Structural Basis for Small Molecule NDB (N-Benzyl-N-(3-(tert-butyl)-4-hydroxyphenyl)-2,6-dichloro-4-(dimethylamino) Benzamide) as a Selective Antagonist of Farnesoid X Receptor \(\alpha\) (FXR\(\alpha\)) in Stabilizing the Homodimerization of the Receptor*

Received for publication, December 17, 2014, and in revised form, June 9, 2015. Published, JBC Papers in Press, June 22, 2015, DOI 10.1074/jbc.M114.630475

Xing Xu †, Xin Xu ‡, Peng Liu †, Zhi-yuan Zhu †, Jing Chen †, Hai-an Fu †, Li-li Chen †, Li-hong Hu ‡, and Xu Shen ‡,‡

From the †CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zouchongzhi Road, Shanghai 201203, China, ‡Shanghai Key Laboratory for Bone and Joint Diseases, Shanghai Institute of Traumatology and Orthopaedics, Shanghai Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China, ‡Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Background: The pharmacological mechanism for FXR antagonist is still unclear.

Results: Crystal structure of hFXR\(\alpha\) ligand binding domain and NDB as an antagonist of hFXR\(\alpha\) was determined.

Conclusion: NDB promotes the formation of FXR homodimerization, inhibits FXR/RXR heterodimer, and decreases gluconeogenic genes of db/db mice.

Significance: The hFXR\(\alpha\)-LBD-NDB structure may help understand the antagonistic mechanism of FXR.

Farnesoid X receptor \(\alpha\) (FXR\(\alpha\)) as a bile acid sensor plays potent roles in multiple metabolic processes, and its antagonist has recently revealed special interests in the treatment of metabolic disorders, although the underlying mechanisms still remain unclear. Here, we identified that the small molecule N-benzyl-N-(3-(tert-butyl)-4-hydroxyphenyl)-2,6-dichloro-4-(dimethylamino) benzamide (NDB) functioned as a selective antagonist of human FXR\(\alpha\) (hFXR\(\alpha\)), and the crystal structure of hFXR\(\alpha\) ligand binding domain (hFXR\(\alpha\)-LBD) in complex with NDB was analyzed. It was unexpectedly discovered that NDB induced rearrangements of helix 11 (H11) and helix 12 (H12, AF-2) by forming a homodimer of hFXR\(\alpha\)-LBD, totally different from the active conformation in monomer state, and the binding details were further supported by the mutation analysis. Moreover, functional studies demonstrated that NDB effectively antagonized the GW4064-stimulated FXR/RXR interaction and FXR\(\alpha\) target gene expression in primary mouse hepatocytes, including the small heterodimer partner (SHP) and bile-salt export pump (BSEP); meanwhile, administration of NDB to db/db mice efficiently decreased the gene expressions of phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase (G6-pase), small heterodimer partner, and BSEP. It is expected that our first analyzed crystal structure of hFXR\(\alpha\)-LBD-NDB will help expound the antagonistic mechanism of the receptor, and NDB may find its potential as a lead compound in anti-diabetes research.

Farnesoid X receptor \(\alpha\) (FXR\(\alpha\)) is a bile acid-activated nuclear receptor responsible for the regulation of the specific target genes involved in a broad spectrum of biological processes including bile acids, glucose and lipid homeostasis, liver regeneration, bacterial growth, and tumor growth (1). FXR\(\alpha\) highly expresses in the liver, intestine, kidney, and adrenals (2, 3), and its modulator has revealed promising therapeutic potential against metabolic disorders. In most cases FXR\(\alpha\) forms a heterodimer with retinoid X receptor \(\alpha\) (RXR\(\alpha\)) to modulate the expression of its target genes. Primary bile acids such as chenodeoxycholic acid (CDCA) and cholic acid (CA) have been identified as the endogenous ligands of this receptor (4). Bile acids as signaling molecules activate not only FXR\(\alpha\) but also a cluster of receptors, such as the pregnane X receptor, vitamin D receptor, the constitutive androstane receptor, and the bile acid receptor TGR5 (5), and are toxic to cells at high concentrations. Thus, discovery of specific non-bile acid modulator of FXR\(\alpha\) is expected to be valuable in the regulation of the diverse metabolic pathways for treating the related metabolic disorders.

In recent years, FXR\(\alpha\) agonists have received much attention for the beneficial effects of FXR\(\alpha\) activation in lowering plasma lipogenesis, repressing very low density lipoprotein production, increasing plasma triglyceride clearance, improving insulin sensitivity, and promoting the storage of glycogen (6). To date increasing numbers of FXR\(\alpha\) agonists have been discovered, such as GW4064, fexaramine, 6a-ethyl-CDCA, MFA-1, XL335, and ivermectin (7–12). However, it was determined that FXR\(\alpha\)

The abbreviations used are: FXR\(\alpha\), farnesoid X receptor \(\alpha\); hFXR\(\alpha\), human FXR\(\alpha\); RXR\(\alpha\), retinoid X receptor \(\alpha\); CDCA, chenodeoxycholic acid; CA, cholic acid; NDB, N-benzyl-N-(3-(tert-butyl)-4-hydroxyphenyl)-2,6-dichloro-4-(dimethylamino) benzamide; LBD, ligand binding domain; SRC-1, steroid receptor coactivator-1; PGC-1\(\alpha\), peroxisome proliferator-activated receptor \(\gamma\) coactivator-1\(\alpha\); SPT, surface plasmon resonance; BSEP, bile-salt export pump; TR-FRET, time-resolved FRET; FXRE, FXR\(\alpha\) response element; LBP, ligand binding pocket; SHP, small heterodimer partner.

* This work was supported by National Natural Science Foundation of China Grants 91413102, 81373462, and 81173105.

The atomic coordinates and structure factors (code 4QRV) have been deposited in the Protein Data Bank (http://wwpdb.org/).

1 To whom correspondence should be addressed. Tel. and Fax: 86-21-58468631; E-mail: lilichen@simm.ac.cn.

2 To whom correspondence should be addressed. Tel. and Fax: 86-21-20231965; E-mail: lhhu@simm.ac.cn.

3 To whom correspondence should be addressed. Tel. and Fax: 86-21-50806918; E-mail: xshen@mail.shcnc.ac.cn.

© 2015 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.
activation by full agonist may also cause undesired side effects in animals. For example, long term treatment of GW4064 even accelerated obesity and exacerbated diabetes in diet-induced obesity mice through reduction of bile acid pool (13). In addition, agonists such as bile acids, GW4064, and XL335 reduced high density lipoprotein cholesterol (14, 15). By contrast, FXRα blockage may find its beneficial effect on the improvement of hypercholesterolemia. For example, the natural product gugulsterone extracted from the guggul tree as an FXRα antagonist FXRα-dependently decreased hepatic cholesterol in mice fed with a high cholesterol diet (16), and the nonsteroidal FXRα antagonist 12u also functioned in lowering hepatic cholesterol in the high cholesterol diet-induced C57BL/6 mice (17). Moreover, the results from FXRα-deficiency mice confirmed that FXRα suppression showed potential effects on the improvement of obesity and diabetes (18–20). Additionally, AMP-activated protein kinase (AMPK) was recently identified as a co-regulator of FXRα, and AMPK activator metformin as an anti-diabetic agent showed potently antagonistic effect on FXRα (21). It is thus believed that discovery of selective antagonist of FXRα will render special interests in anti-metabolic disorders research.

In structure, similar to most of the other nuclear receptors (1), FXRα is divided into several major domains: an N-terminal region containing a ligand-independent transactivation function domain 1 (AF1), a highly conserved DNA binding domain (DBD), and a hinge region linking DBD to the C-terminal LBD that also contains a strong transactivation function domain 2. To date, dozens of the structures of FXRα-LBD in complex with different agonists have been determined (7, 10, 12, 22). The results indicated that in the active conformation of FXRα, FXRα-LBD folds into a canonical three-layer helical sandwich that embeds a hydrophobic pocket for ligand binding, and function domain 2 interacts with the coactivator, including steroid receptor coactivator-1 (SRC-1), SRC-2, SRC-3, and peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α). However, no data have as yet been obtained concerning the molecular mechanism of FXRα antagonism due to the lack of the structural information of either unliganded FXRα (apoLBD) or antagonist-bound FXRα.

In the current work we determined the small molecule N-benzyl-N-(3-(tert-butyl)-4-hydroxyphenyl)-2,6-dichloro-4-(dimethylamino) benzamide (NDB, Fig. 1A) as a selective antagonist of human FXRα (hFXRα), and the crystal structure of hFXRα-LBD in complex with NDB was successfully analyzed. It was discovered that the binding of NDB caused an unusual rearrangement of helix 11 (H11) and H12 (AF-2) of the receptor by forming a homodimer of hFXRα-LBD. The binding details between two hFXRα-LBD monomers were also supported by the mutation analysis and co-repressor binding assay. Moreover, animal-based assays have highlighted the potential of NDB in the prevention of glucose and lipid metabolism dysfunction. It is expected that our first analyzed crystal structure of hFXRα-LBD binding to its antagonist NDB may help to largely expound the antagonistic mechanism of the receptor, and NDB will find its potential as a lead compound in anti-diabetes research.

**Experimental Procedures**

**Materials**—All reagents were of analytical grade. Dimethyl sulfoxide (DMSO), CDCA, GW4064, and collagenase IV were purchased from Sigma. Reverse transcript reagents and SYBR Green PCR Master Mix were from Takara. Series S sensor chip CMS, N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide, N-hydroxysuccinimide, and ethanalamine HCl were from GE Healthcare. Biotin-labeled SRC-1 peptide (Bio-SRC-1, Bio-CPPSHSLTERHKILHRLLIQEGSPS), Biotin-labeled SRC-2 peptide (Bio-SRC-2, Bio-SLTRHKILHRLLQEGSPS), Biotin-labeled SRC-3 peptide (Bio-SRC-3, Bio-KENNALLRYLDRDD), Biotin-labeled PGC-1α peptide (Bio-PGC-1α, Bio-AEPESSLKLLLAP), and Biotin-labeled NCoR-2 peptide (Biotin-NCoR-2, Bio-SFADPA-SNLGLEDIIRKALMG5) were synthesized by Shanghai Shenggong Biotechnology. The histidine detection kit for AlphaScreen assays was purchased from PerkinElmer Life Sciences. Cell culture medium Williams’ E medium, Dulbecco’s modified eagle medium (DMEM), and fetal bovine serum (FBS) were from Gibco. The Dual-Luciferase Assay System kit was obtained from Promega. Anti-GST-Tb was purchased from Cisbio Bioassays.

**Animal Experiments**—Male C57BL/6J db/db mice at 8 weeks of age were fed with normal diet and intraperitoneally injected with vehicle or NDB (24 mg/kg) once a day for 4 weeks. The animals were then killed after fasting overnight, and mice livers were collected and frozen in liquid nitrogen for real-time PCR.

**Cell Cultures**—The human hepatoma HepG2 and HEK293T cells were obtained from ATCC (Manassas, VA). Primary mouse hepatocytes were isolated from male C57/BL6 mice in 8–12 weeks of age (Shanghai SLAC Laboratory Animal Co. Ltd) using a two-step collagenase perfusion and low speed centrifugation (23). Cells were washed and suspended in Williams’ E medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% humidified CO2 incubator and allowed to attach for 4–6 h. The unattached cells were then removed followed by medium changing to fresh DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin with the addition of indicated concentrations of compounds.

**Protein Preparations**—Residues 244–476 of hFXRα-LBD were subcloned into Nde1/XhoI sites of pET15b to generate N-terminal His-hFXRα-LBD protein expression vector. His-hFXRα-LBD for AlphaScreen assay was purified with a nickel-nitrilotriacetic acid column followed by a Superdex 75 column according to the published approach (24). To obtain enough stable protein for crystallization, the plasmid of mutant hFXRα-LBD-C436E/C470E was constructed according to the previously published method (24). The mutant protein was purified by the method similar to that for the wild type His-hFXRα-LBD protein, with the tag removed by incubation with thrombin overnight at 4 °C.

**Surface Plasmon Resonance (SPR) Technology-based Assay**—Binding of NDB to hFXRα-LBD was investigated by using an SPR technology-based Biacore T200 instrument (GE Healthcare). Purified hFXRα-LBD protein was immobilized on Series
NDB Stabilizes hFXRα Homodimerization

S sensor chip CM5 by the standard primary amine coupling reaction followed by injection of different concentrations of NDB to the chip.

All experiments were carried out at 25 °C with HBS-EP (10 mM Hepes, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20, pH 7.5) as the running buffer at a constant flow of 30 μl/min. The equilibrium dissociation constant \( K_d \) was achieved by fitting the data using 1:1 binding model based on the Biacore T200 Evaluation Software Version 1.0.

**Fluorescence Quenching Analysis**—Fluorescence quenching analysis was determined according to our published approach (24). All fluorescent spectra were recorded on F-2500 fluorescence spectrophotometer (Hitachi) equipped with 1.0-cm path length. All final spectra were corrected by deducting the buffer contribution, and the obtained results were the average of three parallel measurements.

**AlphaScreen-based Protein-Peptide Interaction Assay**—The effect of NDB on the interaction of co-factor peptide with hFXRα-LBD was investigated by an AlphaScreen technology-based assay. Briefly, the experiment was conducted with 100 nM His-hFXRα-LBD and ~30–100 nM biotinylated co-factor peptide in the buffer containing 25 mM Hepes, 100 mM NaCl, and 0.1% BSA, pH 7.4. The mixture was incubated in the dark at room temperature followed by a fluorescence measurement on an Envision microplate analyzer (PerkinElmer Life Sciences). The binding signals were detected with NDB combined with GW4064 (0.5 μM) or CDCA (50 μM) and with GW4064 (0.5 μM) or CDCA (50 μM) alone as a positive control and DMSO as a negative control. All values were reported as the mean ± S.D. of triple measurements, and Z’-factor was used to validate the assay.

**Luciferase Reporter Assay**—HepG2 cells plated to 40–50% confluence in 48-well plate in DMEM were cultured overnight. Transient transfection was conducted by Lipofectamine 2000 (Life Technologies) according to the instructions of the manufacturer. In the transactivation system, 100 ng of the plasmids of pcDNA3.1-FXR-LBD and full-length hFXRα, pGL3-FXRE-Luc, and 50 ng of pRL-SV40 were transfected into the cells. After transfection for 6 h, the medium was changed into DMEM and incubated with different concentrations of NDB overnight. In the assay of the selectivity of NDB over the other tested nuclear receptors, the corresponding plasmids were transfected into HepG2 cells and incubated with the indicated compounds for 24 h. The firefly and renilla luciferase activities were assayed with Dual-Luciferase Reporter assay system (Promega). The firefly luminescence was normalized based on the Renilla luminescence, and the ratio of treatment over control is expressed as -fold activation.

**RNA Isolation and Quantitative Real-time PCR**—The total RNA in primary mice hepatocytes and the livers of db/db mice were isolated with TRIzol reagent (Invitrogen), and 1 μg of total RNA was reverse-transcribed into cDNA using PrimeScript RT reagent kit. mRNA levels of BSEP, SHP, phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase (G6-pase), and GAPDH were quantified by quantitative real-time-PCR using specific primers. mRNA levels were all normalized to GAPDH mRNA. The primers of genes were listed in Table 1. All values were reported as the mean ± S.D. of triple measurements of each cDNA sample, and the thermal cycling conditions were 95 °C for 10 min followed by 43 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C.

**Crystallization and Data Analysis**—For preparation of hFXRα-LBD-NDB complex, a 5-fold molar excess of NDB was added into hFXRα-LBD and concentrated to 5 mg/ml. hFXRα-LBD-NDB crystals were grown at 4 °C in hanging drops containing 1 μl of protein-ligand solutions and 1 μl of well buffer (22% polyethylene glycol 2000MME, 0.2 M NaCl, 0.1 M MES, pH 5.9). Crystals grew up within 1 week. Diffraction data were collected at BL17U of Shanghai Synchrotron Radiation Facility (China) and integrated with HKL2000. The structure was determined by molecular replacement methods (MOLREP) with CCP4 using the structure of FXRα-LBD-XL335 complex (PBD code 3FLI) as the initial model. Phasing and refinement were carried out with Refmac5 in CCP4. Model building was manually performed with COOT. The statistics of the structure and data sets are summarized in Table 2. Molecular images were made with PyMOL. Atomic coordinates and structure factors of hFXRα-LBD-NDB have been deposited to Protein Data Bank under accession code 4OIV.

**Mutation Analysis**—The mutants (S457A and W458F) of hFXRα-LBD and full-length hFXRα were prepared with the fast mutagenesis kit (Transgene Biotech) using WT-FXRα-LBD and WT-FXRα as templates. All mutants were validated by sequencing.

**Circular Dichroism Spectroscopy**—Secondary structural changes in the mutants of FXR-LBD were monitored using a thermostated Applied Photophysics Chirascan spectrophotometer (Applied Photophysics). A high quality quartz cell with 1-mm path length was used. Protein samples were prepared in the solution of 50 mM NaCl, 2.7 mM KCl, 10 mM Na3HPO4, and 2 mM KH2PO4. Far-UV CD spectra were collected from 200 to 280 nm. Experimental data were the average values of three measurements and corrected by subtracting the blank obtained under the same conditions in the absence of protein.

**Time-resolved Fluorescence Resonance Energy Transfer (TR-FRET) Assay**—TR-FRET assay was performed according to the published approach (25). HEK293T cells were plated in a 6-well plate and cultured overnight in DMEM. Transfection was performed by Lipofectamine 2000 according to the instructions of

| Gene | Sequence(5‘-3’) |
|------|----------------|
| mSHP | Forward primer  |
| mBSEP | Reverse primer  |
| mPEPCK | Reverse primer  |
| mG6-pase | Reverse primer  |
| mGAPDH | Reverse primer  |

**TABLE 1**

The sequences of all the primers in quantitative RT-PCR analysis are listed above.
TABLE 2

Data collection and refinement statistics

| Statistic                                   | Resolution (Å) | No. of reflections (working/test set) | Completeness (%) | Rmerge (%) | Ramachandran plot (%) |
|---------------------------------------------|----------------|--------------------------------------|------------------|------------|-----------------------|
| Resolution (Å)                              | 50.00-1.70 (1.73-1.70)* | 63,144 /3,383                        | 99.7             | 24.35 (2.69) | 96.93                  |
| Space group                                 | P4,2,2         |                                      |                  | 23.45      | 96.93                 |
| Molecules per ASU 2                        | 2              |                                      |                  | 23.45      | 96.93                 |
| Unit-cell parameters                        |                |                                      |                  | 23.45      | 96.93                 |
| a (Å)                                       | 84.63          |                                      |                  | 23.45      | 96.93                 |
| b (Å)                                       | 84.63          |                                      |                  | 23.45      | 96.93                 |
| c (Å)                                       | 172.23         |                                      |                  | 23.45      | 96.93                 |
| α (°)                                       | 90             |                                      |                  | 23.45      | 96.93                 |
| β (°)                                       | 90             |                                      |                  | 23.45      | 96.93                 |
| γ (°)                                       | 90             |                                      |                  | 23.45      | 96.93                 |
| No. of unique reflections                   | 68,951 (3311)  |                                      |                  | 23.45      | 96.93                 |
| Data redundancy (Å 2)                       | 18.9 (8.4)     |                                      |                  | 23.45      | 96.93                 |
| Completeness (%)                            | 96.32          |                                      |                  | 23.45      | 96.93                 |
| Rmerge (%)                                  | 24.35 (2.69)   |                                      |                  | 23.45      | 96.93                 |
| Rfree (%)                                   | 24.35 (2.69)   |                                      |                  | 23.45      | 96.93                 |

Refinement statistics

| Statistic                        | Resolution (Å) | No. of reflections (working/test set) | Completeness (%) | Rmerge (%) |
|----------------------------------|----------------|--------------------------------------|------------------|------------|
| Resolution (Å)                   | 38.38-1.70     |                                      | 99.7             | 24.35      |
| No. of reflections (working/test set) | 63,144 /3,383 |                                      | 99.7             | 24.35      |
| Completeness (%)                 | 96.32          |                                      |                  | 23.45      |
| Rmerge (%)                       | 24.35 (2.69)   |                                      |                  | 23.45      |
| Rfree (%)                        | 24.35 (2.69)   |                                      |                  | 23.45      |

Ramachandran plot (%)

| Region                        | 96.93 | 96.93 | 96.93 |
|------------------------------|-------|-------|-------|
| In favored region             |       |       |       |
| In allowed region             |       |       |       |
| In outlier region             |       |       |       |

Values in parentheses are for the highest resolution shell.

r.m.s.d. bond lengths and bond angles, root-mean-square deviations from the parameter set for ideal stereochemistry.

First carried out, and the small molecule active compound NDB (Fig. 1A) was thus determined. To quantitatively evaluate the binding feature of NDB to hFXRα-LBD, the association (k\text{on}) and dissociation (k\text{off}) rate constants and the dissociation equilibrium constant (K\text{D}) were determined by fitting the sensorgrams with a 1:1 binding fitting model. As demonstrated in Fig. 1B, NDB bound to hFXRα-LBD with a k\text{on} of 3.63E+4 M\text{-1 s\text{-1}}, k\text{off} of 0.301 s\text{-1}, and K\text{D} of 8.29 μM. Given the two contained tryptophan residues of Trp-454 and Trp-469 in hFXRα-LBD, an intrinsic fluorescence-quenching based assay was also performed to further confirm the binding of NDB to hFXRα-LBD (24). In the assay, different concentrations of NDB were incubated with hFXRα-LBD (1 μM), and intrinsic fluorescence spectra were detected. As shown in Fig. 1C, hFXRα-LBD displayed the maximal fluorescence at 337 nm, and treatment of NDB resulted in fluorescence quenching of hFXRα-LBD in a dose-dependent manner, which indicated NDB binding to hFXRα-LBD. As shown by the dashed line, NDB exhibited no fluorescence absorption at this wavelength.

NDB Is a Selective Antagonist of hFXRα—As NDB has been determined to be the ligand of hFXRα, we next explored the potential agonistic or antagonistic ability of NDB against hFXRα by an AlphaScreen-based assay. As shown in Fig. 2A, in the presence of a synthetic agonist GW4064 (GW, 0.5 μM) or a natural agonist CDCA (50 μM) of hFXRα, the peptide containing the coactivator SRC-1 LXXLL binding motif was specifically recruited to hFXRα-LBD, and NDB (25 μM) suppressed the corresponding agonistic effect induced by GW4064 or CDCA. NDB alone exhibited no agonistic activity on hFXRα coactivator recruitment (data not shown). Similarly, the results shown in Fig. 2, B–D, demonstrated that NDB also exhibited antagonistic activities against GW4064 or CDCA in the activation of hFXRα-LBD-SRC-2, hFXRα-LBD-SRC-3, and hFXRα-LBD-PGC-1α interactions. As a representative, shown in Fig. 2E is the fitted IC\text{50} of 3.4 μM for NDB in the inhibition of the CDCA (50 μM)-induced hFXRα-LBD-SRC1 (peptide) activation with different concentrations of NDB.

Therefore, all of the above results indicated that NDB functioned as an antagonist of hFXRα. Next, a luciferase report assay was further carried out to validate the antagonistic activity of NDB against hFXRα in HepG2 cells. Given that binding of FXRα/RXRα heterodimer to FXRα response element (FXRE) is required for activation of the downstream gene transcription, we examined the effect of NDB on the agonist-induced transactivation of FXRα/RXRα in the cells. It was observed that NDB antagonized either the CDCA (50 μM)- or GW4064 (50 nM)-activated transactivation activity with IC\text{50} at 4.5 or 2.6 μM (Fig. 3, A and B). These results thereby confirmed the antagonistic activity of NDB against hFXRα. In the investigation of the selectivity of NDB against hFXRα, we examined the transactivation activities of NDB toward a group of nuclear receptors, including liver X receptor α/β (LXRα/β), Mineralocorticoid receptor (MR), RXRα, peroxisome proliferator-activated receptor γ (PPARγ), progestosterone receptor B (PR-B), and estrogen receptor α/β (ERα/β). As indicated in Fig. 3C, NDB had no effects on the transactivation activities of these tested nuclear receptors, thereby implying that NDB is a selective antagonist of hFXRα.
**NDB Modulated FXRα Target Genes SHP and BSEP**—As NDB has been determined as a selective antagonist of FXRα, we next evaluated the potential regulation of NDB against the key FXRα target genes of SHP and BSEP in mouse primary hepatocytes by quantitative RT-PCR. In the assay the primary hepatocytes were treated with different concentrations of NDB in combination with GW4064 (GW, 10 μM). As shown in Fig. 4, A and B, NDB could effectively reverse the GW4064-induced stimulation of either BSEP or SHP mRNA expression. This result thus revealed the antagonistic effects of NDB on FXRα target genes.
Crystal Structure of hFXRα-LBD-NDB Revealed the Homodimeric Mode of the Receptor—The crystal structure of hFXRα-LBD-NDB complex was determined at a resolution of 1.7 Å in the P41212 space group by using FXRα-LBD-XL335 (PDB code 3FLI) structure as the model for molecular replacement. The overall structure of hFXRα-LBD-NDB complex was shown in Fig. 5, A and B. As indicated, the complex was in a homodimeric mode formed by two hFXRα-LBD monomers in the asymmetric unit, with either monomer consisting of an hFXRα-LBD molecule and one NDB (Fig. 5, A and B). The buried surface area formed by the two monomers was calculated at 1818.7 Å² (calculated with PDBePISA). Either monomer contained 12 helices and 1 β strand (denoted as S1 and S1') (Fig. 5B), which is different from the active conformation that adopts a 12-α-helix bundle (7, 10). It is noted that the backbones of Asn-461 and Arg-459 in S1 formed two hydrogen bonds with the corresponding backbones in S1' from the other monomer (Asn-461 and Arg-459', Fig. 5C). At the joint of the antiparallel β sheet, residue Ser-457 (Ser-457') formed H-bonds with both His-463’ (His-463) of the backbone and the side chain Asp-462’ (Asp-462) of the adjacent monomer, and Trp-458 (Trp-458') formed an H-bond with Glu-286’ (Glu-286) in H3’ (H3) of the other monomer (Fig. 5D). In addition, hydrophobic interac-
NDB Stabilizes hFXRα Homodimerization

FIGURE 5. Crystal structure of hFXRα-LBD-NDB suggested the dimeric mode of the receptor. A, overall architecture of hFXRα-LBD-NDB. The surfaces of the two monomers are labeled as monomer A (blue) and monomer B (gray) in different colors. B, the two monomers and the β strands in different colors (blue and gray) are depicted in cylinder mode. The atoms of NDB located in the two canonical binding sites of hFXRα-LBD are colored in yellow (carbon atoms), red (oxygen atoms), blue (nitrogen atoms), and green (chlorine atoms). The circle and the arrow show the positions of β strands and the new formed helices (H122 and H122’; Fig. 6) in the structure. C, representation of the four H-bonds between the two β strands (S1 and S1’) involving the interactions of Asn-461 (Asn461’) with Arg-459 (Arg459’); H-bonds were indicated with black dashed lines. The two monomers are shown in blue and gray, respectively. D, representation of the three H-bonds formed by the joint of the two β strands with S’ and H3’. Ser-457 of the joint formed two hydrogen bonds with His-463 and Asp-462’ of S’ and Trp-458 of the joint formed a hydrogen bond with the side chain of Glu-286’ in H3’ by the indicated distances. H-bonds are indicated with black dashed lines. The two monomers were shown in blue and gray, respectively. E, representation of hydrophobic interaction of Trp-458 of the joint with Leu-289’ of H3’. The two monomers are shown in blue and gray, respectively.

NDB Binding Pocket Analysis—In the structure NDB was completely enclosed within hFXRα-LBD (Fig. 6A), and 2Fo − Fc map in the ligand binding pocket (LBP) of hFXRα-LBD-NDB structure revealed a clear electron density of NDB (Fig. 6A). As indicated in Fig. 6B, NDB bound to the LBP of hFXRα-LBD by interacting with a cluster of hydrophobic residues, including Trp-458, Thr-292, Met-456, Leu-455, Ala-452, Phe-333, Ala-295, Ile-366, Met-332, Met-369, Leu-291, Ser-336, His-298, and Tyr-373, and forming one hydrogen bond through Tyr-365 of the backbone in H8 and two bridged-water molecules.

Superposition of the structures of hFXRα-LBD-NDB and hFXRα-LBD-GW4064 (PDB code 3DCT) revealed that both NDB and GW4064 occupied a similar binding pocket (Fig. 6C), although NDB took a smaller size in the LBP compared with GW4064 for the shorter molecule of NDB than GW4064 (Fig. 6D). Such a similar LBP occupation for NDB and GW4064 might suggest that the antagonistic effect of NDB possibly results from the competitive inhibition of NDB against the agonists, including GW4064. In comparison with the hFXRα-LBD-GW4064 structure (Fig. 6C), the hydrophobic interactions of NDB within the LBP made residues of Leu-455’ and Met-456’ closer to the compound thus causing severe conformation changes for H11’ and H12’, with new formations of β strand (S1’) and two small helices (H112’ and H122’) (Fig. 6C), whereas the new formed H112’ at the end of H11’ moved closely to and sealed the LBP (Fig. 6A). In the structure, interaction of S1’ in one monomer with S1 in the other forms an antiparallel β sheet as a bridge to send the small helix His-122’ to the co-factor binding domain of the other monomer (Fig. 5B).

Mutation Assay and Co-repressor Binding Analysis Supported the Dimerization of hFXRα-LBD-NDB Complex—As determined in the crystal structure analysis, residues Arg-459, Asn-461, Ser-457, and Trp-458 played potent roles in the homodimerization of hFXRα-LBD-NDB complex (Fig. 5, C and D). Thus, a mutation assay was next performed trying to validate the dimerization interface. In the assay, considering that residues Asn-461 and Arg-459 are within the backbone of one monomer (Fig. 5C), only mutagenesis of S457A and W458F was designed. First, the structure changes of hFXRα-LBD mutants were detected by CD (Fig. 7A). We found that the structure of the mutant Trp-458 made a rearrangement nevertheless mainly adopting α-helix conformation similar to those of WT-FXRα-LBD and the mutant Ser-457. The results indicated that Trp-458 played an important role in hFXRα-LBD conformation. Next, we also identified the transcriptional activities of FXR mutants induced by GW4064 alone or GW4064 with NDB using luciferase reporter assay. As shown in Fig. 7B, GW4064 increased the transcriptional activation of either mutant, and the capability of NDB in antagonizing the GW4064-induced transactivation activity against the mutant S457A or W458F was obviously reduced (from 100% to 93 and 100% to 77%, respectively) (Fig. 7C). Collectively, these results thereby supported that the residue of Ser-457 actually was important in hFXRα-LBD dimerization, and residue Trp-458 was significant for both hFXRα-LBD conformation and dimerization. As demonstrated in Fig. 7D, His-122’ of one monomer was recruited by
the groove formed by H3 and H5 of the other monomer, involving a group of H-bonds formed by Leu-469 of His-122' with Lys-307 of H3, Glu-470' of His-122' with His-317 of H5, and Glu-471' of His-122' with Arg-459 of S1. These data may imply the inactivity of the homodimer in accommodation of the cofactors. Actually, the results in Fig. 2, A–D, have already indicated that NDB inhibited co-activator binding to hFXR-LBD. As also shown in the AlphaScreen assay results (Fig. 7E), the co-repressor NCoR-2 was not recruited by hFXR-LBD, whereas as a positive control, ivermectin as an FXR agonist promotes the binding of FXRα-LBD to recruit NCoR-2 (11). In addition, the classical antagonist guggulsterone also showed similar effect to NDB. Therefore, all the above-mentioned mutation assay and co-repressor binding analyses have supported the dimeric mode of hFXRα-LBD-NDB complex. Taken together, our results thereby suggested that NDB exerted antagonistic activity against hFXRα-LBD by inducing the formation of the auto-repressed conformation of the receptor. As indicated in Fig. 8A, GW4064 as an FXRα agonist promotes the binding of FXRα/RXRα heterodimer to FXRE to the regulatory regions of target genes for initiation of FXRα target gene expression. Binding of NDB to FXRα causes FXRα homodimer formation leading to the release of co-activators and further repression of the target genes. To confirm the model, we performed the TR-FRET assay, attempting to identify the effect of NDB on the disruption of FXRα/RXRα interaction. As shown in Fig. 8B, FXRα and FXRβ could form a heterodimer compared with FXRα or RXRα alone in the cell lysate. In the presence of GW4064 (5 μM), the interaction of FXRα with RXRα was greatly increased, whereas NDB suppressed the interaction induced by GW4064 in a dose-dependent manner. Therefore, these results further confirmed the mechanism for NDB antagonizing FXRα function.

**NDB Modulated the Key Genes Related to Metabolic Processes in the Liver of db/db Mice**—Given that FXRα antagonists have revealed their great potential in the treatment of metabolic diseases, we next investigated whether NDB as a selective FXRα antagonist could improve metabolic dysfunctions in vivo. In the assay, db/db mice were treated with vehicle or 24 mg/kg NDB for 4 weeks. After the animals were killed, we detected the levels of the metabolism-related genes in the liver of the mice, including the gluconeogenesis-related genes phosphoenolpyruvate carboxykinase and glucose 6-phosphatase and the bile acid-related genes SHP and BSEP. As shown in Fig. 9, A–D, administration of NDB evidently depressed these four genes in vivo. Therefore, our results suggested that FXRα antagonist NDB was effective in regulating FXRα activity and exhibited potential in improving metabolic dysfunctions in vivo.

**FIGURE 6. Investigation of NDB binding details to hFXRα-LBD.** A, the ribbon diagram shows the structure of NDB in complex with monomer with indication of helixes H11', H112, S1', and His-122'. The atoms of NDB are colored in yellow (carbon atoms), red (oxygen atoms), blue (nitrogen atoms), and green (chlorine atoms). The electron density map for NDB is shown as a blue mesh contoured at 1σ. B, Ligplots showed the related interaction residues within hFXRα-LBD-NDB complex based on the crystal structure. The residues were generated using LigPlot program. Hydrophobic interaction amino acids are shown in red, whereas the hydrogen interaction amino acid (Tyr-365) interacting strongly with the two water molecules (Hoh11 and Hoh24) is shown in black sticks. H-bonds are indicated with green dashed lines. C, superposition of hFXRα-LBD-NDB (hFXRα-LBD in gray, NDB in yellow) with hFXRα-LBD-GW4064 (PDB code 3DCT) (FXRα-LBD in violet, co-activator peptide in pink, and GW4064 in magenta) illustrating the conformation difference between agonistic and antagonistic state of hFXRα-LBD. Both the agonistic and antagonistic ligands occupied the same binding site in LBP. D, superposition of NDB (yellow) and GW4064 (magenta).
Discussion

In the current work we determined the small molecule NDB as a selective antagonist of hFXR\alpha/H9251 and successfully analyzed the crystal structure of hFXR\alpha/H9251-LBD/NDB complex. Based on the structure, a new potential antagonistic mechanism of FXR\alpha/H9251 has been suggested. Previously, the reported results demonstrated that FXR\alpha antagonists such as guggulsterone (26) and compound 1 (27) stabilized the complex of co-repressor NCoR with FXR\alpha/H9251 on the promoters, thus failing to recruit co-activators to exert agonistic effects. However, NDB exerted its distinct antagonistic activity against FXR\alpha compared with these two reported FXR antagonists. It was found that NDB at first competitively occupied the same LBP as the agonists, causing the hydrophobic part of H11 bended to the LBP to stabilize this inactive conformation of LBD, which seems to be common in other nuclear receptors such as RXR\alpha-LBD and photoreceptor cell-specific nuclear receptor gene (PNRLBD) (28), and H12 protruded and occupied the co-activators binding site to inhibit their bindings (Fig. 2, A–E). Next, we found that NDB rendered no effect on the interaction of co-repressor NCoR-2 with hFXR\alpha-LBD (Fig. 7E), which demonstrated that NDB possessed the distinct antagonistic activity compared with guggulsterone and compound 1. Based on the crystal structure, we inferred that NDB antagonized FXR\alpha function by inducing FXR\alpha into homodimeric mode and further decreasing the conformation of FXR\alpha/RXR\alpha heterodimer (Fig. 8A). Next, we attempted to assay the effect of NDB on FXR homodimer using TR-FRET. Unfortunately, we failed to find the FXR homodimer induced by NDB. We tentatively suggested that the transformation between monomer and homodimer of FXR is dynamic and the conformation of homodimer is physiologically weak, although we could observe the inhibitory effect of NDB on FXR dimerization by mutation and reporter assay. In addition it is suggested that the dimeric inactive state of hFXR\alpha-LBD might apply to the other FXR species, as the amino acid sequence in dimer interface is highly conserved in different species (Fig. 10).
NDB binding and lock NDB in the LBP. Therefore, His-122 seems to play an important role in stabilizing the antagonistic conformation of the receptor.

As also demonstrated from hFXRα-LBD-NDB structure, the co-factor binding site was occupied by His-122 itself as seen in passive antagonist conformation. Such passive antagonist con-
NDB Stabilizes hFXRα Homodimerization

S1

Homo sapiens  S W R V N
Rattus norvegicus  S W R V N
Mus musculus  S W R V N
Bos taurus  S W R V N
Gallus gallus  S W R V N
Oryctolagus cuniculus  S W R V N
Myotis brandtii  S W R V N
Anas platyrhynchos  S W R V N
Columba livia  S W R V N
Pteropus alecto  S W R V N

FIGURE 10. Sequence alignment of FXRα-LBD dimer interface revealed the high conservation in different species. The secondary structural element of the sequence is displayed above. The residues participating in FXRα-LBD dimerization are labeled in red. All sequences were downloaded from the NCBI database. The sequences belong to Homo sapiens (NP_001193908.1), Rattus norvegicus (NP_068513.1), Mus musculus (NP_001157172.1), Bos taurus (NP_001029880.1), Gallus gallus (AAH90896.1), Oryctolagus cuniculus (NP_001076195.1), Myotis brandtii (EPQ17707.1), Anas platyrhynchos (EOA97707.1), Columba livia (EMC82228.1), and Pteropus alecto (ELK10341.1). NCBI accession number codes are indicated in the parentheses.

formation may not be unique to NDB binding to FXRα. For example, in the domain C of GR-LBD, H12 also occupied the co-factor binding site thus preventing the binding of GR-interacting co-repressor and co-activators (29). In addition, the example of specific passive antagonist of androgen receptor to treat advance prostate cancer revealed that a passive antagonist might be superior to an active antagonist in some cases (29, 30). Therefore, such a passive antagonist might bring a new thought for FXRα-targeted agent discovery.

Currently, several reports have been published regarding other nuclear receptors forming homodimers. For example, vitamin D receptor keeps stable homodimers after binding to the coactivators and increases the ligand-induced vitamin D receptor keeps stable homodimers after binding to vitamin D receptor. The residues participating in FXRα-LBD dimerization are labeled in red. All sequences were downloaded from the NCBI database. The sequences belong to Homo sapiens (NP_001193908.1), Rattus norvegicus (NP_068513.1), Mus musculus (NP_001157172.1), Bos taurus (NP_001029880.1), Gallus gallus (AAH90896.1), Oryctolagus cuniculus (NP_001076195.1), Myotis brandtii (EPQ17707.1), Anas platyrhynchos (EOA97707.1), Columba livia (EMC82228.1), and Pteropus alecto (ELK10341.1). NCBI accession number codes are indicated in the parentheses.

Previously, the study on FXRα response to a fasting-refeeding high carbohydrate diet showed that FXRα deletion displayed a more accelerated response through repression on gluconeogenic genes, such as glucose-6-phosphatase and phosphoenolpyruvate carboxykinase. In our work we detected that NDB could also regulate the glucose metabolism in vivo by reducing the expression of gluconeogenic genes including phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (20). Our results have provided additional understanding of pharmacological functions for FXRα antagonist as an antidiabetes agent.

In conclusion, we discovered a new selective FXRα antagonist NDB that is effective in modulating transcription of FXRα downstream genes. The analyzed crystal structure of hFXRα-LBD in complex with NDB revealed the homodimeric mode of hFXRα-LBD-NDB, and the potential antagonistic mechanism of NDB against the receptor was proposed. It is expected that our results may help better understand the mechanism of FXRα antagonism, thereby providing a new framework for developing novel FXRα-targeted therapeutic agents to treat metabolic disease.

Author Contributions—Xing Xu designed, performed, and analyzed experiments and wrote the first draft of the manuscript. Xin Xu performed and analyzed experiments and revised the manuscript. P. L. synthesized NDB. Z.-y. Z., J. C., and H.-a. F. discussed the experiments. L.-I. C., L.-h. H., and X. S. conceived and coordinated the study.

Acknowledgment—We thank BL17U of Shanghai Synchrotron Radiation Facility (SSRF) in China for data collection.

References
1. Zhang, Y., and Edwards, P. A. (2008) FXR signaling in metabolic disease. Feders Lett. 582, 10–18
2. Seol, W., Choi, H. S., and Moore, D. D. (1995) Isolation of proteins that interact specifically with the retinoid X receptor: two novel orphan receptors. Mol. Endocrinol. 9, 72–85
3. Forman, B. M., Goode, E., Chen, J., Oro, A. E., Bradley, D. J., Perlmann, T., Noonan, D. J., Burk, L. T., McMorris, T., and Lamph, W. W. (1995) Identification of a nuclear receptor that is activated by farnesol metabolites. Cell 81, 687–693
4. Parks, D. J., Blanchard, S. G., Bledsoe, R. K., Chandra, G., Consl, T. G., Kliwer, S. A., Stimmel, J. B., Willson, T. M., Zavacki, A. M., and Moore, D. D. (1999) Bile acids: natural ligands for an orphan nuclear receptor. Science 284, 1365–1368
5. Lee, F. Y., Lee, H., Hubbert, M. L., Edwards, P. A., and Zhang, Y. (2006) FXR, a multipurpose nuclear receptor. Trends Biochem. Sci. 31, 572–580
6. Prawitt, J., Caron, S., and Stael, S. (2009) How to modulate FXR activity to treat the Metabolic Syndrome. Drug Discov. Today Dis. Mech. 6, e55–e64
7. Flatt, B., Martin, R., Wang, T. L., Mahaney, P., Murphy, B., Gu, X. H., Foster, P., Li, J., Pircher, P., and Petersow, M. (2009) Discovery of XL335 (WAY-362450), a highly potent, selective, and orally active agonist of the farnesoid X receptor (FXR). J. Med. Chem. 52, 904–907
8. Maloney, P. R., Parks, D. J., Haffner, C. D., Fivush, A. M., Chandra, G., Plunket, K. D., Creech, K. L., Moore, L. B., Wilson, J. G., and Lewis, M. C. (2000) Identification of a chemical tool for the orphan nuclear receptor FXR. J. Med. Chem. 43, 2971–2974
9. Fiorucci, S., Clerici, C., Antonelli, E., Orlandi, S., Goodwin, B., Sadeghpour, B. M., Sabatino, G., Russo, G., Castellani, D., and Willson, T. M. (2005) Protective effects of 6-ethyl chenodeoxycholic acid, a farnesoid X receptor ligand, in estrogen-induced cholestasis. J. Pharmacol. Exp. Ther. 313, 604–612
10. Downes, M., Verdecia, M. A., Roecer, A. J., Hughes, R., Hogenesch, J. B., Kast-Woelbern, H. R., Bowman, M. E., Ferv, J. L., Anifield, A. M., Ed- wards, P. A., Rosenfeld, J. M., Alvarez, J. G., Noel, J. P., Nicolaou, K. C., and Evans, R. M. (2003) A chemical genetic, and structural analysis of the nuclear bile acid receptor FXR. Mol. Cell 11, 1079–1092
11. Jin, L., Feng, X., Hong, H., Pan, Z., Inaba, Y., Qiu, L., Zheng, W., Lin, S., Wang, R., and Wang, Z. (2013) The antiparasitic drug ivermectin is a novel FXR ligand that regulates metabolism. Nat. Commun. 4, 1937
12. Soisson, S. M., Parhasarathy, G., Adams, A. D., Sahoo, S., Silitani, A., Sparrow, C., Cui, J., and Becker, J. W. (2008) Identification of a potent synthetic FXR agonist with an unexpected mode of binding and activation. Proc. Natl. Acad. Sci. U.S.A. 105, 5337–5342
13. Watanabe, M., Horai, Y., Houten, S. M., Morimoto, K., Sugizaki, T., Arita, E., Matakai, C., Sato, H., Tanigawara, Y., and Schoonjans, K. (2011) Lower-
ing bile acid pool size with a synthetic farnesoid X receptor (FXR) agonist induces obesity and diabetes through reduced energy expenditure. J. Biol. Chem. 286, 26913–26920

14. Claudel, T., Sturm, E., Duez, H., Torra, I. P., Sirvent, A., Kosykh, V., Fruchart, J. C., Dallongeville, J., Hum, D. W., and Kuipers, F. (2002) Bile acid-activated nuclear receptor FXR suppresses apolipoprotein AI transcription via a negative FXR response element. J. Clin. Invest. 109, 961–971

15. Evans, M. J., Mahaney, P. E., Borges-Marcucci, L., Lai, K., Wang, S., Krueger, J. A., Gardell, S. J., Huard, C., Martinez, R., and Vlasuk, G. P. (2009) A synthetic farnesoid X receptor (FXR) agonist promotes cholesterol lowering in models of dyslipidemia. Am. J. Physiol. Gastrointest. Liver Physiol. 296, G543–G552

16. Urizar, N. L., Liverman, A. B., Dodds, D. T., Silva, F. V., Ordentlich, P., Yan, Y., Gonzalez, F. J., Heyman, R. A., Mangelsdorf, D. J., and Moore, D. D. (2002) A natural product that lowers cholesterol as an antagonist ligand for FXR. Science 296, 1703–1706

17. Huang, H., Yu, Y., Gao, Z., Zhang, Y., Li, C., Xu, X., Jin, H., Yan, W., Ma, R., and Zhu, J. (2013) Discovery and optimization of 1,3,4-trisubstituted-pyrazolone derivatives as novel, potent, and nonsteroidal farnesoid X receptor (FXR) selective antagonists. J. Med. Chem. 56, 7037–7053

18. Zhang, Y., Ge, X., Heemstra, L. A., Chen, W. D., Xu, J., Smith, J. L., Ma, H., Kasim, N., Edwards, P. A., and Novak, C. M. (2012) Loss of FXR protects against diet-induced obesity and accelerates liver carcinogenesis in ob/ob mice. Mol. Endocrinol. 26, 272–280

19. Prawitt, J., Abdelkarim, M., Strobeel, J. H., Popescu, I., Duez, H., Velagapudi, V. R., Dumont, J., Bouchaert, E., van Dijk, T. H., Lucas, A., Dorchies, E., Daoudi, M., Lestavel, S., Gonzalez, F. J., Oresic, M., Cariou, B., Kuipers, F., Caron, S., and Staels, B. (2011) Farnesoid X receptor deficiency improves glucose homeostasis in mouse models of obesity. Diabetes 60, 1861–1871

20. Duran-Sandoval, D., Cariou, B., Percevault, F., Hennuyer, N., Greffhorst, A., van Dijk, T. H., Gonzalez, F. J., Fruchart, J. C., Kuipers, F., and Staels, B. (2005) The farnesoid X receptor modulates hepatic carbohydrate metabolism during the fasting-refeeding transition. J. Biol. Chem. 280, 29971–29979

21. Lien, F., Berthier, A., Bouchaert, E., Gheeraert, C., Alexandre, J., Porez, G., Prawitt, J., Dehondt, H., Ploton, M., and Colin, S. (2014) Metformin interferes with bile acid homeostasis through AMPK-FXR crosstalk. J. Clin. Invest. 124, 1037–1051

22. Mi, L. Z., Devarakonda, S., Harp, J. M., Han, Q., Pellicciari, R., Willson, T. M., Khorasanizadeh, S., and Rastinejad, F. (2003) Structural basis for bile acid binding and activation of the nuclear receptor FXR. Mol. Cell 11, 1093–1100

23. Song, Y., Shan, S., Zhang, Y., Liu, W., Ding, W., Ren, W., Xia, H., Li, X., Zhang, Q., and Zhao, L. (2012) Ligand-dependent corepressor acts as a novel corepressor of thyroid hormone receptor and represses hepatic lipogenesis in mice. J. Hepatol 56, 248–254

24. Xu, X., Lu, Y., Chen, L., Chen, J., Luo, X., and Shen, X. (2013) Identification of 15d-PGJ2 as an antagonist of farnesoid X receptor: molecular modeling with biological evaluation. Steroids 78, 813–822

25. Puckett, M. C., Goldman, E. H., Cockrell, L. M., Huang, B., Kasinski, A. L., Du, Y., Wang, C. Y., Lin, A., Ichijo, H., Khuri, F., and Fu, H. (2013) Integration of apoptosis signal-regulating kinase 1-mediated stress signaling with the akt/protein kinase B-B kinase cascade. Mol. Cell. Biol. 33, 2252–2259

26. Wu, J., Xia, C., Meier, J., Li, S., Hu, X., and Lala, D. S. (2002) The hypolipidemic natural product guggulsterone acts as an antagonist of the bile acid receptor. Mol. Endocrinol. 16, 1590–1597

27. Sepe, V., Bifulco, G., Renga, B., D’Amore, C., Fiorucci, S., and Zampella, A. (2011) Discovery of sulfated sterols from marine invertebrates as a new class of marine natural antagonists of farnesoid-X-receptor. J. Med. Chem. 54, 1314–1320

28. Tan, M. H., Zhou, X. E., Soon, F. F., Li, X., Li, J., Yong, E. L., Melcher, K., and Xu, H. E. (2013) The crystal structure of the orphan nuclear receptor NR2E3/PNR ligand binding domain reveals a dimeric auto-repressed conformation. PloS ONE 8, e74359

29. Schoch, G. A., D’Arcy, B., Stihle, M., Burger, D., Bär, D., Benz, J., Thoma, R., and Ruf, A. (2010) Molecular switch in the glucocorticoid receptor: active and passive antagonist conformations. J. Mol. Biol. 395, 568–577

30. Tran, C., Ou, S., Clegg, N. J., Chen, Y., Watson, P. A., Arora, V., Wongvipat, J., Smith-Jones, P. M., Yoo, D., and Kwon, A. (2009) Development of a second-generation antiandrogen for treatment of advanced prostate cancer. Science 324, 787–790

31. Koszewski, N. J., Kiessling, S., and Malluche, H. H. (2001) Isolation of genomic DNA sequences that bind vitamin D receptor complexes. Biochem. Biophys. Res. Commun. 283, 188–194

32. Kim, J. Y., Son, Y. L., Kim, J. S., and Lee, Y. C. (2010) Molecular determinants required for selective interactions between the thyroid hormone receptor homodimer and the nuclear receptor corepressor N-CoR. J. Mol. Biol. 396, 747–760

33. Zhang, H., Chen, L., Chen, J., Jiang, H., and Shen, X. (2011) Structural basis for retinoic X receptor repression on the tetramer. J. Biol. Chem. 286, 24593–24598