Pharmacophore Analysis of the Nuclear Oxysterol Receptor LXRα

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Received November 8, 2000

A cell-free assay was developed for the orphan nuclear receptor LXRα that measures the ligand-dependent recruitment of a peptide from the steroid receptor coactivator 1 (SRC1) to the nuclear receptor. Using this ligand-sensing assay (LiSA), the structural requirements for activation of the receptor by oxysterols and related compounds were studied. The minimal pharmacophore for receptor activation was shown to be a sterol with a hydrogen bond acceptor at C24. 24(*S*),25-Epoxycholesterol (**1**), which meets this criterion, is among the most efficacious of the oxysterols and is an attractive candidate as the LXRα natural hormone. Cholenic acid dimethylamide (**14**) showed increased efficacy compared to **1**, whereas the unnatural oxysterol 22(*S*)-hydroxycholesterol (**4**) was shown to be an antagonist of **1** in the LiSA. The structural requirements for SRC1 recruitment in the LiSA correlated with the transcriptional activity of compounds in a cell-based reporter assay employing LXRα-GAL4 chimeric receptors. Site-directed mutagenesis identified Trp^{443} as an amino acid critical for activation of LXRα by oxysterol ligands. This information was combined with the structure−activity relationship developed from the LiSA to develop a 3D homology model of LXRα. This model may aid the design of synthetic drugs targeted at this transcriptional regulator of cholesterol homeostasis.

Introduction

The steroid, retinoid, and thyroid hormones function as chemical messengers through activation of nuclear receptors within cells.[1] These receptors are ligand-activated transcription factors which couple the level of their cognate hormones to changes in gene expression. To date, the nuclear receptor gene family encodes approximately 50 sequence-related proteins, the majority of which were isolated as orphan receptors prior to the identification of their cognate hormones.[2] The liver X receptors, LXRα (NR1H3) and LXR*β* (NR1H2), together with the farnesoid X receptor, FXR (NR1H4), form a subfamily of these orphan receptors.[3,4] Recently, we and others have reported that LXR and FXR are hormone receptors for oxysterols[5−7] and bile acids,[8−10] respectively. Unlike the classical steroid hormone receptors, LXR and FXR bind to their cognate hormones with relatively low affinity. Among the oxysterols that activate LXRα most effectively is 24(*S*),25-epoxycholesterol (**1**).[5,11] The concentrations of this oxysterol in the liver[12−14] are consistent with its proposed role[5] as an endogenous activator of LXRα in that organ. LXRα functions as a heterodimer with the 9-*cis*-retinoic acid receptor RXR (NR2B) to regulate gene expression.[4] Recently, several genes involved in cholesterol metabolism and homeostasis have been shown to be regulated by the LXRα/RXR heterodimer, including *CYP7A*,[5,11] *CETP*,[15] and *ABCA1*.[16−19] On the heels of these exciting results, LXRα has emerged as a promising target for development of drugs for the treatment of cardiovascular disease.[2,20]

Initial studies of the structural requirements for activation of LXRα employed cell-based reporter assays.[5−7] Data from these assays suggested that the presence of an oxygen atom on the sterol side chain, which can function as a hydrogen bond acceptor, was important for receptor activation. Unfortunately, the low potency of oxysterol ligands in cell-based assays has hampered the generation of precise structure−activity relationships (SARs). A subsequent study using a competition binding assay confirmed the preference for a hydrogen bond acceptor at C24 of the sterol side chain.[21] Notably, oxysterols bind to LXRα with higher affinity than was anticipated from their potency in cell-based assays.

Nuclear receptors regulate gene transcription through interaction with cellular cofactors, known as coactivators and corepressors.[22,23] Agonist ligands promote displacement of corepressors and recruitment of coactivators, which can remodel chromatin and modulate the assembly of the basal transcriptional machinery within cells.[22,23] Structural and biochemical studies have revealed that a short α-helical LxxLL sequence on the coactivator protein is critical for its interaction with the C-terminal domain of the nuclear receptor. In the present study, we developed a ligand-sensing assay (LiSA) that measures ligand-dependent recruitment of

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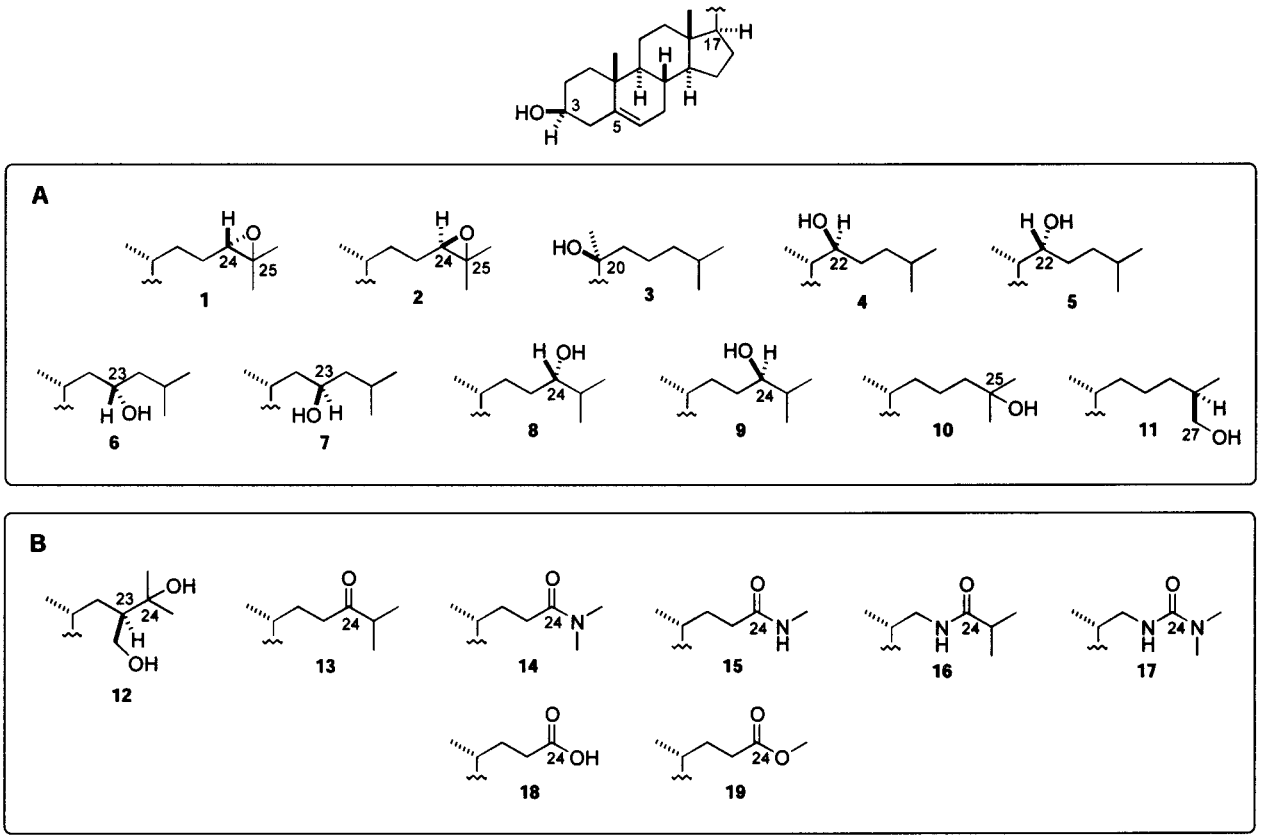
10.1021/jm0004749 CCC: $20.00
Published on Web 02/03/2001

Chart 1. Structures of Oxysterols (A) and Related Compounds (B)[a]



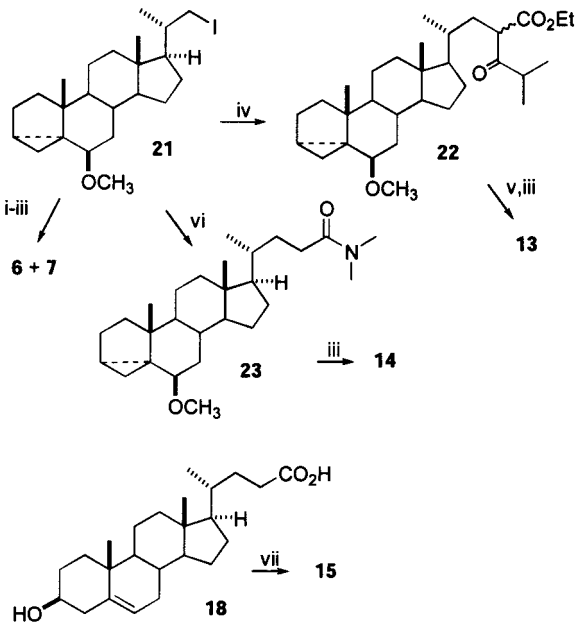
[a] All compounds contain the 3β-hydroxy-Δ^5-androstane skeleton with the respective side chains at C17. The carbon atom at the site of the hydrogen bond donor substitution on the sterol side chain is numbered.

a peptide from the steroid receptor coactivator 1 (SRC1)[24] to LXRα by fluorescence resonance energy transfer. The structural requirement for activation of LXRα was evaluated using a set of oxysterols, which included all the potential regio- and stereoisomers between C22 and the end of the sterol side chain, and a set of nitrogen-containing analogues with potentially excellent hydrogen bond acceptor capability at C24 (Chart 1). The resulting SARs were used to develop a pharmacophore model of LXRα that may aid the design of new ligands for this orphan receptor.

Results

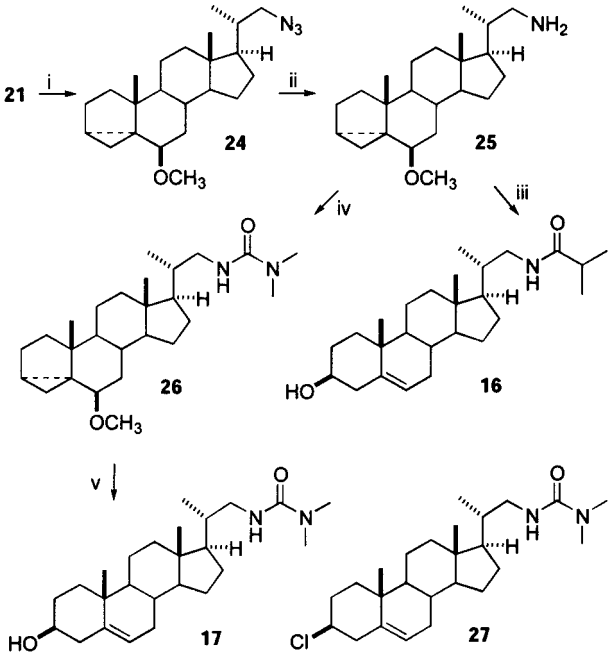
Synthesis of Oxysterols and Related Analogues. The prospective ligands that were not commercially available were prepared as follows. 24(*S*),25-Epoxycholesterol (**1**) and its 24(*R*)-epimer (**2**) were synthesized as recently described.[25] 23(*S*)- and 23(*R*)-Hydroxycholesterol (**6** and **7**) were prepared (Scheme 1) from *i*-steroid iodide **21**,[26] which had been employed in the synthesis of **1** and **2**, via lithiation[27] and reaction with isovaleraldehyde. Treatment of the resulting product mixture with aqueous acid gave **6** and **7** which were separated chromatographically, as has previously been accomplished.[28,29] 24(*S*)- and 24(*R*)-Hydroxycholesterol (**8** and **9**) were prepared as we have recently described[14] by modification of the approach used by Ourisson.[30] 27-Hydroxycholesterol (**11**) was synthesized by the method of Schroepfer.[31] The unusual oxysterol **12** with an unnatural, modified side chain structure was available from a study of the synthesis of **1**.[32] 24-Ketocholesterol (**13**) was synthesized, as shown in Scheme 1, by alkylation of iodide **21** with the anion of ethyl isovalerate to afford **22**, which was converted to **13** by treatment with Al_2O_3 in aqueous dioxane at reflux,[33] followed by treatment with aqueous acid.

Scheme 1[a]



[a] Reagents: (i) *t*-BuLi, pentane; (ii) $(CH_3)_2CHCH_2CHO$, −78 °C; (iii) *p*-TsOH, H_2O, dioxane; (iv) abs. EtOH, Na, $(CH_3)_2CHCH_2CO_2Et$; (v) Al_2O_3, H_2O, dioxane, Δ; (vi) $LiCH_2CON(CH_3)_2$, THF, −78 to 0 °C; (vii) 1. *t*-$Bu(CH_3)_2SiCl$, imidazole, DMAP, 2. $(COCl)_2$, CH_2Cl_2, DMF, 3. CH_3NH_2.

Scheme 2[a]



[a] Reagents: (i) NaN_3, benzene, DMF; (ii) $LiAlH_4$, Et_2O; (iii) 1. $(CH_3)_2CHCOCl$, pyridine, Et_2O, 2. *p*-TsOH, H_2O, dioxane; (iv) $(CH_3)_2NCOCl$, pyridine, Et_2O; (v) *p*-TsOH, H_2O, dioxane.

Dimethylamide **14** had previously been prepared from cholenic acid (**18**),[34,35] but since we had in hand[25,32] substantial quantities of C22 iodide **21**, we chose to develop an alternate synthesis. Alkylation of **21** with the anion of *N*,*N*-dimethylacetamide afforded 93% of *i*-steroid **23**, which upon treatment with aqueous acid yielded 68% of **14**, as shown in Scheme 1. Monomethylamide **15** was prepared in 63% overall yield from cholenic acid (**18**) by treatment successively with TBDMS chloride, oxalyl chloride, and methylamine without isolation of intermediates. This procedure afforded **15** directly, and it was not investigated whether the TBDMS derivative of the C3 hydroxyl group of **18** had not formed or was cleaved by HCl generated during the reaction with oxalyl chloride. Iodide **21** was also used to prepare inverse amide **16** and urea **17** (Scheme 2). Treatment of **21** with NaN_3 gave 93% of azide **24**, which was reduced with $LiAlH_4$ to amine **25** in 79% yield. Treatment of **25** with isobutyryl chloride, followed by aqueous acid treatment of the crude product, afforded the desired amide **16** in 44% overall yield. An analogous reaction sequence with **25** using carbamyl chloride afforded 22% of urea **17** and 37% of 3β-chloro compound **27**. The latter presumably resulted from the presence of chloride ion during the aqueous acid deprotection step. When the intermediate *i*-steroid intermediate **26** was isolated and purified before conversion to **17**, the overall yield of **17** from **25** was 56%.

Development of a Cell-free Assay for LXRα Activation. To study the structural requirements for LXRα activation by oxysterols, we chose to develop a cell-free assay that monitors the ligand-dependent recruitment of SRC1. A related cell-free LiSA has recently been employed to study the structural requirements for activation of FXR by bile acids and synthetic ligands.[8,36] The purified human LXRα ligand-binding domain was expressed from *E. coli*, purified, biotinylated, and complexed with the streptavidin-labeled fluorophore allophycocyanin. A peptide encompassing the second LxxLL motif[8] (amino acids 676−700) of SRC1[24] was synthesized, biotinylated, and complexed with streptavidin-labeled europium chelate. Time-resolved fluorescence resonance energy transfer was used to monitor the interaction between the SRC1 peptide and the LXRα ligand-binding domain. A basal interaction was detected, which increased in the presence of increasing concentrations of known LXRα agonists.

Table 1. Activity of Compounds in the LXRα LiSA[a]

compound		LXRα	
no.	name	EC_{50} (nM)	RE
1	24(*S*),25-epoxycholesterol	460 ± 80	1.0
2	24(*R*),25-epoxycholesterol	670 ± 60	1.0
3	20(*S*)-hydroxycholesterol	470 ± 20	1.0
4	22(*S*)-hydroxycholesterol		ia
5	22(*R*)-hydroxycholesterol	325 ± 60	0.85
6	23(*S*)-hydroxycholesterol	5900 ± 470	0.70
7	23(*R*)-hydroxycholesterol		ia
8	24(*S*)-hydroxycholesterol	130 ± 30	1.0
9	24(*R*)-hydroxycholesterol	220 ± 50	0.85
10	25-hydroxycholesterol	1160 ± 20	0.35
11	27-hydroxycholesterol	250 ± 50	0.30
12	23(*R*)-CH_2OH-25-bisnorergoster-3β,24-diol		ia
13	24-ketocholesterol	180 ± 20	1.3
14	cholenic acid dimethylamide	170 ± 20	1.4
15	cholenic acid monomethylamide	720 ± 80	0.55
16	22-[*N*-(2′-methylpropionyl)amino]-23,24-bisnorchol-5-en-3β-ol		ia
17	22-[*N*-(*N*,*N*-dimethylformamido)amino]-23,24-bisnorchol-5-en-3β-ol		ia
18	cholenic acid	3000 ± 1000	0.2
19	cholenic acid methyl ester	105 ± 5	1.1
20	cholesterol		ia

[a] EC_{50} = concentration of the test compound that leads to half-maximal activity ± standard error, $n = 3$; RE = relative efficacy of the test compound determined by the maximal increase in relative fluorescence compared to 24(*S*),25-epoxycholesterol (**1**); ia = RE < 0.1.

Activation of LXRα by Oxysterols. The putative LXRα natural ligand 24(*S*),25-epoxycholesterol (**1**) showed a saturable dose-dependent increase in fluorescence in the LiSA with an EC_{50} of 460 nM (Table 1 and Figure 1A). This value is in good agreement with the reported K_i of 200 nM for binding to LXRα[21] but is lower than the estimated EC_{50} of 4−7 μM in cell-based reporter assays.[5,21] Notably, the increase in SRC1 recruitment is consistent with the agonist activity of **1** in these cell-based reporter assays. The unnatural epimer 24(*R*),25-epoxycholesterol (**2**) exhibited a higher EC_{50} but equivalent efficacy for recruitment of the SRC1 peptide to LXRα (Figure 1A). Both **1** and **2** were previously shown to be full agonists in a cell-based reporter assay,[5] and the difference in EC_{50} for SRC1 recruitment may contribute to the lower potency of **2** compared to **1** in cells. The oxysterols **3**−**11** were assayed to evaluate the effect of regio- and stereochemistry of hydroxyl substitution between C20−C27 of the cholesterol side chain (Chart 1). At C20, oxysterol **3** with the *S*-stereochemistry showed equivalent activity to **1**. At C22, only the *R*-isomer **5** was active, although it was less efficacious than **1**. At C23, only the *S*-isomer **6** was active, although it was less efficacious and less potent than **1**. At C24, both isomers were active, but the *S*-isomer **8** was more efficacious than its *R*-epimer **9**.

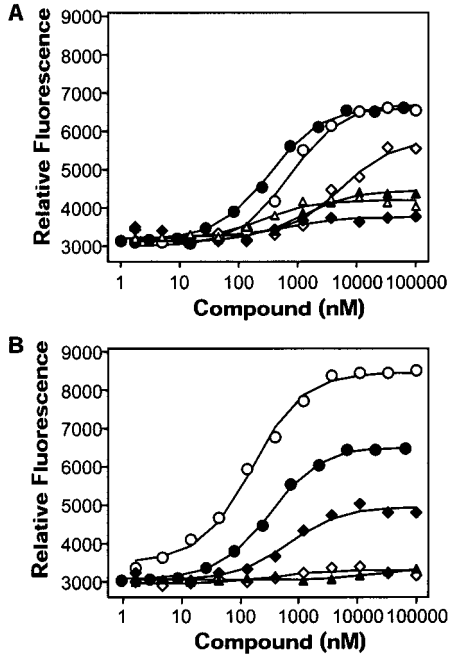


Figure 1. Dose−response analysis of selected oxysterols and related compounds in the LXRα LiSA. (A) Oxysterols: 24(*S*),25-epoxycholesterol (**1**; ●), 24(*R*),25-epoxycholesterol (**2**; ○), 23(*S*)-hydroxycholesterol (**6**; ◇), 23(*R*)-hydroxycholesterol (**7**; ◆), 25-hydroxycholesterol (**10**; ▲), and 27-hydroxycholesterol (**11**; △). (B) Nitrogen-containing analogues: cholenic acid dimethylamide (**14**; ○), cholenic acid monomethylamide (**15**; ◆), 22-[*N*-(2′-methylpropionyl)amino]-23,24-bisnorchol-5-en-3*β*-ol (**16**; ◇), and 22-[*N*-(*N*,*N*-dimethylformamido)amino]-23,24-bisnorchol-5-en-3*β*-ol (**17**; ▲). 24(*S*),25-Epoxycholesterol (**1**; ●) is shown for comparison. Data are representative of 3 independent experiments.

Both the C25- and C27-substituted oxysterols **10** and **11** showed low efficacy in the LiSA. The weak activity of 25-hydroxycholesterol (**10**) on LXRα contrasts with its activity as a suppressor of SREBP signaling,[37] which suggests that these two regulators of cholesterol metabolism may be under the control of different pools of oxysterols.

The effect of the regio- and stereochemistry of hydroxyl substitution measured in the LiSA is in general agreement with the SAR observed in cell-based assays.[5−7,21] However, the increased potency of oxysterols in the cell-free LiSA allows a more precise measurement of relative potency and efficacy (Figure 1 and Table 1). The activity of 24(*R*)-hydroxycholesterol (**9**) in the LXRα LiSA was surprising, since it did not displace [^{3}H]-**1** in a competition binding assay.[21] However, we note that **9** has been reported to activate LXRα in a cell-based assay,[5] which is consistent with the LiSA data. The C20- and C24-substituted oxysterols (Chart 1) showed the best activity in the LiSA, with **1**−**3** and **8** demonstrating the most efficacy for recruitment of SRC1. Of these oxysterols, 24(*R*),25-epoxycholesterol (**2**) has not been detected as a naturally occurring compound[38] and 20(*S*)-hydroxycholesterol (**3**), like 22(*R*)-hydroxycholesterol (**5**), is only found in the adrenals as an intermediate in pregnenolone biosynthesis.[39] By contrast, both 24(*S*),25-epoxycholesterol (**1**) and 24(*S*)-hydroxycholesterol (**8**) have been detected in liver extracts at physiologically relevant concentrations.[12,14,40] Importantly, the hepatic level of these oxysterols is raised by cholesterol feeding in rats,[13,14] making **1** and **8** the leading candidates for physiological regulators of LXRα activity in the liver.[13,14]

Activation of LXRα by 24-Ketocholesterol (13) and Related Compounds. To further refine the pharmacophore for LXRα activation, a series of side chain-modified cholesterol derivatives was studied. All of the analogues were designed to have a hydrogen bond acceptor at C24 (Chart 1B). 24-Ketocholesterol (**13**) showed comparable activity to **1** (Table 1), as was previously shown using a cell-based assay.[5] Among a series of nitrogen-substituted analogues, dimethylamide **14** was notable for its increased efficacy for SRC1 recruitment compared to **1**. The secondary amide **15** showed low efficacy, and the inverse amide **16** and urea **17** were inactive (Table 1 and Figure 1B). Thus, nitrogen substitution appears to be tolerated at C25 but not at C23 on the cholesterol side chain. Cholenic acid (**18**) was weakly active, but its methyl ester **19** showed activity comparable to **1**. These results demonstrate that a hydrogen bond acceptor at C24 fulfills the minimal structural requirement for activation of LXRα by steroidal ligands. The best synthetic activators are substituted with a carbonyl function at this position, which may function as an isosteric replacement for the epoxide in **1**.

Increased Efficacy of LXRα Activation by Cholenic Acid Dimethylamide (14). Since dimethylamide **14** showed increased recruitment of the SRC1 peptide in the LXRα LiSA compared to **1**, we decided to evaluate its activity in an established cell-based reporter assay.[5,21] CV-1 cells were transfected with a plasmid expressing a chimera of the human LXRα ligand-binding domain fused to the yeast transcription factor GAL4 and a reporter plasmid containing the GAL4 upstream activation sequence (UAS) fused to a minimal promoter and the gene for secreted alkaline phosphatase. It was previously established that oxysterols show equivalent activity on either the chimeric LXRα-GAL4 or full-length LXRα receptors.[5,21] The induction of alkaline phosphatase expression was measured by a standard colorimetric assay in the presence and absence of 10 μM amides **14**−**16** and 24(*S*),25-epoxycholesterol (**1**) (Figure 2A). The dimethylamide **14** showed greater activation of the reporter gene compared to **1**, while the secondary amides **15** and **16** were much less active. Notably, the relative activities of **1** and **14**−**16** in cells paralleled their efficacy for SRC1 recruitment in the LXRα LiSA (Figure 1B). Full dose−response analysis showed that the increased activity of **14** was due to its greater efficacy on LXRα rather than increased potency compared to **1** (Figure 2B). Thus, the increase in recruitment of SRC1 by **14** measured in the LiSA translates to increased efficacy for activation of LXRα in cells.

Identification of an LXRα Antagonist. All but one of the oxysterols that had been reported to bind to LXRα[21] were also active in the LiSA. The exception was 22(*S*)-hydroxycholesterol (**4**), which binds to LXRα with a K_i of 180 nM[21] but did not recruit SRC1 in the LiSA assay at concentrations up to 100 μM (Table 1). Since this oxysterol binds but does not activate LXRα, we determined whether it functioned as an antagonist in the LiSA. As shown in Figure 3A, 100 μM **4** was able to

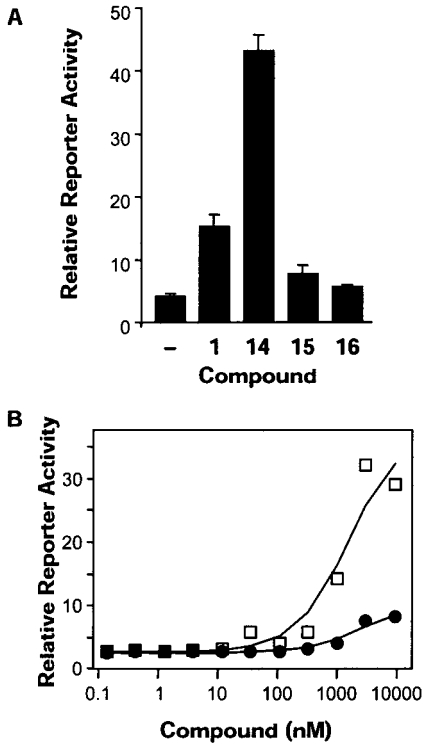


Figure 2. Activity of selected compounds on LXRα-GAL4 chimeric receptors in a cell-based reporter assay. (A) 24(*S*),25-Epoxycholesterol (**1**) and the amides **14**−**16** were assayed at a concentration of 10 μM; (−) vehicle control. Data are the mean of 3 independent experiments ± standard error. (B) Dose−response analysis of 24(*S*),25-epoxycholesterol (**1**; ●) and cholenic acid dimethylamide (**14**; □). Data are representative of 2 independent experiments.

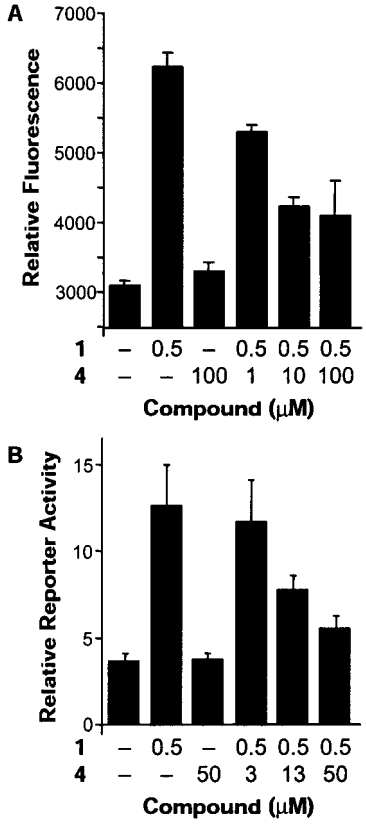


Figure 3. Antagonist activity of 22(*S*)-hydroxycholesterol (**4**). The concentrations of **1** and **4** are indicated; (−) vehicle control. (A) LXRα LiSA. Data are the mean of 3 independent experiments ± standard error. (B) LXRα-GAL4 cell-based assay. Data are the mean of 8 independent experiments ± standard error.

partially block the recruitment of the SRC1 peptide induced by 500 nM 24(*S*),25-epoxycholesterol (**1**). To confirm these results, **4** was tested in the LXRα-GAL4 cell-based assay (Figure 3B). A 50 μM dose of **4** did not induce the reporter gene but was able to block the activity of **1**. Thus, 22(*S*)-hydroxycholesterol (**4**) is a functional antagonist in the LXRα LiSA and LXRα-GAL4 assay.

Trp^{473} Is Required for LXRα Activation. The data from the LiSA and cell-based assays are consistent with a minimum pharmacophore in which the oxysterol side chain acts as a hydrogen bond acceptor from the LXRα protein. The C-termini of all ligand-activated nuclear receptors contain a region known as the activation function 2 (AF2) that is required for interaction with SRC1. The X-ray crystal structures of nuclear receptor ligand-binding domains have shown that AF2 forms an α-helix.[41] With the aid of these structures we generated a sequence alignment of several nuclear receptors, including LXRα, LXRβ, and FXR (Figure 4A). The alignment shows a conserved Glu in the center of the AF2 helix for each receptor. The PPARγ crystal structure[42,43] revealed that this amino acid (Glu^{471}) forms half of a "charge clamp" that directs the interaction of SRC1 with PPARγ. The crystal structure also revealed that Tyr^{473} in the PPARγ AF2 helix forms a hydrogen bond with the agonist rosiglitazone, which serves to stabilize the charge clamp interaction between the receptor and SRC1.[42,43] Notably, our sequence alignment (Figure 4A) suggests that LXRα, LXRβ, and FXR contain a Trp at the analogous position in the AF2 helix.

To test whether Trp^{443} in LXRα could play a role in activation of the receptor by oxysterols, it was changed to Phe, Tyr, Leu, Cys, Ala, or Thr by site-directed mutagenesis of the LXRα-GAL4 expression plasmid. The resulting wild-type and mutant LXRα-GAL4 chimeras were transiently expressed in CV-1 cells. Western blot analysis of the transfected cell extracts, using an antibody to GAL4, showed that all of the receptors were expressed at comparable levels (Figure 4B). Therefore, point mutation of Trp^{443} did not appear to affect the stability or expression level of the protein. The activation of the wild-type and mutant receptors was examined using the GAL4-responsive reporter. As shown in Figure 4C, the wild-type LXRα-GAL4 chimera activated the reporter gene by 15−20-fold in the presence of 10 μM 24(*S*),25-epoxycholesterol (**1**). By contrast, none of the mutant LXRα-GAL4 chimeras were activated by (**1**). These results demonstrate that Trp^{443} plays a critical role in ligand-mediated activation of LXRα and suggests that this residue may be the hydrogen bond donor that interacts with oxysterol ligands.

Construction of a Homology Model of LXRα. A model of the LXRα ligand-binding domain was built from the crystal structure of the retinoic acid receptor γ (RARγ)[44] (Figure 5). 24(*S*),25-Epoxycholesterol (**1**) was docked manually in the ligand-binding site in an orientation which was consistent with the SAR generated in the LXRα LiSA and the results of the pharmacophore analysis. In the resulting model (Figure 5A,B), the side chain of 24(*S*),25-epoxycholesterol (**1**) adopts a

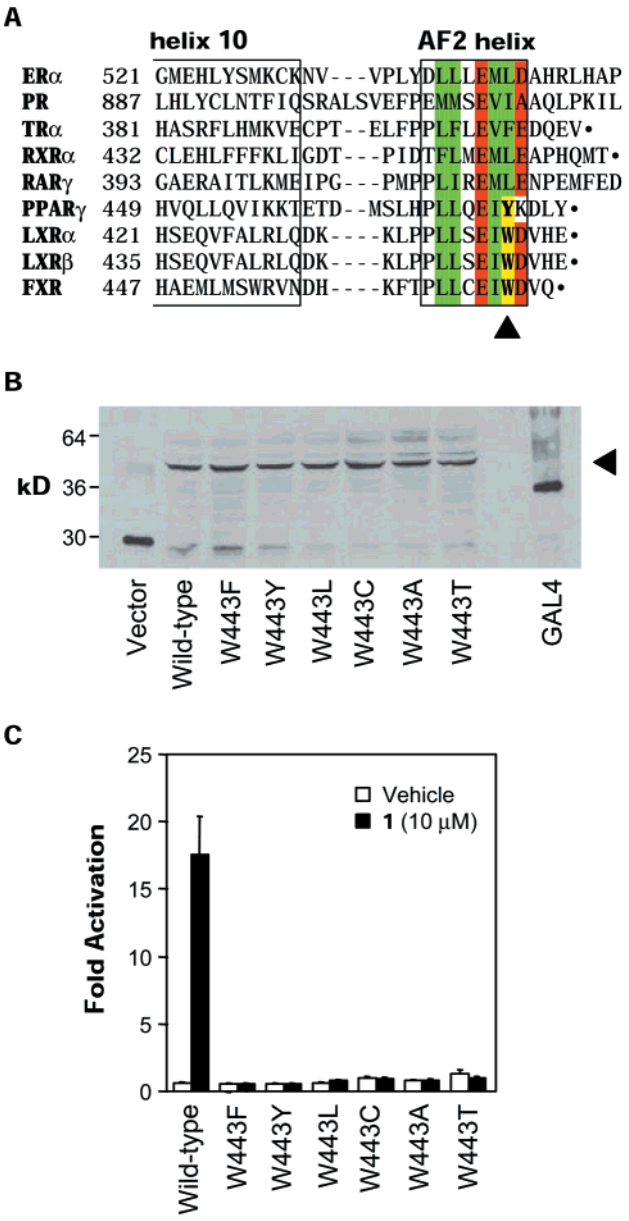


Figure 4. Pharmacophore analysis of LXRα. (A) Sequence alignment of the C-termini of nuclear receptor ligand-binding domains. The AF2 helix and part of helix 10 are boxed; (●) indicates the C-terminus of a receptor. Within the AF2 helix, conserved acidic amino acids are highlighted in red and hydrophobic amino acids in green. The black arrow indicates the position of the polar amino acids (highlighted in yellow) that are proposed to bind to agonist ligands in the NR1 subfamily. (B) Western blot analysis of extracts of CV-1 cell transfected with expression plasmids containing wild-type and mutant LXRα-GAL4 receptors. The black arrow indicates the LXRα protein. (C) Activation of wild-type and mutant LXRα-GAL4 chimeric receptors by 24(*S*),25-epoxycholesterol (**1**) in CV-1 cells. Data are the mean of 3 independent experiments ± standard error.

low-energy extended conformation that permits a hydrogen bond interaction between Trp^{443} and the epoxide oxygen. The model also identifies Arg^{305} as the amino acid that may interact with the C3 hydroxyl group of the sterol A-ring at the other end of the ligand-binding pocket. The orientation of the sterol side chain is consistent with the diastereoselectivity observed with the C22- and C23-substituted sterols **4**−**7**. The hydroxyl groups of the active 22(*R*)-hydroxycholesterol (**5**), 23(*S*)-hydroxycholesterol (**6**), and 24(*S*)-hydroxycholesterol (**8**) would all lie between 2.0−2.5 Å from Trp^{443} (Figure 5B). Only the activity of 20(*S*)-hydroxycholesterol (**3**) is not explained by the current model. The hydroxyl group of **3** would lie 4 Å from Trp^{443}, which requires that either the ligand or protein be shifted slightly to form a strong hydrogen bond. Notably, the relative lack of stereoselectivity with C24-substituted sterols (**1** vs **2** and **8** vs **9**) is explained by this model, since both pairs of diastereomers place a hydrogen bond acceptor within 2.5 Å of Trp^{443} (Figure 5B). The model also provides a possible explanation for the LXRα antagonist activity of 22(*S*)-hydroxycholesterol (**4**). As shown in Figure 5C, the C22 hydroxyl group of **4** can accept a hydrogen bond from His^{421}, which lies on helix 10 of the ligand-binding domain. This interaction could allow **4** to bind to the receptor without stabilizing the AF2 helix. Thus, the homology model is generally consistent with the SAR generated in the LiSA by both LXRα agonists and an antagonist.

Discussion

The orphan nuclear receptor LXRα is a potential new target for the development of drugs for the treatment of cardiovascular disease.[2,20] In the absence of a crystal structure for this nuclear receptor, we opted to develop a homology model of the ligand-binding domain to aid the design of LXRα agonists. Use of the cell-free LiSA has allowed us to extend the SARs for oxysterol activation of LXRα that had been generated using cell-based reporter assays.[5−7] The cell-free LiSA has several advantages over other techniques for studying the structural requirements for activation of nuclear receptors. Compared to a competition binding assay,[21] the LiSA assay does not require the synthesis of a radioligand. The LiSA also generates information about the relative efficacy of a test ligand, which cannot be derived from a binding assay. Notably, the LiSA can be run in a 384-well format, which is particularly useful in high-throughput screening for synthetic nonsteroidal ligands. Cell-based reporter assays[5−7] can be affected by the ability of the ligand to cross cell membranes as well the metabolism of the ligand within the cell. This may explain the lower potency of oxysterols in cell-based compared to cell-free assays. The data from cell-based assays can also vary due the nature of the host cell and the structure of the reporter gene. For example, our data indicate that cholenic acid dimethylamide (**14**) is more efficacious than 24(*S*),25-epoxycholesterol (**1**) in the cell-free LiSA (Figure 1B) and in CV-1 cells employing a LXRα-GAL4 chimeric receptor (Figure 2). A recent report also described dimethylamide **14** as one of the most potent and efficacious activators, compared to natural and unnatural cholanoic acids, of the full-length LXRα/RXRα heterodimer in HEK-293 cells using a *c-fos* gene reporter.[45] By contrast, it was reported that dimethylamide **14** was less efficacious than 24(*S*),25-epoxycholesterol (**1**) in CV-1 cells employing full-length LXRα and a *CYP7A* gene reporter.[21] The different results generated with various cell-based assays underscore the value of the LXRα LiSA as an independent method for studying the structural requirements for receptor activation.

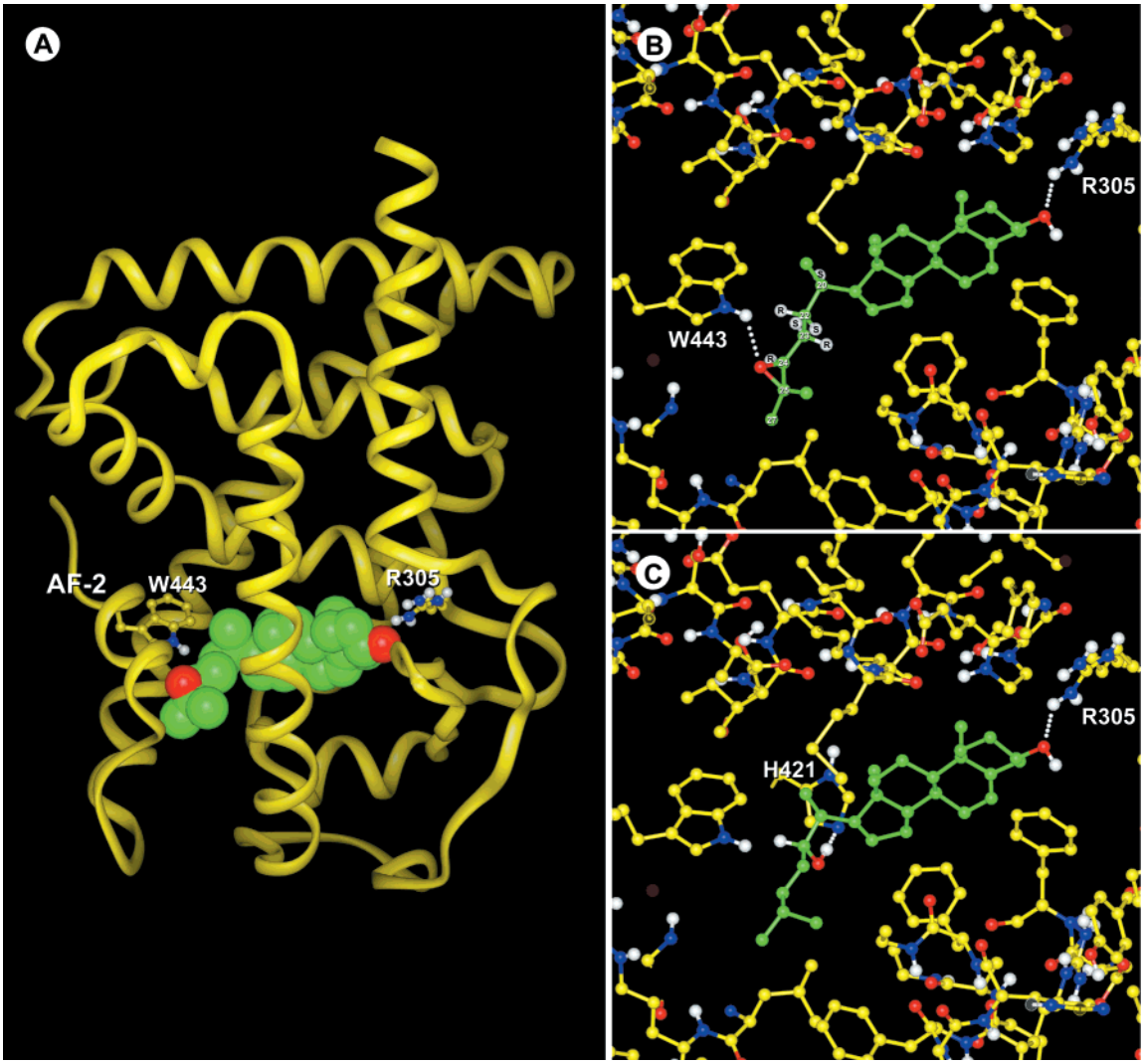


Figure 5. Homology model of the LXRα ligand-binding domain. (A) Protein backbone is shown as a yellow worm, with the amino acids Trp^{443} and Arg^{305} highlighted. 24(*S*),25-Epoxycholesterol (**1**) is shown as a van der Waals space-filling representation with carbon atoms colored green and oxygen atoms red. The C-terminal AF2 helix is labeled. (B) View of the ligand-binding pocket showing the proposed hydrogen bonds between 24(*S*),25-epoxycholesterol (**1**) and Trp^{443} and Arg^{305}. The hydrogens of C20−C24 are highlighted to show that one face of the sterol side chain is pointed toward Trp^{443}. (C) View of the ligand-binding pocket showing the proposed hydrogen bonds between 22(*S*)-hydroxycholesterol (**4**) and His^{421} and Arg^{305}.

On the basis of the SARs from the LiSA, we have developed a homology model of the LXRα ligand-binding domain that incorporates the critical role Trp^{443} plays in activation of the receptor. Since the other members of the NR1 subfamily, LXR*β* and FXR, also contain a Trp at the analogous position in AF2 (Figure 4A), it will be intriguing to see if they use a similar mechanism of activation by their cognate ligands. Trp^{443} in LXRα lies in an analogous position to Tyr^{473} of PPAR*γ* (Figure 4A). Like LXRα, PPAR*γ* binds to DNA as a heterodimer with the 9-*cis*-retinoic acid receptor RXR.[4] Notably, the PPAR*γ*/RXR and LXRα/RXR heterodimers can each be activated by the binding of 9-*cis*-retinoic acid to the RXR side of the dimer.[46,47] The molecular basis of this phenomenon, known as "permissive activation", was recently explained by analysis of the X-ray crystal structure of the PPAR*γ*/RXRα heterodimer.[43] A salt bridge, which is formed between Lys^{431} of RXRα and the C-terminal carboxylate of PPAR*γ*, provides a mechanism by which 9-*cis*-retinoic acid can promote recruitment of SRC1 to its heterodimeric partner. The sequence alignment in Figure 4A shows that the C-terminus of LXRα also lies at the end of its AF2 helix. Thus, the LXRα AF2 helix, like PPAR*γ*, may be held in a quasi-active state by a salt bridge with RXR. Our model (Figure 5B) proposes that agonists bind to LXRα in an orientation that generates a hydrogen bond between the ligand and Trp^{443}. This hydrogen bond provides a mechanism by which oxysterols can further stabilize the AF2 helix and increase the level of SRC1 recruitment to the LXRα/RXR heterodimer.

22(*S*)-Hydroxycholesterol (**4**) failed to recruit SRC1 in the LiSA but was able to block the activity of 24(*S*),25-epoxycholesterol (**1**) (Figure 3A). Oxysterol **4** also blocked the activity of **1** in the cell-based assay employing the LXRα-GAL4 chimeric receptor (Figure 3B). Notably, this oxysterol has the unnatural stereochemistry at C22, and there is no evidence that it is formed in vivo. Consistent with the antagonist activity of **4** in these assays, our model (Figure 5C) shows that the hydroxyl group of **4** is on the wrong face of the sterol side chain to form a hydrogen bond with Trp^{443}. Instead, **4** may form a hydrogen bond with His^{421} that results in ligand binding without promoting recruitment of SRC1.

Interestingly, a modulator of PPARγ (GW0072) has been identified that binds to this receptor without interacting with Tyr[473] and fails to recruit SRC1.[48] GW0072 is an antagonist of the PPARγ-GAL4 chimera and of adipocyte differention but displays tissue-specific PPARγ agonist activity in animal models of type 2 diabetes.[48,49] Since oxysterol **4** binds to LXRα without stabilizing AF2 or recruiting SRC1, it may also profile as a tissue-specific modulator of receptor activity. Unfortunately, the physical properties of this oxysterol have not allowed us to test this hypothesis in vivo. However, the observation that the structure and the position of the hydrogen bond acceptor affect SRC1 recruitment and receptor activation augurs well for the use of rational design to develop drugs with modified LXRα agonist activity.

Our model of the LXRα ligand-binding domain requires a functional group on one face of the cholesterol side chain to accept a hydrogen bond from Trp[443] to effect activation of the receptor (Figure 5B). The LiSA demonstrated that placement of a carbonyl group at C24 was sufficient to activate the receptor. For example, the ketone **13** was a full agonist, and the dimethylamide **14** was even more efficacious. This suggests that placement of a hydroxyl group on the sterol side chain, which can both donate and accept a hydrogen bond, offers no additional advantage for activation of LXRα. Notably, 24(*S*),25-epoxycholesterol (**1**), which has only a hydrogen bond acceptor in the sterol side chain, is among the most effective oxysterols at promoting recruitment of the SRC1 peptide (Figure 1A). Thus, the requirement for an agonist to accept, but not donate, a hydrogen bond is consistent with the structure of the efficacious natural (e.g. **1**) and synthetic (e.g. **14**) LXRα ligands. As mentioned earlier, **1** is found in the liver at sufficient concentration to activate LXRα[12−14] and (along with **8**) increases in concentration in the nuclei of rat liver cells after administration of an atherogenic diet.[13,14] Of possible relevance also is the fact that, among all the oxysterols, 24(*S*),25-epoxycholesterol (**1**) is formed by a unique biosynthetic pathway involving the squalene dioxide shunt.[13] In concert with our pharmacophore analysis, these observations suggest that LXRα may have evolved as an epoxycholesterol receptor[5] rather than a generalized oxysterol sensor.[6]

Conclusion

We have developed a homology model for the nuclear oxysterol receptor LXRα. The model incorporates the structural requirements for activation of the receptor by oxysterols and analogues, and the critical role that Trp[443] plays in this process. The model explains the LXRα agonist activity of 24(*S*),25-epoxycholesterol (**1**) as well as the antagonist activity of 22(*S*)-hydroxycholesterol (**4**). Since LXRα plays an important role in the regulation of cholesterol homeostasis, this model may be useful in the design of new drugs for the treatment of cardiovascular disease.[2,20] The recent disclosure of a nonsteroid LXRα agonist[18] provides a template for development of these analogues.

Experimental Section

Oxysterols and Related Compounds. 22(*S*)-Hydroxycholesterol (**4**), 22(*R*)-hydroxycholesterol (**5**), 25-hydroxycholesterol (**10**), cholenic acid (**18**), cholenic acid methyl ester (**19**), and cholesterol (**20**) were purchased from Sigma. 24(*S*)-Epoxycholesterol (**1**),[25] 24(*R*),25-epoxycholesterol (**2**),[25] 24(*S*)-hydroxycholesterol (**8**),[14] 24(*R*)-hydroxycholesterol (**9**),[14] 27-hydroxycholesterol (**11**),[31] and 23(*R*)-hydroxymethyl-26,27-dinorergost-5-en-3β-ol (**12**)[32] were prepared as previously described.

Synthesis. General. NMR spectra were run in $CDCl_3$, unless otherwise indicated, on a 300-MHz Varian spectrometer. The chemical shifts are reported in units of δ. Melting points (mp) were determined using a Thomas-Hoover apparatus in capillary tubes and are uncorrected. Thin-layer chromatography (TLC) was carried out on EM plastic sheets precoated with silica gel 60 F-254 (Whatman). Visualization was obtained by exposure to 5% phosphomolybdic acid in 2-propanol. Flash chromatography was carried out on EM Reagent silica gel 60 (230−400 mesh), unless otherwise noted. $MgSO_4$ was used to dry all organic layers.

CH_2Cl_2 was distilled from calcium hydride. Tetrahydrofuran and ether were distilled from sodium/benzophenone. Pyridine was distilled from calcium hydride onto 3 Å molecular sieves. Benzene was dried over 4 Å molecular sieves for 6 h and then distilled onto 4 Å molecular sieves. All reagents, unless otherwise noted, were obtained from Aldrich Chemical Co.

6β-Methoxy-3α,5-cyclo-23-carboxyethyl-5α-cholest-24-one (22). To a solution of 23 mg (222 mmol) of sodium in 10 mL of absolute ethanol was added dropwise 35 mg (222 mmol) of ethyl isobutyrylacetate with stirring at room temperature under nitrogen. This mixture was then added dropwise to a suspension of 78 mg (171 mmol) of **21**, prepared by the method of Partridge[26] as modified previously,[25] in 10 mL of absolute ethanol at 45 °C and the mixture was heated at reflux (oil bath temperature 78−80 °C) with stirring for 24 h (**21** only becomes soluble at reflux). The ethanol was evaporated and the residue was dissolved in 25 mL of ether and washed with 25 mL of 10% HCl solution, 25 mL of saturated $NaHCO_3$ solution, and 25 mL of brine. The ether layer was dried, filtered, and evaporated to afford 99 mg of residue that was chromatographed (1:19 EtOAc/hexanes) to yield 36 mg (43%) of yellow oily **22**: ^{1}H NMR 4.21−4.09 (m, 2H), 3.72−3.66 (m, 1H), 3.30 (s, 3H), 2.80−2.74 (m, 1H), 1.04−1.02 (d, J = 7.2 Hz, 6H), 1.04−1.02 (d, J = 7.2 Hz, 3H), 0.95 (s, 3H), 0.62 (s, 3H); EI-HRMS (M+) calcd for $C_{31}H_{50}O_4$ 486.3709, found 486.3709.

3β-Hydroxycholest-5-en-24-one (13). According to a procedure by Greene[33] a mixture of 36 mg (74 mmol) of **22** and 600 mg of Al_2O_3 in 2 mL of 1% aqueous dioxane was heated at reflux (oil bath temperature 100−105 °C) with stirring under nitrogen for 96 h. Vacuum filtration through a pad of Celite followed by evaporation of the solvent afforded 30 mg of crude decarboxylated *i*-steroid, which was mixed with 1.4 mg (9.00 mmol) of *p*-toluenesulfonic acid monohydrate in 1 mL of 25% aqueous dioxane and heated (oil bath temperature 78−80 °C) with stirring for 5 h. The mixture was cooled to room temperature, quenched with 10 mL of saturated $NaHCO_3$ solution, and extracted with 3 × 10 mL of ether. The ether was dried, filtered, and evaporated to afford 20 mg of crude residue that was chromatographed (1:4 EtOAc/hexanes) to yield 10 mg (34%) of colorless **13**: mp 134−137 °C (lit.[30,50] mp 137−138 °C); ^{1}H NMR 5.28 (m, 1H), 3.52−3.39 (m, 1H), 1.04−1.02 (d, J = 7.2 Hz, 6H), 1.04−1.02 (d, J = 7.2 Hz, 3H), 0.95 (s, 3H), 0.62 (s, 3H); ^{13}C NMR ($CDCl_3$) 215.8, 141.0, 121.7, 71.8, 56.9, 56.0, 50.2, 42.5, 42.4, 41.0, 39.9, 37.4, 36.7, 35.6, 32.0, 31.7, 30.0, 29.9, 28.3, 24.4, 21.2, 19.6, 18.7, 18.5, 18.5, 12.0, 1.2.

23(*S*)- and 23(*R*)-Cholest-5-en-3β,23-diol (6 and 7). According to a procedure by Bailey[27] to a solution of 106 mg (0.23 mmol) of **21** in 3 mL of dry ether was added dropwise 0.37 mL (0.50 mmol) of 1.36 M *t*-BuLi in pentane at −78 °C with stirring under nitrogen. After stirring for 5 min, the mixture was allowed to warm slowly and stand at room temperature over 1 h. The mixture was cooled to −78 °C and treated dropwise with 40 mg (0.46 mmol) of isovaleraldehyde, stirred for 3 h, slowly warmed to room temperature, and stirred for 12 h. The mixture was quenched with saturated NH_4Cl solution, extracted with 2 × 10 mL of ether, and the ether was washed with 20 mL of $NaHCO_3$, 20 mL of brine, dried, filtered,

and evaporated to give 200 mg of crude residue. According to a procedure by Partridge,[26] this residue (200 mg) was combined with 13 mg (0.068 mmol) of *p*-toluenesulfonic acid in 4 mL of 25% aqueous dioxane and heated (oil bath temperature 79−80 °C) for 5 h. The mixture was cooled to room temperature, quenched with 10 mL of saturated $NaHCO_3$ solution, and extracted with 3 × 25 mL of CH_2Cl_2. The CH_2Cl_2 was dried, filtered, and evaporated to afford 160 mg of crude residue that was chromatographed (gradient: 1:9, 1:4, 1:2 EtOAc/hexanes) to yield 44 mg (48% from **21**) of a ca. 1:1 epimeric mixture of **6** and **7**. Pure samples were obtained by HPLC on a 5-μm chiral phase Bakerbond DNBPG column (4.6 × 25 mm, Beckman) using 96:4 hexanes:2-propanol. 23(*R*)-Hydroxycholesterol (more polar[29]) (**7**): mp 168.1−168.7 (lit.[29] mp 163−166 °C); ^{1}H NMR 5.32 (m, 1H), 3.79−3.70 (m, 1H), 3.56−3.45 (m, 1H), 0.99 (s, 3H), 0.92 (d, J = 7.5 Hz, 6H), 0.67 (s, 3H). 23(*S*)-Hydroxycholesterol (**6**): mp 160.8−161.4 (lit.[29] mp 160−162 °C); ^{1}H NMR ($CDCl_3$) 5.32 (m, 1H), 3.81−3.70 (m, 1H), 3.56−3.44 (m, 1H), 0.99 (s, 3H), 0.89 (d, J = 6.6 Hz, 6H), 0.69 (s, 3H).

***N,N*-Dimethyl-6*β*-methoxy-3α,5-cyclo-5α-cholanamide (23).** To a solution of 0.15 mL (1.07 mmol) of diisopropylamine in 1 mL of dry THF at −78 °C was added 0.55 mL (1.10 mmol) of 2.0 M *n*-BuLi under N_2. After the solution was stirred at −78 °C for 30 min, a solution of 98.3 mg (1.13 mmol) of *N*,*N*-dimethylacetamide in 1 mL of THF was added. The resulting mixture was stirred at −78 °C for 20 min, then at 0 °C for 20 min, cooled to −78 °C and a solution of 201 mg (0.440 mmol) of **21** in 2 mL of THF was added dropwise. The reaction mixture was stirred at −78 °C for 6 h, then at 0 °C overnight, diluted with 25 mL of saturated NH_4Cl, and extracted with 3 × 15 mL of CH_2Cl_2. The combined organic layers were washed with 10 mL of brine, dried, filtered, and evaporated to give 225 mg of residue which was chromatographed (1:4 EtOAc:hexane) to give 170.2 (93%) of oily **23**: ^{1}H NMR 3.28 (s, 3H), 2.97 (s, 3H), 2.89 (s, 3H), 2.73 (t, J = 2.7 Hz, 1H), 0.98 (s, 3H), 0.90 (d, J = 6.3 Hz, 3H), 0.68 (s, 3H); ^{13}C NMR 173.8, 82.6, 56.7, 56.3, 48.2, 43.6, 43.0, 40.5, 37.5, 35.9, 35.6, 35.5, 35.2, 33.6, 31.4, 30.7, 30.6, 28.5, 25.2, 24.4, 23.0, 21.7, 19.5, 18.7, 13.3, 12.5; EI-HMRS (M^+) calcd for $C_{27}H_{45}NO_2$ 415.3450, found 415.3445.

***N,N*-Dimethyl-3*β*-hydroxycholenamide (14).** To a solution of 132.7 mg (0.320 mmol) of **23** in 3.5 mL of dioxane and 1.0 mL of H_2O was added 29.5 mg of *p*-toluenesulfonic acid monohydrate. The resulting mixture was heated at reflux for 5 h, diluted with 10 mL of H_2O, and extracted with 3 × 20 mL of CH_2Cl_2. The combined organic layers were washed with 10 mL of saturated $NaHCO_3$, 10 mL of H_2O, dried, filtered, and evaporated to give 140 mg of residue which was chromatographed (3:17 EtOAc:hexane) to give 86.8 mg (68%) of **14**: mp 187.5−189.5 °C (lit.[34] mp 189.5−190 °C); ^{1}H NMR 5.34 (m, 1H), 3.52 (m, 1H), 3.00 (s, 3H), 2.94 (s, 3H), 1.00 (s, 3H), 0.94 (d, J = 6.6 Hz, 3H), 0.68 (s, 3H); ^{13}C NMR 174.0, 141.1, 121.9, 72.0, 57.0, 56.2, 50.4, 42.6, 42.5, 40.0, 37.6, 37.5, 36.8, 36.0, 35.7, 32.2, 31.9, 31.5, 30.7, 28.5, 24.6, 21.4, 19.7, 18.8, 12.2.

***N*-Methyl-3*β*-hydroxycholenamide (15).** According to a procedure by Okamoto,[51] to a solution of 98.3 mg (0.262 mmol) of **18** (Sigma) in 2 mL of dry DMF were added 136.3 mg (0.899 mmol) of *t*-BuMe_2SiCl, 146.0 mg (2.17 mmol) of imidazole and 71.0 mg (0.585 mmol) of 4-(dimethylamino)pyridine at 0 °C. The mixture was stirred at room temperature for 6 h under N_2, diluted with 10 mL of H_2O, and extracted with 3 × 10 mL of EtOAc. The combined organic layers were washed with 10 mL of H_2O, 10 mL of brine, dried, filtered, and evaporated to give 144 mg of residue. According to procedures by Firestone[52] and Cope,[53] to a solution of 140 mg of this material in 2 mL of dry CH_2Cl_2 were added slowly 0.2 mL (2 mmol) of $(COCl)_2$ and 1 drop of dry DMF. The resulting mixture was stirred at room temperature under N_2 for 2 h and the solvent was evaporated to give a yellow solid residue, to which was added ca. 120 mmol of CH_3NH_2, generated using the method of Russel[32] by dripping 10 mL of 40 wt % aqueous CH_3NH_2 onto solid KOH pellets and passing the resultant gas through a KOH drying tube before being condensed with a coldfinger at −78 °C. The mixture was stirred at −78 °C overnight, then at room temperature for 2 h, diluted with 20 mL of H_2O, and extracted with 2 × 20 mL of Et_2O, and 3 × 20 mL of CH_2Cl_2. The combined organic layers were dried, filtered and evaporated to give 124 mg of solid which was chromatographed (3:1 EtOAc:hexane), then recrystallized from acetone to give 63.8 mg (63% from **18**) of **15**: mp 223−224 °C; IR ν_{max} 3312, 1642, 1555 cm^{-1}; ^{1}H NMR 5.37 (m, 2H), 3.53 (m, 1H), 2.81 (d, J = 4.8 Hz, 3H), 1.01 (s, 3H), 0.93 (d, J = 6.3 Hz, 3H), 0.69 (s, 3H); ^{13}C NMR (CD_3OD) 177.5, 142.4, 12.6, 72.6, 58.3, 57.4, 51.9, 43.7, 43.2, 41.3, 38.7, 37.8, 37.0, 34.1, 33.5, 33.4, 33.2, 32.4, 29.3, 26.5, 25.5, 22.3, 20.0, 19.0, 12.5; HRMS (MH^+) calcd for $C_{25}H_{42}NO_2$ 388.3216, found 388.3204. Anal. ($C_{25}H_{41}NO_2$) C, H, N.

22-Azido-6*β*-methoxy-3α,5-cyclo-5α-23,24-bisnorcholane (24). According to a modification of a procedure by Koziara,[54] to a solution of 201 mg (0.441 mmol) of **21** in 2 mL of DMF and 2 mL of benzene was added 74.8 mg (1.15 mmol) of NaN_3. The mixture was heated at reflux overnight under N_2, diluted with 10 mL of H_2O, and extracted with 3 × 10 mL of benzene. The combined organic layers were dried, filtered, and evaporated to give 213.5 mg of a colorless oil, which was chromatographed (3:97 EtOAc:hexane) to give 155.4 mg (95%) of oily **24**: IR ν_{max} 2096 cm^{-1}; ^{1}H NMR 3.35 (dd, J = 3.3, 11.7 Hz, 1H), 3.31 (m, 3H), 3.03 (dd, J = 7.2 Hz, J = 11.7 Hz, 1H), 2.76 (m, 1H), 1.03 (d, J = 6.6 Hz, 3H), 1.01 (s, 3H), 0.71 (s, 3H); ^{13}C NMR 82.6, 58.3, 56.8, 56.5, 53.6, 48.2, 43.6, 43.2, 40.3, 37.2, 35.5, 33.6, 30.8, 28.2, 25.2, 24.5, 23.0, 21.7, 19.5, 18.0, 13.4, 12.6; Anal. ($C_{23}H_{37}N_3O$) C, H, N.

22-Amino-6*β*-methoxy-3α,5-cyclo-5α-23,24-bisnorcholane (25). According to a procedure by Bose,[55] to a solution of 119 mg (0.320 mmol) of azide **24** in 5 mL of dry ether was added 86.7 mg (2.29 mmol) of $LiAlH_4$. The mixture was heated at reflux for 3 h under N_2, diluted with 10 mL of wet ether and 10 mL of H_2O at 0 °C, and extracted with 3 × 20 mL of ether. The combined organic layers were dried, filtered, and evaporated to give 122 mg of yellow oil which was chromatographed (88:12:2 EtOAc:MeOH:concd NH_4OH) to give 86.8 mg (79%) of colorless oily **25**: IR ν_{max} 3384 cm^{-1}; ^{1}H NMR 3.28 (s, 3H), 2.73 (m, 1H), 2.70 (m, 1H), 2.36 (dd, J = 12.6 Hz, 7.2 Hz, 1H), 0.98 (s, 3H), 0.94 (d, J = 6.6 Hz, 3H), 0.69 (s, 3H); ^{13}C NMR 82.5, 56.7, 56.5, 53.8, 48.1, 47.7, 43.5, 42.9, 40.3, 39.1, 35.4, 35.2, 33.5, 30.6, 28.0, 25.1, 24.4, 22.9, 21.6, 19.4, 17.0, 13.2, 12.5; EI-HRMS (M^+) calcd for $C_{23}H_{39}NO$ 417.3032, found 417.3030.

22-[*N*-(2′-Methylpropionyl)amino]-23,24-bisnorchol-5-en-3*β*-ol (16). To a solution of 39.7 mg (0.115 mmol) of **25** in 4 mL of dry ether were added dropwise 0.3 mL (3.75 mmol) of dry pyridine and 0.8 mL (7.6 mmol) of isobutyryl chloride in −78 °C under N_2. The mixture was stirred at −78 °C for 1 h, then at room temperature overnight, diluted with 10 mL of H_2O, and extracted with 3 × 20 mL of ether. The combined organic layers were washed with 10 mL of brine, dried, filtered, and evaporated to give 53.0 mg of an oil, to which was added 23.7 mg (0.125 mmol) of *p*-toluenesulfonic acid monohydrate in 3 mL of dioxane and 1 mL of H_2O. The resulting mixture was heated at reflux for 5 h, diluted with 10 mL of H_2O, and extracted with 3 × 20 mL of CH_2Cl_2. The combined organic layers were washed with 10 mL of saturated $NaHCO_3$, 10 mL of H_2O, dried, filtered, and evaporated to give 47.0 mg of residue which was chromatographed (3:2 EtOAc:hexane) to give 21.1 mg (44% from **25**) of **16**: mp 180.5−181.5 °C; ^{1}H NMR 5.46 (m, 1H), 5.34 (m, 1H), 3.52 (m, 1H), 3.34 (m, 1H), 2.95 (m, 1H), 2.30 (m, 3H), 1.15 (d, J = 6.6 Hz, 6H), 1.01 (s, 3H), 0.96 (d, J = 6.6 Hz, 3H), 0.69 (s, 3H); ^{13}C NMR (CD_3OD) 177.2, 140.1, 121.8, 71.9, 56.7, 54.3, 50.3, 45.0, 42.7, 42.5, 39.8, 37.5, 36.8, 36.7, 36.1, 32.1, 32.0, 31.8, 28.1, 24.6, 21.2, 20.0, 19.9, 19.6, 17.6, 12.1; FAB-HRMS (MH^+) calcd for $C_{26}H_{44}NO_2$ 402.3372, found 402.3379. Anal. ($C_{26}H_{43}NO_2$) C, H, N.

22-[*N*-(*N,N*-Dimethylformamido)amino]-23,24-bisnorchol-5-en-3*β*-ol (17). To a solution of 67.7 mg (0.196 mmol) **25** in 3 mL of ether and 0.3 mL (4 mmol) of dry pyridine at −78 °C was added dropwise 0.5 mL (6 mmol) of dimethylcarbamyl chloride under N_2. The resulting mixture was stirred

at −78 °C for 1 h, then at room temperature for 17 h, diluted with 10 mL of H_2O, and extracted with 3 × 20 mL of CH_2Cl_2. The combined organic layers were washed with 3 × 10 mL of 10% KOH, 10 mL of brine, dried, filtered, and evaporated to give 135 mg of red-yellow residue, which was chromatographed (1:1 EtOAc:hexane) to give 57.4 mg (70%) of oily **26**: ^{1}H NMR 4.39 (m, 1H), 3.35 (m, 1H), 3.30 (s, 3H), 2.89 (s, 6H), 2.83 (m, 1H), 2.75 (t, J = 2.7 Hz, 1H), 1.00 (s, 3H), 0.95 (d, J = 6.6 Hz, 3H), 0.72 (s, 3H); ^{13}C NMR 158.8, 82.5, 56.7, 56.5, 54.5, 48.2, 46.6, 43.5, 43.1, 40.3, 37.2, 36.4, 35.4, 35.2, 33.5, 30.6, 28.2, 25.1, 24.4, 22.9, 21.7, 19.4, 17.6, 13.2, 12.5; EI-HRMS (M^+) calcd for $C_{26}H_{44}N_2O_2$ 416.3403, found 416.3406. To 52.1 mg (0.125 mmol) of this **26** was added 25.5 mg (0.134 mmol) of *p*-toluenesulfonic acid monohydrate and 3 mL of dioxane and 1 mL of H_2O. The resulting mixture was refluxed overnight, diluted with 10 mL of H_2O, and extracted with 3 × 15 mL of CH_2Cl_2. The combined organic layers were washed with 10 mL of saturated $NaHCO_3$, 10 mL of H_2O and 10 mL of brine, dried, filtered, and evaporated to give 63 mg of residue which was crystallized twice from CH_2Cl_2−hexane to give 40.4 mg (80%) of **17**: mp 203−204 °C; ^{1}H NMR 5.35 (m, 1H), 4.38 (m, 1H), 3.52 (m, 1H), 3.38 (m, 1H), 2.91 (s, 6H), 2.85 (m, 1H), 1.00 (s, 3H), 0.97 (d, J = 6.6 Hz, 3H), 0.70 (s, 3H); ^{13}C NMR 158.8, 141.1, 121.8, 71.9, 56.8, 54.3, 50.3, 46.6, 42.7, 42.5, 39.9, 37.5, 37.2, 36.7, 36.4, 32.1, 31.8, 29.9, 28.1, 24.6, 21.3, 19.6, 17.6, 12.2; EI-HRMS (M^+) calcd for $C_{25}H_{42}N_2O_2$ 402.3246, found 402.3247.

22-[*N*-(*N*,*N*-Dimethylformamido)amino]-3β-chloro-23,24-bisnorchol-5-ene (27). To a solution of 99.8 mg (0.289 mmol) of **25** in 4 mL of ether and 0.4 mL (5 mmol) of dry pyridine at −78 °C was added dropwise 0.7 mL (8 mmol) of dimethylcarbamyl chloride under N_2. The resulting mixture was stirred at −78 °C for 1 h, then at room temperature for 17 h, diluted with 10 mL of H_2O, and extracted with 3 × 20 mL of CH_2Cl_2. The combined organic layers were washed with 10 mL of saturated $NaHCO_3$, 10 mL of brine, dried, filtered, and evaporated to give 331 mg of red-yellow residue, to which was added 38.8 mg (0.204 mmol) of *p*-toluenesulfonic acid monohydrate, 3 mL of dioxane, and 1 mL of H_2O. The resulting mixture was refluxed overnight, diluted with 10 mL of H_2O, and extracted with 3 × 15 mL of CH_2Cl_2. The combined organic layers were washed with 10 mL of saturated $NaHCO_3$, 10 mL of H_2O and 10 mL of brine, dried, filtered, and evaporated to give 125 mg of residue which was chromatographed (3:1 EtOAc:hexane) to give 25.4 mg (22%) of **17** and 45.4 mg (37%) of **27**: mp 229−231 °C; ^{1}H NMR 5.37 (m, 1H), 4.38 (m, 1H), 3.76 (m, 1H), 3.39 (ddd, J = 3.6 Hz, 5.4 Hz, 13.2 Hz, 1H), 2.91 (s, 6H), 2.85 (m, 1H), 1.03 (s, 3H), 0.97 (d, J = 6.6 Hz, 3H), 0.70 (s, 3H); ^{13}C NMR 158.8, 140.9, 122.6, 60.5, 56.6, 54.3, 50.2, 46.6, 43.6, 42.7, 39.7, 39.3, 37.2, 36.5, 36.4, 33.5, 32.0, 31.9, 28.1, 24.5, 21.1, 19.4, 17.6, 12.1. Anal. ($C_{25}H_{41}ClN_2O$) C, H, N.

LiSA. A modified polyhistidine tag (MKKGHHHHHHG) was fused in frame of the human LXRα ligand-binding domain (amino acids 183−447 of GenBank accession number U22662, with the 14th amino acid corrected to A from R). The LXRα fusion protein was expressed in *E. coli* and purified as previously described.[8,21] The purified protein was diluted to approximately 10 μM in PBS and a 5-fold molar excess of NHS-LC-Biotin (Pierce) was added in a minimal volume of PBS. This solution was incubated with gentle mixing for 30 min at ambient room temperature. The biotinylation reaction was stopped by the addition of 2000-fold molar excess of Tris-HCl, pH 8. The modified LXRα protein was dialyzed against 4 buffer changes, each of at least 50 volumes, with PBS containing 5 mM DTT, 2 mM EDTA and 2% sucrose. The biotinylated LXRα protein was subjected to mass spectrometric analysis to reveal the extent of modification by the biotinylation reagent. In general, approximately 95% of the protein had at least a single site of biotinylation; the overall extent of biotinylation followed a normal distribution of multiple sites, ranging from 1 to 9.

The biotinylated protein was incubated for 20−25 min at a concentration of 20 nM in assay buffer (50 mM NaF, 50 mM MOPS, pH 7.5, 0.1 mM CHAPS, 0.1 mg/mL FAF-BSA, 10 mM DTT) with equimolar amounts of streptavidin-AlloPhycoCyanin (APC, Molecular Probes). At the same time, a biotinylated peptide comprising amino acids 675−699 of SRC1 (CPSSH-SSLTERHKILHRLLQEGSPS-$CONH_2$) at a concentration of 20 nM was incubated in assay buffer with an equimolar amount of streptavidin-labeled europium (Wallac) for 20−25 min. After the initial incubations were completed, a 20 molar excess (400 nM) of biotin was added to each of the solutions to block the unattached streptavidin reagents. After 20 min at room temperature, the solutions were mixed, yielding a concentration of 10 nM for the dye-labeled LXRα protein and SRC1 peptide. 49 μL of the protein/peptide mixture was added to each well of an assay plate containing 1 μL of test compound. The final volume in each well was 0.05 mL, and the concentration in the well for the dye-labeled protein and peptide was 10 nM. The final test compound concentrations were between 1 nM and 100 μM. The plates were incubated at room temperature for 2−4 h and then counted on a Wallac Victor fluorescent plate reader in a time-resolved mode. The relative fluorescence was measured at 665 nm.

Plasmids. Expression vectors were generated by insertion of cDNA encoding human LXRα (amino acids 162−447) fused in-frame with the GAL4 DNA binding domain in the pSG5 vector (Stratagene, La Jolla, CA). The Trp^{443} codon (TGG nucleotide sequence) of LXRα was altered to a Phe (TCC), Tyr (TAC), Leu (CTG), Cys (TGC), Ala (GCG), or Thr (ACG) codon by Quick-Change Mutagenesis (Stratagene, La Jolla, CA).

Cell-Based Reporter Assay. Cell-based assays for LXRα activity were performed as previously described[5] using CV-1 cells transfected with a chimeric LXRα-GAL4 receptor expression plasmid and a $(UAS)_5$-tk-SPAP reporter plasmid. Transfections were performed using Lipofectamine (Life Technologies, Grand Island, NY) according to manufacturer's instructions. A β-galactosidase expression plasmid was included in each transfection for use as a normalization control.

Western Blotting. Equal amounts of total cellular protein were electrophoresed on 12% SDS−PAGE and transferred to Trans-Blot nitrocellulose membranes (Bio-Rad, Hercules, CA). LXRα-GAL4 chimeric proteins were visualized using an ECL detection system (Amersham Pharmacia Biotech, Piscataway, NY) after incubation with mouse monoclonal anti-GAL4 DNA binding domain antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a horseradish peroxidase-linked goat anti-mouse secondary antibody (Southern Biotech Associates, Birmingham, AL).

Homology Model. A model for the LXRα ligand-binding domain was built from the crystal structure of RARγ bound to all-*trans*-retinoic acid[44] using the MVP program.[56] Compared with RARγ, LXRα has one single-residue insertion, located between the predicted helices 6 and 7, and two single-residue deletions, located in the loops leading into helix 3 and the AF2 helix. The insertion and deletions all lie 10 Å or more from the proposed ligand-binding site, and no special effort was made to model their effects. The MVP buildup procedure was used to obtain low-energy conformations for mutated side chains in the binding site region. This involved a search of 90 side chain torsional coordinates, with torsional coordinate energy minimization during the buildup process and Cartesian coordinate energy minimization after the buildup was complete. The all-*trans*-retinoic acid molecule from RARγ was included in these calculations to bias side chains away from the ligand-binding site. 24(*S*),25-Expoxycholesterol (**1**) was manipulated into the LXRα binding site graphically, considering several alternative conformations of the sterol side chain and several orientations of the steroid ring system. The initial conformation of **1** was taken from subunit A of the small-molecule crystal structure.[25]

Acknowledgment. The research at Dartmouth College was supported by NIH Grant HL52069.

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JM0004749