Identification of a Novel Liver X Receptor Agonist that Regulates the Expression of Key Cholesterol Homeostasis Genes with Distinct Pharmacological Characteristics

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ABSTRACT

Activation of liver X receptor (LXR) is associated with cholesterol metabolism and anti-inflammatory processes, which makes it beneficial to antiatherosclerosis therapy. Nevertheless, existing agonists that target LXR, for example TO901317, are related to unwanted side effects. In the present study, using a screening method we identified IMB-808, which displayed potent dual LXRα/β agonistic activity. In vitro, IMB-808 effectively increased the expression quantity of genes related to reverse cholesterol transport process as well as those associated with cholesterol metabolism pathway in multiple cell lines. Additionally, IMB-808 remarkably promoted cholesterol efflux from RAW264.7 as well as THP-1 macrophage cells and reduced cellular lipid accumulation accordingly. Interestingly, compared with TO901317, IMB-808 almost did not increase the expression quantity of genes related to lipogenesis in HepG2 cells, which indicated that IMB-808 could exhibit fewer internal lipogenic side effects with a characteristic of selective LXR agonist. Furthermore, in comparison with the full LXR agonist TO901317, IMB-808 recruits coregulators differently and possesses a distinct predictive binding pattern for the LXR ligand-binding domain. In summary, our study demonstrated that IMB-808 could act as an innovative partial LXR agonist that avoids common lipogenic side effects, providing insight for the design of novel LXR modulators. Our data indicate that this compound might be used as a promising therapeutic agent for the prospective treatment of atherosclerosis in the future.

Introduction

Atherosclerosis, as the dominant cause of mortality in developed nations, is gradually becoming a health issue around world (Roger et al., 2012). The liver X receptor (LXR) was capable of inhibiting inflammatory reactions driven by macrophages and promoting the process of reverse cholesterol transport (RCT) which made it a prospective target for treating atherosclerosis (Joseph et al., 2003; Naik et al., 2006; Zhang et al., 2012). LXRs, including LXRα and LXRβ, bind to response elements of their target genes to modulate gene expression (Edwards et al., 2002). Although the expression range of LXRβ is ubiquitous, LXRα is expressed exclusively in kidney, lung, intestine, adipose tissue, liver, as well as certain kinds of immunocytes (Auboeuf et al., 1997; Heine et al., 2009). Activation or repression of LXR depends on the presence or absence of its ligands. In the absence of ligands, LXRs are in a nonactive state, combining to corepressors, for example, the nuclear receptor corepressor (NcoR) (Chen and Evans, 1995). The binding of ligands results in a change of the conformation of LXRs that enables corepressors to be released, coactivators to be recruited, and the target genes to be transactivated (Wiebel et al., 1999).

LXRs serve as a sensor of cholesterol that protects cells from the adverse effect of overloaded cholesterol by inducing the expression of target genes. RCT is a primary mechanism for removing cholesterol from cells and transferring it into the liver, which can be stimulated by LXRs (Beltowski, 2008). Several proteins of the ATP-binding cassette (ABC) transporter family contribute to cholesterol metabolism regulation and are regarded as target genes of LXRs. ABCA1 and ABCG1 play important roles in cholesterol efflux, and ABCG5 and

ABBREVIATIONS: ABC, ATP-binding cassette; ApoA-I, apolipoprotein A-I; ApoE, apolipoprotein E; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; FAS, fatty acid synthase; FL, fluorescein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione S-transferase; HDL, high-density lipoprotein; LBD, ligand-binding domain; LXR, liver X receptor; NcoR, nuclear receptor corepressor; NPC1L1, Niemann-Pick C1 like 1; ox-LDL, oxidized low-density lipoprotein; PBS, Phosphate-buffered saline; PCR, polymerase chain reaction; PDB, Protein Data Bank; RCT, reverse cholesterol transport; SCD-1, stearoyl-coenzyme A desaturase 1; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; SREBP-1c, sterol response element binding protein 1c; Tb, terbium; TR-FRET, time-resolved fluorescence resonance energy transfer.
ABCG8 influence hepatic cholesterol excretion and intestinal absorption (Yu et al., 2003; Cavelier et al., 2006; Wang, 2007). LXR can also increase apolipoprotein E (ApoE) expression, which serves as a critical regulator for atherogenesis to maintain cholesterol homeostasis (Laffitte et al., 2001; Parikh et al., 2014). Moreover, Niemann-Pick C1 like 1 (NPC1L1) is indispensable for absorbing cholesterol in intestine. According to reports, the expressing of NPC1L1 can be downregulated by LXR activators in human intestinal and mouse cells (Duval et al., 2006).

A previous report (Schuster et al., 2002) demonstrated that either LXRα or LXRβ could exert an effort on the antiatherosclerosis role of macrophage cells while lacking LXRα and LXRβ led to accumulating lipids in the foam cells of lesions in arteries. Several synthesized LXR ligands, for example full agonists TO901317 and GW3966, were widely focused on and studied substantially for many years (Janowski et al., 1996; Houck et al., 2004; Geyeregger et al., 2006). Nevertheless, these ligands have not yet been developed as drugs because of their undesirable side effects. Through the activation of hepatic sterol regulatory element–binding protein-1c (SREBP-1c), the ligands could induce lipogenesis and hypertriglyceridemia (Peet et al., 1998; Schultz et al., 2000). Consequently, particular LXR activators that induced no hepatic synthesis of fatty acids interested us. It was reported that such ligands, for example N, N-dimethyl-3β-hydroxy-cholenamide and WAY-252623, could reduce atherosclerosis without lipogenesis increasing and SREBP-1c activating (Kratzer et al., 2009; Quinet et al., 2009). This finding increased the probability that some LXR agonists with the effect of treating atherosclerosis might reduce atherosclerosis without lipogenesis increasing and ABCG8 influence hepatic cholesterol excretion and intestinal absorption (Yu et al., 2003; Cavelier et al., 2006; Wang, 2007). LXR can also increase apolipoprotein E (ApoE) expression, which serves as a critical regulator for atherogenesis to maintain cholesterol homeostasis (Laffitte et al., 2001; Parikh et al., 2014). Moreover, Niemann-Pick C1 like 1 (NPC1L1) is indispensable for absorbing cholesterol in intestine. According to reports, the expressing of NPC1L1 can be downregulated by LXR activators in human intestinal and mouse cells (Duval et al., 2006).

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In our current study, we identified IMB-808, which was an innovative analog of benzo-dioxepine-carboxamide exhibiting impressive activity of LXR agonist using a cell-based luciferasen reporter assay. We found that IMB-808 had an effect on LXR target genes and influenced some cholesterol metabolism–related pathways in multiple cells. Moreover, the molecular docking result provided us with a theoretical basis to study the interaction site between this compound and the construction of both LXRα and LXRβ ligand-binding domains (LBDs). Furthermore, based on the coregulator recruitment and site mutation activation assays, the possible mechanism of LXRα/β interaction with IMB-808 was clarified.

Materials and Methods

Reagents. TO901317, which was also called T1317 in the current study, accompanied by phorbol-12-myristate-13-acetate and Oil Red O stain were obtained from Sigma-Aldrich (St. Louis, MO). IMB-808 was obtained from the compound library of the National Laboratory for Screening New Microbial Drugs, Institute of Medicinal Biotechnology, Peking Union Medical College (Beijing, People’s Republic of China). Opti-MEM Reduced Serum Medium as well as fetal bovine serum were obtained from Invitrogen (ThermoFisher Scientific, Carlsbad, CA). RPMI 1640 medium, Dulbecco’s modified Eagle’s medium as well as modified Eagle’s medium were obtained from Hyclone (Thermo Scientific, Rockford, IL). 22-NBD-cholesterol and Lipofectamine 2000 were obtained from Invitrogen. Oxidized low-density lipoprotein (ox-LDL), apolipoprotein A-I (ApoA-I) as well as high-density lipoprotein (HDL) were bought from Union Biology Company (Beijing, People’s Republic of China).

Plasmids. The wild-type genes of human LXRα-LBD and LXRβ-LBD were obtained by polymerase chain reaction (PCR) from HepG2 cells and cloned into pBIND vector (Promega, Madison, WI), in which GAL4 DNA binding domain existed. GAL4-pGL4-luc plasmids were prepared as similarly described before (Li et al., 2013).

The method of mutagenesis directed by sites was used to create mutations in pBIND-LXRα-LBD or pBIND-LXRβ-LBD using the Fast Mutagenesis System (TransGen Biotech, Beijing, People’s Republic of China). A few important amino acids of LXRα-LBD were converted into other molecules. The plasmids were mutated according to the following pattern: F257Y (Phe257 to Tyr), T302I (Thr302 to Ile), R305G (Arg305 to Gly), H421D (His421 to Asp), and W443G (Trp443 to Gly). Meanwhile, key amino acids in LXRβ-LBD were changed to alanines accordingly: F271A (Phe271 to Ala), M312A (Met312 to Ala), T316A (Thr316 to Ala), H435A (His435 to Ala), and W457A (Trp457 to Ala). Sequencing was used to verify the successful pBIND-LXRα-LBD and pBIND-LXRβ-LBD mutated plasmids.

Human LXRα-LBD (amino acids 182–447) and LXRβ-LBD cDNA (amino acids 196–461) were cloned into pET30a vector separately. To construct the mutation expression plasmids, Arg305 was subsequently mutated to Gly in pET30a-LXRα-LBD (named pET30a-LXRα-R305G), whereas Phe271 was mutated to Ala in pET30a-LXRβ-LBD (named pET30a-LXRβ-P271A).

The Reporter Assay of LXR-GAL4 Chimera and Cell Culture. In brief, RAW264.7 macrophages, HepG2, HEK293T, Caco-2 cells as well as the human monocyte cell line THP-1 were cultured in different media separately, as described previously (Li et al., 2014). Phorbol-12-myristate-13-acetate 100 nM was added to THP-1 cells and incubated for 24 hours to produce fully differentiated macrophages, after which serum-free medium was used to replace the former medium.

IMB-808 was identified through the screening of a synthesized compound library that contained 20,000 drug-like constructions, as described previously (Li et al., 2016). In the activity assay of IMB-808, HEK293T cells were transfected by pBIND-LXRα-LBD (or pBIND-LXRβ-LBD) expression plasmid as well as GAL4-pGL4-luc reporter plasmid by Lipofectamine 2000 and incubated for 6 hours before being treated by compounds for 18 hours. The Luciferase Assay System (Promega) was used to determine luciferase activity using a microplate reader (PerkinElmer, Waltham, MA).

Real-Time Quantitative Reverse-Transcription PCR Analysis. HepG2 and Caco-2 cells were inoculated in six well plates with the density of × 104 cells/ml. RAW264.7 macrophages and human THP-1 monocytes were cultured in six-well plates with densities of 6 × 104 and 1.5 × 105 cells/well, respectively. TRIzol reagent (ThermoFisher Scientific) was used to extract total RNA of the cells, whereas reverse transcriptional kits (TransGen Biotech) were used to reverse transcribe it. The real-time quantitative PCR assay was performed on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) by using SYBR Green (Roche Diagnostics, Lewes, UK) detecting reagents. Table 1 shows the primer sequences used in this study. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level was used to normalize all mRNA expression quantities, and the ΔΔCt method was used to conduct quantitative measurement.

Western Blotting. RAW264.7 macrophages, HepG2 cells, THP-1 human monocytes, and Caco-2 cells were cultured as described above. Varied concentrations of IMB-808 were added into cells after which the cells had established attachment (24 hours). Cells were collected after 18 hours of incubation, and protein samples were extracted according to the above-mentioned protocol. The protein samples were detected using the corresponding primary antibodies and then incubated with secondary anti-rabbit and anti-mouse IgG antibodies (1: 5000; Novus, Littleton, CO). An Enhanced Chemiluminescence reaction kit (EMD Millipore, Billerica, MA) was used for blot detection. The following primary antibodies were used: anti-GAPDH (1:2000; Abmart; Shanghai, People’s Republic of China), anti-ApoE (1:1000; Abcam, Cambridge, UK), anti-ABCG1 (1:500; Novus), anti-ABCA1 (1:1000; Novus), anti-ABCG5 (1:1000; Abcam), anti-ABCG8 (1:500; Novus).
Novus), anti-NPC1L1 (1:1000; Abcam), and anti-SREBP-1c (1:1000; Novus). GAPDH was used to normalize entire proteins.

**Oil Red O Staining.** Oil Red O staining was used to evaluate accumulated cellular lipids in RAW264.7 macrophages. The cells were cultured in 96-well plates and supplemented with 60 μg/ml ox-LDL after attachment. After 12 hours, they were stained with varied concentrations of IMB-808 for 18 hours then fixed and stained with Oil Red O according to previous methods, and observed by light microscopy (Li et al., 2013). To extract Oil Red O, isopropanol was added to each well. The samples were measured at 510 nm by a microplate reader 10 minutes after that (Zou and Shen, 2007).

**Cholesterol Efflux Experiment.** 22-NBD-cholesterol was used to conduct a cell cholesterol efflux experiment on RAW264.7 macrophages and THP-1 monocytes separately (Li et al., 2014). Briefly, the cells were seeded into 96-well clear-bottom black plates (Costar; Corning, Corning, NY) and marked using 22-NBD-cholesterol with a final concentration of 2.0 μM in serum-free medium that contained 0.2% (w/v) bovine serum albumin (medium A; Sigma-Aldrich) for 24 hours. Phosphate-buffered saline (PBS) was used to wash cells twice, after which the cells were incubated with IMB-808 or vacant control for an additional 18 hours. Subsequently, the receptor protein of ApoA-I or HDL with final concentrations of 10 or 50 μg/ml was added into cells and incubated for 6 hours. A microplate reader was used to test the quantities of cholesterol in cells as well as of the medium separately (PerkinElmer; excitation 485 nm, emission 535 nm). The calculating mode of the 22-NBD-cholesterol efflux percentage was (medium)/(medium - cell) × 100%. Each efflux efflux was conducted in triplicate.

**Expression and Purification of LRXα-R305G and LRXβ-F271A.** The pET30a-LRXα-R305G and pET30a-LRXβ-F271A plasmids were transformed into Rosetta (DE3) Escherichia coli cells separately. The expression of the two proteins was induced by the addition of 0.2 mM isopropyl β-D-thiogalactopyranoside at 20°C overnight. The supernatants of LRXα-R305G and LRXβ-F271A were harvested by centrifugation then filtered through a 0.45 μM filter and loaded onto a Ni²⁺ His Trap chelating column (GE Healthcare, Little Chalfont, UK). The binding buffer contained 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 20 mM imidazole. LRXα-R305G protein was eluted by 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 200 mM imidazole, whereas LRXβ-F271A protein was eluted by 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 250 mM imidazole. The purified proteins were concentrated to 1 mg/ml by ultrafiltration and subsequently stored in the buffer of 50 mM potassium phosphate (pH 8.0), 150 mM KCl, 0.5 mM EDTA, 0.5% CHAPS, 5 mM dithiothreitol, and 20% glycerol at -80°C.

**LanthaScreen Time-Resolved Fluorescence Energy Transfer (LTXR-coregulator Peptide Interaction assays).** LanthaScreen time-resolved fluorescence energy transfer (TR-FRET) LXR-Coactivator Assay Kit (PV4665; ThermoFisher Scientific) and LXRβ-Coactivator Assay Kit (PV4658; ThermoFisher Scientific) were used to perform TR-FRET LXR-coregulator peptide interaction assays according to the manufacturer instructions separately. Human glutathione S-transferase (GST)-LXRα-LBD, GST-LXRβ-LBD, terbium (Tb)-labeled anti-GST tag antibody, fluorescein (FL)-labeled peptides, including FL-TRAP220/DRIP2 (PV5439), FL-DCD2 (PV4387), FL-NeoR (Neo ID2, PV4624), and FL-silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) (SMRTID2, PV4423), as well as all of the buffers were included in the current study. TO901317 or IMB-808 was diluted and first added to 384-well black plates (Costar) according to the kit instructions. LXRα-LBD or LXRβ-LBD protein was then added, followed by mixed coregulator and FL-peptide/Tb-anti-GST, which was added last.

Human LXRα-R305G and LXRβ-F271A mutation proteins were obtained as described before. Tb-labeled anti-His tag antibody (PV5866, Invitrogen) was purchased from Invitrogen. TR-FRET assay of mutation LXR and coregulator interaction was performed under the similar condition. TO901317 or IMB-808 was diluted and added to the plate, LRXα-R305G (final concentration 30 nM) or LXRβ-F271A (final concentration 100 nM) was then added, followed by coregulator and FL-peptide/Tb-anti-His mixed together.

The plates were shaken in darkness under ambient temperature for 2 hours. A PerkinElmer EnVision plate reader was used to measure the TR-FRET ratio (520/495 nm) of all assay wells, and the emission signal at 520 nm was divided by the emission signal at 495 nm to obtain the data. Each assay for each FL peptide was performed four times separately (n = 4).

**Virtual Molecular Docking.** The ligand action of IMB-808 was evaluated by the docking program Discovery Studio 4.1 (Accelrys, San Diego, CA) with the crystal structure of LRXα [Protein Data Bank (PDB) code: 1UHL, LRXα with TO901317] and LRXβ (PDB code: 1PQC, LRXβ with TO901317) separately. After removing all crystallized H2O molecules from the former construction, hydrogen was added into the DS CDOCKER module. An optimized start conformation was obtained by minimizing the compound to achieve the lowest energy level ahead of docking in silico.

**Statistical Analysis.** The software of GraphPad Prism 5.0 (GraphPad, San Diego, CA) was used to calculate statistics as well as best-fit curves. The data were represented as the mean ± SEM. One-way analysis of variance and Student’s t test were used to analyze results with SPSS version 11.0 (SPSS Inc., Chicago, IL). A P value of <0.05 was regarded as statistically significant (*P < 0.05; **P < 0.01; and ***P < 0.001).

**Results**

**IMB-808 Displays LXRα/β Dual-Agonist Activity.** In this study, IMB-808, an LRXβ agonist with an analogous structure of benzodioxepine-carboxamide (Fig. 1A) was verified using LXRβ-GAL4 luciferase reporter screening according to the above-mentioned protocol. The chemical name of IMB-808 is N-methyl-N-(2-oxo-2-(2,3,4-trifluorophenyl)amino) ethyl) -3,4-dihydro-2H-benzo[b][1,4] dioxine-7-carboxamide, and this compound has not been reported to display any activity previously. IMB-808 significantly dose-dependently induced

**TABLE 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primers</th>
<th>Reverse Primers</th>
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<tr>
<td>hABCa1</td>
<td>5′-TTCCCGCATATTCTGGAAGC-3′</td>
<td>5′-CAAGGTCCATTTCTTGCTGT-3′</td>
</tr>
<tr>
<td>hABCg1</td>
<td>5′-ATTCCAGGACCTTTCTACTGCAG-3′</td>
<td>5′-CTCCACTATGGAACCTTCCCG-3′</td>
</tr>
<tr>
<td>hApoe</td>
<td>5′-GTGTCGTCATCACTTGCCTG-3′</td>
<td>5′-GCAGAGTTACCCAAAAAGGAC-3′</td>
</tr>
<tr>
<td>hFas</td>
<td>5′-TGGAATGGCAGTCTAGAAGCCA-3′</td>
<td>5′-GCCCAATAGCAGAATACCC-3′</td>
</tr>
<tr>
<td>hGAPDH</td>
<td>5′-AGCCACATGGCTGAGACAC-3′</td>
<td>5′-CAAGCAGTAGAGUCCCTGGGCA-3′</td>
</tr>
<tr>
<td>hNPC1l1</td>
<td>5′-TCACTUGGCTGTGTCGTGC-3′</td>
<td>5′-CAACGCCATGCAGCUCCCTGGCA-3′</td>
</tr>
<tr>
<td>hScd-1</td>
<td>5′-TGCTGCGGCTCTGG-3′</td>
<td>5′-CATACCCCAAGTCCCTGTCAC-3′</td>
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<tr>
<td>hSreb1p-c</td>
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<td>5′-GATGCTCATGGGACCTAAGCTTC-3′</td>
</tr>
<tr>
<td>mAbca1</td>
<td>5′-AAAAACCGACAGCTCCTCTCAC-3′</td>
<td>5′-GATACCCAAACGTCCTGTCAC-3′</td>
</tr>
<tr>
<td>mGAPDH</td>
<td>5′-AGTCCCGTGTGAAAGCAGTTTG-3′</td>
<td>5′-GAGGTGTTGATGTCGACA-3′</td>
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LXRβ activation under concentrations ranging from 0.001 to 30 μM, with an EC_{50} of 0.53 μM, and displayed a maximized activity of nearly 2-fold (Fig. 1D). In this model, TO901317 showed approximately 3-fold LXRβ activation (Fig. 1E). Subsequently, we examined the activity of IMB-808 using LXRα-GAL4 luciferase reporter assays. It was revealed that IMB-808 showed approximately 3-fold LXRβ activation (Fig. 1E). Subsequently, we examined the activity of IMB-808 using LXRα-GAL4 luciferase reporter assays. It was revealed that IMB-808 showed approximately 3-fold LXRβ activation (Fig. 1E).
also could dose-dependently activate LXRα with a lower EC_{50} of 0.15 μM (Fig. 1B), and TO901317 showed 3.3-fold LXRα activation with an EC_{50} of 0.05 μM (Fig. 1C).

**IMB-808 Could Induce the Expression of ABCG1, ABCA1, and ApoE In Vitro.** ABCA1 and ABCG1 are important target genes of LXR that were associated with the RCT pathway in macrophages (Geyeregger et al., 2006). The influence of IMB-808 on the expressing profiles of ABCA1 and ABCG1 in murine and human macrophages was first detected by Western blot and real-time quantitative PCR assays. IMB-808 significantly increased both protein and mRNA levels of ABCG1 as well as ABCA1 in RAW264.7 macrophages (Fig. 2, A and D) and THP-1–derived macrophages (Fig. 2, B and E).

ApoE is another crucial target gene of LXR, which is involved in cholesterol homeostasis and is beneficial for the protection of atherosclerosis (Laffitte et al., 2001). In the present study, the expressing quantities of mRNA and the protein of ApoE were slightly increased after treatment with IMB-808 in THP-1–derived macrophages. Moreover, these genes were induced to a greater extent while treating the cells with full agonist TO901317 (Fig. 2, C and F).

**IMB-808 Promotes Cholesterol Efflux from Macrophages.** ABCG1 and ABCA1 were crucial transporter proteins for facilitating cholesterol efflux out of macrophages to plasma HDL and ApoA-I (Repa and Mangelsdorf, 2000). Subsequently, the influence on the cholesterol efflux of macrophages derived from THP-1 and RAW264.7 was studied.

HDL (50 μg/ml) or ApoA-I (10 μg/ml) was added with the aim of promoting cholesterol efflux separately. It was also discovered that IMB-808 could dose-dependently promote cholesterol efflux toward ApoA-I and HDL and reduce the cellular cholesterol concentration in these two cell lines (Fig. 3).

**IMB-808 Reduces Cellular Lipid Accumulation.** To determine the potential effect of IMB-808 on the inhibition of lipid accumulation and foam cell formation, assays of foam cells were carried in RAW264.7 cells. According to Fig. 4, C–G, IMB-808 could effectively reduce the quantity of accumulated lipid in comparison with that of a single ox-LDL (Fig. 4B). Furthermore, foam cell formation was significantly inhibited after stimulation with 3 μM IMB-808 (Fig. 4F), resulting in levels comparable to those of the control group (Fig. 4A) and inferior to that seen after treatment with TO901317 (Fig. 4H).

Then, the content of lipid in cells was quantitated. The result showed that IMB-808 was capable of significantly reducing accumulated lipid in cells from 0.1 to 10 μM (Fig. 4I).

**IMB-808 Regulates the Expressing Profiles of ABCG5, ABCG8, and NPC1L1, Which Are Related to the Absorbing and Secreting Process of Cholesterol.** It was proposed by previous studies that LXRs influenced cholesterol level by reducing the quantity of cholesterol

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**Fig. 3.** IMB-808 promoted cholesterol efflux. (A and B) RAW264.7 macrophages were preincubated with 22-NBD-cholesterol for 24 hours. The cells were then washed with PBS and incubated with IMB-808 (0, 0.01, 0.1, 1, or 10 μM). After 18 hours, 10 μg/ml ApoA-I or 50 μg/ml HDL (final concentration) was added and incubated for 6 hours at 37°C. The amount of cholesterol in the medium and cells was measured separately. Relative 22-NBD-cholesterol efflux to ApoA-I or HDL induced by IMB-808 was calculated as described in the Materials and Methods. (C and D) Similar cholesterol efflux assays were performed in THP-1–derived macrophages. Similar results were obtained in three independent experiments. Data are reported as the mean ± SEM (n = 3, *P < 0.05 versus control, **P < 0.01 versus control).
fatty acid synthase (FAS) as well as SREBP-1c did not increase obviously after treatment with 0.01–10 μM IMB-808, in contrast with treatment with TO901317 (Fig. 6B).

**IMB-808 Shows Distinct Recruitment of Coregulators in Comparison with TO901317.** The expression of genes was regulated by LXR, which was transcriptionally activated in a ligand-dependent manner through dissociating corepressors and subsequently recruiting coactivators (Leo and Chen, 2000). The specific conformation alteration of LXR induced by IMB-808 might be due to coregulator interactions, which probably explains the different regulating patterns on genes that were discovered during lipogenesis. It was probably caused by distinct patterns of ligand binding with LXR-LBD, which consequently led to an alternating capacity of dissociating corepressors and/or recruiting coactivators. To further analyze the molecular mechanism of compound binding with LXR-LBD, the TR-FRET assay was conducted with the aim of comparing the capacity of TO901317 and IMB-808 to adjust the interacting activity of LXRα and LXRβ with corepressors or coactivators, respectively.

For LXRα, IMB-808 displayed a weak displacement with the corepressor NcoR dose dependently compared with that of TO901317 (approximately 42%) (Fig. 6C) and showed a more moderate ability to recruit coactivator TRAP220 (approximately 38%) compared with that of TO901317 (Fig. 6D). At the same time, IMB-808 also exhibited an inferior influence compared with that of TO901317 to displace the corepressor SMRT (approximately 40%) (Fig. 6E) and recruit coactivator D22 (approximately 44%) (Fig. 6F) for LXRβ.

**IMB-808 Dock to the LXR-LBD in Silico.** To investigate the supposed binding pattern and possible interaction between the ligand and pocket of IMB-808, the structure of IMB-808 was virtually docked to the crystal structures of LBD of LXRα (PDB code: 1UHL) and LXRβ (PDB code: 1PQC) using the docking program DS CDOCKER separately. The virtual binding result suggested that IMB-808 can fit well in either LBD compared with those of TO901317, we performed the

**IMB-808 Almost Does Not Induce Lipogenic Gene Expression.** Another crucial target gene of LXR, SREBP-1c, which can induce hepatic fatty acid synthesis, was analyzed (Wagner et al., 2003). Interestingly, our results demonstrated that IMB-808 almost did not increase the protein expression of SREBP-1c in comparison with that after TO901317 treatment (approximately 4-fold for 1 μM TO901317) (Fig. 6A). Moreover, the mRNA levels of stearoyl-coenzyme A desaturase-1 (SCD-1),

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### Fig. 4. IMB-808 reduced ox-LDL–induced lipid accumulation in RAW264.7 macrophages. RAW264.7 macrophages were preincubated with PBS (vehicle) (A) or ox-LDL (60 μg/ml) (B–H). After 24 hours, these cells were treated with 0.1% dimethylsulfoxide (B), IMB-808 (0.1, 0.3, 1, 3, or 10 μM) (C–G), or T1317 (1 μM) (H) for 18 hours. Cells were fixed with 4% paraformaldehyde and stained with 0.5% Oil Red O to detect lipid accumulation. Representative images of the eight study groups are displayed (original magnification, 400×). (I) After Oil Red O staining, bound dye was solubilized and quantified spectrophotometrically at 510 nm. Similar results were obtained in three independent experiments. Data are reported as the mean ± SEM (n = 3, *P < 0.05 versus control, **P < 0.001 versus control).
site-directed mutagenesis and then examined the luciferase activity of mutation plasmids on the LXRα or LXRβ agonist screening model, respectively.

Five critical amino acid residues of LXRα LBD, which played an essential role in binding IMB-808 or TO901317, were replaced with others respectively, and the obtained mutant proteins were tested to figure out whether they could be activated by IMB-808 or TO901317 (Fig. 7, C and D, respectively). The activation levels of W443G, H421D, and T302I mutants conferred were low and the increasing extent was slight, whereas the R305G mutant was barely capable of activating LXRα. Interestingly, the mutant and the wild-type LXRα plasmids had nearly the same activity when Phe257 was replaced with Tyr (Fig. 7C). In addition, T302I and F257Y mutants were slightly activated whereas W443G and H421D were barely activated by TO901317 (Fig. 7D). Furthermore, five different amino acids of LXRβ that were determined to be crucial residues for IMB-808 binding were individually replaced with alanine residue, and the obtained mutant proteins were tested to determine whether they could be activated by IMB-808 or TO901317 (Fig. 8, C and D, respectively). F271A, M312A, as well as T316A mutants displayed significant decreases in agonistic activation by IMB-808, which indicated that these residues played an important role in transcriptional activation. Interestingly, when H435 and W457 were transformed into alanine, the mutants that were obtained displayed activity similar to that of the wild-type LXRβ plasmids (Fig. 8C). On the contrary, T316, H435, and W457 mutants barely displayed any activation, whereas M312 mutants were only mildly activated by TO901317 (Fig. 8D). Conforming to these findings, various mutants displayed different agonist activity compared with the wild-type group after stimulation by 10 mM IMB-808 (Fig. 7E; Fig. 8E) or 1 μM TO901317 (Fig. 7F; Fig. 8F).

IMB-808 Shows Distinct Effect of the LXR-LBD Mutation on Coregulator Recruitment. To investigate the differential effect of IMB-808 and T1317 on LXR mediated by the interaction of LXR-LBD and cofactors, experiments to

Fig. 5. IMB-808 regulated ABCG5, ABCG8, and NPC1L1 expression. (A and B) HepG2 cells were treated with IMB-808 at various concentrations or T1317 (1 μM) for 18 hours, and ABCG5 and ABCG8 protein levels were determined by Western blot assays. (C and D) Caco-2 cells were incubated with various concentrations of IMB-808 or T1317 for 18 hours. ABCG5 and ABCG8 protein levels were then measured. Western blotting (E) and real-time PCR (F) were performed on Caco-2 cells that were incubated with IMB-808 or T1317 for 18 hours. NPC1L1 protein and mRNA levels were then determined. Induction factors were normalized against GAPDH, and the control groups were treated with dimethylsulfoxide (0.1%). Similar results were obtained in four independent experiments. Data are reported as the mean ± SEM (n = 4, *P < 0.05 versus control, **P < 0.01 versus control).
determine the effect of the mutation on cofactor recruitment was performed by TR-FRET. According to the effects of the luciferase activity of mutation plasmids on LXRα or LXRβ, LXRα-R305G and LXRβ-F271A were chosen as representative mutation proteins for the TR-FRET assay.

For LXRα-R305G, IMB-808 exhibited moderate displacement with the corepressor NcoR compared with that of TO901317 (approximately 31%) (Fig. 9A) and showed a weak ability to recruit coactivator TRAP220 (approximately 18%) compared with that of TO901317 (Fig. 9B). Moreover, IMB-808 also displayed an influence that was inferior to that of TO901317 to displace the corepressor SMRT (approximately 23%) (Fig. 9C) and recruit coactivator D22 (approximately 13%) (Fig. 9D) for LXRβ-F271A.

**Discussion**

LXRs were known as nuclear receptors with key functions on the regulation of lipid and cholesterol homeostasis in tissues and recently have attracted attention because they
also display anti-inflammatory activities (Willy et al., 1995; Miyata et al., 1996). Synthesized LXR agonists have drawn much attention in the development of new drugs for treating atherosclerosis (Hong and Tontonoz, 2014). However, full LXR agonism commonly leads to lipid accumulation in the liver because it activates the LXRα subtype by increasing the expressing quantity of SREBP-1c genes associated with the lipogenesis pathway (Baranowski, 2008). Therefore, our current study aimed to find an innovative partial LXR agonist that had antiatherosclerotic activity and fewer lipogenic side effects as determined by screening.

Here, we found that IMB-808 is an activator of LXRβ using a cell-based screening method that revealed a novel structural agonist with an EC50 of 0.53 μM. Subsequently, we found that IMB-808 was not only a partial modulator for LXRβ, it could also dose-dependently activate LXRα with a lower EC50 of 0.15 μM. LXRs were important transcription factors while regulating RCT. In fact, it was proposed by the following studies that LXRs could influence almost every part of this pathway. The first step of RCT was cholesterol efflux out of cells that were mainly regulated by ABCG1 and ABCA1 transporters. Here, we found that the levels of both protein and mRNA expression of ABCG1 as well as of ABCA1 were improved by IMB-808 dose dependently in two macrophage cell lines. Furthermore, IMB-808 could reduce cellular lipid accumulation and inhibit foam cell formation in RAW264.7 macrophages. ABCA1 and ABCG1 are responsible for transferring both phospholipid and/or cholesterol molecules through plasma membranes toward ApoA-I without lipids or HDL (Dean et al., 2001; Kennedy et al., 2005). At the same time, we determined that IMB-808 could significantly increase cholesterol efflux toward HDL or ApoA-I and reduce cholesterol levels inside cells dose dependently in both macrophage cell lines. Moreover, as one of other important LXR target genes, the transcription of ApoE, which maintains cholesterol homeostasis in plasma, is
promoted through loading cholesterol (Laffitte et al., 2001). Therefore, we concluded that the improvement in ABCA1, ABCG1, and ApoE protein expression was conducive to preventing atherosclerosis via the activation of both LXR<sub>a</sub> and LXR<sub>b</sub> by IMB-808.

LXR could regulate systematic cholesterol homeostasis by reducing cholesterol absorbed in the intestine and promoting the excretion of bile cholesterol through regulating transporters on the membrane, which included ABCG5, ABCG8, as well as NPC1L1 (Repa et al., 2002). It was revealed in this article that IMB-808 efficaciously upregulated ABCG8 and ABCG5 protein expression of HepG2 and Caco-2 cells dose dependently. Additionally, after stimulation with IMB-808, both NPC1L1 mRNA and protein were downregulated. Consequently, we suggest that IMB-808 could increase the expression level of LXR-related target genes in vitro and regulate the entire cholesterol metabolism pathway by activating LXR<sub>a</sub>/<b/alpha>.

To date, several LXR agonists with various structures from different pharmaceutical companies have been reported and investigated, such as GW3965 (GlaxoSmithKline, Brentford, UK) and TO901317 (Tularik, South San Francisco, CA), which are regarded as classic potent full LXR<sub>a</sub>/<b/alpha> agonists (Collins et al., 2002). In response to natural or synthetic ligands, LXRs stimulate SREBP-1c, acetyl-CoA carboxylase, SCD-1, and FAS expression in the liver, leading to increased fatty acid synthesis and plasma triglyceride levels. However, many studies have suggested that LXR<sub>a</sub> is the dominant isoform in this pathway (Repa et al., 2000). In this study, it was discovered that IMB-808 neither induced the expression of lipogenesis genes nor exhibited any toxicity at 200 μM in RAW 264.7 macrophages and HepG2 cells (data not shown). The
crystal structures of the two LXR subtypes revealed that most of the crucial residues in the LBD are conserved, suggesting that the discovery of subtype-selective LXR ligands may be challenging (Williams et al., 2003; Fradera et al., 2010; Li et al., 2010). Surprisingly, our study indicated that IMB-808 has a number of advantages with good selectivity for gene expression regulation, and it may have few lipogenic side effects in vivo. Furthermore, we speculate that the selective activity of IMB-808 is due to a distinct mode of interacting with LXRα and LXRβ compared with TO901317. Coregulator recruiting capacity was typically analyzed to study the ligand qualitatively as an agonist. We wanted to elucidate how IMB-808 displayed specificity for LXRα and LXRβ compared with TO901317. First, in the TR-FRET analysis, IMB-808 weakly displaced the corepressors SMRT and NcoR, and recruited coactivator D22 and TRAP220 at approximately 40% of that of TO901317. Thus, IMB-808 action was characteristic of a partial agonist of LXR rather than of the full agonist, like TO901317. Recently, some groups demonstrated that one of the coactivators that interacted with LXRα was specifically recruited to SREBP-1c, which was the responding element of LXR rather than ABCA1 (Kim et al., 2015). This theory indicated that the mechanism of coactivator specificity was probably associated with modulating the specified expression level of other critical regulating genes. Therefore, this led to our conclusion that IMB-808 regulated cholesterol metabolism without significant lipogenic side effects compared with TO901317.

Moreover, virtual docking was performed to analyze IMB-808 ligand characteristics. Key amino acid residues were predicted according to the result of docking and were subsequently replaced with other different residues by site-directed mutagenesis. Interestingly, we determined that the amino acids in LXRα-LBD or LXRβ-LBD interacting with IMB-808 differed from those of TO901317. In LXRα-LBD, Phe257 formed a Pi-alkyl interaction with IMB-808, but a hydrophobic interaction with TO901317. Moreover, Arg305 significantly influenced the activity of IMB-808 but not of TO901317. Three amino acids, Phe271, Met312, and Thr316, in LXRβ form critical interactions with IMB-808. In contrast, H421 and W443 in LXRα (H435 and W457 in LXRβ), which are important for binding TO901317, interact with IMB-808 in a moderate level. Partial agonists may form interaction with a portion of the crucial amino acids in the LBD-active pocket of the nuclear receptor, such as LXR and peroxisome proliferator–activated receptor, resulting in diminished stabilization of LBD AF-2 surface (Bruning et al., 2007). In the study by Liu et al. (2015), they also found that alterations in the conformation of peroxisome proliferator–activated receptor γ could lead to the process of recruiting differentiated sets of cofactors and subsequently reduce the side effects of the compound, which were possibly associated with such a specific interaction.

Fig. 9. (A) The TR-FRET assay was used to examine corepressor peptide NcoR ID2 displacement from human LXRα-R305G treated with IMB-808 or TO901317. (B) The TR-FRET assay was performed to examine coactivator TRAP220/DRIP2 recruitment to human LXRα-R305G treated with IMB-808 or TO901317. (C) The TR-FRET assay was used to examine corepressor peptide SMRT ID2 displacement from human LXRβ-F271A treated with IMB-808 or TO901317. (D) The TR-FRET assay was performed to examine coactivator D22 recruitment to human LXRβ-F271A treated with IMB-808 or TO901317. The data are expressed as the mean ratio of the emission signal at 520 nm and the signal at 405 nm. All assays were repeated four times independently (n = 4). Max, maximum.
Novel LXR Agonist with Distinct Characteristics

According to the virtual docking result and the luciferase activity assay of mutation plasmids on LXRe or LXRβ, we adopted LXRa-R305G and LXRβ-F271A as being representative to further discuss the differential effect of the LXR-LBD mutation on coregulator recruitment between IMB-808 and TO901317 by TR-FRET. In the TR-FRET analysis, IMB-808 weakly displaced the corepressors SMRT and NcoR, and recruited coactivator D22 and TRAP220 at approximately 10–30% that of TO901317. The mutations LXRa-R305G and LXRβ-F271A significantly influenced the activity of IMB-808 on coregulator recruitment but not of TO901317. Consequently, it was suggested that IMB-808 had a unique mechanism as an innovative partial dual agonist of LXRα/β with unique regulatory pattern for different target genes, and its interaction mod e differs from that of the traditional LXR agonist TO901317.

Overall, IMB-808 is a novel potent LXR agonist that could regulate the gene expression involved in the pathway of metabolizing cholesterol by relying on the activation of LXRe/β. IMB-808 remarkably promoted cholesterol efflux out of macrophages and reduced the accumulated lipids in foam cells. Moreover, in comparison with TO901317, our data showed that IMB-808 had an obvious advantage because it almost did not increase lipogenesis gene expression, which suggested that IMB-808 may have lower lipogenic side effects in vivo. The findings in this study provide us with directions for the design of innovative drugs targeting LXR for the treatment of atherosclerosis in the future.

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Participated in research design: Li and Si Conducted experiments: Li, Wang, Xu, and Liu Contributed new reagents or analytic tools: Lin, Zhu, and Lu Performed data analysis: Li and Wang Wrote or contributed to the writing of the manuscript: Li and Si

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