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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Abbreviation list:

ABC, ATP-binding cassette; ApoA-I, apolipoprotein A-I; ApoE, apolipoprotein E; FAS, fatty acid synthase; FRET, fluorescence resonance energy transfer; GAPDH, glyceraldehyde-phosphate dehydrogenase; HDL, high-density lipoprotein; LBD, ligand-binding domain; LXR, liver X receptor; NPC1L1, Niemann-Pick C1 like 1; ox-LDL, oxidized low-density lipoprotein; RCT, reverse cholesterol transport; SCD-1, stearoyl-coenzyme A desaturase 1; SREBP-1c, sterol response element binding protein 1c
Abstract:

Activation of Liver X receptor (LXR) is associated with cholesterol metabolism and anti-inflammatory processes, which makes beneficial to anti-atherosclerosis. Nevertheless, existing agonists which 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 expressing 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 expressing 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 to the full LXR agonist TO901317, IMB-808 recruits co-regulators differently and possesses 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 avoiding 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.
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Introduction

Atherosclerosis, as the dominant mortality cause in developed nations, is gradually becoming a health issue around world (Roger et al., 2012). The liver X receptor 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). LXRα and LXRβ, bind to response elements of their target genes to modulate gene expression (Edwards et al., 2002). Although the expressing 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, LXRα are in a non-active state, combining to co-repressors, for example the Nuclear Receptor Corepressor (NCoR) (Chen and Evans, 1995). The binding of ligands results a change of the LXRα conformation that enables co-repressors to be released, co-activators to be recruited and the target genes to be trans-activated (Wiebel et al., 1999).

LXRα serves as a sensor of cholesterol which protects cells from adverse effect of overloaded cholesterol by inducing the target genes expression. RCT is a primary mechanism for removing cholesterol from cells and transferring it into liver which can be stimulated by LXRα (Beltowski, 2008). Several proteins of the ATP-binding cassette (ABC) transporter family contribute to cholesterol metabolism regulation and are regarded as target genes of LXRα. ABCA1 and ABCG1 play important roles in cholesterol efflux, and ABCG5 and ABCG8 influence hepatic cholesterol excretion and intestinal absorption (Cavelier et al., 2006; Wang, 2007; Yu et al., 2003). 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 to absorbing cholesterol in intestine. According to reports, the expressing of NPC1L1 can be down-regulate by LXR activators in human intestinal and mice cells (Duval et al., 2006).

A previous report demonstrated that either LXRα or LXRβ could exert an effort to the anti-atherosclerosis role of macrophage cells while lacking LXRα and LXRβ leaded to accumulating lipid in foam cells of lesions in arteries (Schuster et al., 2002). Several synthesized LXR ligands, for example full agonists TO901317 and GW3965, were focused widely and studied substantially for many years (Geyeregger et al., 2006; Houck et al., 2004; Janowski et al., 1996). Nevertheless, these ligands have not yet been developed as drugs because of their undesirable side effects. Through the activation of hepatic sterol regulating 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 which induced no hepatic synthesis of fat acids interested us. It was reported that such ligands, for example N,N-dimethyl-3b-hydroxy-cholena-mide 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 enhance reverse cholesterol transport while do not significantly cause lipid accumulation in the liver. Consequently, it was our target to identify LXR agonists which exhibited satisfactory selectivity.
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 luciferase reporter assay. We found that IMB-808 had effect on LXR target genes and influenced some cholesterol metabolism-related pathways in multiple cells. Moreover, the molecular docking result provided us a theoretical basis to study the interaction site between this compound and both LXRα/β ligand-binding domain (LBD) construction. Furthermore, based on the co-regulators recruitment and site-mutation activation assays, the possible mechanism of LXRα/β interacting with IMB-808 was clarified.
Materials and Methods

Reagents

TO901317, which was called T1317 as well in current study, accompanied with phorbol-12-myristate-13-acetate (PMA) and Red Oil O stain were obtained from Sigma (St. Louis, MO, USA). IMB-808 was obtained from compound library of the National Laboratory for Screening New Microbial Drugs, Institute of Medicinal Biotechnology, PUMC (Beijing, China). Opti-MEM® Reduced Serum Medium as well as fetal bovine serum (FBS) were obtained from Gibco (Invitrogen, Carlsbad, CA, USA). RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM) as well as Modified Eagle's medium (MEM) were obtained from Hyclone (Thermo Scientific, Rockford, USA). 22-NBD-cholesterol and Lipofectamine 2000 and 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, China).

Plasmids

The wild type gene of human LXRα-LBD and LXRβ-LBD were obtained by PCR from HepG2 cells and cloned into pBIND vector (Promega, Madison, WI, USA), in which GAL4 DNA binding domain (GAL4-DBD) 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 utilizing Fast Mutagenesis System.
(TransGen Biotech, Beijing, China). A few important amino-acids of LXRα-LBD were converted into other molecules. The plasmids were mutated according to 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), while Phe271 was mutated to Ala in pET30a-LXRβ-LBD (named pET30a-LXRβ- F271A).

The reporter assay of LXR -GAL4 chimera and cell culture

In brief, RAW264.7 macrophages, HepG2, HEK293T, Caco-2 cells as well as human monocyte cell line THP-1 were cultured in different medium separately as described before (Li et al., 2014). 100 nM PMA was added into THP-1 cells and incubated for 24h in order to produce fully differentiated macrophages, after which serum free medium was utilized to replace the former medium.

IMB-808 was identified through screening of a synthesized compound library which contained 20000 drug-like constructions as described before (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 h before being treated by compounds for 18 h. Luciferase Assay System (Promega) was utilized to determine the luciferase activity using a microplate reader (PerkinElmer, Waltham, MA, USA).

**Real time quantitative RT-PCR analysis**

HepG2 and Caco-2 cells were inoculated in six well plates with the density of 4×10^5 cells/ml. RAW264.7 macrophages and human THP-1 monocytes were cultured in six well plates with the densities of 6×10^5 cells/ml and 1.5×10^6 cells/ml respectively. TRIzol® reagent (Invitrogen) was used to extract total RNA of the cells while reverse transcriptional kits (TransGen Biotech) were utilized to reversely transcribe it. The real time quantitative PCR assay was performed on a CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA) by using SYBR Green (Roche Diagnostics, Lewes, UK) detecting reagents. Table 1 shows the primer sequences utilized in this study. GAPDH level was used to normalize all mRNA expression quantities and ΔΔCt method was utilized 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 had established attachment (24 h). Cells were collected after 18 h of incubation and protein samples were extracted according to 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). An Enhanced Chemiluminescence (ECL) reaction kit (Millipore) was used for blot detection. The following primary antibodies were used: anti-GAPDH (1:2000, Abmart, Shanghai, China), anti-ApoE (1:1000, Epitomics, Abcam), anti-ABCG1 (1:500, Novus, Littleton, CO, USA), anti-ABCA1 (1:1000, Novus), anti-ABCG5 (1:1000, Epitomics), anti-ABCG8 (1:500, Novus), anti-NPC1L1 (1:1000, Epitomics,) 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 h, they were stimulated with varied concentrations of IMB-808 for 18 h 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 used as addition into each well. The samples were measured at 510 nm by a micro-plate reader 10 minutes after that (Zou and Shen, 2007).

Cholesterol efflux experiment

22-NBD-cholesterol was used to conduct 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) and marked using 22-NBD-cholesterol with the final concentration of 2.0 µM at the final concentration in serum free medium which contained 0.2% (w/v) bovine serum albumin (BSA) (Sigma Chemical) (medium A) for 24 h. PBS was used to wash cells twice which were then incubated with IMB-808 or vacant control for an additional 18 h. Subsequently the receptor protein of ApoA-I or HDL with the final concentrations of 10 µg/ml or 50 µg/ml was added into cells and incubated for 6 h. A micro-plate reader was used to test the quantities of cholesterol in cells as well as 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 test was conducted in triplet.

Expression and purification of LXRα-R305G and LXRβ-F271A

The pET30a-LXRα-R305G and pET30a-LXRβ-F271A plasmid were transformed into Rosetta (DE3) E. coli cells separately. The expression of the two proteins was induced by the addition of 0.2 mM IPTG at 20°C overnight. The supernatant of LXRα-R305G and LXRβ-F271A were harvested by centrifugation, then filtered through a 0.45-µM filter and loaded onto a Ni²⁺ His Trap chelating column (GE Healthcare). The binding buffer contained 20 mM Tris-HCl pH 7.4, 500 mM NaCl, and 20 mM imidazole. LXRα-R305G protein was eluted by 20 mM Tris-HCl pH 7.4, 500 mM NaCl, 200 mM imidazole, while LXRβ-F271A protein was eluted by 20 mM Tris-HCl pH 7.4, 500 mM NaCl, 250 mM imidazole. The purified proteins were concentrated to 1mg/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 DTT, and 20% glycerol at −80°C.

**LanthaScreen TR-FRET LXR-coregulator peptide interaction assays**

LanthaScreen TR-FRET LXRα-Coactivator Assay Kit (PV4655, Invitrogen) and LXRβ-Coactivator Assay Kit (PV4658, Invitrogen) were used to perform TR-FRET LXR-coregulator peptide interaction assays according to the manufacturer’s instruction separately. Human GST-LXRα-LBD, GST-LXRβ-LBD, terbium (Tb)-labeled anti-GST tag antibody, fluorescein (FL)-labeled peptides, including FL-TRAP220/DRIP2 (PV4549), FL-D22 (PV4387), FL-NcoR (NCoR ID2, PV4624), FL-SMRT (SMRTID2, PV4423) as well as all of the buffers were included in current study. TO901317 or IMB-808 was diluted and first added to 384-well black plates (Costar) according to the kits’ instructions. LXRα-LBD or LXRβ-LBD protein was then added, following 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. Terbium (Tb)-labeled anti-His tag antibody (PV5863, Invitrogen) was purchased from Invitrogen. TR-FRET assay of mutation LXR and co-regulator interaction was performed under the similar condition. TO901317 or IMB-808 was diluted and added to the plate, LXRα-R305G (final concentration 30 nM) or LXRβ-F271A (final concentration 100 nM) was then added, following by co-regulator and FL-peptide/Tb-anti-His mixed together.

The plates were shaking away from light under ambient temperature for two hours. A PerkinElmer EnVision plate reader was used to measure the TR-FRET ratio (520
nm/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 for 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 Inc., CA, USA) with the crystal structure of LXRα (PDB code: 1UHL, LXRα with TO901317) and LXRβ (PDB code: 1PQC, LXRβ with TO901317) separately. After removing all crystallized H$_2$O molecules from the former construction, hydrogen was added into DS CDOCKER module. An optimized start conformation was obtained by minimizing the compound to achieve the lowest energy level ahead of docking in silica.

**Statistic analyzing**

The software of Graphpad Prism 5.0 (San Diego, CA, USA) was used to calculate statistics as well as best fit curves. The data was represented as means ± SEM. One way ANOVA and Student’s $t$-test were utilized to analyze result with SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). $P$ value < 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 LXRβ agonist with an analogous structure of benzo-dioxepine-carboxamide (Fig. 1A) was verified using LXRβ-GAL4 luciferase reporter screening according to 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]dioxepine-7-carboxamide, and this compound has not been reported to display any activity previously. IMB-808 significantly dose-dependently induced LXRβ activation under the concentrations ranged from 0.001 μM to 30 μM, with an EC₅₀ of 0.53 μM, and displayed a maximized activity of nearly two folds (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 also could dose-dependently active LXRα with a lower EC₅₀ of 0.15 μM (Fig. 1B), and TO901317 showed 3.3-fold LXRα activation with an EC₅₀ of 0.05 μM (Fig. 1C).

IMB-808 could induces the expression of ABCG1, ABCA1 and ApoE in vitro

ABCA1 and ABCG1 are important target genes of LXR that were associated with RCT pathway in macrophages (Geyeregger et al., 2006). The influence of IMB-808 upon the expressing profiles of ABCA1 and ABCG1 in murine and human macrophages were 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. 2A and D) and THP-1 derived macrophages (Fig. 2B and E).

ApoE is another crucial target gene of LXR which involved in cholesterol homeostasis and make beneficial for the protection of atherosclerosis (Laffitte et al., 2001). In present paper, the expressing quantities of mRNA and protein of ApoE were slightly increased following 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. 2C 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) were added with the aim of promoting cholesterol efflux separately. It was also discovered that IMB-808 could promote cholesterol efflux towards ApoA-I and HDL dose-dependently 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. 4C–G, IMB-808 could effectively reduce the quantity of accumulated lipid in comparison to that of single ox-LDL (Fig. 4B). Furthermore, foam cell formation was significantly inhibited following stimulation with 3 μM IMB-808 (Fig. 4F), resulting in levels comparable to control group (Fig. 4A) and inferior to that following the treatment with TO901317 (Fig. 4H). At the same time, we used a method to quantitate the content of lipid in cells. 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 cholesterol quantity absorbed in intestine through NPC1L1, ABCG8 as well as ABCG5 regulation. Moreover, LXR up-regulate the expression of ABCG8 as well as ABCG5 to promote cholesterol efflux into bile (Yu et al., 2003). In this study, IMB-808 increased the expression of ABCG5 and ABCG8 protein in HepG2 cells by 1.70 folds as well as 1.82 folds separately (Fig. 5A and B). We also found that IMB-808 significantly induced the expression of ABCG5 and ABCG8 proteins of Caco-2 cells dose-dependently (Fig. 5C and D). Furthermore, in Caco-2 cells, both
protein and mRNA expression quantities of NPC1L1 decreased after treatment with IMB-808 (Fig. 5E and F).

**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 to that following TO901317 treatment (approximately 4-fold for 1μM TO901317) (Fig. 6A). Moreover, the mRNA levels of stearoyl-coenzyme A desaturase-1 (SCD-1), fatty acid synthase (FAS) as well as SREBP-1c did not increase obviously after treatment with 0.01-10 μM IMB-808, in contrast to treatment with TO901317 (Fig. 6B).

**IMB-808 shows distinct recruitment of co-regulators in comparison to TO901317**

The expression of genes were regulated by LXR which was transcriptionally activated in ligand dependent manner through dissociating co-repressors and subsequently recruiting co-activators (Leo and Chen, 2000). The specific conformation alteration of LXR induced by IMB-808 might be due to coregulator interactions, which probably explained the different regulating patterns on genes which were discovered during lipogenesis. It was probably caused by distinct patterns of ligand binding with
LXR-LBD, which consequently led to alternated capacity of dissociating co-repressors and/or recruiting co-activators. 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 co-repressors or co-activators respectively.

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

**IMB-808 docks to the LXR-LBD in silica**

To investigate the supposed binding pattern and possible interaction between 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 LXRα LBD (Fig. 7A and B) or LXRβ LBD (Fig. 8A and B). First, for the LXRα LBD model, the fluorine atom in the trifluorophenyl
ring formed a hydrogen bonding with Arg305 and the phenyl ring of the benzo-dioxepine system formed a hydrogen bonding with Trp443. IMB-808 forms a \( \pi - \pi \) bond with the imidazole ring of His421 and an amide-Pi bond with Thr302. In addition, IMB-808 is surrounded by Phe257, Ala261, Met298 and Glu301 through Pi-alkyl interaction, hydrophobic interaction and van der Waals forces.

Secondly, for the LXR\( \beta \) LBD model, IMB-808 virtually docked into the pocket including two hydrogen bonds, two \( \pi - \pi \) stacking interactions, some van der Waals force, and hydrophobic interactions with amino acids around. Specifically, the oxygen atom of IMB-808 separately forms two hydrogen bonds with Met312 and Thr316 in a conjugated structure. While, the benzene ring of IMB-808 is close to Phe271 and Phe329 of the LXR\( \beta \) LBD and forms \( \pi - \pi \) stacking interactions with these amino acids. In addition, IMB808 is also surrounded by Thr272, Ala275, Phe340, Leu345 and Phe349 of the LXR\( \beta \) LBD through van der Waals forces or hydrophobic interactions to form a complete agonistic conformation.

**IMB-808 displays distinct interacting sites from those of TO901317**

With the aim of investigating the interaction sites of IMB-808 with LXR\( \alpha \) LBD or LXR\( \beta \) LBD compared to those of TO901317, we carried the site-directed mutagenesis and then examined the luciferase activity of mutation plasmids on LXR\( \alpha \) or LXR\( \beta \) agonist screening model respectively.

Five critical amino acid residues of LXR\( \alpha \) 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 (Figure 7C and D, separately). The activation levels W443G, H421D and T302I mutants conferred were low and the increasing extent was slight while R305G mutant was barely capable of activating LXRα. Interestingly, the mutant and the wild-type LXRα plasmid nearly had the same activity when Phe257 was replaced with Tyr (Fig. 7C). In addition, T302I and F257Y mutants were slightly while 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 figure out whether they could be activated by IMB-808 or TO901317 (Figure 8C 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, obtained mutants displayed similar activity to the wild type LXRβ plasmids (Fig. 8C). On the contrary, T316, H435, and W457 mutants barely displayed any activation while M312 mutants were only mildly activated by TO901317 (Fig. 8D). Conforming to these findings, various mutants displays different agonist activity in comparison to wild type group after stimulating by 10 μM IMB-808 (Fig. 7E and 8E) or 1 μM TO901317 (Fig. 7F and 8F).
IMB-808 shows distinct effect of the LXR-LBD mutation on co-regulator recruitment

To investigate the differential effect of IMB-808 and T1317 on LXR is mediated by the interaction of LXR-LBD and co-factor, experiments to determine the effect of the mutation on co-factor recruitment was performed by TR-FRET. According to the result of the luciferase activity of mutation plasmids on LXRα or LXRβ, LXRα-R305G and LXRβ-F271A were chose as representative mutation proteins for TR-FRET assay.

For LXRα-R305G, IMB-808 exhibited moderate displacement with the co-repressor NcoR in comparison to that of TO901317 (approximately 31%) (Fig. 9A), and showed a weak ability to recruit co-activator TRAP220 (approximately 18%) compared with that of TO901317 (Fig. 9B). Moreover, IMB-808 also displayed an inferior influence than that of TO901317 to displace the co-repressor SMRT (approximately 23%) (Fig. 9C) and recruit co-activator D22 (approximately 13%) (Fig. 9D) for LXRβ-F271A.
LXRs were known as nuclear receptors with key function on the regulation of lipid and cholesterol homeostasis in tissues and have attracted recent attention because they also display anti-inflammatory activities (Miyata et al., 1996; Willy et al., 1995). Synthesized LXR agonists drawn much attention to develop new drugs for treating atherosclerosis (Hong and Tontonoz, 2014). However, full LXR agonism commonly leads to hepatic accumulating lipid because it activates LXRα subtype by increasing the expressing quantity of SREBP-1c genes associated with the lipogenesis pathway (Baranowski, 2008). Therefore, in our current study, it was aimed to find out an innovative partial LXR agonist which had anti-atherosclerotic activity and fewer lipogenic side effects by screening.

Here, we found that IMB-808 is an activator of LXRβ using a cell-based screening method revealing a novel structural agonist with an EC\textsubscript{50} 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 EC\textsubscript{50} of 0.15 μM. LXRs were important transcription factors while regulating RCT. In fact, it was proposed by following studies that LXRs could almost influence every part of this pathway. The first step of RCT was cholesterol efflux out of cells which were mainly regulated by ABCG1 and ABCA1 transporters. Here, we found that both protein and mRNA expression levels of ABCG1 as well as ABCA1 were improved by IMB-808 dose-dependently in two macrophage cell lines. Furthermore, IMB-808 could reduce cellular lipid accumulating and inhibit foam cell forming in RAW264.7 macrophages.
ABCA1 and ABCG1 are responsible for transferring both phospholipid and/or cholesterol molecules through plasma membranes towards ApoA-I without lipids or to HDL (Dean et al., 2001), (Kennedy et al., 2005). At the same time, we determined that IMB-808 could significantly increase cholesterol efflux towards 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 improvement of ABCA1, ABCG1, and ApoE protein expression was conducive to preventing atherosclerosis via activation of both LXRα and LXRβ by IMB-808.

LXR could regulate systematic cholesterol homeostasis by reducing cholesterol absorbed in intestine and promoting the excretion of bile cholesterol through regulating transporters on membrane which included ABCG5, ABCG8 as well as NPC1L1 (Repa et al., 2002). It was revealed in this paper that IMB-808 efficaciously up-regulated 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 down-regulated. 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 LXRα/β.

To date, several LXR agonists with various structures from different pharmaceutical companies have been reported and investigated, such as GlaxoSmithKline’s GW3965 and Tularik’s TO901317, which are regarded as classic
potent full LXRα/β 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 triglycerides. However, many studies have suggested that LXRα 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 discovery of subtype-selective LXR ligands may be challenging (Fradera et al., 2010; Li et al., 2010; Williams et al., 2003). Surprisingly, our study indicated that IMB-808 has a number of advantages with good selectivity for gene expression regulation, and it may have little 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.

Co-regulator 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 corepressor 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 the full agonist like TO901317. Recently, some groups demonstrated that one of co-activators
which 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 co-activator specificity was probably associated with modulating the specified expressing quantity of other critical regulating genes. Therefore, this provided the possibility for our conclusions that IMB-808 regulated cholesterol metabolism without significant lipogenic side effects compared to 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 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 PPAR, resulting in diminished stabilization of LBD AF-2 surface (Bruning et al., 2007). In Liu’s study, they also found that alterations in conformation of PPARγ could lead to the process of recruiting differentiated sets of co-factors and
subsequently reduce side-effects of the compound, which was possibly associated with such specific interaction (Liu et al., 2015).

According to the virtual docking result and the luciferase activity assay of mutation plasmids on LXRα or LXRβ, we adopted LXRα-R305G and LXRβ-F271A as representative to further discuss the differential effect of the LXR-LBD mutation on co-regulator recruitment between IMB-808 and TO901317 by TR-FRET. In the TR-FRET analysis, IMB-808 weakly displaced the corepressor SMRT and NcoR, and recruited coactivator D22 and TRAP220 at approximately 10%-30% of that of TO901317. The mutation LXRα-R305G and LXRβ-F271A significantly influenced the activity of IMB-808 on co-regulator recruitment but not 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 mode differs from the traditional LXR agonist TO901317.

Overall, IMB-808 is a novel potent LXR agonist which could regulate gene expression involved in the pathway of metabolizing cholesterol relying on the activation of LXRα/β. IMB-808 remarkably promoted cholesterol efflux out of macrophages and reduced the accumulated lipids of foam cells. Moreover, in comparison to TO901317, our data showed that IMB-808 has 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 finding in this study provides us designing directions of innovative drugs targeting LXR for treating atherosclerosis in the future.
Conflict of interest statement

The authors have declared no conflict of interest.

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Authorship Contributions

Participating in research design: N. Li, S. Si.

Conducting experiments: N. Li, X. Wang, Y. Xu, P. Liu.

Contributing new analyzing tools or reagents: Y. Lin, N. Zhu, D. Lu.

Performing data analysis: N. Li, X. Wang.

Writing or contributing to composing the manuscript: N. Li, S. Si.
MOL #105213

References


Li N, Wang X, Liu P, Lu D, Jiang W, Xu Y and Si S (2016) E17110 promotes reverse cholesterol...


Footnotes

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2. N. L. and X. W. contributed equally to this work.
MOL #105213

**Fig. 1.** IMB-808 regulated LXRβ. (A) Structure of IMB-808. (B) LXRα activation by IMB-808. HEK293T cells were transfected with the GAL4-pGL4-luc reporter plasmid and the pBIND-LXRα expression plasmid. IMB-808 displayed significant LXRα agonistic activity in the luciferase activity assay. (C) LXRα activation by T1317. (D) LXRβ activation by IMB-808. HEK293T cells were transfected with the GAL4-pGL4-luc reporter plasmid and the pBIND-LXRβ expression plasmid. (E) LXRβ activation by T1317. Similar results were obtained in three independent experiments. Data are mean ± SEM (n = 3).

**Fig. 2.** Effect of IMB-808 on ABCA1, ABCG1, and ApoE protein expression. (A) RAW264.7 macrophages were incubated with IMB-808 at various concentrations or T1317 for 18 h, and ABCA1 and ABCG1 protein levels were determined by western blotting. (B and C) THP-1 derived macrophages were treated with IMB-808 (1 μM or 10 μM) or T1317 (1 μM) for 18 h, and ABCA1, ABCG1, and ApoE protein levels were determined by western blotting. (D) The mRNAs levels of ABCA1 and ABCG1 were measured by real-time quantitative PCR in RAW264.7 macrophages. (E and F) ABCA1, ABCG1, and ApoE mRNA levels were determined by real-time quantitative PCR separately in THP-1 derived macrophages. Induction factors were normalized against GAPDH, and the control groups were treated with DMSO (0.1%). Similar results were obtained in four independent experiments. Data are mean ± SEM (n = 4, *P < 0.05 vs. control, **P < 0.01 vs. control, ***P < 0.01 vs. control).
**Fig. 3.** IMB-808 promoted cholesterol efflux. (A and B) RAW264.7 macrophages were preincubated with 22-NBD-cholesterol for 24 h. The cells were then washed with PBS and incubated with IMB-808 (0, 0.01, 0.1, 1, or 10 µM). After 18 h, 10 µg/ml ApoA-I or 50 µg/ml HDL (final concentration) was added and incubated for 6 h at 37°C. The amount of cholesterol in the medium and cells were 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 mean ± SEM (n = 3, *P < 0.05 vs. control, **P < 0.01 vs. control).

**Fig. 4.** IMB-808 reduced ox-LDL-induced lipid accumulation in RAW264.7 macrophages. RAW264.7 macrophages were preincubated with (A) PBS (vehicle) or (B–H) ox-LDL (60 µg/ml). After 24 h, these cells were treated with (B) 0.1% DMSO, (C–G) IMB-808 (0.1 µM, 0.3 µM, 1 µM, 3 µM, or 10 µM), or (H) T1317 (1 µM) for 18 h. 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 (×400 magnification). (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 mean ± SEM (n = 3, *P < 0.05 vs. control, ***P < 0.001 vs. control).
**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 h, 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 h. ABCG5 and ABCG8 protein levels were then measured. (E) Western blotting and (F) real-time PCR were performed on Caco-2 cells that were incubated with IMB-808 or T1317 for 18 h. NPC1L1 protein and mRNA levels were then determined. Induction factors were normalized against GAPDH, and the control groups were treated with DMSO (0.1%). Similar results were obtained in four independent experiments. Data are mean ± SEM (n = 4, *P < 0.05 vs. control, **P < 0.01 vs. control).

**Fig. 6.** (A) Effect of IMB-808 on SREBP-1c expression. HepG2 cells were incubated with IMB-808 or T1317 for 18 h, and SREBP-1c protein levels were determined by western blotting. (B) Effects of IMB-808 on SREBP-1c, FAS, and SCD-1 mRNA expression. HepG2 cells were treated with IMB-808 at various concentrations, and T1317 was used as a positive control. After 18 h, the SREBP-1c, FAS, and SCD-1 mRNA expression levels were determined by real-time PCR. Four independent experiments were performed and representative graphs are shown. Data are mean ± SEM (n = 4, *P < 0.05 vs. control, **P < 0.01 vs. control). (C) The TR-FRET assay was used to examine corepressor peptide NcoR ID2 displacement from human
LXRα-LBD treated with IMB-808 or TO901317. (D) The TR-FRET assay was performed to examine coactivator TRAP220/DRIP2 recruitment to human LXRα-LBD treated with IMB-808 or TO901317. (E) The TR-FRET assay was used to examine corepressor peptide SMRT ID2 displacement from human LXRβ-LBD treated with IMB-808 or TO901317. (F) The TR-FRET assay was performed to examine coactivator D22 recruitment to human LXRβ-LBD 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 495 nm. All assays were repeated four times independently (n = 4).

**Fig. 7.** (A and B) The result of IMB-808 virtually docking into the active site of the LXRα ligand-binding domain based on the X-ray co-crystal structure of TO901317. (C and D) Activation of various LXRα mutants by IMB-808 or T1317, using the LXRα-GAL4 reporter assay. Data are displayed as mean values. (E) IMB-808 (10 μM) or (F) T1317 (1 μM) displayed different LXRα agonist activities between the wild-type group (WT) and the different mutants in the LXRα-GAL4 chimera reporter assays. Similar results were obtained in four independent experiments. Data are mean ± SEM (n = 4).

**Fig. 8.** (A and B) The result of IMB-808 virtually docking into the active site of the LXRβ ligand-binding domain based on the X-ray co-crystal structure of T1317. (C and D) Activation of various LXRβ mutants by IMB-808 or T1317, using the LXRβ-GAL4 chimera reporter assay. Data are displayed as mean values. (E)
IMB-808 (10 μM) or (F) T1317 (1 μM) displayed different LXRβ agonist activities between the wild-type group (WT) and the different mutants in the LXRβ-GAL4 chimera reporter assays. Similar results were obtained in four independent experiments. Data are mean ± SEM (n = 4).

**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 495 nm. All assays were repeated four times independently (n = 4).
# Table 1. Primers for real-time quantitative PCR

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Fig. 1.

A. IMB-808

B, C. EC$_{50}$ values for IMB-808 and T1317.

D, E. EC$_{50}$ values for IMB-808 and T1317.
Fig. 3.

A) RAW264.7

B) RAW264.7

C) THP-1

D) THP-1

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Fig. 4.

A-B: Vehicle and oxLDL treated groups.
C-D: Different concentrations of IMB-808 (0.1 μM and 0.3 μM).
E-H: Different concentrations of IMB-808 (1 μM, 3 μM, 10 μM).

I: Bar graph showing OD at 510 nm for Vehicle, oxLDL, and different concentrations of IMB-808 (0.1, 0.3, 1, 3, 10 μM) and T1317 (1 μM).
Fig. 5.

A

HepG2 cells

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ABCG8

ABCG5

GAPDH

B

HepG2

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Relative protein expression

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Caco-2 cells

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ABCG8

ABCG5

GAPDH

D

Caco-2

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Relative protein expression

E

Caco-2 cells

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NPC1L1

GAPDH

F

Relative mRNA expression

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Relative mRNA expression
Fig. 9.

A. NCoR ID2

B. TRAP220/DRIP2

C. SMRT ID2

D. D22

% of Max T1317 Recruitment for LXRα-R305G vs. compound (μM)

% of Max T1317 Recruitment for LXRβ-F271A vs. compound (μM)

31% 18% 23% 13%