A Structure-based Approach to Retinoid X Receptor-α Inhibition*

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In this paper we describe a structure-based approach designed to identify novel ligands for retinoid X receptor-α (RXRα). By using a virtual approach based on a modified scoring function, we have selected 200 potential candidates on the basis of their predicted ability of docking into the ligand-binding site of the target. Subsequent experimental verification of the compounds in vitro and cell-based assays led to the identification of a number of novel high affinity ligands for RXRα. The compounds are capable of displacing 9-cis-retinoic acid with IC50 values in the 10 nM and 5 μM range and exhibit marked antagonistic activity in cellular assays. The inhibitory scaffolds discovered with this method form the basis for the development of novel RXRα ligands with potential therapeutic properties.

Retinoid X receptor-α (RXRα) is a member of the nuclear receptor superfamily, which mediates the biological effects of many hormones, vitamins, and drugs (1–5). A unique property of RXRα is its exceptional ability to form heterodimers with other nuclear receptors, including retinoic acid receptors (RARs), thyroid hormone receptor, vitamin D receptor, and peroxisome proliferator-activated receptor (1–5). RXRα can homodimerize in response to its ligands (1–5). Heterodimerization of RXRα with its partners can mediate diverse endocrine signaling pathways that when altered can lead to the development of cancer (6). Genetic disruption of RXRα targeted to the prostatic epithelium results in intraepithelial neoplasia in mice (7), whereas diminished RXRα protein expression may represent an early event in the development of human cancer (8). Like other nuclear receptors, RXRα acts as a transcriptional factor to positively or negatively regulate gene targeting to induce apoptosis (9–11) and its interaction with β-catenin to inhibit the Wnt/β-catenin signaling (12). RXRα was found to cotranslocate with NGFI-B (also known as Nur77 or TR3) from the nucleus to the cytoplasm in response to nerve growth factor treatment (13), a process implicated in the differentiation of PC12 pheochromocytoma cells. In response to apoptotic stimuli, RXRα and Nur77 associate with mitochondria as a Nur77/RXRα heterodimer in LNCaP prostate cancer and H460 lung cancer cells (10). RXRα also cotranslocates with Nur77 from the nucleus to mitochondria in response to IGFBP-3 (insulin-like growth factor-binding protein-3) (9), presumably its interaction with IGFBP-3.

A number of natural and synthetic molecules with different structural features and diverse biological effects have been identified as ligands for RXRα. 9-cis-Retinoic acid (9-cis-RA) was the first compound known to bind RXRα. Recently, several dietary fatty acids were found to bind RXRα and appear to act as natural RXRα ligands (14–16). A nonsteroidal anti-inflammatory drug, (R)-etodolac, also binds RXRα and acts as an RXRα antagonist to inhibit its transactivation, an event which is associated with its tumor-selective induction of apoptosis in animal (11). The ability of RXRα to bind various ligands that have diverse biological effects suggests that RXRα acts as an important intracellular mediator regulating multiple signal transduction pathways. Such molecules could be useful for their therapeutic properties but also will facilitate mechanistic studies on the role of RXRα in signal transduction. In this study, we undertook a structure-based approach for the identification of new RXRα ligands. By using a virtual docking approach based on the three-dimensional structure of RXRα, we identified a number of potential RXRα ligands. Following experimental in vitro and in-cell-based assays with top scoring compounds, we have finally identified ligands with nanomolar binding affinity to RXRα ligand binding domain (LBD) and selectivity when tested against RARα.

EXPERIMENTAL PROCEDURES

Virtual Docking—A target binding site was derived from the crystal structure of the ternary complex involving BMS-649 compound in complex with (h)RXRα-LBD (Protein Data Bank code 1MV C) (17). The protein active site was defined, including those residues within 6.5 Å from the benzoic acid substructure of BMS-649 found in the complex. Hydrogen atoms were calculated using FlexX (BioSolveIT, Sankt Augustin, Germany) (18, 19), and water molecules were eliminated.

Docking geometries were obtained by using FlexX (five solutions per molecule) implemented on a 20 × 3.2-GHz CPUs Linux cluster. Different parameters of the scoring functions were varied (Table 1), and a collection of random 1026 compounds that included the BMS-649 compound was used to estimate the ranking ability of each function. 50,000 compounds (ChemBridge, San Diego, CA) were subsequently docked and ranked according to the software FlexX using D score. Top 200 ranking compounds were selected (Chembridge, San Diego) and experimentally tested in the in vitro displacement assay. Hit compounds

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3 The abbreviations used are: RXRα, retinoid X receptor-α; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate; LBD, ligand binding domain; RA, retinoic acid; RAR, retinoic acid receptor; CAT, chloramphenicol acetyltransferase; h, human; PDB, Protein Data Bank; BMS-49, 4-[(2-(5,5,8,8-tetramethyl-5,6,7,8-tetrayhydrodronaphthalen-2-yl)-1,3-dioxolan-2-yl)benzoic acid.
were repurchased and tested in additional NMR binding and cell-based assays. When the six validated hits were included in the collection of 1025 compounds, however, the average ranking of the compounds was only slightly better when using DJ score compared with FlexX, although both functions performed much better than Screenscore and Chemscore, with this particular set.

**Ligand Binding Assays**—His-tagged (h)RXRα-LBD (amino acids 223–462) was expressed in *Escherichia coli* and purified as a polyhistidine-tagged fusion protein for use in competition binding assays (20, 21). Briefly, (h)RXRα-LBD (100 ng) was incubated in binding buffer (0.15 M KCl in 10 mM Tris-HCl, pH 7.4, containing 0.5% CHAPS detergent (Roche Diagnostics) and 8% glycerol; 300 liters) with 7.5 nM 9-cis-[3H]RA (44 Ci/mmol; PerkinElmer Life Sciences) in the absence or presence of increasing concentrations of nonlabeled compound for 16–18 h at 4 °C. Next, His-Bind resin (500 μg; Novagen) was added, and incubation with shaking was continued for 1 h at room temperature. The His-Bind resin was washed (three times with 1 ml of binding buffer) to separate receptor-bound from nonbound label, suspended (500 μl of buffer), and then transferred for scintillation counting (3.5 ml of Ecolume liquid scintillation fluid, ICN; Beckman Coulter LS 3801 counter). Experiments were performed in duplicate, and specific binding was calculated as the average of the percentage of the total bound

![FIGURE 1. Comparison of docking geometries for the compound BMS-649 generated by different scoring functions: FlexX, green; Screen score, blue; Chemscore, yellow; DJ score, red. The docked geometry obtained via x-ray crystallography (Protein Data Bank code 1MVC) is shown in orange.](image)

**TABLE 1** Comparison of parameters used with FlexX, Screening, Chemscore, and DJ score functions and the results using the BMS-649 as a test compound

| Parameters             | FlexX | Screening | Chemscore | DJ score |
|------------------------|-------|-----------|-----------|----------|
| Match*                 | 1.00  | 1.00      | 1.00      | 3.00     |
| Lipo_contacts*         | −0.17 | −0.07     | −0.12     | −0.12    |
| Ambig_contacts*        | −0.17 | −0.07     | 0.00      | 0.00     |
| Weights of plp*        | 1.00  | 0.30      | 1.00      | 1.00     |
| plp_hbond*             | 2.00  | 2.00      | 2.00      | 2.00     |
| plp_steric*            | 0.40  | 0.40      | 0.40      | 0.40     |
| plp_rep*               | 20.00 | 20.00     | 20.00     | 20.00    |
| Entropy*               | 1.11A | 1.00A     | 0.97A     | 1.00A    |
| r.m.s.d.*              | 6.00  | 4.00      | 8.00      | 1.00     |
| Ranking†               | 4.00  | 8.00      | 1.00      | 1.00     |

*Match indicates matched interaction term.
*Lipo_contacts indicate lipophilic atom-atom contacts.
*Ambig_contacts indicate hydrophilic-hydrophobic atom contacts.
*Weights of plp indicate weights of plp terms.
*plp_hbond indicates hbond components of PLP scoring.
*plp_steric indicates steric components of PLP scoring.
*plp_rep indicates repulsive components of PLP scoring.
*Entropy indicates the term used.
*r.m.s.d. indicates the root mean square deviation between all heavy atoms of the crystal structure and docked structures generated by the different scoring functions.
 Ranking indicates the ranking of the crystal structure of the BMS-649 compound when included in a data base of randomly selected 1025 compounds. The ranking was obtained by using the following free energy of binding: ΔGbind = (a) × Fmatch + (b) × Flipo + (c) × Fambig + (d) × Fplp + 1.69 × log Fplp = (e) × Fplp_hbond + (f) × Fplp_lipo + 20 × Fplp_rep, where a–g represent the factors of parameters listed in this table used by each scoring function.

**TABLE 2** Structures of hit compounds and their *in vitro* characterization

| #  | Structure | IC50 | NMR-T1ρ |
|----|-----------|------|---------|
| 1  | ![Structure image](image) | 10 nM | N.D.    |
| 2  | ![Structure image](image) | 50 nM | N.D.    |
| 3  | ![Structure image](image) | 75 nM | N.D.    |
| 4  | ![Structure image](image) | 300 nM | +++     |
| 5  | ![Structure image](image) | 500 nM | ++      |
| 6  | ![Structure image](image) | 5 μM | +       |
counts/min remaining defined as counts/min/(total bound counts/min) × 100.

NMR Experiments—All experiments were carried out at 298 K by using a 600- or a 500-MHz Bruker Avance spectrometer, equipped with four and three rf channels and pulse-field gradients along the z axis, respectively. NMR samples were consisted of 100 μM of compound in presence or absence of 10 μM of protein, in 40 mM D₂O phosphate buffer, pH 7.5.

A spin-lock pulse of variable length (1, 10, 100, and 200 ms) was used for the acquisition of one-dimensional ¹H T₁ series (22, 23). Each spectrum was acquired with 256 scans. Water suppression was achieved by means of a WATERGATE pulse scheme. Spectra were processed and analyzed with the software Mestrec-C.

RXRa Transactivation Assays—CV-1 cells were maintained in Dulbecco’s minimal essential medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 g/ml streptomycin. For transfection assays, cells were seeded at 1.0 × 10⁵ cells/ml in 24-well plates for 16–24 h before transfection. Cells were then transfected using FuGENE 6 Transfection Reagent (Roche Diagnostics) with (TREpal)₂- tk-CAT with either RXRa or RARα (25 ng). In addition, cells were also transfected with β-galactosidase expression vector (pCH 110, Amersham Biosciences) and carrier DNA (pBluescript, Stratagene) to a final concentration of 1000 mg/well. At 20 h after transfection, the medium was changed to Dulbecco’s minimal essential medium containing charcoal-stripped fetal calf serum, and cells were treated for 24 h with either 10 nM 9-cis-RA for RXRa or 10 nM all-trans-RA for RARα with varying concentrations of a compound of interest. CAT activity was expressed relative to β-galactosidase activity to normalize for transfection efficiency (1, 2). Reactions were performed in triplicate and the results averaged.

Western Blotting—Cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, and 150 mM NaCl with 0.1% Triton X-100, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate; Sigma). Equivalent protein extracts from each sample were separated on 8% SDS-polyacrylamide gels. Protein was quantitated by a total protein assay (Bio-Rad). Proteins were transferred onto nitrocellulose membranes (Trans-Blot, Bio-Rad). Nitrocellulose membranes were preblocked with 5% nonfat milk powder in phosphate-buffered saline containing 0.05% Tween 20 detergent for 1 h at room temperature. Following phosphate-buffered saline/Tween washes, preblocked membranes were incubated with 1 μg/ml equivalent of anti-RARβ polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). RARβ protein was detected by horseradish peroxidase-conjugated secondary antibody against immunoglobulins (Amersham Biosciences) after 1 h of incubation at room temperature, and specific bands were visualized by ECL (Amersham Biosciences). Equivalent loading of samples was deter-

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FIGURE 2. Binding affinity of compound 4 to (h)RXRa-LBD. Competitive radioligand binding assays were performed as described under Experimental Procedures. Binding was conducted in duplicate. The data represent the relative percentage of bound counts/min compared with counts/min bound in the absence of competitor ligand.

FIGURE 3. NMR-based binding assays. A, comparison of T₁ₚ spectra (spin-lock duration of 200 ms) of compound 6 (100 μM) in the absence (blue) and presence of 10 μM (h)RXRa-LBD (red). Peaks marked with an asterisk indicate extra signals present in the protein buffer. B, comparison of T₁ₚ spectra (200 ms relaxation time) of compound 4 (100 μM) acquired in the presence (red) and absence (blue) of protein. C, T₁ₚ spectra (200 ms spin-lock duration) of compound 5 (100 μM) in the free (blue) and bound (red) state.

FIGURE 4. Docked structures of novel ligands into RXRa-LBD (PDB code 1MVC). Superposition of the x-ray structure of the known RXRa inhibitor compound BMS-649 (shown in orange in each panel) and the conformation of each of the novel ligands reported in Table 2. A–F, report ligands 1–6. In each panel, intermolecular hydrogen bondings between a given docked inhibitor and the ligand binding domain of RXRa are highlighted.
RESULTS AND DISCUSSION

In order to identify novel RXRα ligands, we adopted a structure-based approach based on virtual docking of a large library of drug-like small organic molecules followed by experimental verification of top scoring compounds. This approach is based on first obtaining reliable docking geometries for each compound and subsequently being able to rank order the compounds for their predicted binding affinity according to a scoring function.

Although most of the available virtual docking software packages have demonstrated their utility in several different cases (25–27), in practical terms, it has been shown that their success and failures are
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In conclusion, we report here a successful virtual docking approach in which 5 of 200 selected compounds showed RXRα transcriptional antagonistic activity in vitro and in cells with binding affinities in the nanomolar range in vitro and low micromolar cellular activity. These compounds with their structural features differing from the existing RXR ligands may represent valuable tools to assist with the dissection of the complex RXR signal transduction pathways. The novel structural scaffolds reported here could be useful for the development of potential lead compounds targeting members of the nuclear receptor superfamily in the development of novel cancer therapeutics.

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