Discovery of diverse thyroid hormone receptor antagonists by high-throughput docking

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Treatment of hyperthyroidism, a common clinical condition that can have serious manifestations in the elderly, has remained essentially unchanged for >30 years. Directly antagonizing the effect of the thyroid hormone at the receptor level may be a significant improvement for the treatment of hyperthyroid patients. We built a computer model of the thyroid hormone receptor (TR) ligand-binding domain in its predicted antagonist-bound conformation and used a virtual screening algorithm to select 100 TR antagonist candidates out of a library of >250,000 compounds. We were able to obtain 75 of the compounds selected in silico and studied their ability to act as antagonists by using cultured cells that express TR. Fourteen of these compounds were found to antagonize the effect of T3 on TR with IC50 ranging from 1.5 to 30 μM. A small virtual library of compounds, derived from the highest affinity antagonist (1-850) that could be rapidly synthesized, was generated. A second round of virtual screening identified new compounds with predicted increased antagonist activity. These second generation compounds were synthesized, and their ability to act as TR antagonists was confirmed by transfection and receptor binding experiments. The extreme structural diversity of the antagonist compounds shows how receptor-based virtual screening can identify diverse chemistries that comply with the structural rules of TR antagonism.

Overproduction of thyroid hormone (hyperthyroidism or thyrotoxicosis) is an extremely common clinical entity caused by a number of different pathological conditions of the thyroid gland. Approximately 0.5% of women will experience some clinical manifestation of hyperthyroidism in their lifetime (3–5 times more frequent than men), with potentially life-threatening effects on the cardiovascular system (e.g., cardiac arrhythmias, heart failure, angina, and myocardial infarction), particularly in the elderly (1–3).

The treatment of hyperthyroidism has essentially remained unchanged for the past 30 years and includes the use of radioactive [131I]iodine, surgery, or the use of antithyroid drugs, such as propylthiouracil, that inhibit thyroid hormone synthesis by blocking the iodination of thyroglobulin (1–3). Each approach has its own intrinsic limitations and/or side effects. Propylthiouracil and related drugs, which block thyroid hormone synthesis, act slowly and can take up to 6–8 weeks to fully deplete the thyroid gland and intrathyroidal stores of iodinated thyroglobulin, during which time hyperthyroidism can have severe consequences in certain individuals. Radiochemical destruction of thyroid tissues by [131I]iodine is associated with the development of the auditory system and in the pituitary (11, 12), whereas TRβ1 plays a role in the future treatment of hyperthyroidism. Such molecules would act rapidly by directly antagonizing the effect of thyroid hormone at the receptor level, a significant improvement for individuals with hyperthyroidism who require surgery, have cardiac disease, or are at risk for life-threatening thyrotoxic storm.

The crystal structure of the ligand-binding domain (LBD) of several NRs has been solved in the absence of ligand or in the presence of bound agonists or antagonists and has provided a detailed model for the structural mechanism of activation and inhibition of members of the NR family (14–19). Although most of the LBD remains relatively static, regardless of the activation state of the receptor, the most C-terminal helix (referred to as H12) is rather dynamic and can adopt a variety of conformations when no ligand is bound to the receptor. Binding of an agonist stabilizes a conformation of the receptor where H12 folds like a lid onto the agonist, contributing to the formation of a hydrophobic cavity at the surface of the receptor, involved in the binding of coactivator proteins. Antagonists, on the other hand, destabilize this coactivator-recruitment state by preventing H12 from folding on the ligand-binding pocket. The crystal structure of ERα bound to the partial antagonist roxiloxifene shows that H12 relocates onto the coactivator binding site (14). Alternatively, the pure antagonist ICI 164,384 presents an arm that extends out of the ligand-binding pocket and onto the coactivator binding site (18). The crystal structure of a PPARγ/antagonist/corepressor peptide complex also recently showed that binding of corepressor proteins can further destabilize the active conformation of the receptor (19). In most cases, two important properties of antagonist molecules apply: (i) they bind within the ligand-binding pocket, making specific interactions with pocket-lining residues in a fashion similar to that for agonists; (ii) they present an extension that protrudes [slightly in the case of the PPARγ antagonists GW6471 (19), more extensively in the case of ICI 164,384 (18)] out of the pocket and antagonizes the active conformation of H12. The recent crystal structure of the ERβ-selective antagonist (R,R)-5,11-cis-diyethyl-5,6,11,12-tetrahydrocrysine-2,8-diul bond to the inactive form of the ERβ-LBD depicts an alternate mode of antagonism, where the ligand induces nonproductive conformations of binding pocket residues that destabilize the active state of the H12 helix (20).

Abbreviations: ER, estrogen receptor; ICM, internal coordinate mechanics; LBD, ligand-binding domain; NR, nuclear hormone receptor; PPARγ, peroxisome proliferator-activated receptor-γ agonist; RAR, retinoic acid receptor; T3, L-triiodothyronine; TR, thyroid hormone receptor; VLS, virtual library screening.

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unclear whether this interesting “passive-antagonism” mechanism is an isolated case. On the other hand, the mechanism whereby a protruding extension directly induces relocation of helix H12 was experimentally observed in a variety of complexes (14, 18, 19, 21–23).

Based on such observations, we built a low-energy model of the retinoic acid receptor-α (RARα) LBD where H12 adopts a conformation similar to raloxifene-bound ERα and showed that such a model can be used for structure-based discovery of novel RAR antagonists (24). This rationale was recently confirmed by another group which designed a TR antagonist by adding a steric moiety to T3 that would destabilize the transcriptionally active conformation of H12 (25). In this report, we demonstrate that, by deriving molecules from the structure of the receptor, rather than the structure of known agonists, a large array of TR antagonists can be identified, that present striking structural diversity. Such an approach could accelerate the discovery of new chemical entities for the treatment of hyperthyroidism.

Materials and Methods

Model Building of a TR Antagonist Structure. The crystal structure of raloxifene-bound ERα (14) was used as a template to derive from the structure of agonist-bound TRβ-LBD (26) a predicted model of the receptor bound to an antagonist. The conformation of the N-terminal domain of the TRβ-LBD was left unchanged from the agonist-bound form, up to residue Leu-440 whereas the C-terminal domain (residues His-441 to Asp-461) was remodeled in a two-step process, as described for RAR (24). This model is based on the hypothesis (derived from sequence alignment with the ER and the crystal structure of the ER/raloxifene complex) that the FXXVF site. Each of the 250,000 flexible compounds of the Available Chemicals Directory database (MDL Information Systems, San Leandro, CA) that passed the Lipinski filters (35) was docked to the receptor grids by the ICM method (29) and assigned a score that reflects the quality of the complex and includes grid energy, continuum electrostatic, and entropy terms (refs. 30 and 31 for review). Docking took an average of 1 min per processor and per ligand. The whole process was conducted four times in parallel, and the lowest score assigned to each ligand was retained. Compounds scoring below (i.e., better than) the ICM VLS threshold used for RAR (24) and therefore most likely to bind the TR ligand binding pocket were then tested in silico for their predicted antagonist activity. Although all NR antagonists do not necessarily present a bulky moiety protruding out of the binding pocket (20), all known ligands that do present such moiety are antagonists (14, 18, 19, 21–23). Based on this observation, only the top 1,000 ligands that had a bound conformation incompatible with the active state of the receptor were retained (docked compounds that were further than 3 Å away from Phe-455 or Phe-459 of the active form of helix H12 were discarded). The structure of this top 1,000 selection was rapidly refined with both ligand and receptor side-chains flexible, according to the ICM local energy minimization method that uses internal coordinates and analytical derivatives (28, 29), and the refined structures presenting unacceptable receptor/ligand van der Waals repulsions were filtered out. Each of the remaining 300 top-scoring complexes was visually inspected; parameters taken into account were shape complementarity, hydrogen bond network and flexibility of the ligand. Based on this final subjective selection step, 100 compounds were retained to be characterized in vitro. It is difficult to evaluate the relevance and enrichment generated by the different automatic or visual filters added after the initial high-throughput docking; to better judge the efficiency of the first screening step, the score distribution of all compounds screened as well as that of the active compounds is provided (Fig. 6, which is published as supporting information on the PNAS web site, www.pnas.org); of 14 active TR antagonists identified, 9 ranked in the top 600 of ~250,000 compounds and 3 ranked in the top 300 [the source library included ~250,000 ligands but only 190,000 passed the Lipinski filters (35) and were assigned a score].

Effect of Antagonist 1-850 and Derivatives on the T3-Mediated Binding of TRs to NRC in Vitro. Full-length TRα was synthesized in the presence of L-[35S]methionine by using TNT reticulocyte lysates (Promega). Approximately 2.5–5 × 10⁶ cpm of [35S]-labeled TRα (20 fmol) in 2 µl of lysate was incubated with 500 ng of GST fused to the receptor interaction region of the coactivator NRC (NRC15) immobilized on glutathione-agarose beads (33). The samples were incubated at room temperature for 15 min in 500 µl of binding buffer (20 mM Hepes (pH 7.8)/1 mM MgCl₂/100 mM KCl/0.05% Triton X-100/1 mM DTT/10%...
(vol/vol) glycerol/100 μg/ml ovalbumin/0.1 μM ZnCl₂] with the indicated concentrations of 1-850 and the 1-850 derivatives D1 and D4. The samples were then chilled on ice and incubated with 1 nM T3 for an additional 60 min at 4°C. Control samples contained no T3 or antagonists or received only T3. The beads were collected by centrifugation (~500 × g) at 4°C for 5 min and washed three times with 1 ml of binding buffer. The bound [³⁵S]TRα was electrophoresed in an SDS/10% polyacrylamide gel followed by analysis and quantitation of the amount of [³⁵S]TRα bound by using a Molecular Dynamics PhosphorImager and IMAGEQUANT software. The percent inhibition of T3-mediated binding of [³⁵S]TRα to GST-NRC15 by 1-850, D1, and D4 was determined after subtracting the amount of [³⁵S]TRα bound to GST-NRC15 in the absence of T3.

**Generation of a Virtual Focused Library.** A virtual library of derivatives of the antagonist 1-850 (the best hit derived from first-generation VLS) was generated, based on the chemical synthesis scheme described below, so that all molecules generated in silico could be easily and rapidly synthesized. All phenylisocyanates commercially available from Sigma–Aldrich were extracted from virtual structure libraries with the ICM “find molecule” command (27) and attached to the nonvariable moiety of 1-850. Each molecule was then assigned ECEPP3 potentials and mmff partial charges, energy-minimized, and stored in a focused library of 101 compounds. All chemo-informatics procedures were carried out with ICM (27).

**Preparation of 1-850 Active Analogs D1–D57.** Selected compounds from the series of 1-850 analogs D1–D101 were prepared by coupling of compound 4 (see Supporting Text, which is published as supporting information on the PNAS web site, for more details) with commercially available phenylisocyanates (Fig. 3). The general procedure involved adding 0.5 mmol of phenylisocyanate to a solution of 0.13 g of compound 4 (0.5 mmol) in 1 ml of dry CH₂Cl₂, stirring at room temperature for 2 h, and then separation of the final product by filtration. The purity of the all library compounds was determined by LC-MS. The most active compounds D1–D4 were characterized more fully by ¹H NMR and MS.

**Results**

**Modeling of the Antagonist-Bound Conformation of TR.** A low-energy model of the antagonist structure of the TRβ-LBD was built by homology to agonist-bound TRβ-LBD (26) and raloxifene-bound ERα (14). Following a strategy devised with RAR (14), we docked the C-terminal helix H12 of the TRβ LBD to the coactivator recruitment site as described (34), and remodeled the 20 C-terminal residues of the receptor by an extensive global energy minimization (27, 38).

**Virtual Screening.** A virtual database of 250,000 commercially available compounds was rapidly screened in silico. All continuously flexible ligands were docked to a grid representation of the receptor and assigned a score reflecting the quality of the complex, according to the ICM method (Molsoft). Briefly, a series of grid potentials representing the shape, hydrophobicity, hydrogen bonding profile, and electrostatic potential of the receptor was generated. Each ligand was then docked to the grid representation of the receptor by a Monte Carlo simulation in the internal coordinates space, and the complex was optimized with flexible receptor side chains (see Materials and Methods). Putative ligands that did not extend out of the binding pocket in the docked conformation were automatically filtered out. After visual inspection of the 300 best scoring compounds, 100 were selected as potential TR antagonists, 75 of which were still commercially available (chemicals listed in the Available Chemicals Directory are often not resynthesized once they run out of stock).

### Table 1. Name, structure, and relative activity of the 14 TR antagonists identified after the first round of virtual screening

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Concentration, Inhibition, μM</th>
<th>%</th>
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<tr>
<td>1-850</td>
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<td>90</td>
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<tr>
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<td><img src="image14.png" alt="Structure" /></td>
<td>4*</td>
<td>33</td>
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*Compound 2-1060 was toxic at 20 μM.*
Based on the model, we synthesized derivatives of 1-850 that might display higher affinity for the receptor. 1-850 was divided into one core unit that was synthesized separately and one variable extremity, where polysubstituted phenylisocyanates could be used as building blocks to easily derive the hydrophobic, trifluoromethylated moiety of 1-850 (Fig. 3 and Supporting Text). A virtual library of all such building blocks available from the Sigma–Aldrich catalog was generated, and all corresponding derivatives of 1-850 were constructed in silico with ICM. The resulting chemical library of 101 compounds was docked to the receptor as described above, and each 1-850 derivative was assigned a score reflecting its predicted fit with the receptor. To address the relevance of this virtual automatic parallel synthesis and docking strategy, we synthesized 8 of the 57 top-ranking 1-850 analogs and compared calculated scores with observed activities. A dose–response inhibition of T3-stimulation comparing 1-850 with the two antagonist derivatives (D4 and D1) is shown in Fig. 4, and the results of the 8 compounds are shown in Table 2. None of the 1-850 derivatives exhibited partial agonist activity in the absence of T3. The best two inhibitors (D4 and D1), were among the top four scoring compounds whereas the two less active molecules were the lowest scoring ones (D37 and D57). One derivative (D4) reached IC50 in the nanomolar range (0.75 μM), and all compounds inhibited TR from 10% to 84% at 5 μM (Table 2). According to our docking simulation, the methyl and isopropyl groups of D4’s hydrophobic end would make more extensive hydrophobic interactions with the receptor than the trifluoromethyl of 1-850 (Fig. 7, lower).

**Fig. 1.** Inhibition of [125I]T3 binding to TRs by 1-850 in intact cells. The GH4C1 pituitary cell line, which contains endogenous TRs (TRα, TRβ1, and TRβ2), was incubated with 0.1 nM [125I]T3 alone and with the indicated concentrations of unlabeled T3 and 1-850 as described in Materials and Methods. After incubation for 60 min at 37°C, the cells were chilled and washed, and the nuclei were isolated as described in Materials and Methods. The results indicate the inhibition of binding of [125I]T3 by T3 and 1-850.

**Fig. 2.** Predicted conformation of 1-850 (gold) bound to the TRβ ligand-binding pocket. (Upper) A hydrogen bond between His-435 and a carbonyl oxygen of 1-850 and possibly between Arg-282 and a nitro oxygen of 1-850 constitute the only polar interactions. All other contacts are hydrophobic (not shown for clarity). (Lower) 1-850 superimposes with the crystal structure of T3 (green), bound to active TR and clashes with the active conformation of helix H12 (cyan). Color code is red for oxygen, blue for nitrogen, and magenta for fluoride/iodide in 1-850/T3, respectively.

**Fig. 3.** Chemical synthesis of 1-850 derivatives was conducted by preparing a nonvariable core structure and rapidly linking commercially available polysubstituted phenylisocyanate building blocks in a simple step of parallel synthesis (see Materials and Methods for details).
which is published as supporting information on the PNAS web site.) To document that D4 and D1 acted through inhibition of T3 binding to receptor, we compared their activity to 1-850 using a T3-dependent in vitro coactivator binding assay (Fig. 5). In this study, we incubated rabbit reticulocyte labeled [35S]TRα with GST-NRC15 immobilized on glutathione-agarose beads. NRC15 is a region of the nuclear receptor coactivator NRC that interacts with agonist-bound TR through the NRC LXXLL-1 motif (33). GST-NRC15 was incubated with [35S]TRα with the indicated concentrations of D4, D1, and 1-850 followed by incubation with 1 nM T3 (a concentration that leads to a maximal effect in the absence of antagonist). After incubation and washing as indicated in Materials and Methods, the samples were electrophoresed in a 10% SDS/polyacrylamide gel, followed by imaging and quantitation by using a PhosphorImager. The results shown in Fig. 5 indicate that the antagonists block the T3-mediated interaction of TRα with NRC, and the extent of inhibition generally parallels the relative ability of these compounds to inhibit T3-mediated stimulation of gene expression in cells (D4 > D1 > 1-850; Fig. 4).

**Discussion**

In this study, we used the crystal structure of agonist-bound TRβ and raloxifene-bound ERα to construct a computer model of the predicted antagonist form of TRβ and used this model as a template to discover in silico small molecule inhibitors of TR. Using the most active initial hit, we generated a small virtual library that can be easily and rapidly synthesized with commercially available building blocks. High-throughput docking of this library with TR identified a derivative with increased activity.

First, this work confirms a conceptually important observation that we made with RAR (24), documenting that it is possible to...
rationally design NR antagonists by building a computer model of the predicted antagonist-bound form of the receptor. This result reinforces the hypothesis that the structural mechanism for NR inhibition initially depicted for ER (14) can be generalized to other domains of the chemistry space that would be overlooked by ligand-based pharmacophore approaches. This work demonstrates that high-throughput docking can be used to rapidly identify NR antagonists that differ from known ligands and to prioritize subsequent lead optimization. The extreme structural diversity of the antagonist molecules discovered in this work illustrates the power of receptor-based virtual screening and demonstrates that diverse chemistry can comply with strict structural rules. Further optimization of TR antagonists could open the way for improved modalities for the treatment of hyperthyroidism.

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