PROCEDURES

*Labeled Peptides*—Peptide SRC2-2 (CLKEKHKILHRLLQDSSSPV) labeled with 5-iodoacetamidofluorescein (Molecular Probes) was kindly provided by Jamie M. R. Moore (probe) (17); $\alpha$-helical proteomimetics **3** (positive control) and **11** (negative control) were kindly provided by Timothy R. Geistlinger (26).

*Vector*—hTR$\beta$ LBD ($His_6$ T209-D461) was cloned into the BamHI and HindIII restriction sites downstream of the hexahistidine tag of the expression vector pET DUET-1 (Novagen). The replacement of C309 for A in the hTR$\beta$ LBD construct was performed with the QuikChange XL site-directed mutagenesis kit (Stratagene). The sequence of both constructs was verified by DNA sequencing (Elim Biopharmaceuticals, Inc., Hayward, CA).

*Protein Expression and Purification*—hTR$\beta$ LBD ($His_6$; residues T209-D461) was expressed in BL21(DE3) (Invitrogen) (10 × 1L culture) at 20 °C, 0.5 mM isopropyl-1-thio-b-D-galactopyranoside added at $A_{600}$ = 0.6 (17). When the $A_{600}$ reached 4, cells were harvested, resuspended in 20 ml of buffer/1 liter of culture (20 mM Tris, 300 mM NaCl, 0.025% Tween 20, 0.10 mM phenylmethylsulfonyl fluoride, 10 mg of lysozyme, pH 7.5), incubated for 30 min on ice, and then sonicated for 3 × 3 min on ice. The lysed cells were centrifuged at 100,000 × *g* for 1 h, and the supernatant was loaded onto Talon resin (20 ml, Clontech). Protein was eluted with 500 mM imidazole (3 × 5 ml) plus ligand (3,3′,5-triiodo-L-thyronine (Sigma)). Protein purity (>90%) was assessed by SDS-PAGE and high pressure size exclusion chromatography, and protein concentration was measured by the Bradford protein assay. The protein was dialyzed overnight against assay buffer (3 × 4 liters, 50 mM sodium phosphate, 150 mM NaCl, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol). The protein functionality was determined by a direct binding assay of SRC2-2 (see Fig. 3*A*) giving a $K_d$ for SRC2-2 of 0.44 $\mu$M, agreeing with prior results. hTR$\alpha$ LBD ($His_6$; residues $Glu^{148}$-$Val^{410}$) was expressed using the same procedure as hTR$\beta$ with the exception that 0.5 mM isopropyl-1-thio-$\beta$-D-galactopyranoside was added at $A_{600}$ = 1.2. Unliganded protein was eluted with 100 mM imidazole. Purity assessment and buffer exchange were carried out as described. The functionality was determined in a direct binding assay (see Fig. 3*A*) giving a $K_d$ for SRC2-2 of 0.17 $\mu$M, agreeing with prior results. hTR$\beta$ LBD C309A ($His_6$; residues $Thr^{209}$-$Asp^{461}$) was expressed in BL21 cells (Stratagene) at 18–20 °C by using the pET DUET1-hTR$\beta$ LBD (41). General procedures were as described above. The functionality was determined in a direct binding assay (see Fig. 3*A*) giving a $K_d$ for SRC2-2 of 0.17 $\mu$M.

*Direct Binding Assay*—The protein was serially diluted from 70 to 0.002 $\mu$M in binding buffer (50 mM sodium phosphate, 150 mM NaCl, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol) containing 1 $\mu$M ligand T3 in 96-well plates (17). Then 10 $\mu$l of diluted protein was added to 10 $\mu$l of labeled SRC2-2 (20 nM) in 384-well plates yielding final protein concentrations of 35–0.001 $\mu$M and 10 nM fluorescent peptide concentration. The samples were allowed to equilibrate for 30 min. Binding was then measured using fluorescence polarization (excitation $\lambda$ 485 nm, emission $\lambda$ 530 nm) on an Analyst AD plate reader (Molecular Devices). Two independent experiments, each in quadruplicate, were carried out for each state. Data were analyzed using SigmaPlot 8.0 (SPSS, Chicago, Il), and the $K_d$ values were obtained by fitting data to the following equation ($y$ = min + (max − min)/1 + ($x/K_d$)Hill slope).

*Screening Procedure*—The small molecule screen was carried out at the Bay Area Screening Center (BASC) at the California Institute for Quantitative Biology (QB3). A library comprised of 138,000 compounds (ChemRX, 28,000; ChemDiv, 53,000; ChemBridge, 24,000; SPECS, 31,000; Microsource, 2,000) was screened in 384-well format. The complete composition of this library is available from the BASC website (ucsf.edu/basc). First, 384-well dilutions plates (costar 3702) were prepared by addition of 34 $\mu$l of dilution buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 5.9% $Me_2SO$) to each well by using a WellMate (Matrix) followed by addition of 6 $\mu$l compound solutions (1 mM compound in dimethyl sulfoxide ($Me_2SO$)) using a Multimek (Beckman) equipped with a 96-channel head and mixing by subsequent aspiration and dispensing. Second, 5 $\mu$l from the dilution plates were transferred to 384-well assay plates (Costar 3710) using a Multimek followed by the addition of 24 $\mu$l of protein mixture (20 mM Tris-HCl, 100 mM NaCl, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 1 $\mu$M TR$\beta$ LBD, 1 $\mu$M T3, 0.025 $\mu$M labeled SRC2-2 using a WellMate. The final concentration of compound was 30 $\mu$M with 4% $Me_2SO$ content. Each plate was monitored by the addition of a positive control **3** and negative control **11**. After an incubation time of 2 h the binding was measured using fluorescence polarization (excitation $\lambda$ 485 nm, emission $\lambda$ 530 nm) on an Analyst AD plate reader (Molecular Devices). Additionally the fluorescence intensity was measured. All data relevant to the project (plate and compound information, screening data, annotation info, etc.) was deposited directly into a mySQL data base (v. 4.1.7). Data were manipulated and analyzed using protocols written in Pipeline Pilot 4.5.1 (Scitegic, Inc). Our protocols automated the process of joining experimental data to compound information, flagging suspicious plates based on low Z-factors, extracting compounds with statistically significant activity, and annotating hits with additional information (*i.e.* chemical similarity to known bioactive compounds, known genotoxic/cytotoxic molecules, or available compounds, and profiles from ADME models).

*Dose-response Experiments*—The small molecules were serially diluted from 1000 to 4.88 $\mu$M in $Me_2SO$ into a 96-well plate (Costar

3365). 10 μl of each concentration was transferred into 100 μl of binding buffer (20 mM Tris-HCl, 100 mM NaCl, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol) and mixed by subsequent aspiration and dispensing. Then 10 μl of diluted compound was added to 10 μl of protein mixture (20 mM Tris-HCl, 100 mM NaCl, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 2 μM TRβ LBD, 2 μM T3, 0.02 μM labeled SRC2-2 in 384-well plates yielding final compound concentration of 50–0.024 μM. The samples were allowed to equilibrate for 3 h. Binding was then measured using fluorescence polarization (excitation λ 485 nm, emission λ 530 nm) on an Analyst AD (Molecular Devices). Two independent experiments, in quadruplicate, were carried out for each compound. Data were analyzed using SigmaPlot 8.0, and the $K_d$ values were obtained by fitting data to the following equation ($y$ = min + (max − min)/1 + ($x/K_d$)Hill slope).

*Thyroid Hormone Competition Binding Assay*—Full-length hTRβ was produced using a TnT T7 quick-coupled transcription translation system (Promega). Competition assays for binding of unlabeled T3 and **L1** were performed using 1 nM [$^{125}$I]T3 in gel filtration binding assay as described (46).

*Binding Assay with **L8** and **L9***—TRβ or TRβ C309A (5 μM) and T3 (20 μM) were incubated in binding buffer (100 μM) with different concentrations **L8** and **L9**, respectively. After 3 h at room temperature an aliquot of 20 μM was treated with a denaturing buffer (10 μM), boiled for 2 min, and separated using 10% SDS-polyacrylamide gel electrophoresis and visualized by a fluorescence spectrometer.

*Pull-down Assays*—GST fusions to the thyroid hormone receptor (full-length) were expressed in *Escherichia coli* BL21. Cultures were grown to $A_{600}$ 1.2–1.5 at 22 °C and induced with 0.5 mM isopropyl-D-thiogalactoside for 4 h. The cultures were centrifuged (1000 × *g*), and bacterial pellets were resuspended in 20 mM Hepes, pH 7.9, 80 mM KCl, 6 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors and sonicated. Debris was pelleted by centrifugation (100,000 × *g*). The supernatant was incubated with glutathione-Sepharose 4B beads (Amersham Biosciences) and washed as previously described. Protein preparations were stored at −20 °C in 20% glycerol until use. [$^{35}$S]methionine-labeled SRC2 was produced by using coupled *in vitro* transcription-translation (TnT kit, Promega). The binding reactions were carried out on ice in a volume of 150 μl composed of 137.5 μl of protein-binding buffer along with 10 μl of GST-bead slurry corresponding to 3 μg of fusion protein, 1 μl of *in vitro* translated protein, and 1.5 μl of ligand or vehicle. The protein-binding buffer composed of 20 μl of A-150 (20 mM Hepes, 150 mM KCl, 10 mM, $MgCl_2$, 1% glycerol) and 2 μl each of phosphate-buffered saline supplemented with 1% Triton X-100 and 1% Nonidet P-40. Phenylmethylsulfonyl fluoride, dithiothreitol, bovine serum albumin, and protease inhibitor mixture (Novagen) was freshly prepared. The mix was incubated at 4 °C with gentle agitation; the beads were pelleted, washed four times with protein-binding buffer containing no bovine serum albumin, and dried under vacuum for 20 min. The sample was taken up in SDS-PAGE loading buffer and then subjected to SDS-PAGE and autoradiography.

*Transient Transfection Assays*—Human bone osteosarcoma epithelial cells (U2OS) cells (Cell Culture Facility, UCSF) were grown to ~80% confluency in Dulbecco's modified Eagle's/H-21, 4.5 g/liter glucose medium containing 10% newborn calf serum (heat-inactivated), 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells (~1.5 × $10^6$) were collected and resuspended in 0.5 ml of electroporation buffer (Dulbecco's phosphate-buffered saline containing 0.1% glucose, 10 mg/ml bioprene). 5 μg of a TR expression vector (full-length hTRβ-CMV) and 1.5 μg of a reporter plasmid contained a synthetic TR response element (DR-4) containing two copies of a direct repeat spaced by four nucleotides (AGGTCAcaggAGGTCA) cloned immediately upstream of a minimal (−32/+45) thymidine kinase promoter linked to luciferase coding sequence (35). Cells were electroporated using a Bio-Rad gene pulser at 350 V and 960 microfarads, pooled in growth medium (DME H-21 with 10% charcoal-treated, hormone-stripped, newborn bovine serum), and plated in 96-well dishes. After a 3-h incubation compounds were added to the cell culture medium as $Me_2SO$ solutions so as to yield a final $Me_2SO$ concentration of 1%. After additional 18 h of incubation, cells were harvested and assayed for luciferase activity using the Promega dual luciferase kit (Promega) and an Analyst AD (Molecular Devices). Data were analyzed using SigmaPlot 8.0, and the IC values were obtained by fitting data to the following equation ($y$ = min + (max − min)/1 + ($x/K_d$)Hill slope).

## RESULTS

The high-throughput screen was carried out using a 384-well plate format. A total of 300 compounds as single points together with quadruple positive and quadruple negative controls were dispensed in each 384-well plate followed by the addition of TRβ LBD and the labeled SRC2-2 peptide. The SRC2-2 peptide was utilized because it had the tightest binding (0.44 μM) of all the NR box peptides investigated (17). After incubation for 2 h the fluorescence polarization and fluorescence intensity was measured. From the 138,000 compounds screened 27 hit compounds inhibited the interaction between TRβ LBD and the SRC2-2 coactivator peptide with at least 50% efficacy at a concentration of 30 μM and had a fluorescence intensity variation of less then 10%. The structures of these hits, along with the percent inhibitions at 30 μM are shown in Fig. 1. The molecules are divided into six groups depending on their chemical properties. Group A represents electrophilic molecules with a medium sized alkyl substituent. Based on our results at least two of them are irreversible inhibitors of the TR-CoA interaction.

All hits shown in Fig. 1 were evaluated by performing a dose to the response of inhibition study over a range of compound concentrations of 0.024–30 μM to allow the calculation of the $IC_{50}$ values. Only two compounds (Fig. 2*B*, *L1* and *L2*) had $IC_{50}$ values less than 10 μM (*C*, entries *1* and *2*), with a clear saturation at a higher concentration (*A*). These were designated validated hits. The remaining compounds were all sufficiently weak in potency to call their validity into question. This represents an overall hit rate of 0.00145%.

Both of the validated hits are β-aminoketones. These compounds are better known as Mannich bases, first synthesized in the 19th century and systematically studied by Carl Mannich in the beginning of last century (47). Several biological activities have been discovered for this compound class including anticancer, antimicrobial, and cytotoxic activities (48). These activities have been attributed to the liberation of α,β-unsaturated ketones by internal elimination of the amino group. Although this reaction proceeds very slowly under physiological pH in water it has been reported that protein surfaces are able to catalyze this reaction very efficiently (49). Such soft electrophiles, termed Michael addition acceptors, can alkylate protein nucleophiles such as cysteine, tyrosine, and serine. Because of the strong nucleophilicity of organic sulfides, cysteine residues are the most reactive toward this class of Michael acceptors.

To investigate the probability that a similar mechanism underlay inhibition of coactivator binding to the TRβ LBD we tested the unsaturated ketone **L3** (Fig. 2*B*). Interestingly, it showed a similar inhibitory ability of the coactivator recruitment suggesting that indeed the liberated unsaturated ketone **L3** is the active species for compounds **L1** and **L2** (Fig. 2*C*, entry *3*). To determine whether the binding is based on the

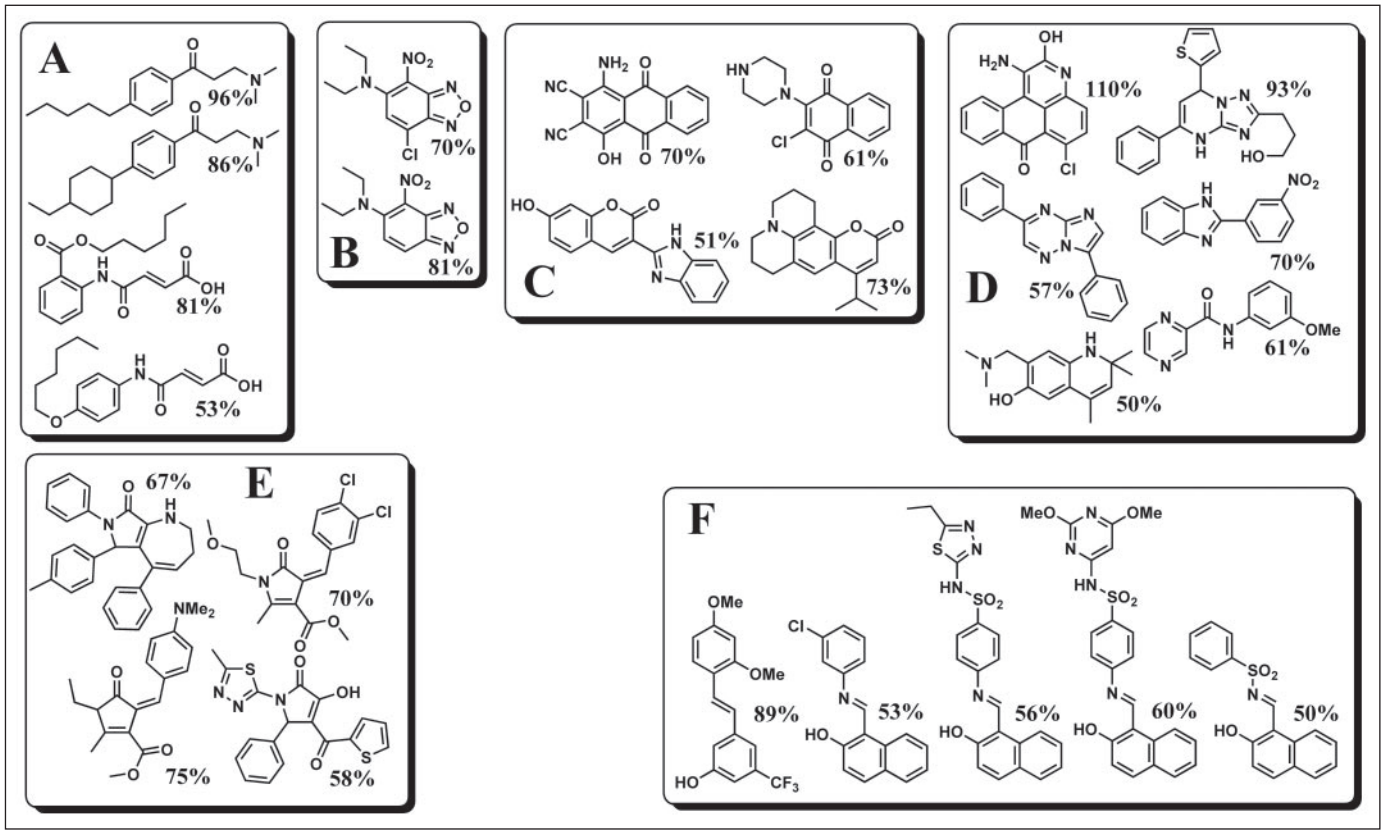


FIGURE 1. **Hit structures from HTS for inhibitors of the interaction of hTR$\beta$ and SRC2-2.** Structures of hits are shown, grouped by chemotype, and annotated with the percent inhibition of SRC2-2 binding at 30 $\mu$M concentration of compound; *A*, electrophilic molecules with alkyl substituents; *B*, 7-nitrobenz-2-oxa-1,3-diazole derivatives; *C*, quinone and coumarin derivatives; *D*, *N*-heterocycles; *E*, highly substituted pyrrolidone derivatives; *F*, stilbene derivatives.

electrophilic nature of the molecule **L3** and not on steric effects, a saturated ketone **L4** was tested. This compound exhibited no competitive ability in the polarization assay (Fig. 2*C*, entry *4*). Subsequently we investigated the importance of the alkyl substituent. Compound **L5**, with an elongated alkyl chain and compound **L6**, with no substituent, both failed to compete with SRC2-2 for binding to the TR$\beta$ LBD (Fig. 2*C*, entries *5* and *6*). Taken together, these results argue for a receptor templated covalent inactivation mechanism.

To ascertain some details of the deamination reaction presumably producing **L3**, several compounds with different alkyl nitrogen substituents that should possess different propensities for elimination were synthesized and investigated in the coactivator binding assay with no significant change in the $IC_{50}$ values.[4] Point mutations of the charged amino acids $Lys^{306}$ and $Glu^{457}$ of the TR$\beta$ LBD diminish the binding of SRC2 (24). This property prevented using these mutants in the competitive coactivator binding assay to investigate whether the deamination reaction of **L1** takes place at the coactivator binding pocket of TR$\beta$ LBD or elsewhere on the TR$\beta$ protein surface.

A thyroid hormone binding assay in the presence of **L3** was conducted to rule out the possibility that the small molecule is competing with T3, which would also result in the release of the labeled coactivator in case of an antagonistic behavior (46). No competition of **L3** with the hormonal ligand was detected in a range of 0.1–10 $\mu$M **L3** using [$^{125}$I]T3 (Fig. 2*D*).

The probability of the formation of a covalent bond between **L1** and TR$\beta$ LBD was investigated by several independent methods. We synthesized Bodipy®-labeled compounds **L8** and **L9** (Fig. 2*E*). To prove that such acrylate analogs have similar activity as compound **L3**, we first investigated the activity of a 4-alkyl-substituted aromatic acrylate **L7** (Fig. 2*B*). This compound showed a similar activity in the competition assay as the unsaturated ketone **L3** (Fig. 2*C*, entry *7*).

Compound **L8** was incubated in different concentrations (10, 5, and 1 $\mu$M) with TR$\beta$ LBD (5 $\mu$M) in the presence of T3 (20 $\mu$M). Separation by a SDS-polyacrylamide gel showed a strong fluorescent band corresponding to TR$\beta$ LBD-**L8** (Fig. 2*F*, *lanes 7–9*). In contrast, incubation with **L9** resulted in no detectable band under the same conditions (Fig. 2*F*, *lanes 4–6*).

Mass spectroscopy is used extensively to detect modified biomolecules like labeled proteins. We observed different MS spectra for the **L1** treated and untreated TR$\beta$ LBD (Fig. 2*G*). The difference of 200–250 *m*/*z* indicates that a covalent adduct is formed and that of one molecule of TR$\beta$ LBD reacts with one molecule of compound **L1**. The exact difference would be theoretically 217 *m*/*z*, well within the experimental error of the method.

The formation of a covalent bond between the **L1** and TR$\beta$ LBD implies that the binding is irreversible. In general irreversible inhibitors show a significant time dependence, which varies with their concentration. Therefore a competition assay with **L1** in the presence of TR$\beta$ LBD and fluorescent coactivator peptide was followed in time (Fig. 2*H*). At a high concentration (50 $\mu$M) **L1** almost instantly inhibited binding of SRC2-2 to the TR$\beta$ LBD coactivator site. A time dependence of inhibition over 4 h was discovered with concentrations of **L1** between 25 and 1.5 $\mu$M. At 0.33 $\mu$M **L1**, no significant inhibition was observed. This indicates that the inhibition is time dependent and requires a stoichiometric amount of **L1**, to the limits of accuracy of the determination of protein concentration.

[4] L. A. Arnold, unpublished results.

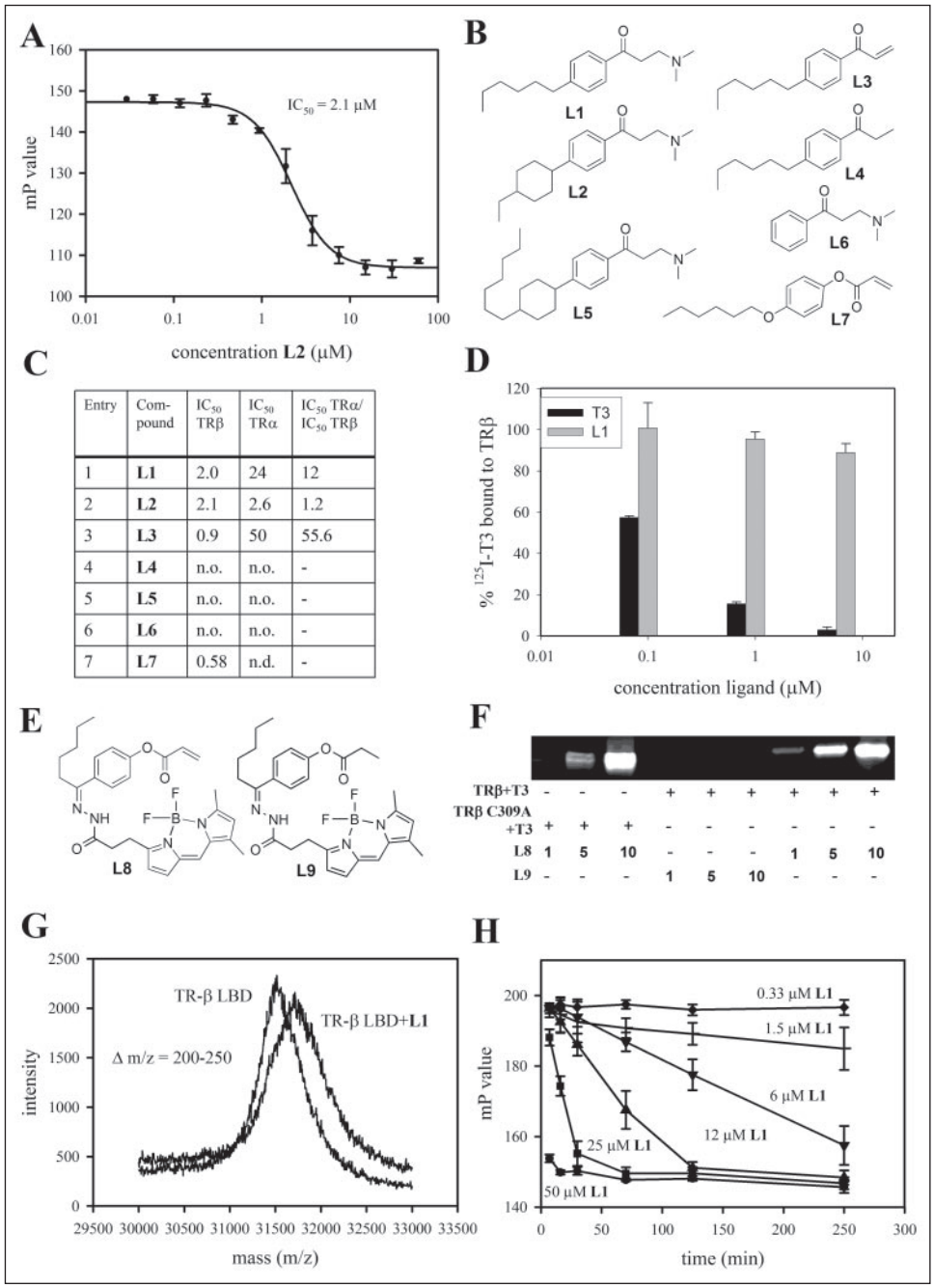


FIGURE 2. **Activities and structures of inhibitors.** *A*, competitive fluorescence polarization assay of **L2** in the presence of TR$\beta$ LBD (1 $\mu$M), T3 (1 $\mu$M), and fluorescence labeled SRC2-2 peptide (10 nM). The data were recorded after 4 h, and the $IC_{50}$ is extracted by fitting to the equation ($y$ = min + (max − min)/1 + ($x/K_d$)Hill slope). *B*, small molecule analogs of **L1** synthesized to test mechanistic hypotheses. *C*, summary of $IC_{50}$ values of compounds **L1–L7** for TR$\alpha$ and TR$\beta$. Additionally the ratio (selectivity) between TR$\alpha$ LBD and TR$\beta$ LBD are given for compound **L1–L3**. *n.o.*, none observed; *n.d.*, none detected. *D*, competition ligand binding of **L1** in the presence of 1 nM[$^{125}$I]T3 and full-length TR$\beta$ in a gel filtration binding assay. *E*, structure of labeled small molecules. *F*, fluorescent image of a 10% SDS-polyacrylamide gel showing labeled small molecules covalent bound to TR$\beta$. *Lanes 1–3*, TR$\beta$ C309A and **L8**; *4–6*, TR$\beta$ and **L9**; *7–9*, TR$\beta$ and **L8**. *G*, matrix-assisted laser desorption ionization-mass spectrometry spectra of untreated TR$\beta$ LBD and TR$\beta$ LBD treated with **L1**. *H*, time dependence of inhibition of TR$\beta$ LBD coactivator binding by **L1** at different concentrations; competitive fluorescence polarization assay was followed over time.

For **L1** to inhibit coactivator binding to TR$\beta$ LBD there must be accessible nucleophilic residues at the coactivator binding site. The LBD of TR$\beta$ has seven cysteine residues. Most of them are exposed on the surface of the protein. There are three cysteine residues near the coactivator binding site. One is freely exposed at the surface ($Cys^{298}$) and a pair of two adjoining cysteine residues ($Cys^{308}$ and $Cys^{309}$) is buried deeply in the binding pocket (Fig. 4*A*) (23). These cysteines are a unique feature of the TR coactivator binding pocket relative to other NR. Based upon our expectation of binding mode for the compounds, we hypothesized that $Cys^{309}$ was the most likely to be involved in the alkylation reaction.

To test the hypothesis that $Cys^{309}$ was forming the covalent adduct with **L1**, we prepared a C309A TR$\beta$ LBD mutant. The mutant was fully functional with respect to SRC2-2 binding in the presence of T3 measured by a direct binding assay ($K_d$ = 0.17 $\mu$M), in comparison to the wild type TR$\beta$ LBD ($K_d$ = 0.44 $\mu$M) (Fig. 3*A*). Using this mutant in a competition binding assay showed that the $IC_{50}$ value of **L1** was increased by more than 50-fold suggesting that $Cys^{309}$ plays a crucial role in the inhibition of the coactivator recruitment of wild type TR$\beta$ by **L1** (Fig. 3*B*). This hypothesis was supported by the fact that the labeling of TR$\beta$ C309A, employing **L8**, was significantly less efficient in comparison to the wild type (Fig. 2*F*, *lanes 1–3*).

The ability of **L1** to compete with intact coactivator SRC2, containing all three SRC2 NR boxes, was tested using a semiquantitative glutathione *S*-transferase assay (Fig. 3*C*). Control experiments indicated that the SRC2 bound to full-length hTR$\beta$ in the presence of T3 (Fig. 3*C*, *lane 4*) and failed to bind in the absence of T3 (*lane 3*). This interaction was blocked by **L1** at concentrations between 200 and 7 $\mu$M (Fig. 3*C*, *lanes 5–8*). At lower concentrations (2–0.7 $\mu$M, Fig. 3*C*, *lanes 9* and *10*) no inhibition was observed. The control experiment with compound **L4**

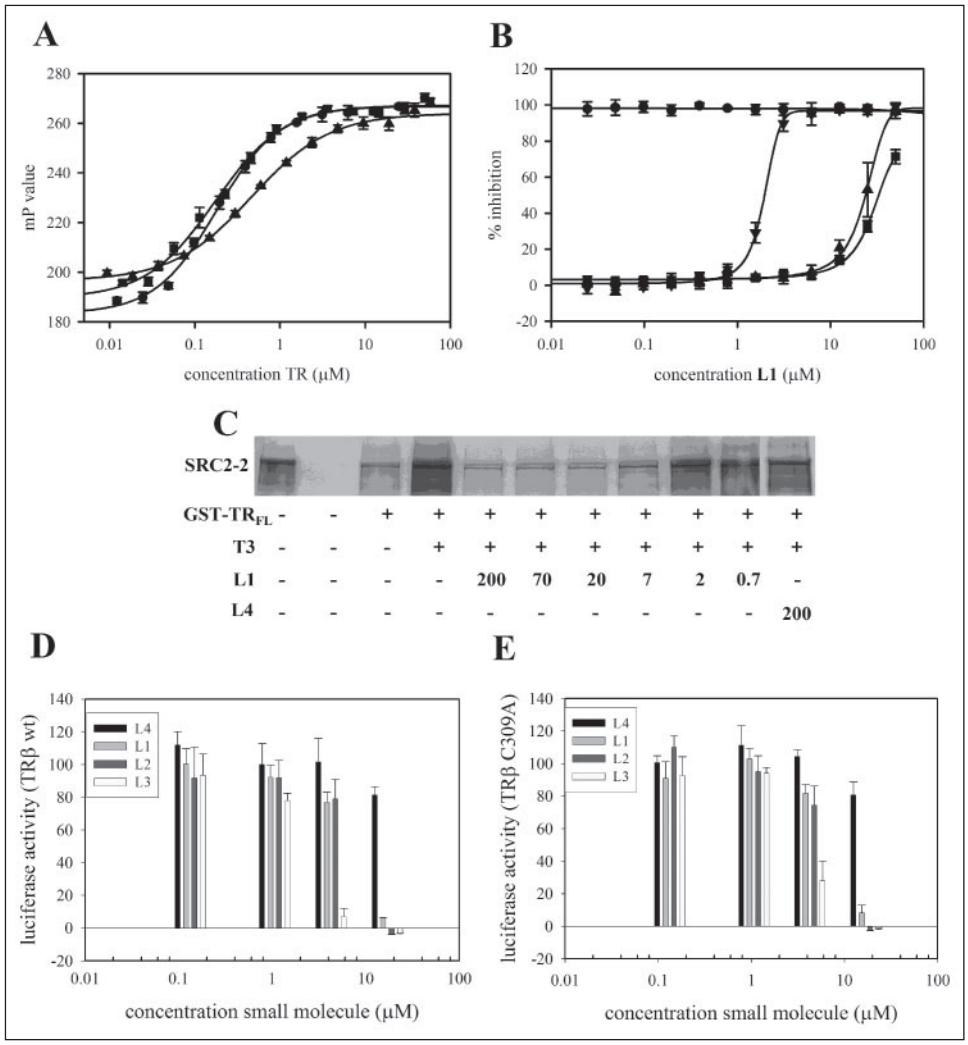


FIGURE 3. **Detailed mechanistic studies of inhibitors.** *A*, direct binding assay of labeled SRC2-2 peptide to hTR$\alpha$ LBD, hTR$\beta$ LBD, and hTR$\beta$ LBD (C309A). The protein was serially diluted and treated with 1 $\mu$M ligand T3 and 0.01 $\mu$M of fluorescent SRC2-2. The binding was measured after 30 min using fluorescence polarization. The $K_d$ values were obtained by fitting data to the following equation ($y$ = min + (max − min)/1 + ($x/K_d$)Hill slope). ●, TR$\alpha$ LBD = 0.17 $\mu$M; ▲, TR$\beta$ LBD = 0.46 $\mu$M; ■, TR$\beta$ LBD (C309A) = 0.17 $\mu$M. *B*, competition polarization assay with labeled SRC2-2 peptide (10 nM), L1, and TR (1 $\mu$M). **L1** was serially diluted and equilibrated with all components for 4 h prior to analysis. ●, TR$\alpha$ LBD, no T3; ▲, TR$\alpha$ LBD with T3; ▼, TR$\beta$ LBD with T3; ■, TR$\beta$ LBD (C309A) with T3. *C*, autoradiogram of 10% SDS-polyacrylamide gel showing products of *in vitro* binding reactions between $^{35}$S-labeled SRC2 and GST fusion to full-length TR$\beta$. *Lane 1*, 10% input labeled SRC2; *2*, GST control; *3*, no T3 hormone, $^{35}$S-labeled SRC2 binding is ligand-dependent; *4*, no **L1** inhibitor, maximal binding of $^{35}$S-labeled SRC2 to hTRb in the presence of T3 (10 $\mu$M); *5–10*, different concentrations of **L1** in the presence of T3; *11*, **L4**, no inhibition. *D*, inhibition of expression of a thyroid response element-driven luciferase reporter enzyme by **L1–L4** at different concentrations in the presence of a constant, fully inducing, concentration of T3. U2OS cells were transfected with a TR$\beta$ expression vector. The data are normalized to basal expression (treatment with equal amounts of $Me_2SO$, but no T3) and fully induced expression (treatment with equal amounts of $Me_2SO$ and T3). *E*, inhibition of expression of a thyroid response element-driven luciferase reporter enzyme by **L1–L4** at different concentrations in the presence of a constant, fully inducing, concentration of T3. U2OS cells were cotransfected with a TR$\beta$ C309A expression vector. The data are normalized to basal expression (treatment with equal amounts of $Me_2SO$, but no T3) and fully induced expression (treatment with equal amounts of $Me_2SO$ and T3).

showed no inhibition at 200 $\mu$M (Fig. 3*C*, *lane 10*). Thus, the inhibition of interaction of full-length hTR$\beta$ and SRC2 by **L1** exhibited dose dependence, similar to the peptide binding studies described above.

The specificity of **L1** inhibition of SRC2 binding was examined with respect to both TR isoforms, TR$\alpha$ and TR$\beta$. Both isoforms were used under the same conditions in a competition polarization assay. **L1** competes with SRC2 for binding to TR$\alpha$ with 12-fold lower apparent affinity giving an $IC_{50}$ of 24 $\mu$M (Fig. 2*C*, entry *1*). In the absence of T3 no SRC2 is recruited (Fig. 3*B*). For **L3** this difference was even higher with 50-fold decrease in affinity for TR$\alpha$ (Fig. 2*C*, entry *3*). Surprisingly **L2** showed similar affinities for both TR$\alpha$ and TR$\beta$, 2.6 and 2.1 $\mu$M, respectively (Fig. 2*C*, entry *2*). As expected compounds **L4**, **L5**, and **L6** showed no binding to either isoform (Fig. 2*C*, entries *4–6*).

To examine the influence of **L1–L4** on transcriptional transactivation of a consensus thyroid response element, U2OS cells were cotransfected with an expression vector TR$\beta_1$ and a thyroid response element-driven luciferase reporter plasmid. After incubation for 18 h the luciferase activity was determined for cells exposed to a fixed concentration of T3 and different concentrations of compounds **L1–L4** (Fig. 3*D*). The compounds **L1–L3** showed full inhibition of transcription at 17 $\mu$M. **L4**, used as a control, had almost no influence on the luciferase activity in comparison to $Me_2SO$ alone. Minor inhibition of transcription was observed at 4 $\mu$M applying **L1** and **L2**. **L3** in contrast fully suppressed transcription at concentration of 4 $\mu$M and had minor effects at 1 $\mu$M.

At the concentrations measured, the inhibition of transcription using an expression vector TR$\beta$ C309A was similar for **L1** and **L2** in comparison to the wild type TR$\beta$ (Fig. 3*E*). Major differences were observed for **L3** showing no inhibition at 1 $\mu$M and only moderate potency at 4 $\mu$M. The viability of the cells was monitored with no significant cell death taking place in any experiment at these concentrations.

## DISCUSSION

A HTS of small molecules was successfully applied to find a hit that led to the first cell active modulators of nuclear hormone receptor coactivator interactions. The screen was based on the ability of liganded TR to recruit coregulator proteins capable of enhancing transcriptional regulation. We evaluated small molecules capable of inhibiting this protein-protein interaction by using fluorescence polarization with a peptide probe representing the coregulator. During the initial screen we identified 27 hit compounds showing an inhibition of more than 50% at a concentration of 30 $\mu$M. The study of the dose response of inhibition of these 27 compounds revealed two validated hits with an $IC_{50}$ value of less then 10 $\mu$M. The overall hit rate of 0.00145% is unusually low for a target-based HTS campaign. We hypothesize that this is because of the absence of molecules with the correct chemotypes in a library whose construction was biased toward current philosophy of "drug-like" character for enzymatic and cell surface receptor targets.

The two validated hit compounds **L1** and **L2**, with $IC_{50}$ values of 2.0 and 2.1 $\mu$M, respectively, are $\beta$-aminoketones. The biological activities

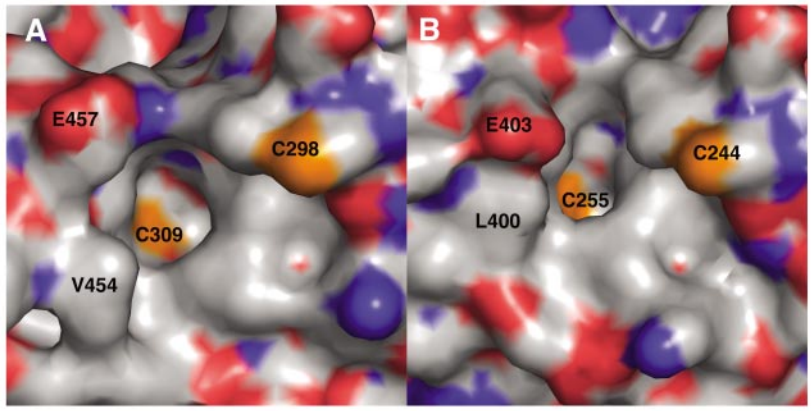


FIGURE 4. **Surface display of TR coactivator binding pocket.** *A*, TR$\beta$; *B*, TR$\alpha$. The TR LBD coactivator binding site is represented by a *solid surface* indicating in *gray* the hydrophobic residues, in *blue* the positively charged residues, in *red* the negatively charged residues, and in *yellow* the cysteines. The thyroid receptor AF-2 transactivation function is a surface exposed hydrophobic cleft comprised of residues from helices 3, 5, and 12. Some of these residues important for coactivator binding are labeled in both TR$\beta$ and TR$\alpha$. Both are depicted in identical orientation.

of this class of compounds have been attributed to the fact that they can liberate a corresponding unsaturated ketone capable of alkylating biological nucleophiles. A binding study with the corresponding unsaturated ketone **L3** showed an $IC_{50}$ value of 0.9 $\mu$M. This result suggests that the unsaturated ketone is the active species. To exclude the possibility that steric properties of **L3** are important for inhibition we tested saturated compound **L4**. This compound was not able to inhibit the TR$\beta$-CoA binding.

We hypothesize that the deamination reaction producing the active **L3** *in situ* is catalyzed on the protein surface because of the unlikelihood of an intramolecular mechanism at physiological pH. The small variation of $IC_{50}$ values based on aminoketones with different alkyl nitrogen substituents suggests a hydrophobic and fairly rigid catalytic site. However, direct investigation of the most likely catalytic residues of the TR$\beta$ coactivator binding domain is prevented because these residues are necessary for binding of the coactivator.

The electrophilic functionality of the active inhibitor species **L3** has been found to be an essential property of the TR antagonists suggesting that the inhibition is based on the alkylation of nucleophilic residues forming the TR$\beta$-CoA interface. Binding studies with compounds **L5** and **L6** showed no inhibition, concluding that a medium-sized hydrophobic group at the 4 position of the aromatic $\beta$-aminoketones is necessary for interaction. Taken together, these studies strongly imply that the active species of inhibitors are actually $\alpha,\beta$-unsaturated ketones acting as direct alkylators of nucleophilic residues on the surface of the thyroid receptor. This is supported by the fact that the natural ligand T3 is not released by the addition of **L1**, which implies that the conformation of TR$\beta$ is not altered in the presence of **L1**.

Covalent inhibitors have several unique properties. 1) They produce an adduct with the target that has increased molecular weight. This feature can be used to permanently label the corresponding binding partner; 2) they require stoichiometric, but not largely superstoichiometric amounts of inhibitor for full activity, and 3) they exhibit strong time dependence when acting in modest excess relative to the target concentration. After the treatment of TR$\beta$ LBD with **L1** we could detect a new species with a 200–250 $m/z$ higher mass. We assigned this mass to TR$\beta$-**L1** proving that TR$\beta$ is selectively alkylated by one equivalent of **L1**. In addition we followed the inhibition of TR$\beta$-CoA by **L1** in time. A significant time dependence of inhibition was found between **L1** concentrations of 25 and 1.5 $\mu$M when interacted with TR at a concentration of 1 $\mu$M. The time dependence altered with the **L1** concentration suggesting an irreversible inhibition. A covalent complex was formed when TR$\beta$ was treated with fluorescently labeled analog **L8**, in contrast to the inactive compound **L9**, lacking the electrophilic properties of **L8**, which did not. In summary, the detection of the mono-alkylated TR$\beta$, its time-dependent formation, and the fact that TR$\beta$ could be covalently labeled with a fluorescent inhibitor supports the postulated mechanism that **L1** forms the unsaturated ketone **L3**, alkylating irreversibly one of the residues of TR$\beta$ LBD.

Based upon the expected chemical reactivity of **L1**, as predicted by frontier molecular orbital theory, we would expect that **L1** is most likely to react with a solvent-exposed cysteine residue. The fact that we observe a single alkylation event is exceptional because there are seven cysteine residues present in TR$\beta$ LBD. Most of cysteine residues are exposed to the surface of the protein. We hypothesized that the selectivity might be driven by a preassociation event that positions the antibonding orbital of the electrophile **L1** near a nucleophilic cysteine. The coactivator binding site has three cysteine residues (Fig. 4, $Cys^{308}$, $Cys^{309}$, and $Cys^{298}$). Of these, $Cys^{309}$ seemed most likely to be reacting with **L1** based upon our expected mode of binding. To support this hypothesis three independent experiments were carried out in systems where cysteine residue $Cys^{309}$ was replaced by an alanine: 1) competitive coactivator binding studies using TR$\beta$ C309A revealed that **L1** had a 50-fold reduced $IC_{50}$ value in comparison with the wild type TR$\beta$; 2) direct labeling of TR$\beta$ C309A using **L8** was less efficient in comparison with the wild type; and 3) inhibition of transfection by **L3** using U2OS cells cotransfected by a TR$\beta$ C309A expression vector was significantly reduced in comparison with the wild type. Although a direct comparison with C308A and C298A clones is missing, we think that $Cys^{309}$ is the most likely target for **L1**. $Cys^{309}$ is exposed in a defined hydrophobic pocket capable of activation through nearby charged residues. The residues forming the coactivator binding surface of TR$\alpha$ and TR$\beta$ LBD are identical (Fig. 4). Although crystal structures of the binding pockets of the two isoforms of TR (TR$\alpha$ LBD and TR$\beta$ LBD) are very similar, there are distinct differences in the region immediately surrounding the pocket. We think that these differences in the hydrophobic relief are the reason for the significant differences in $IC_{50}$ values for **L1** and **L3** for TR$\alpha$ and TR$\beta$ LBD. The decrease in affinity for TR$\alpha$ was 12-fold for **L1** and 50-fold for **L3**. On the other hand, **L2** showed the same affinity for both isoforms. This selectivity is very important for future studies targeting specific tissues with differently expressed levels of TR$\alpha$ and TR$\beta$.

The ability to inhibit a protein peptide interaction does not guarantee that the same inhibitor will block the interaction of the full-length proteins. In this case, **L1** fully inhibited the interaction of full-length SRC2, containing three NR boxes, and full-length TR$\beta$. A concentration of 7 $\mu$M **L1** was sufficient for blocking this receptor coactivator interaction. The fact that the potency of **L1** in this semiquantitative glutathione *S*-transferase assay matched that in the protein-peptide interaction increased the likelihood that **L1** would block this interaction between the full-length transcription factors in a cellular environment.

A reporter gene transfection assay, carried out in cultured U2OS cells, showed indeed that compounds **L1**, **L2**, and **L3** were able to reduce transcriptional activation to basal levels. **L3** showed highly increased potency in comparison to **L1** and **L2** with almost full inhibition of transcription at 4 $\mu$M. We concluded that **L3** can penetrate the cell membrane and is transported to the nucleus. Furthermore it can inhibit coregulator recruitment and has a direct impact on the transcriptional activity of TR$\beta$.

In summary, we report that small molecules are able to inhibit the interaction between the liganded thyroid hormone receptor and its coactivator SRC2. To our knowledge this is the first irreversible inhibitor of the nuclear receptor coregulator binding that has been reported. Molecules like **L1** are a new class of TR antagonist, active in the presence of T3 but silencing its hormone-induced signaling. They open the door to understand the coupling of multiple thyroid hormone-regulated signaling events and the potential for treatment of hyperthyroidism using approaches that do not affect thyroid hormone levels. Compounds **L1** and **L3** exhibit exceptional TR$\beta$ selectivities making them potentially useful for the study of tissue selective thyroid activities. We are currently investigating the effects of these compounds in cell-based assays and *in vivo* studies.

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## REFERENCES


1. Yen, P. M. (2001) *Physiol. Rev.* **81,** 1097–1142
2. Malm, J. (2004) *Curr. Pharm. Des.* **10,** 3525–3532
3. Aranda, A., and Pascual, A. (2001) *Physiol. Rev.* **81,** 1269–1304
4. Harvey, C. B., and Williams, G. R. (2002) *Thyroid* **12,** 441–446
5. Williams, G. R. (2000) *Mol. Cell. Biol.* **20,** 8329–8342
6. Mangelsdorf, D. J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, R. M. (1995) *Cell* **83,** 835–839
7. Xu, J. M., and Li, Q. T. (2003) *Mol. Endocrinol.* **17,** 1681–1692
8. Hu, X., and Lazar, M. A. (2000) *Trends Endorinol. Metab.* **11,** 6–10
9. Moore, J. M. R., and Guy, R. K. (2005) *Mol. Cell. Proteomics* **4,** 475–482
10. Onate, S. A., Tsai, S. Y., Tsai, M. J., and Omalley, B. W. (1995) *Science* **270,** 1354–1357
11. Voegel, J. J., Heine, M. J. S., Zechel, C., Chambon, P., and Gronemeyer, H. (1996) *EMBO J.* **15,** 3667–3675
12. Hong, H., Kohli, K., Garabedian, M. J., and Stallcup, M. R. (1997) *Mol. Cell. Biol.* **17,** 2735–2744
13. Suen, C. S., Berrodin, T. J., Mastroeni, R., Cheskis, B. J., Lyttle, C. R., and Frail, D. E. (1998) *J. Biol. Chem.* **273,** 27645–27653
14. Ito, M., and Roeder, R. G. (2001) *Trends Endorinol. Metab.* **12,** 127–134
15. Puigserver, P., Wu, Z. D., Park, C. W., Graves, R., Wright, M., and Spiegelman, B. M. (1998) *Cell* **92,** 829–839
16. Ko, L., Cardona, G. R., and Chin, W. W. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 6212–6217
17. Moore, J. M. R., Galicia, S. J., McReynolds, A. C., Nguyen, N. H., Scanlan, T. S., and Guy, R. K. (2004) *J. Biol. Chem.* **279,** 27584–27590
18. Kalkhoven, E. (2004) *Biochem. Pharmacol.* **68,** 1145–1155
19. Heinlein, C. A., Ting, H. J., Yeh, S. Y., and Chang, C. S. (1999) *J. Biol. Chem.* **274,** 16147–16152
20. Treuter, E., Albrektsen, T., Johansson, L., Leers, J., and Gustafsson, J. A. (1998) *Mol. Endocrinol.* **12,** 864–881
21. Zhang, H., Thomsen, J. S., Johansson, L., Gustafsson, J. A., and Treuter, E. (2000) *J. Biol. Chem.* **275,** 39855–39859
22. Seol, W., Choi, H. S., and Moore, D. D. (1996) *Science* **272,** 1336–1339
23. Darimont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushner, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) *Genes Dev.* **12,** 3343–3356
24. Feng, W. J., Ribeiro, R. C. J., Wagner, R. L., Nguyen, H., Apriletti, J. W., Fletterick, R. J., Baxter, J. D., Kushner, P. J., and West, B. L. (1998) *Science* **280,** 1747–1749
25. Geistlinger, T. R., and Guy, R. K. (2001) *J. Am. Chem. Soc.* **123,** 1525–1526
26. Geistlinger, T. R., and Guy, R. K. (2003) *J. Am. Chem. Soc.* **125,** 6852–6853
27. Leduc, A. M., Trent, J. O., Wittliff, J. L., Bramlett, K. S., Briggs, S. L., Chirgadze, N. Y., Wang, Y., Burris, T. P., and Spatola, A. F. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 11273–11278
28. Galande, A. K., Bramlett, K. S., Burris, T. P., Wittliff, J. L., and Spatola, A. F. (2004) *J. Peptide Res.* **63,** 297–302
29. Rodriguez, A. L., Tamrazi, A., Collins, M. L., and Katzenellenbogen, J. A. (2004) *J. Med. Chem.* **47,** 600–611
30. Dietrich, S. W., Bolger, M. B., Kollman, P. A., and Jorgensen, E. C. (1977) *J. Med. Chem.* **20,** 863–880
31. Briel, D., Pohlers, D., Uhlig, M., Vieweg, S., Scholz, G. H., Thormann, M., and Hofmann, H. J. (1999) *J. Med. Chem.* **42,** 1849–1854
32. Stanton, J. L., Cahill, E., Dotson, R., Tan, J., Tomaselli, H. C., Wasvary, J. M., Stephan, Z. F., and Steele, R. E. (2000) *Bioorg. Med. Chem. Lett.* **10,** 1661–1663
33. Ye, L., Li, Y. L., Mellstrom, K., Mellin, C., Bladh, L. G., Koehler, K., Garg, N., Collazo, A. M. G., Litten, C., Husman, B., Persson, K., Ljunggren, J., Grover, G., Sleph, P. G., George, R., and Malm, J. (2003) *J. Med. Chem.* **46,** 1580–1588
34. Webb, P., Nguyen, N. H., Chiellini, G., Yoshihara, H. A. I., Lima, S. T. C., Apriletti, J. W., Ribeiro, R. C. J., Marimuthu, A., West, B. L., Goede, P., Mellstrom, K., Nilsson, S., Kushner, P. J., Fletterick, R. J., Scanlan, T. S., and Baxter, J. D. (2002) *J. Steroid Biochem. Mol. Biol.* **83,** 59–73
35. Chiellini, G., Apriletti, J. W., Yoshihara, H. A., Baxter, J. D., Ribeiro, R. C. J., and Scanlan, T. S. (1998) *Chem. Biol.* **5,** 299–306
36. Mishra, M. K., Wilson, F. E., Scanlan, T. S., and Chiellini, G. (2004) *J. Comp. Physiol. B* **174,** 471–479
37. Grover, G. J., Egan, D. M., Sleph, P. G., Beehler, B. C., Chiellini, G., Nguyen, N. H., Baxter, J. D., and Scanlan, T. S. (2004) *Endocrinology* **145,** 1656–1661
38. Freitas, F. R. S., Moriscot, A. S., Jorgetti, V., Soares, A. G., Passarelli, M., Scanlan, T. S., Brent, G. A., Bianco, A. C., and Gouveia, C. H. A. (2003) *Am. J. Physiol.* **285,** E1135–E1141
39. Manzano, J., Morte, B., Scanlan, T. S., and Bernal, J. (2003) *Endocrinology* **144,** 5480–5487
40. Trost, S. U., Swanson, E., Gloss, B., Wang-Iverson, D. B., Zhang, H. J., Volodarsky, T., Grover, G. J., Baxter, J. D., Chiellini, G., Scanlan, T. S., and Dillmann, W. H. (2000) *Endocrinology* **141,** 3057–3064
41. Wagner, R. L., Huber, B. R., Shiau, A. K., Kelly, A., Lima, S. T. C., Scanlan, T. S., Apriletti, J. W., Baxter, J. D., West, B. L., and Fletterick, R. J. (2001) *J. Mol. Endocrinol.* **15,** 398–410
42. Arkin, M. R., and Wells, J. A. (2004) *Nat. Rev. Drug Discov.* **3,** 301–317
43. Berg, T. (2003) *Angew. Chem. Int. Ed. Engl.* **42,** 2462–2481
44. Toogood, P. L. (2002) *J. Med. Chem.* **45,** 1543–1558
45. Roehrl, M. H. A., Wang, J. Y., and Wagner, G. (2004) *Biochemistry* **43,** 16056–16066
46. Apriletti, J. W., Baxter, J. D., Lau, K. H., and West, B. L. (1995) *Protein Express. Purif.* **6,** 363–370
47. Arend, M., Westermann, B., and Risch, N. (1998) *Angew. Chem. Int. Ed. Engl.* **37,** 1045–1070
48. Gul, H. I., Gul, M., Vepsalainen, J., Erciyas, E., and Hanninen, O. (2003) *Biol. Pharm. Bull.* **26,** 631–637
49. Davioud-Charvet, E., McLeish, M. J., Veine, D. M., Giegel, D., Arscott, L. D., Andricopulo, A. D., Becker, K., Muller, S., Schirmer, R. H., Williams, C. H., and Kenyon, G. L. (2003) *Biochemistry* **42,** 13319–13330