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

Leggy A. Arnold, Eva Estévez-Pepiná, Marie Togashi, Natalia Jouravel, Anang Shelat, Andrea C. McReynolds, Ellena Mar, Phuong Nguyen, John D. Baxter, Robert J. Fleiterick, Paul Webb, and R. Kiplin Guy

From the Department of Pharmaceutical Chemistry, Department of Molecular and Cellular Pharmacology, Diabetes Center and Department of Medicine, University of California, San Francisco, California 94143

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 (CoRs) 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 IC50 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) 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 THRβ 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 LXXLL 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 (Va234, Lys238, Ile230, Lys236, Leu254, and Gln255) 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 proteoimetics 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 Kd 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...
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 TRβ 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 methods for studying protein-protein interactions 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 (CLKEKHKLHRLLQDSSSPV) labeled with 5-iodoacetamidofluorescein (Molecular Probes) was kindly provided by Jamie M. R. Moore (probe) (17); α-helical peptideimetics (positive control) and 11 (negative control) were kindly provided by Timothy R. Geistlinger (26).

**Vector**—hTRβ LBD (His6 residues T209-D461) was cloned into the BamHII and HindIII restriction sites downstream of the hexahistidinetag of the expression vector pET DUET-1 (Novagen). The replacement of C309 for A in the hTRβ LBD construct was performed with the QuickChange 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**—hTRβ LBD (His6 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 A600 = 0.6 (17). When the A600 reached 4, cells were harvested, resuspended in 20 ml of buffer/1 liter of culture (20 mM Tris, 300 mM NaCl, pH 7.2, 1 mM dithiothreitol, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol) containing 1 mM ligand T3 in 96-well plates (17). Then 10 μl of diluted protein was added to 10 μl of labeled SRC2-2 (20 nM) in 384-well plates yielding final protein concentrations of 35–0.001 μM and 10 nM fluorescent peptide concentration. The samples were allowed to equilibrate for 30 min. Binding was then measured using fluorescence polarization (excitation 485 nm, emission 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 Kd values were obtained by fitting data to the following equation (y = min + (max – min)/1 + (x/Kd)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 μl 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% MeSO) to each well by using a WellMate (Matrix) followed by addition of 6 μl compound solutions (1 mM compound in dimethyl sulfoxide (MeSO)) using a Multimek (Beckman) equipped with a 96-channel head and mixing by subsequent aspiration and dispensing. Second, 5 μl from the dilution plates were transferred to 384-well assay plates (Costar 3710) using a Multimek followed by the addition of 24 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, 1 μM TRβ LBD, 1 μM T3, 0.025 μM labeled SRC2-2 using a WellMate. The final concentration of compound was 30 μM with 4% MeSO content. Each plate was monitored by the addition of a positive control and negative control. After an incubation time of 2 h the binding was measured using fluorescence polarization (excitation 485 nm, emission 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 database (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 μM in MeSO into a 96-well plate (Costar...
TR-CoR Antagonists

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 μl 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 K₅ values were obtained by fitting data to the following equation (y = min + (max − min)/1 + (x/K₅)H₁Hill slope).

Thyroid Hormone Competition Binding Assay—Full-length hTRβ was produced using a TntT T7 quick-coupled transcription translation system (Promega). Competition assays for binding of unlabeled T3 and L1 were performed using 1 nm [35S]methionine-labeled SRC2 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 A₆₀₀ nm 1.2–1.5 at 22 °C and induced with 0.5 mM isopropyl-β-thiogalactoside for 4 h. The cultures were centrifuged (1000 x g), and bacterial pellets were resuspended in 20 mM Hepes, pH 7.9, 80 mM KCl, 6 mM MgCl₂, 1 mM DTT, 1 mM ATP, 0.2 mM phenylmethylsulfonyl fluoride, and protease inhibitors and sonicated. Debris was pelleted by centrifugation (100,000 x g). The supernatant was incubated with glutathione-Sepharose 4B beads (Amershams Biosciences) and washed as previously described. Protein preparations were stored at −20 °C in 20% glycerol until use. [35S]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, MgCl₂, 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 x 10⁶) were collected and resuspended in 0.5 ml of electroporation buffer (Dulbecco's phosphate-buffered saline containing 0.1% glucose, 10 mg/ml bovine serum albumin). 5 μg of a TR expression vector (full-length hTRβ-CMV) and 1.5 μg of a reporter plasmid containing 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 Me₆SO solutions so as to yield a final Me₆SO 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 IC₅₀ values were obtained by fitting data to the following equation (y = min + (max − min)/1 + (x/K₅)H₁Hill 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 than 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 IC₅₀ values. Only two compounds (Fig. 2B, L1 and L2) had IC₅₀ 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,ω-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. 2B). 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. 2C, entry 3). To determine whether the binding is based on the
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. 2C, 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 β LBD (Fig. 2C, 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 IC₅₀ values. Point mutations of the charged amino acids Lys¹⁰⁶ and Glu⁴⁵⁷ of the TR α LBD diminish the binding of L1 coactivator site. A time dependence of inhibition over 4 h was discovered with concentrations of 25 and 1.5 μM. At 0.33 μM L1, no significant inhibition was observed. This 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 β LBD and fluorescent coactivator peptide was followed in time (Fig. 2G). At a high concentration (50 μM) L1 almost instantly inhibited binding of SRC2-2 to the TR β LBD coactivator site. A time dependence of inhibition over 4 h was discovered with concentrations of L1 between 25 and 1.5 μM. At 0.33 μM 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.

FIGURE 1. Hit structures from HTS for inhibitors of the interaction of hTRβ and SRC2-2. Structures of hits are shown, grouped by chemotype, and annotated with the percent inhibition of SRC2-2 binding at 30 μM 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.

4 L. A. Arnold, unpublished results.
For L1 to inhibit coactivator binding to TR β LBD there must be accessible nucleophilic residues at the coactivator binding site. The LBD of TR β 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 (Cys298) and a pair of two adjoining cysteine residues (Cys308 and Cys309) is buried deeply in the binding pocket (Fig. 4A) (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 Cys309 was the most likely to be involved in the alkylation reaction.

To test the hypothesis that Cys309 was forming the covalent adduct with L1, we prepared a C309A TR β H9252 LBD mutant. The mutant was fully functional with respect to SRC2-2 binding in the presence of T3 measured by a direct binding assay (Kd = 0.17 μM), in comparison to the wild type TR β LBD (Kd = 0.44 μM) (Fig. 3A). Using this mutant in a competition binding assay showed that the IC50 value of L1 was increased by more than 50-fold suggesting that Cys309 plays a crucial role in the inhibition of the coactivator recruitment of wild type TR β by L1 (Fig. 3B). This hypothesis was supported by the fact that the labeling of TR β C309A, employing L8, was significantly less efficient in comparison to the wild type (Fig. 2, 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. 3C). Control experiments indicated that the SRC2 bound to full-length hTR β in the presence of T3 (Fig. 3C, 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 μM (Fig. 3C, lanes 5–8). At lower concentrations (2–0.7 μM, Fig. 3C, lanes 9 and 10) no inhibition was observed. The control experiment with compound L4...
showed no inhibition at 200 μM (Fig. 3C, lane 10). Thus, the inhibition of interaction of full-length hTRβ 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 isofoms, TRα and TRβ. Both isofoms were used under the same conditions in a competition polarization assay. L1 competes with SRC2 for binding to TRα with 12-fold lower apparent affinity giving an IC50 of 24 μM (Fig. 2C, entry 3). In the absence of TR3 no SRC2 is recruited (Fig. 2B). For L3 this difference was even higher with 50-fold decrease in affinity for TRα (Fig. 2C, entry 3). Surprisingly, L2 showed similar affinities for both TRα and TRβ, 2.6 and 2.1 μM, respectively (Fig. 2C, entry 2). As expected compounds L4, L5, and L6 showed no binding to either isoform (Fig. 2C, 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 TRβ 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 L1–L4 at different concentrations of compounds L1–L4 (Fig. 3D). The compounds L1–L3 showed full inhibition of transcription at 17 μM, L4, used as a control, had almost no influence on the luciferase activity in comparison to MeSO4 alone. Minor inhibition of transcription was observed at 4 μM applying L1 and L2. L3 in contrast fully suppressed transcription at concentration of 4 μM and had minor effects at 1 μM.

At the concentrations measured, the inhibition of transcription using an expression vector TRβ C309A was similar for L1 and L2 in comparison to the wild type TRβ (Fig. 3E). Major differences were observed for L3 showing no inhibition at 1 μM and only moderate potency at 4 μM. 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 μM. The study of the dose response of inhibition of these 27 compounds revealed two validated hits with an IC50 value of less than 10 μM. 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 IC50 values of 2.0 and 2.1 μM, respectively, are β-aminoketones. The biological activities...
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 IC50 value of 0.9 μM. 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 TRβ-CoA binding.

We hypothesize that the deamination reaction producing the active L3 in situ is catalyzed on the protein surface because of the inability of an intramolecular mechanism at physiological pH. The small variation of IC50 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 TRβ 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 TRβ-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 β-aminoketones is necessary for interaction. Taken together, these studies strongly imply that the active species of inhibitors are actually α,β-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 TRβ 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 stoichiometric 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 TRβ LBD with L1 we could detect a new species with a 200–250 m/z higher mass. We assigned this mass to TRβ-L1 proving that TRβ is selectively alkylated by one equivalent of L1. In addition we followed the inhibition of TRβ-CoA by L1 in time. A significant time dependence of inhibition was found between L1 concentrations of 25 and 1.5 μM when interacted with TR at a concentration of 1 μM. The time dependence altered with the L1 concentration suggesting an irreversible inhibition. A covalent complex was formed when TRβ 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 TRβ, its time-dependent formation, and the fact that TRβ 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 TRβ 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 TRβ 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 anti-binding orbital of the electrophile L1 near a nucleophilic cysteine. The coactivator binding site has three cysteine residues (Fig. 4, Cys308, Cys309, and Cys309). Of these, Cys309 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 Cys309 was replaced by an alanine: 1) competitive coactivator binding studies using TRβ C309A revealed that L1 had a 50-fold reduced IC50 value in comparison with the wild type TRβ; 2) direct labeling of TRβ C309A using L8 was less efficient in comparison with the wild type; and 3) inhibition of transfection by L3 using U2OS cells cotransfected with a TRβ 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 Cys309 is the most likely target for L1. Cys309 is exposed in a defined hydrophobic pocket capable of activation through nearby charged residues. The residues forming the coactivator binding surface of TRα and TRβ LBD are identical (Fig. 4). Although crystal structures of the binding pockets of the two isoforms of TR (TRα LBD and TRβ 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 IC50 values for L1 and L3 for TRα and TRβ LBD. The decrease in affinity for TRα 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 TRα and TRβ.

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 TRβ. A concentration of 7 μM 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 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 μM. We concluded that L3 can penetrate the cell membrane and is transported to the nucleus. Furthermore it inhibits coregulator recruitment and has a direct impact on the transcriptional activity of TRβ.

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 TRβ 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 vitro studies.

Acknowledgments—The HTS was carried out in the Bay Area Screening Center (QB3/UCSF) with support from UCSF and the Sandler Research Foundation. We thank J. Williams, M. Uehara-Bingen, B. Wolff, and L. Hicks for their help with the HTS and C. Ocasio for assistance in cell culture and the TR competition assay.

REFERENCES

1. Yen, P. M. (2001) Physiol. Rev. 81, 1097–1142
2. Malin, J. (2004) Curr. Pharm. Des. 10, 3555–3552
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., Champon, 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 Endocrinol. Metab. 11, 6–10
9. Roehrl, M. H. A., Wang, J. Y., and Wagner, R. L. (2004) Protein Expr. Purif. 35, 363–370
10. Treuter, E., Albrektsson, T., Johansson, L., Leers, J., and Gustafsson, J. A. (1998) Mol. Endocrinol. 12, 864–881
11. Zhang, H., Thomsen, J. S., Johansson, L., Gustafsson, J. A., and Treuter, E. (2000) J. Biol. Chem. 275, 39855–39859
12. Seol, W., Choi, H. S., and Moore, D. D. (1996) Science 272, 1336–1339
13. Darmont, B. D., Wagner, R. L., Apriletti, J. W., Stallcup, M. R., Kushnir, P. J., Baxter, J. D., Fletterick, R. J., and Yamamoto, K. R. (1998) Genes Dev. 12, 3343–3356
14. Fong, W. J., Ribeiro, R. C. J., Wagner, R. L., Nguyen, H., Apriletti, J. W., Fletterick, R. J., Baxter, J. D., Kushnir, P. J., and West, B. L. (1998) Science 280, 1747–1749
15. Geistlinger, T. R., and Guy, R. K. (2001) J. Am. Chem. Soc. 123, 1525–1526
16. Geistlinger, T. R., and Guy, R. K. (2003) J. Am. Chem. Soc. 125, 6852–6853
17. 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
18. Galande, A. K., Bramlett, K. S., Burris, T. P., Wittliff, J. L., and Spatola, A. F. (2004) J. Peptide Res. 63, 297–302
19. Rodriguez, A. L., Tamrazi, A., Collins, M. L., and Katzenellenbogen, J. A. (2004) J. Med. Chem. 47, 600–611
20. Dietrich, S. W., Bolger, M. B., Kollman, P. A., and Jorgensen, E. C. (1977) J. Med. Chem. 20, 863–880
21. Brief, D., Pohlers, D. U., Uhlig, M., Vieweg, S., Scholz, G. H., Thommarn, M., and Hoffmann, H. J. (1999) J. Med. Chem. 42, 1849–1854
22. Stanton, J. L., Caihle, E., Dotson, R. T., Tan, J., Tomaselli, H. C., Wasyv, J. M., Stephan, Z. F., and Steele, R. E. (2000) Bioorg. Med. Chem. Lett. 10, 1661–1663
23. Ye, L., Li, Y. T., Mellstrom, K., Mellin, C., Bladh, L. G., Koehler, K., Garg, N., Collazo, A. M. G., Liten, C., Husman, B., Persson, K., Ljunggren, J., Grover, G., Sleph, P. G., George, R., and Malm, J. (2003) J. Med. Chem. 46, 1580–1588
24. Webb, P., Nguyen, N. H., Chiellini, G., Yoshihara, H. A. I., Lima, S. T. C., Apriletti, J. W., Ribeiro, R. C. J., Marimunhu, A., West, B. L., Goeede, P., Mellstrom, K., Nilsson, S., Kushnir, P. J., Fletterick, R. J., Scanlan, T. S., and Baxter, J. D. (2002) J. Steroid Biochem. Mol. Biol. 83, 59–73
25. 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
26. Mishra, M. K., Wilson, F. E., Scanlan, T. S., and Chiellini, G. (2004) J. Comp. Physiol. B 174, 471–479
27. 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
28. Freitas, F. 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
29. Manzano, J., Morte, B., Scanlan, T. S., and Bernal, J. (2003) Endocrinology 144, 5480–5487
30. Trost, S. U., Swanson, E., Glos, 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
31. Wagner, R. L., Huber, B. R., Shuai, A. K., Kelly, A., Lima, S. T. C., Apriletti, J. W., Baxter, J. D., West, B. L., and Fletterick, R. J. (2001) J. Mol. Endocrinol. 15, 398–410
32. Arkin, M. R., and Wells, J. A. (2004) Nat. Rev. Drug Discov. 3, 301–317
33. Berg, T. (2003) Angew. Chem. Int. Ed. Engl. 42, 2462–2481
34. Toogood, P. L. (2002) J. Med. Chem. 45, 1543–1558
35. Roehrl, M. H. A., Wang, J. Y., and Wagner, G. (2004) Biochemistry 43, 16056–16066
36. Apriletti, J. W., Baxter, J. D., Lau, K. H., and West, B. L. (1995) Protein Express. Purif. 6, 363–370
37. Arend, M., Westermann, B., and Risch, N. (1998) Angew. Chem. Int. Ed. Engl. 37, 1045–1070
38. Gul, H. I., Gul, M., Vepsalainen, J., Erciyas, E., and Hanninen, O. (2003) Biol. Pharm. Bull. 26, 631–637
39. Davioud-Charvet, E., McLeish, M. J., Veine, D. M., Giegel, D., Arscock, L. D., Andricopulo, A. D., Becker, K., Muller, S., Schirmer, R. H., Williams, C. H., and Kenyon, G. L. (2003) Biochemistry 42, 13319–13330