Cinnamamides, Novel Liver X Receptor Antagonists Inhibit Ligand-induced Lipogenesis and Fatty Liver* 

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Running Title: Cinnamamide derivatives inhibit steatosis in mice

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Abbreviations: ABCA1, ABCG1, ATP-binding cassette transporter A1 or G1; ACC, acetyl-coenzyme A carboxylase; ALT, alanine aminotransferase; ChREBP, carbohydrate response element binding protein; CoIP, co-immunoprecipitation; DRIP, vitamin D receptor interacting protein; FAS, fatty acid synthase; HDL, high density lipoprotein; H&E, hematoxylin and eosin; HFD, high fat diet; LBD, ligand binding domain; LXR, liver X receptor; NAFLD,
nonalcoholic fatty liver disease; NCoR, nuclear receptor corepressor; qRT-PCR, quantitative real-time polymerase chain reaction; SCD-1, stearoyl-CoA desaturase-1; SMRT, silencing mediator of retinoic acid and thyroid receptor; SPA099, N-(2,3-dihydro-benzo[1,4]dioxin-6-yl) 2-naphthamide; SPA109, N-(3,4-dimethoxylphenyl) 3-methoxy-4-methoxymethylloxycinnamamide; SREBP, sterol regulatory element binding protein; T090, T0901317 (N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide); TFCA, N-(4-trifluoromethylphenyl) 3,4-dimethoxycinnamamide; TG, triglyceride; TRAP, thyroid hormone receptor-associated proteins; TR-FRET, time-resolved fluorescence resolution energy transfer; VLDL, very low density lipoprotein

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Abstract

Liver X receptor (LXR) is a member of the nuclear receptor superfamily and regulates various biological processes including de novo lipogenesis, cholesterol metabolism, and inflammation. Selective inhibition of LXR may aid the treatment of nonalcoholic fatty liver diseases. In the present study, we evaluated the effects of three cinnamamide derivatives on ligand-induced LXRα activation, and explored whether these derivatives could attenuate steatosis in mice. N-(4-trifluoromethylphenyl) 3,4-dimethoxycinnamamide (TFCA) decreased the luciferase activity in LXRE-tk-Luc-transfected cells, and also suppressed ligand-induced lipid accumulation and expression of the lipogenic genes in murine hepatocytes. Furthermore, it significantly attenuated hepatic neutral lipid accumulation in a ligand-induced fatty liver mouse system. Modeling study indicated that TFCA inhibited activation of the LXRα ligand-binding domain by hydrogen bonding to Arg305 in the H5 region of that domain. It regulated the transcriptional control exerted by LXRα by influencing coregulator exchange; this process involves dissociation of the TRAP/DRIP coactivator and recruitment of the NCoR corepressor. These results show that TFCA has the potential to attenuate ligand-induced lipogenesis and fatty liver by selectively inhibiting LXRα in the liver.
Introduction

Nuclear receptors are the second largest family of drug targets; these receptors are involved in diverse physiological functions linked to a wide range of diseases. Most importantly, the receptors are usually “drugable,” in the sense that it is often possible to identify small molecule modifiers suitable for use in humans. Such materials today account for 10-15% of the total $400 billion global pharmaceutical market (Gronemeyer et al., 2004).

Liver X receptors (LXRs) are nuclear oxysterol receptors that serve as physiological master regulators of lipid and cholesterol metabolism (Jakobsson et al., 2012). LXRβ (NR1H2) is expressed ubiquitously, but LXRα (NR1H3) is restricted to the metabolically active tissues including the liver, adipose tissue, and macrophages. The role played by LXR in peripheral cholesterol metabolism has been intensively studied. Agonistic LXRα activation lowers peripheral cholesterol levels by increasing reverse cholesterol transport from macrophages (via the ATP-binding cassette transporter G1 (ABCG1) or A1 (ABCA1)). Such agonists also have anti-inflammatory effects, rendering them suitable candidates for anti-atherogenic drug development (van der Hoorn et al., 2011). However, LXRα activation induces the development of fatty liver; the hepatic lipogenic pathway mediated by the sterol regulatory element binding protein (SREBP) and carbohydrate response element binding protein (ChREBP) is also activated (Repa et al., 2000; Cha et al., 2007).

Nuclear receptors regulate many metabolic processes via recruitment of cofactors. Such receptors are required for remodeling of chromatin structure near promoters, thus modulating the actions of the transcriptional machinery (McKenna and O’Malley, 2002). Uncomplexed LXRs bind to response elements associated with corepressors, including the nuclear receptor corepressor (NCoR)/silencing mediator of retinoic acid and thyroid receptor (SMRT) (Wagner et al., 2003). Histone deacetylase is also recruited to such complexes; the
end result is that overall transcription is repressed (Jones et al., 2001). Ligand binding triggers a conformational change in the receptor, facilitating the dissociation of corepressors and association of coactivators. The latter include switch/sucrose nonfermentable (SWI/SNF) nucleosome-remodeling complex, CREB binding protein/Steroid receptor coactivator-1 (CBP/SRC-1), and p300/CBP-associated factor (P/CAF), which exhibit histone acetyltransferase activities, and thyroid hormone receptor-associated proteins (TRAP)/vitamin D receptor interacting protein (DRIP)/activator-recruited cofactor (ARC), which recruits core transcription factors (Glass and Rosenfeld, 2000). Such coregulator exchange relieves chromatin-mediated repression and initiates the transcription of target genes. For example, the grapefruit flavonoid naringenin inhibits ligand-induced LXRα activation by suppressing the action of Trap220 coactivator (Goldwasser et al., 2010). The synthetic LXR inverse agonist SR9238 increases the interaction between NCoR and LXR, and decreases the Trap220 interaction (Griffett et al., 2013).

LXRα-dependent regulation of lipogenesis features transcriptional activation of the lipogenic transcription factor SREBP-1c (Repa et al., 2000). Analyses of liver biopsy samples from patients with nonalcoholic fatty liver disease (NAFLD) have revealed that the hepatic expressions of LXRα, SREBP1-c, acetyl-CoA carboxylase (ACC), and fatty acid synthase (FAS) are significantly upregulated (Higuchi et al., 2008). Although several risk factors for NAFLD are known, the best-studied relevant molecular pathway is that involving inhibition of AMP-activated protein kinase and/or activation of both the mammalian target of rapamycin (mTOR) and downstream lipogenic signals including SREBP-1c. Recently, we showed that dibenzylbutane lignan meso-dihydroguairetic acid isolated from Machilus thunbergii had potent LXRα antagonistic effects by binding to the ligand-binding domain (LBD) of the protein, thus inhibiting Trap220/Drip-2 recruitment. The M. thunbergii isolate
attenuates LXR\(\alpha\) expression and hepatic neutral lipid accumulation in fatty livers induced by ethanol-containing diets and high-fat diets (HFDs) (Sim et al., 2014). N-(4-trifluoromethylphenyl) 3,4-dimethoxycinnamamide (TFCA) is a new chemical entity with a cinnamamide structure. In the present study, we evaluated the effects of TFCA and structurally related compounds on LXR\(\alpha\)-associated fatty liver, and identified a possible mechanism of action in terms of inhibition of LXR\(\alpha\) activation. We found that TFCA acts as an antagonist, inhibiting ligand-activated LXR\(\alpha\) co-activation and transcriptional expression of the downstream target genes involved in fatty acid synthesis, thereby reducing lipid accumulation in mice given the LXR\(\alpha\) ligand T0901317 (T090).
Materials and Methods

Materials

TFCA, N-(2,3-dihydro-benzo[1,4]dioxin-6-yl) 2-naphthamide (SPA099), and N-(3,4-dimethoxylphenyl) 3-methoxy-4-methoxymethyloxycinnamamide (SPA109) (Fig. 1) were synthesized and analyzed by NMR spectroscopy. The purities of TFCA, SPA099, and SPA109 measured by LC/MS were > 97.0%. Nile Red, Oil Red O, Oleic acid, T090, 22S-hydroxycholesterol, GW4064, and Rifampicin were purchased from Sigma Chemical Co. (St. Louis, MO).

Synthesis of Cinnamamide Derivatives

N-(4-trifluoromethylphenyl) 3,4-dimethoxycinnamamide (TFCA; C$_{18}$H$_{16}$O$_3$NF$_3$)

To a solution of 4-trifluoromethylaniline (313 mg, 1.94 mmol) and NaHCO$_3$ (222 mg, 2.64 mmol) in 10 mL of CH$_2$Cl$_2$ was added 3,4-dimethoxycinnamoyl chloride (400 mg, 1.76 mmol) at 0°C slowly, and the reaction mixture was stirred for 5 h at 0°C. The reaction mixture was diluted with 1N HCl solution and extracted with EtOAc. The organic layer was washed with 1N NaOH solution and brine, and then dried over MgSO$_4$. The solvent was removed in vacuo, and the residue was triturated with Et$_2$O to give 482 mg (78%) of TFCA as a white solid. $^1$H NMR (500 MHz, CDCl$_3$): δ 7.75 (d, $J = 8.5$ Hz, 2H), 7.72 (d, $J = 15.4$ Hz, 1H), 7.61 (bs, 1H), 7.59 (d, $J = 8.5$ Hz, 2H), 7.12 (dd, $J = 1.6$, 8.3 Hz, 1H), 7.04 (d, $J = 1.6$ Hz, 1H), 6.86 (d, $J = 8.3$ Hz, 1H), 6.44 (d, $J = 15.4$ Hz, 1H), 3.92 (s, 3H), 3.90 (s, 3H); $^{13}$C NMR (125 MHz, CDCl$_3$): δ 164.42, 151.16, 149.24, 143.30, 141.25, 127.32, 126.34 (q), 126.12, 125.18, 122.47, 119.42, 117.93, 111.16, 109.87, 56.00, 55.90.

N-(2,3-dihydro-benzo[1,4]dioxin-6-yl) 2-naphthamide (SPA099; C$_{19}$H$_{15}$O$_3$N)

To a solution of 2,3-dihydro-benzo[1,4]dioxin-6-amine (349 mg, 2.31 mmol) and NaHCO$_3$ (265 mg, 3.15 mmol) in 10 mL of CH$_2$Cl$_2$ was added 2-naphthoyl chloride (400 mg,
2.10 mmol) at 0°C slowly, and the reaction mixture was stirred for 5 h at 0°C. The reaction mixture was diluted with 1N HCl solution and extracted with EtOAc. The organic layer was washed with 1N NaOH solution and brine, and then dried over MgSO4. The solvent was removed in vacuo, and the residue was triturated with Et2O to give 580 mg (90%) of SPA099 as a white solid. 1H NMR (500 MHz, CDCl3): δ 8.30 (s, 1H), 8.06 (bs, 1H), 7.83~7.85 (m, 4H), 7.49~7.56 (m, 2H), 7.31 (d, J = 1.9 Hz, 1H), 7.06 (dd, J = 1.9, 8.6 Hz, 1H), 6.82 (d, J = 8.6 Hz, 1H), 4.23 (s, 4H); 13C NMR (125 MHz, CDCl3): δ 165.66, 143.60, 140.70, 134.81, 132.63, 132.23, 131.70, 128.97, 128.68, 127.80, 127.47, 126.89, 123.58, 117.30, 114.01, 110.23, 64.46, 64.33.

**N-(3,4-dimethoxylphenyl) 3-methoxy-4-methoxymethyloxycinnamamide (SPA109; C20H23O6N)**

To a solution of 3,4-dimethoxyaniline (263 mg, 1.72 mmol) and NaHCO3 (197 mg, 2.34 mmol) in 10 mL of CH2Cl2 was added 3-methoxy-4-methoxymethyloxycinnamoyl chloride (400 mg, 1.56 mmol) at 0°C slowly, and the reaction mixture was stirred for 5 h at 0°C. The reaction mixture was diluted with 1N HCl solution and extracted with EtOAc. The organic layer was washed with 1N NaOH solution and brine, and then dried over MgSO4. The solvent was removed in vacuo, and the residue was triturated with Et2O to give 470 mg (81%) of SPA109 as a light yellow solid. 1H NMR (500 MHz, CDCl3): δ 7.73 (s, 1H), 7.67 (d, J = 15.4 Hz, 1H), 7.53 (s, 1H), 7.12 (d, J = 8.2 Hz, 1H), 7.05 (d, J = 8.6 Hz, 2H), 6.99 (d, J = 8.2 Hz, 1H), 6.80 (d, J = 8.6 Hz, 1H), 6.49 (d, J = 15.4 Hz, 1H), 5.25 (s, 2H), 3.87 (s, 6H), 3.85 (s, 3H), 3.50 (s, 3H); 13C NMR (125 MHz, CDCl3): δ 164.17, 149.80, 149.08, 148.19, 145.84, 141.71, 131.99, 129.14, 121.65, 119.41, 115.94, 111.73, 111.38, 110.65, 104.86, 95.25, 56.34, 56.12, 55.90.

**Cell Culture**
AML12 cells were used in the study of ligand-induced lipogenesis and gene expression. We used HEK293 and HepG2 cells in LXRE- and SRE-luc as well as PXRE- and FXRE-luc assay, respectively. All the cell lines were obtained from American Type Culture Collection (Rockville, MD). AML12 cells were maintained in DMEM/F12 medium (Invitrogen, CA) containing 10% fetal bovine serum (FBS, Gibco BRL), insulin-transferrin-selenium (100x, liquid) (Gibco BRL), 40 ng/ml dexamethasone, penicillin (100 units/ml), and streptomycin (100 μg/ml). HepG2 and HEK293 cells were cultured in DMEM (Gibco BRL) and RPMI1640 (Gibco BRL) supplemented with 10% FBS, penicillin/streptomycin. The cells were cultured in a 37°C incubator with humidified 5% CO₂. Cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

**Transient Transfection and Reporter Gene Assay**

HEK293 cells were transfected with the LXRα expression vector and LXRE-tk-Luc reporter construct. HepG2 cells were transfected with FXRE-Luc (Dr. Bart Staels, Université Lille Nord de France) or PXRE-Luc (Dr. Mi-Ock Lee, Seoul National University) reporter or SRE-Luc construct. pCMV-RL plasmids were co-transfected as an internal control. Dual-Luciferase assay detection kit (Promega, Madison, WI) was used to determine luciferase activities according to the manufacturer's protocols.

**Triglyceride (TG) analysis**

TG concentrations in the cells and the tissues were measured by a modified Folch method using TG assay kit (Sigma) following the manufacturers’ protocols.

**Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)**

For qRT-PCR, RNA was prepared from cells and mice liver using the Easy-Blue™ Total RNA Extraction Kit (Intron Biotechnology, Seoul, Korea). The resulting cDNA was amplified by qRT-PCR using iTaq™ Universal SYBR® Green Supermix kit (Bio-Rad,
Animals

The experiments using animals were carried out in accordance with U.S. National Institutes of Health Guidelines for the Care and Use of Laboratory Animals with approval of the Institutional Animal Care and Use Committee of Seoul National University. A total of 40 male C57BL/6 mice were purchased from SLC Inc. (Hamamatsu, Japan) and housed in an air-conditioned room (24°C) with a 12 h light/dark cycle with free access to food and water.

T090-induced fatty liver experiments

Mice were randomized and T090 (10 mg·kg⁻¹; oral) was given to mice once a day for 4 days with oral administration of TFCA, SPA099, and SPA109 (10, 30 mg·kg⁻¹; oral) 30 min before each T090 administration. A cross section of the left lateral lobe of the liver was collected in 10% neutral buffered formalin for histopathology. The remaining liver tissues were collected in RNase-free tubes and snap-frozen in liquid nitrogen. Frozen tissues were stored at -70°C until processed for RNA or protein extraction.

Histological analysis and Oil Red-O staining

The tissues were embedded in paraffin blocks, cut into 5 µm sections and stained with hematoxylin and eosin (H&E) (Park et al., 2013). For Oil Red-O staining, frozen liver tissues were cut into 7 µm and affixed to microscope slides. Sections were reacted with Oil Red-O solution buffer and counterstained with Harris Hematoxylin as described (Jung et al., 2011).

Serum Biochemistry

Serum alanine aminotransferase (ALT), TG, total cholesterol, and high density lipoprotein (HDL) were monitored by standard clinical chemistry assays on a Tokyo Boeki Prestige 24I Chemistry Analyzer.
**Western Blot and Co-immunoprecipitation (CoIP)**

Western blot was performed by established procedures using specific antibodies against FAS, SCD-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH (Cell signaling Technology, Beverly, MA). CoIP was performed using specific antibodies against NCoR (Pierce Antibodies, Rockford, IL) and LXRα (Santa Cruz Biotechnology) and subjected to Western blotting. HRP-conjugated secondary anti-rabbit or anti-mouse antibody was purchased from Cell signaling Technology.

**Molecular Docking**

All computational performances were done by using Tripos Sybyl-X 2.1.1 (Tripos Inc, St Louis, MO, USA) molecular modeling package with CentOS Linux 5.4. operating system. All used ligands including TFCA, SPA109, and SPA 099 except T090 were prepared using sketcher modules embedded in Sybyl-X 2.1.1 software package. T090 was extracted from crystal structure of LXRα (PDB id:1UHL). All hydrogen atoms were added into sketched molecules and Gasteiger-Hückel charges were assigned onto all atoms in ligand. Ligands were energy-minimized with the standard tripos force field with convergence to maximum derivatives of 0.001 kcal·mol⁻¹·Å⁻¹. The X-ray structure of LXRα in complex with agonist T090 was retrieved from Protein Data Bank (PDB id:1UHL) and optimized by biopolymer preparation tool to be used as receptor for docking study. All hydrogen atoms and the Kollman-All atomic charges were added into the receptor by biopolymer module.

A molecular docking study was conducted using the program Surflex-Dock embedded in Tripos Sybyl X 2.1.1 software package (Spitzer *et al.*, 2012). First, the active site was assigned by generating protomol based on the native ligand. Docking performance was done using default parameters value (threshold = 0.60 and bloat = 0 was used) and maximum number of poses per ligand was 50 conformers. All results were analyzed based on score and
visual inspection. Binding affinity of each docking pose was calculated by Surflex-dock score and consensus scoring function (CScore). The total Surflex-Dock score was expressed as $-\log(K_d)$ to represent binding affinities. To select the best docking model, key interactions between candidate compound and active site were investigated.

**Time-Resolved Fluorescence Resolution Energy Transfer (TR-FRET) LXRα Co-regulator Recruitment Assay**

LanthaScreen™ TR-FRET LXRα co-activator assay kit (Invitrogen, Carlsbad, CA) was used according to the manufacturer’s instructions. Details of the TR-FRET assay are reported elsewhere (Sim et al., 2014).

**Statistical Analysis**

Multiple comparisons were evaluated by one-way analysis of variance followed by Tukey's multiple comparison procedure with $p < 0.05$ considered significant.
Results

Cinnamamides inhibit ligand-induced LXRα activation and lipogenesis in vitro

We recently described a dibenzylbutane lignane LXRα antagonist, and we used the chemical scaffold (Sim et al., 2014) of this material as our starting point. We synthesized 84 cinnamamide derivatives and used the LXRE-tk-Luc reporter gene assay to determine whether they inhibited ligand-induced LXRα activation. T090-induced luciferase activity was diminished significantly upon co-treatment with the test substances, and the three most potent in this regard were TFCA, SPA099, and SPA109 (Fig.1 and Fig. 2A). TFCA inhibited T090- and oleic acid-induced lipid accumulation in a concentration dependent manner (Fig. 2B). SPA109 also reduced T090- and oleic acid-induced TG accumulation, but SPA099 decreased only T090-induced TG level (Supplemental Fig. 1). The cytotoxicity IC₅₀ values for TFCA, SPA099, and SPA109 were 534.5, 282.3, and 357.5 μM, respectively. To explore whether the effects of TFCA are specific for LXRα, we performed PXRE and FXRE luciferase assay. TFCA did not affect PXRE-Luc activity but inhibited ligand-induced FXRE-Luc activity (Fig. 2C). Thus, we selected TFCA for further work.

TFCA attenuates T090-induced lipogenic gene expression in vitro

Next, we explored whether TFCA inhibited T090-induced expression of lipogenic genes under the transcriptional regulation of SREBP-1c in AML12 cells. TFCA was not cytotoxic at concentrations of up to 30 μM (Fig. 3A). In the SRE luciferase assay, TFCA decreased SRE-Luc activity in a dose-dependent manner. TFCA at 30 μM also suppressed T090-induced SRE-Luc activity (Fig. 3B). T090 increased the expression levels of mRNAs from Lxrα and Srebf1c, and those from the target genes Fas and Scd-1. However, co-treatment with TFCA significantly reduced such effects (Fig. 3C). LXRα inhibition may
compromise the reverse cholesterol transport that triggers hypercholesterolemia. In THP-1 human monocytic leukemia cells, TFCA did not significantly affect the expression levels of genes encoding the ATP-binding cassette transporter (Abca1 and Abcg1) (Supplemental Fig. 2).

**TFCA inhibits T090-induced lipid accumulation and fatty liver**

We explored whether inhibition of LXRα by TFCA was also evident in vivo. C57BL/6 mice were gavaged once daily with 10 mg·kg⁻¹ T090 for 4 days; TFCA, SPA099, and SPA109 were administered 30 min before each T090 dose. H&E staining, Oil-Red O staining, and hepatic TG analysis revealed that T090 caused hepatic lipid accumulation and development of fatty liver, which were significantly reduced by treatment with 30 mg·kg⁻¹ TFCA alone (Fig. 4A and B). Serum ALT and TG levels were also reduced by TFCA; the TG effect was statistically significant. In line with the effects of TFCA on the expression of reverse cholesterol transport genes in THP-1 cells, TFCA did not increase the total cholesterol level in serum. Notably, TFCA did increase the HDL level significantly (Fig. 4C). The hepatic gene expression profile was very similar to that of AML12 cells. T090 increased the expression of the lipogenic transcription factor Srebf-1 4.6-fold. In turn, expression of the Srebf-1 target genes Fas and Scd-1 were notably increased upon T090 administration. In contrast, TFCA significantly reduced the expression of Srebf-1 and Fas (Fig. 5A). The elevated FAS protein levels in T090-treated mice were restored to normal upon treatment with 30 mg·kg⁻¹ TFCA (Fig. 5B), indicating that TFCA had potent anti-steatotic effects in the mice. The highest dose of TFCA alone did not affect hepatic, serum TG, ALT and other lipid profiles (Supplemental Fig. 3A). TFCA also did not change the expressions of lipogenic genes and proteins (Supplemental Fig. 3B, C).
We measured the TFCA level in the liver from Fig. 4. TFCA was undetectable in the sample but we could find a peak at RT 19.231 min that is supposed to be a glucuronic acid conjugate of the TFCA metabolite produced by the cleavage of the amide bond (Supplemental Fig. 4). To understand the fate of TFCA, we gavaged the same dose and analyzed at 2 h and 6 h. The hepatic concentration of TFCA was 1.39 +/- 0.34 pmole/g liver at 2 h, but undetectable at 6 h implying that TFCA is metabolically unstable (Supplemental Fig. 5 and Supplemental Table 2).

**TFCA binds to the LXRα LBD and induces interaction with NCoR corepressor**

To explore the molecular interactions between the LXRα LBD and the cinnamamides (TFCA, SPA099, and SPA109), we performed docking modeling using the flexible automated docking program Surflex-Dock. The X-ray structure of LXRα LBD in a complex with T090 (PDB id:1UHL) was used as the receptor in docking analysis. As shown in Fig. 6A, all compounds fitted comfortably into the binding pocket for the agonist T090, and the binding positions were similar. This was in line with the general belief that the ligand-binding domains of nuclear receptors accommodate mostly lipophilic ligands in predominantly hydrophobic pockets formed by residues of helices H5, H12, and other regions of the receptors (Wohlfahrt et al., 2009). We examined the docked pose of the most active antagonist (TFCA) in terms of hydrogen-bond interactions with the receptor, and compared this structure to the X-ray pose of agonist T090 (Fig. 6B). T090 forms hydrogen bonds with His421 and Trp443 of H12, and Thr302 of H5; these are the key residues in terms of agonist-binding to LXRα. However, TFCA did not form hydrogen bonds with any of these key residues of the activated form of LBD, although the overall binding pose of TFCA was similar to the X-ray pose of T090. Rather, a fluorine atom of the para-trifluoromethyl group...
of TFCA formed a hydrogen bond with an amino group of the guanidyl moiety (Arg305) of H5 (Fig. 6C).

To further characterize the interaction of TFCA with LXRα, we performed TR-FRET-based co-activator and co-repressor recruitment assays. T090 mediated a clear concentration-dependent increase in binding of the coactivator TRAP/DRIP to LXRα (Supplemental Fig. 6A). Although TFCA alone did not significantly change the interaction of LXRα with a coactivator NR box peptide derived from TRAP220/DRIP2 (Fig. 7A), T090-induced recruitment of this coactivator peptide was inhibited upon cotreatment with TFCA (Fig. 7B). Moreover, TFCA increased the interaction of LXRα with a corepressor NR box peptide derived from NCoR (NCoR ID1 and NCoR ID2) (Fig. 7C). It did not induce recruitment of SMRT (SMRT ID1 and SMRT ID2) (Supplemental Fig. 6B). To confirm the involvement of NCoR recruitment in the TFCA-induced suppression of LXRα activation, AML12 cells were treated with both T090 and TFCA. T090 significantly suppressed corepressor binding to LXRα, which was restored by TFCA (Fig. 7D). Together, these results suggest that TFCA competitively binds to the ligand-binding pocket of T090 and suppresses ligand-induced activation of LXRα.
Discussion

LXR is a member of the nuclear receptor superfamily that regulates various biological events including glucose and lipid homeostasis as well as immune and neurological functions. The purpose of the present study was to identify novel cinnamamide analogs as selective LXR antagonist for the potential treatment of ligand-induced lipogenesis and fatty liver.

Cinnamic acid and derivatives thereof are widely distributed in plants. This aromatic fatty acid, which has an α,β-unsaturated carbonyl moiety, generally exhibits what are termed “Michael acceptor functionalities”. Many phase II enzyme inducers, and anticancer drugs including hydroquinones and phenylpropanoids (chalcones and flavonoids), are cinnamic acid derivatives (Dinkova-Kostova et al., 2007). Recently, cinnamic acid amides and related compounds have attracted increasing attention because of their antimicrobial, antioxidant, and anticancer properties (Patel et al., 2014; Natella et al., 1999; De et al., 2011). Cinnamic acid derivatives can exert antitubercular activities by targeting fatty acid synthase type II (FAS II), a unique bacterial enzyme responsible for the synthesis and elongation of membrane fatty acids (Payne et al., 2001). Although structural and functional differences between mammalian type I FAS and bacterial FAS II are evident, chemical regulation of the two enzymes may have features in common. We recently showed that meso-dihydroguaiaretic acid inhibits ligand-induced LXRα activation by reducing the expression of the hepatic LXRα coactivator, and we thus used the chemical scaffold of this material as a starting point for our work (Sim et al., 2014). We synthesized 84 cinnamamide derivatives in a search for a potent patentable LXRα antagonist. In the LXRE-reporter gene assay, TFCA suppressed the transcriptional activity of LXRα. TFCA inhibited ligand-induced lipid accumulation, and the expression of genes associated with lipogenesis both in vitro and in vivo, without affecting reverse cholesterol transport. We found that TFCA exhibited potent antagonistic activity in
silico, and regulated the transcriptional control mediated by LXRα, by triggering a coregulator exchange involving dissociation of the TRAP/DRIP coactivator and recruitment of the NCoR corepressor. Thus, TFCA potentially attenuates ligand-induced lipogenesis and fatty liver via selective inhibition of LXRα.

LXR plays key roles in the regulation of cholesterol, lipid, and carbohydrate metabolism by controlling the expression levels of specific rate-limiting genes in relevant pathways. Such properties of a nuclear receptor render LXRs promising therapeutic drug targets for the treatment of various diseases (Jakobsson et al., 2012). NAFLD is caused by an imbalance between lipid accumulation and consumption. Hepatic TG accumulates when the uptake and de novo synthesis of fatty acids exceed lipid catabolism via fatty acid β-oxidation and VLDL secretion. LXR is one of the most important regulators of de novo lipogenesis; it induces transcriptional activation of Fas, Acc, and Scd-1, both directly and indirectly, via mechanisms involving SREBP-1c and ChREBP (Liang et al., 2002; Chen et al., 2004). LXRα expression levels in NAFLD patients tend to be higher than those of controls (Ahn et al., 2014). Increases in hepatic fatty acid uptake and enhanced de novo lipogenesis are of prime importance in the development of hepatic steatosis; lipid disposal via oxidation and export are only moderately affected in patients with this condition (Lewis et al., 2002). Therefore, antagonizing LXR action by inducing the recruitment of transcriptional corepressors to promoters would suppress de novo lipogenesis. A recent study found that the LXRα antagonist 22-S-hydroxycholesterol counteracted T090-induced lipogenesis and lipid formation in myotubes from type 2 diabetic patients (Kase et al., 2007). Hepatospecific inhibition of LXR using an inverse agonist also inhibited NAFLD development induced by a HFD (Griffett et al., 2013). Published studies indicate that there is a very good correlation between the level of LXRα expression and NAFLD. Higuchi et al. reported that LXRα acts
as one of the main regulators of lipid metabolism by regulating SREBP-1c expression in patients with NAFLD. According to our recent study investigating the association between LXRα and NAFLD in human, the positive rate of LXRα expression was 30% in control, 50% in NAFLD and 97% in NASH group (Ahn et al., 2014). In high-fat diet-induced steatosis animal model, expression of hepatic LXRα was increased significantly from the first day of high fat feeding that was lasted for weeks (Lee et al., 2011). Taken together, we can expect positive effects of TFCA in high-fat diet model.

As LXRα positively regulates lipogenesis, efforts to develop LXRα agonists that enhance reverse cholesterol transport (and thus inhibit the progression of atherosclerosis) have been hampered by hepatic lipogenic side-effects (Grefhorst et al., 2002). LXR-623, a tissue-specific ligand that activates LXR with limited hepatic liability, was also disappointing; central nervous system-related adverse events were observed in the first-in-humans clinical trial (Katz et al., 2009). Therefore, it is essential to identify selective LXR agonists or antagonists that have beneficial effects on the target tissue with minimal unwanted effects on off-target tissues. During initial in vitro screening, SPA099 was one of the most potent compounds inhibiting T090-induced LXRα luciferase activity (Fig. 2A). However, the inhibitory effect of SPA099 on T090-induced lipid accumulation by AML12 cells was less than the LXRα inhibition exhibited by this material. Where TFCA significantly suppressed oleic acid-induced lipid accumulation, SPA099 did not (over the same dose range) (Fig. 2B and Supplemental Fig. 1). Finally, in the docking study, TFCA bound more strongly to the T090-binding domain than did SPA099. On the basis of these results, we selected TFCA as the primary hit and elucidated its mechanism of action and in vivo effects. Although 30 µM TFCA inhibited agonist-induced luciferase activity in the FXRE-Luc assay, the expression level of the target gene shp-1 was not affected (data not shown). Thus, further study of the
effects of TFCA on FXR and bile acid metabolism is needed.

We used an LXRE reporter gene assay and molecular modeling to show that TFCA significantly inhibited T090-induced LXRα activation, and bound to Arg305 of LXRα. It is already known that certain LXRα antagonists form hydrogen bonds with Arg305 of the LXRα LBD (Hioki et al., 2009; Svensson et al., 2003). Arg305 (located in the H5-binding region of TFCA) may inhibit coactivator binding by rendering the H12 region rather loose; the conformation of the latter region is important in terms of coactivator complex binding (Svensson et al., 2003). The TR-FRET assay indicated that TFCA decreased the interaction between LXRα and the TRAP/DRIP coactivator, and increased recruitment of the corepressor NCoR, in the presence of the synthetic agonist T090 (Fig. 7B, C). TFCA did not affect the interaction of LXRα with SMRT (Supplemental Fig. 6B).

Nuclear receptors have distinct preferences for specific corepressors. Where the retinoic acid receptor (RAR) interacts optimally with SMRT, LXR and the thyroid receptor (TR) prefer NCoR when the receptors are in the unliganded state. The specificity of the receptor-corepressor interaction is controlled by individual nuclear receptors interacting with specific interaction domains within preferred corepressors (Hu et al., 2001). Where LXRα interacts with both CoR NR box peptides, the interaction of LXRα with NCoR ID2 is much stronger, being preferred to the interaction with NCoR ID1 (Hu et al., 2003). When the importance of NCoR ID2 in terms of repressing the transcription of LXRα target genes is considered, the ability of TFCA to recruit a specific corepressor is noteworthy.

Using an LXRE reporter gene assay, we showed that TFCA inhibited agonist-induced LXRα activation. This coregulator recruitment assay supports the suggestion that TFCA, in the presence of T090, significantly and concentration-dependently induces the dissociation of TRAP/DRIP from, and interaction of NCoR with, the ligand-binding domain of LXRα. The
docking results suggest that TFCA may competitively bind to the binding pocket for agonist T090, inhibiting activation of LXRα, in agreement with the results of experiments in mice. TFCA significantly attenuated T090-induced lipid accumulation in murine hepatocytes, and the development of fatty liver in mice. Finally, TFCA selectively inhibited LXRα-mediated hepatic lipogenesis with minimal, if any, effect on the expression of genes associated with reverse cholesterol transport.
Authorship Contributions

Participated in research design: Shin, Jeon, B.H. Lee

Conducted experiments: Sim, D.G. Kim, K.J. Lee, Y.J. Choi\textsuperscript{a}, Y.J. Choi\textsuperscript{b}, S.J. Park, H.J. Park, J.W. Kim

Contributed new reagents: Shin

Performed data analysis: Sim, K.J. Lee, J.W. Kim, W.K. Oh, B.H. Lee

Wrote or contributed to the writing of the manuscript: Sim, B.H. Lee
References


Footnotes

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Figure Legends

Figure 1 The chemical structures of T090, TFCA, SPA099, and SPA109

Figure 2 TFCA inhibits LXRE-luciferase activity and lipid accumulation.
HEK293 cells were transfected with 0.2 μg LXRE-Luc reporter gene and 0.05 μg LXRα expression vector, and treated with TFCA, SPA099, or SPA109 (3, 10, 30 μM), with or without 10 nM T090, for 24 h (A). AML12 cells were treated with T090 (5 μM) or oleic acid (100 μM), and various concentrations of TFCA, for 24 h. Intracellular TG was extracted and measured as described in Method section (B). HepG2 cells were transfected with FXRE- and PXRE-Luc reporter genes and treated with TFCA, at the indicated concentrations, in the absence or presence of agonists, for 24 h (C). Results are expressed as percentages of control. All data are the means ± SDs of those of three independent experiments. Means sharing the same letter are not significantly different from each other; p < 0.05.

Figure 3 TFCA inhibits SRE-luciferase activity and T090-induced lipogenic gene expression.
AML12 cells were treated with increasing concentrations of TFCA for 24 h and cell viability was assessed using the MTT assay (A). HEK293 cells were transfected with 0.2 μg SRE-Luc reporter construct and 0.05 μg LXRα expression vector, and treated with the indicated concentrations of TFCA, in the absence or presence of 1 μM T090 (B). AML12 cells were treated with 5 μM T090 in the absence or presence of various concentrations of TFCA, for 24 h. The expression levels of lxrα, srebf1, fas, and scd1 were measured via quantitative real-time PCR (C). All data are means ± SDs of those of three independent experiments. Means sharing the same letter are not significantly different from each other; p < 0.05.
Figure 4 TFCA ameliorates T090-induced fatty liver development in vivo.

Mice were gavaged with the indicated doses of TFCA, SPA099, SPA109 (in mg·kg⁻¹), and T090 (10 mg·kg⁻¹) for 4 days, as described in the Materials and Methods. Hepatic TG of mice were analyzed as described in the Materials and Methods (A). H&E- and Oil Red O-stained mouse liver sections are shown (original magnification ×400) (B). Serum ALT, TG, total cholesterol, and HDL levels were measured as described in the Materials and Methods (C). The data represent the means ± SDs of values from 4~5 mice. Means sharing the same letter are not significantly different from each other; p < 0.05.

Figure 5 TFGA inhibits the expression of lipogenic genes and their encoded proteins in vivo.

Total RNA was prepared from the livers of 4~5 of the mice described in Fig. 4 and analyzed via qRT-PCR for expression of lxrα, srebf1, fas, and scd1 (A). Proteins were extracted from mice liver homogenates for quantification (via Western blotting) of FAS and SCD1 levels, which are expressed as percentages of control (B). Means sharing the same letter are not significantly different from each other; p < 0.05 or not significant (N.S.).

Figure 6 Docking models of LXRα antagonists.

Superimposition of docked poses of compounds in the LXRα binding pocket of the X-ray structure (PDB id: 1UHL). The mashed MOLCAD surface map indicates the lipophilic potential; lipophilicity increases from blue (hydrophilic) to brown (lipophilic). The LXRα backbone is shown as gray ribbons, and key amino acid residues within the binding site are indicated in the “capped sticks” form (carbon atoms are in gray). The compounds are distinguished by coloring their carbon atoms differently (TFCA: magenta, SPA099: orange...
and SPA109: green); the carbon atoms of the native ligand T090 are colored yellow (A).

Superposition of the docked pose of TFCA (carbon atoms in magenta) and the X-ray pose of the agonist T090 (yellow capped stick) in the active site of LXRα LBD. Hydrogen-bonding interactions are indicated by dashed ellipsoids (B). Docking pose of TFCA in the active site of LXRα LBD (C).

**Figure 7** TFCA inhibits LXRα co-activator binding and increases recruitment of the LXRα co-repressor.

The LanthaScreen™ TR-FRET assay was performed according to the manufacturer’s instructions. Increasing concentrations of TFCA, in the absence (A) or presence (B) of 100 nM T090 were incubated with LBD. Increasing concentrations of TFCA were incubated with the co-activator peptide TRAP/DRIP or the co-repressor peptides NCoR ID1 and ID2 (C). The 520/490 TR-FRET ratios were measured using a Molecular Devices Spectrophotometer M5, as described in the Materials and Methods. AML12 cells were treated with the indicated concentrations of TFCA in the presence of 5 μM T090, for 24 h. Protein extracts were prepared from cell lysates and CoIP was performed using an anti-LXRα antibody; binding of NCoR to LXRα was quantified via Western blotting; the results are expressed as percentages of control (D). Data are the means ± SDs of three independent experiments. Means sharing the same letter are not significantly different from each other; *p* < 0.05.
Table 1. Sequences of PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
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</tbody>
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Figure 1
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7