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Regulation of CYP3A4 and CYP2B6 expression by liver X receptor agonists

Zofia Duniec-Dmuchowski [a], Ewa Ellis [b], Stephen C. Strom [b], Thomas A. Kocarek [a,*]

[a] Institute of Environmental Health Sciences, Wayne State University, Detroit, MI, USA
[b] Department of Pathology, University of Pittsburgh, Pittsburgh, PA, USA

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ABSTRACT

The liver X receptor (LXR) agonists, 24(S),25-epoxycholesterol and T0901317, were previously shown to be capable of inducing CYP3A expression in primary cultured rodent hepatocytes through activation of the pregnane X receptor (PXR). In this study, the abilities of these two LXR agonists to regulate CYP3A4 and CYP2B6 mRNA expression in primary cultures of human hepatocytes were evaluated. Treatment with 10 or 30 μM of the endogenous oxysterol, 24(S),25-epoxycholesterol, had no effect on CYP3A4 mRNA content in five preparations of primary cultured human hepatocytes, while 30 μM 24(S),25-epoxycholesterol treatment increased CYP2B6 mRNA content by approximately two-fold. By comparison, treatment with the synthetic LXR agonist, T0901317, potently increased CYP3A4 and CYP2B6 mRNA levels in the human hepatocyte cultures, producing multi-fold increases at 10 nM. Using a HepG2-based transactivation assay, T0901317 activated human PXR with an $EC_{50} \sim 20$ nM, which was more than 10-fold lower than that of the potent PXR ligand, SR-12813, while treatment with 24(S),25-epoxycholesterol failed to induce reporter expression in this assay. Therefore, while 24(S),25-epoxycholesterol-mediated PXR activation and CYP3A induction does not appear to be conserved from rodent to human, T0901317 is among the most potent known activators of human PXR.

1. Introduction

The liver X receptors, LXRα and LXRβ (NR1H3 and NR1H2), are oxysterol-sensing nuclear receptors that regulate the transcription of genes that function in the maintenance of lipid homeostasis (for recent review see [1]). The oxysterol, 24(S),25-epoxycholesterol, is one of the most potent and efficacious ligand activators of LXRα and LXRβ [2,3]. 24(S),25-Epoxycholesterol is biosynthesized through a pathway parallel to that for cholesterol biosynthesis, which begins with the diversion of squalene 2,3-oxide to squalene 2,3:22,23-dioxide [4], and appreciable amounts of this molecule are detectable in liver extracts [5]. N-(2,2,2-Trifluoroethyl)-N-[4-(2,2,2-trifluoro-1-hydroxy-1-trifluoromethyl-ethyl)-phenyl]-benzenesulfonamide (T0901317) is a synthetic agonist of LXR that is frequently used as an experimental tool for investigating LXR-mediated processes. In the original study characterizing the pharmacological properties of T0901317, the drug was found to activate LXR potently ($EC_{50} \sim 20$ nM) and selectively [6], although treatment with 1 μM T0901317 also produced some activation of the xenobiotic-sensing pregnane X receptor (PXR, NR1I2).

We previously reported that treatment of primary cultured rat hepatocytes with 24(S),25-epoxycholesterol effectively induced CYP3A mRNA and immunoreactive protein levels [7]. CYP3A mRNA induction also occurred in hepatocyte

* *Corresponding author at*: Institute of Environmental Health Sciences, Wayne State University, 2727 Second Avenue, Room 4000, Detroit, MI 48201, USA. Tel.: +1 313 963 7487; fax: +1 313 577 0082.
E-mail address: t.kocarek@wayne.edu (T.A. Kocarek).
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cultures prepared from wild-type or LXR-null mice, but not in hepatocytes from PXR-null mice, demonstrating that the underlying mechanism was PXR, rather than LXR, activation [7]. The 24(S),25-epoxycholesterol concentrations that produced CYP3A induction in the hepatocyte cultures were in the mid micromolar range, not far above the reported EC_{50} for LXR activation by this sterol, as determined by transactivation analysis in CV-1 cells (3-4 μM) [2]. These findings were confirmed by Gnerre et al. [8], who reported that 10 μM concentrations of the oxysterols, 24(S),25-epoxycholesterol, 25-hydroxycholesterol and 27-hydroxycholesterol, activated mouse PXR, also in a CV-1-based transactivation assay [8]. We also found that T0901317 treatment caused CYP3A induction in rat and mouse hepatocyte cultures, and that these effects were also mediated through PXR activation [7].

These findings prompted us to investigate the abilities of 24(S),25-epoxycholesterol and T0901317 to regulate P450 expression and activate PXR in human liver cell systems. As the foundation system, we evaluated treatment effects on CYP3A4 and CYP2B6 mRNA levels in primary cultured human hepatocytes, while as a secondary system we performed transactivation assays in the human hepatoma cell line, HepG2, that was co-transfected with supplemental human PXR and a PXR-responsive reporter. We report that 24(S),25-epoxycholesterol has essentially no ability to activate the human PXR, while T0901317 is among the most potent activators of human PXR yet described.

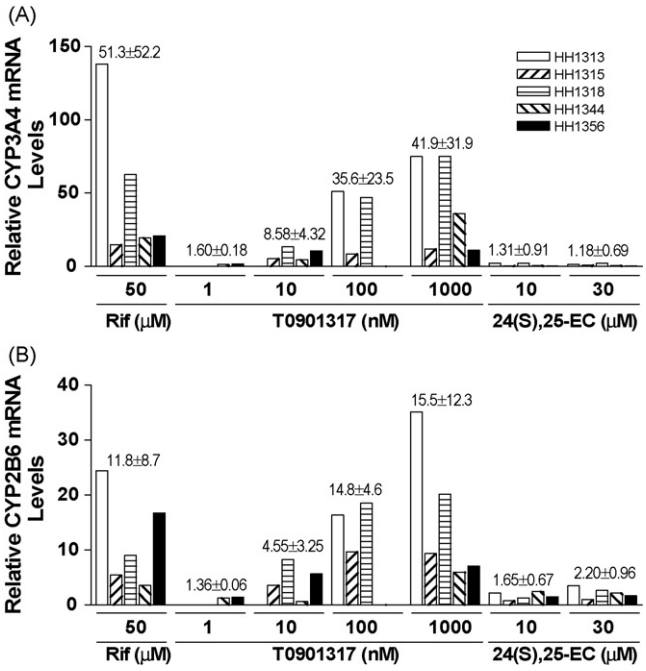

Fig. 1 – Effects of T0901317 or 24(S),25-epoxycholesterol treatments on CYP3A4 and CYP2B6 mRNA levels in primary cultures of human hepatocytes. Five preparations of primary cultured human hepatocytes (HH) were treated for 24 h with medium containing 0.1% DMSO, 50 μM rifampicin (Rif, in DMSO), 1, 10, 100 or 1000 nM T0901317 (in DMSO, not all concentrations were tested in each hepatocyte preparation), 0.1% ethanol, or 10 or 30 μM 24(S),25-epoxycholesterol [24(S),25-EC, in ethanol]. After treatment, the hepatocytes were harvested for measurement of CYP3A4 (A) and CYP2B6 (B) mRNA levels, using TaqMan Gene Expression Assays. Values are expressed as fold changes relative to the respective DMSO-treated or ethanol-treated controls, and results for each individual hepatocyte preparation are shown. The average fold increases (±S.D. for $n = 3$ to 5; ±range for $n = 2$) produced by the treatments are shown above the bars.

2. Materials and methods

2.1. Materials

T0901317 and rifampicin were purchased from Sigma-Aldrich (St. Louis, MO). 24(S),25-Epoxycholesterol and SR-12813 were purchased from BIOMOL International (Plymouth Meeting, PA). Cell culture media, fetal bovine serum and antibiotics were purchased from Invitrogen (Carlsbad, CA). Recombinant human insulin (Novolin R) was purchased from Novo Nordisk Pharmaceuticals, Inc. (Princeton, NJ). Other materials were obtained from the sources indicated below.

2.2. Primary cultured human hepatocytes and HepG2 cell culture

Plated primary cultures of human hepatocytes were obtained from the Liver Tissue Procurement and Distribution System (LTPADS) of the University of Minnesota, in collaboration with Dr. Stephen Strom (University of Pittsburgh). The following human hepatocyte preparations were used in this study: HH1313 (59 year old male), HH1315 (84-year-old female), HH1318 (17-month-old male), HH1344 (44-year-old female) and HH1356 (25-year-old female). After hepatocyte preparation and overnight culture, the hepatocytes, in T25 flasks, were express shipped to Wayne State University. Upon receipt, medium was replaced with Williams' Medium E supplemented with 0.25 U/ml insulin, 0.1 μM triamcinolone acetonide, 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B, and the cells were placed in an incubator and maintained at 37 °C under a humidified atmosphere of 95% air/5% CO_2. The following day, medium was replaced with the supplemented Williams' Medium E also containing 200 μg/ml Matrigel (BD Biosciences, San Jose, CA). The following day, the cultures were treated for 24 h as described in the legend to Fig. 1 and harvested for preparation of total RNA.

HepG2 cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, at 37 °C under a humidified atmosphere of 95% air/5% CO_2. For gene expression analysis, approximately 1 million HepG2 cells were seeded into 60 mm dishes, and two days after plating these cultures (approximately 70% confluent) were treated as described in the legend to Fig. 3 and harvested for preparation of total RNA. For transient transfection analysis, approximately 250,000 HepG2 cells were seeded into the wells of 12-well plates, and the following day these cultures were transfected as described below.

2.3. Gene expression analysis

Total RNA was prepared from individual T25 flasks of human hepatocytes using the ToTALLY RNA kit (Ambion, The Woodlands, TX) or from HepG2 cells using the RNeasy Mini kit (Qiagen, Valencia, CA), according to the manufacturers' instructions. The integrities of the RNA samples were assessed using a 2100 Bioanalyzer and the RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA). Two μg samples of total RNA were reverse transcribed at 37 °C, using the Omniscript RT kit (Qiagen, Valencia, CA) and random primers (Invitrogen) in 20 μl reactions. The levels of CYP1A1, CYP2B6, CYP3A4 and SREBP1c mRNA were measured, using TaqMan Gene Expression Assays Hs00153120_m1, Hs00167937_g1, Hs00430021_m1, and Hs00231674_m1, respectively, in multiplex reactions with a primer-limited primer/probe set for 18S rRNA as endogenous control (Applied Biosystems, Foster City, CA). Briefly, 1 μl of reverse transcription reaction (containing cDNA from 100 ng RNA) was aliquoted in triplicate into the wells of MicroAmp Optical 96-well reaction plates containing 19 μl of TaqMan Universal PCR Master Mix, a target primer/probe set and the 18S primer-limited primer/probe set. Thermal cycling conditions were initial activation for 10 min at 95 °C followed by 40 cycles of melting for 15 s at 95 °C and annealing/extension for 1 min at 60 °C. Cycle threshold values were obtained using Relative Quantification Study Software (Applied Biosystems). For each RNA sample, the target mRNA level was normalized to the 18S rRNA level. These normalized data were then expressed relative to a DMSO- or ethanol-treated control group.

2.4. Transient transfection analysis

The day after HepG2 cell plating, for each transfection the cells (3 wells per treatment group) were incubated with 0.6 ml of OptiMEM (Invitrogen) containing a premixed complex of 6.25 μl Lipofectamine 2000 (Invitrogen) and a plasmid mixture consisting of 800 ng of the XREM-CYP3A4-Luc reporter [construct p3A4-362 (7836/7208ins) described in [9], provided by Dr. Bryan Goodwin, GlaxoSmithKline, Research Triangle Park, NC], 0–200 ng pSG5-hPXR1 (provided by Dr. Steven Kliewer, University of Texas Southwestern Medical Center, Dallas, TX), 0–200 ng pSG5 (Stratagene, La Jolla, CA) to balance the total amount of expression plasmid that was used within an experiment, 1 ng of pRL-CMV (Promega Corporation, Madison, WI) to normalize for differences in transfection efficiency among samples, and sufficient pBlueScript II KS^+ (Stratagene) to adjust total DNA content to 1 μg. After 5 h, the medium was aspirated and replaced with 1 ml Dulbecco's Modified Eagle Medium containing 10% charcoal-stripped fetal bovine serum. The following day, the cells were treated as described in the legends to Figs. 2 and 3. Twenty-four hours later, the cells were harvested for the measurement of firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega) and an LMAX II384 microplate reader (Molecular Devices Corporation, Sunnyvale, CA) equipped with SoftMaxPro software. Normalized luciferase data (firefly/Renilla) were fit with sigmoid curves (variable slopes), using Prism 4 for Windows (GraphPad Software, San Diego, CA).

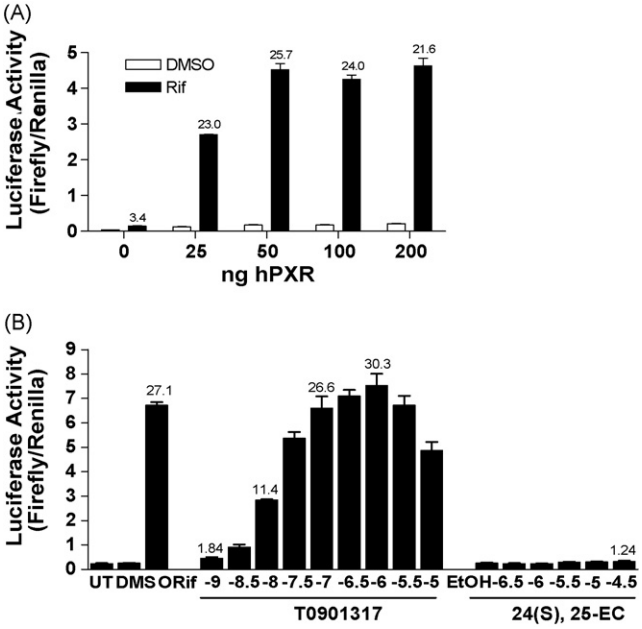

Fig. 2 – Effects of T0901317 and 24(S),25-epoxycholesterol treatments on human PXR-responsive reporter expression. (A) Optimization of transactivation conditions. HepG2 cells were transfected with 0–200 ng of a plasmid expressing human PXR (pSG5-hPXR1) in combination with 800 ng of a PXR-responsive reporter plasmid (XREM-CYP3A4-Luc), treated for 24 h with medium containing 0.1% DMSO or 50 μM rifampicin (Rif), and harvested for measurement of luciferase activities. (B) HepG2 cells were co-transfected with 50 ng pSG5-hPXR1 and 800 ng XREM-CYP3A4-Luc, treated for 24 h with medium alone (UT) or containing 0.1% DMSO, 50 μM Rif (in DMSO), 1 nM to 10 μM T0901317 (in DMSO), 0.1% ethanol (EtOH), or 0.3–30 μM 24(S),25-epoxycholesterol [24(S),25-EC, in EtOH] (concentrations are shown as log molarities), and harvested for measurement of luciferase activities. Values are presented as mean normalized luciferase activities (firefly/Renilla) ± S.D. ($n = 3$ wells per treatment group). The average fold-increases produced by selected treatments relative to the appropriate vehicle-treated controls are shown above the bars.

3. Results

The abilities of T0901317 and 24(S),25-epoxycholesterol to regulate CYP3A4 and CYP2B6 mRNA levels were evaluated in five preparations of primary cultured human hepatocytes (Fig. 1). The hepatocyte cultures were treated for 24 h with 1, 10, 100 or 1000 nM T0901317 (not all concentrations were tested in each hepatocyte preparation) or 24(S),25-epoxycholesterol (10 or 30 μM in each preparation), or with 50 μM rifampicin as a positive control treatment. Treatment with 10 nM or higher T0901317 produced clear increases in CYP3A4 (Fig. 1A) and CYP2B6 (Fig. 1B) mRNA contents in the hepatocyte preparations. Although the fold increases were variable among preparations, average increases in CYP3A4 mRNA levels of 8.58-, 35.6- and 41.9-fold were measured at T0901317

concentrations of 10, 100 and 1000 nM, respectively, while average CYP2B6 mRNA increases of 4.55-, 14.8 and 15.5-fold were measured at these same T0901317 concentrations. The magnitudes of the 1000 nM T0901317-mediated effects were comparable to the average increases that were produced by rifampicin treatment (51.3-fold and 11.8-fold for CYP3A4 and CYP2B6 mRNA, respectively). By contrast, 24(S),25-epoxycholesterol had essentially no effect on CYP3A4 mRNA levels in any of the human hepatocyte cultures (Fig. 1A), although treatment with 30 μM 24(S),25-epoxycholesterol did produce an average increase of 2.20-fold in CYP2B6 mRNA levels (Fig. 1B). Neither T0901317 nor 24(S),25-epoxycholesterol had any effect on CYP1A1 mRNA levels in the human hepatocyte cultures (data not shown).

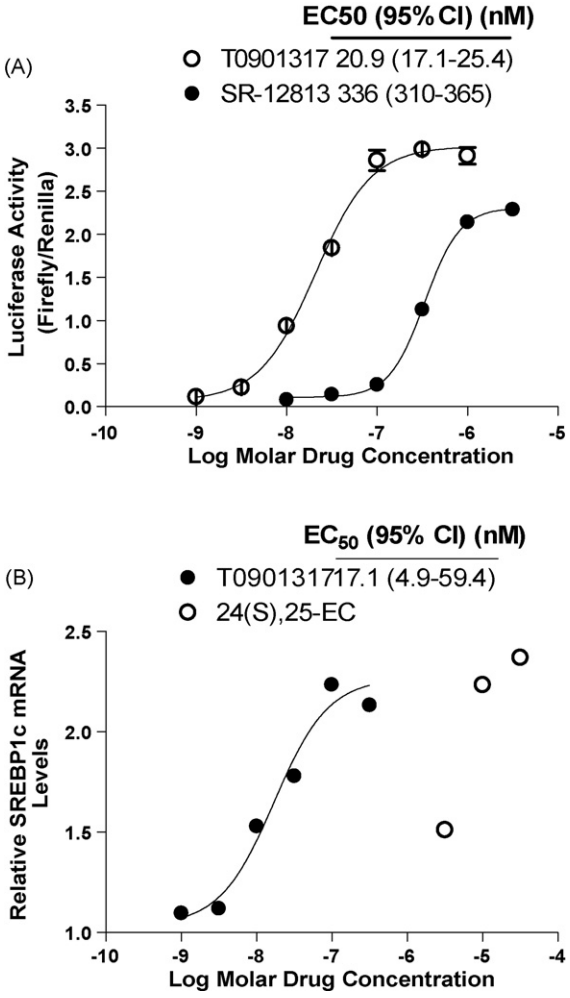

Fig. 3 – Concentration-dependent effects of T0901317 and SR-12813 on human PXR-responsive reporter expression, and of T0901317 and 24(S),25-epoxycholesterol on LXR target gene expression. (A) HepG2 cells were co-transfected with 50 ng pSG5-PXR1 and 800 ng XREM-CYP3A4-Luc, treated for 24 h with medium containing 1 nM to 1 μM T0901317 or 10 nM to 30 μM SR-12813 (concentrations shown as log molarities), and harvested for measurement of luciferase activities. Values are presented as mean normalized luciferase activities (firefly/Renilla) ± S.D. ($n = 3$ wells per treatment group). Fitted sigmoid curves with calculated EC_{50} values and 95% confidence intervals are shown. (B) HepG2 cells were treated for 24 h with 0.1% DMSO, 1–300 nM T0901317 (in DMSO), 0.l% ethanol, or 3–30 μM 24(S),25-epoxycholesterol [24(S),25-EC, in ethanol]. After treatment, the cells were harvested for measurement of SREBP1c mRNA levels, using a TaqMan Gene Expression Assay. Values are presented as fold increases [T0901317/DMSO or 24(S),25-EC/ethanol]. The fitted sigmoid curve for the T0901317 data is shown, together with the calculated EC_{50} value and 95% confidence interval.

Because these findings indicated that T0901317 was a highly potent CYP3A4 and CYP2B6 inducer, while 24(S),25-epoxycholesterol had little to no activity, we evaluated the abilities of these two agents to activate human PXR by performing transactivation assays in HepG2 cells that were transiently co-transfected with a plasmid expressing human PXR in combination with the PXR-responsive reporter plasmid, XREM-CYP3A4-Luc [9] (Fig. 2). Although a clear rifampicin-mediated induction of reporter expression was produced in HepG2 cells containing only basal levels of PXR, supplementation with additional human PXR produced enhancements of basal and rifampicin-inducible reporter expression (Fig. 2A). While the rifampicin/DMSO fold inducibility was essentially maximal in HepG2 cells transfected with 25 ng PXR (23.0-fold), the absolute rifampicin-inducible reporter expression was maximal at 50 ng PXR (Fig. 2A), and we therefore used this amount of PXR plasmid in subsequent experiments. The HepG2-based transactivation system displayed selectivity of response to PXR activation, as assessed by the failures of treatments with activators of other CYP3A4-regulating nuclear receptors to induce reporter expression. Treatment of the transfected HepG2 cells with 6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-3,4-dichlorobenzyl) oxime (CITCO) at a concentration (0.1 μM) sufficient to activate the human constitutive androstane receptor (CAR, NR1I3) [10] and induce CYP2B6 mRNA expression in primary cultured human hepatocytes had no effect on reporter expression (data not shown). Similarly, treatment with 0.1 μM vitamin D3, which can induce CYP3A4, CYP2B6 and CYP2C9 through activation of the vitamin D receptor [11], caused only a slight increase in reporter expression (less than two-fold; data not shown).

T0901317 treatment of PXR- and XREM-CYP3A4-Luc-transfected HepG2 cells produced a concentration-dependent increase in reporter expression (Fig. 2B). The effects of T0901317 treatment on PXR-responsive reporter expression in the HepG2 transactivation assay were comparable to those observed on CYP3A4 expression in the primary cultured hepatocytes, with 1 nM T0901317 producing a slight increase (1.84-fold in the transactivation assay versus 1.60-fold in the human hepatocytes), 10 nM T0901317 producing a clear increase (11.4-fold in the transactivation assay versus 8.58-fold in the human hepatocytes), and 100 and 1000 nM producing near-maximal increases (26.6- to 30.3-fold in the transactivation assay versus 35.6- to 41.9-fold in the human hepatocytes) (Fig. 2B). The increases in reporter expression that were produced by treatment with 100 or 1000 nM T0901317 were comparable to that seen following treatment with 50 μM rifampicin (27.1-fold). By contrast, treatment with 24(S),25-epoxycholesterol treatment had no effect on

PXR-responsive reporter expression, which was again consistent with the lack of CYP3A4 induction that was observed in the primary cultured human hepatocytes (Fig. 2B).

The potency of T0901317 for human PXR activation was evaluated in comparison to that of tetraethyl-2-(3,5-di-t-butyl-4-hydroxyphenyl)-ethenyl-1,1-bisphosphonate (SR-12813), one of the most potent known PXR activators [12] (Fig. 3A). SR-12813 has been reported to activate human PXR with an $EC_{50} \sim 400$ nM, and to bind to PXR in vitro with a $K_d \sim 40$ nM [12]. T0901317 induced PXR-responsive reporter expression with an $EC_{50} \sim 20$ nM (20.9 nM in the data shown in Fig. 3; 21.4, 19.1 and 14.7 in three replicate experiments), a value that was approximately 17-fold lower than the EC_{50} of SR-12813 for PXR-responsive reporter induction (average of 321 in two experiments) (Fig. 3A). In addition to its superior potency, T0901317 produced a 35% (average in two experiments) greater maximal induction of reporter expression than did SR-12813 (Fig. 3A). As an indication of the abilities of T0901317 and 24(S),25-epoxycholesterol to activate human LXR, effects on sterol regulatory element binding protein 1c (SREBP1c) mRNA levels were measured (Fig. 3B). By this index, T0901317 activated human LXR with an $EC_{50} \sim 17.1$, a value that is in agreement with the reported EC_{50} of ~20 nM [6], and that is essentially identical with the potency of this agent for activating human PXR (Fig. 3B). Treatment with 3–30 μM 24(S),25-epoxycholesterol also increased SREBP1c mRNA levels, demonstrating that the failure of this agent to activate human PXR was not due to its inability to evoke an effect in the test system (Fig. 3B).

4. Discussion

24(S),25-Epoxycholesterol is one of the most potent and efficacious endogenous ligand activators of the LXR receptors [2,3], and, as we and others have previously reported, is capable of activating rodent PXRs [7,8]. However, when tested in either of two models of human hepatic PXR activation/CYP3A4 induction, 24(S),25-epoxycholesterol treatment had essentially no effect, despite the fact that the sterol caused the expected induction of SREBP1c in HepG2 cells, indicating effective LXR activation. Therefore, 24(S),25-epoxycholesterol-mediated PXR activation does not appear to be conserved from rodent to human. This finding is reminiscent of the reported preferential abilities of certain bile acid precursor sterols, such as 5β-cholestane-3α,7α,12α-triol, to activate murine relative to human PXR [13,14].

CYP2B6, like CYP3A4, is a PXR target gene [15]. In addition, both P450s are regulated by CAR, although CYP2B6 is the preferred target [16]. The ability of 24(S),25-epoxycholesterol to evoke a small increase in CYP2B6 expression in primary cultured human hepatocytes might suggest that this oxysterol is capable of weakly activating human CAR or that CYP2B6 is a target gene for LXR. 24(S),25-Epoxycholesterol treatment did not increase CYP2B expression in rat hepatocytes in our earlier study [7].

When the effects of T0901317 were evaluated in several preparations of primary cultured human hepatocytes, it was apparent that T0901317 was a very potent inducer of CYP3A4 and CYP2B6 expression, producing clear, multi-fold increases at a concentration of 10 nM. Using the HepG2-based transactivation model of human hepatic PXR activation for detailed concentration-response analysis, the effects of T0901317 were found to be in close agreement with the effects on CYP3A4 expression that were observed in the primary human hepatocyte cultures, demonstrating the validity of the transactivation assay. When the concentration-dependent effects of T0901317 on human PXR activation were compared with those of SR-12813, T0901317 activated PXR with a potency (EC_{50} ~20 nM) that was more than an order of magnitude greater (EC_{50} for SR-12813 ~230). To our knowledge, this makes T0901317 one of the most potent known activators of human PXR. Only hyperforin, a major active antidepressant component of St. John's Wort, appears to have a comparable potency, with a reported EC_{50} of 23 nM for human PXR activation [17]. Also, in our transactivation assay, the maximal PXR activation that was produced by T0901317 treatment exceeded that produced by SR-12813, suggesting that different potent PXR activators may activate the receptor with different efficacies, perhaps in the manner of the selective receptor modifiers that have been described for other nuclear receptors (for recent review see [18]).

Our findings for T0901317 are in close agreement with those reported in two recent studies [19,20]. Xue et al. [19] determined the crystal structure of the human PXR ligand-binding domain containing bound T0901317, and found that T0901317 contacts 15 amino acid side chains that line the PXR ligand-binding pocket, which is the largest number that has yet been observed for a small ligand, although less than the 18 residues contacted by the large ligand, rifampicin. By comparison, T0901317 contacts 16 side chains of LXR, and 9 of these drug-receptor interactions are shared with PXR [19]. In functional studies, T0901317 was found to activate both PXR and LXRβ with high affinities (EC_{50}s of 13 and 100 for PXR and LXRβ, respectively) [19]. These investigators used the structural information to prepare a series of T0901317 analogs, with the purpose of determining whether key modifications would result in the production of a PXR antagonist. While no such result was found, certain modifications to the T0901317 structure were found to produce divergent effects on the affinities for PXR and LXR. For example, replacement of one of the trifluoromethyl groups adjacent to the drug's hydroxyl moiety with the large cyclohexyl group resulted in the production of a molecule with very high affinity for PXR (EC_{50} of 3 nM) but negligible affinity for LXR [19].

Mitro et al. [20] also recently reported that T0901317 potently and efficaciously activates both human PXR and LXR. In transactivation assays, T0901317 activated human PXR and LXR with EC_{50}s of 23 and 15 nM, respectively, while in coactivator recruitment and radioligand displacement in vitro binding assays, T090137 interacted with human PXR with an EC_{50} of 27 nM and a K_d of 26 nM [20]. Although T0901317 was found to activate mouse PXR with considerably lower potency (EC_{50} of 800 nM), the authors demonstrated that treatment of mice with the drug caused hepatic induction of several PXR target genes [20]. In this regard, Langmade et al. [21] previously reported that T0901317 activated mouse PXR with an EC_{50} of 75 nM, and that treatment of BALB/c NPC^{nih} mice with T0901317 caused induction of the PXR target gene, CYP3A13, in cerebellar tissue.

In conclusion, based on some shared structural features of the human PXR and LXR ligand-binding pockets, certain compounds are capable of binding to and activating both receptors. T0901317 is a very clear example of this, as it is not only a potent ligand activator of the LXR, but is an equipotent activator of the human PXR. Compounds that activate LXR but not PXR do exist, however. In the study by Mitro et al. [20], another synthetic LXR ligand, GW3965, was found to have no ability to interact with PXR. In addition, we demonstrate in this study that the endogenous oxysterol, 24(S),25-epoxycholesterol, is unable to activate human PXR. Our findings add to the clear evidence that T0901317 is a highly potent and efficacious activator of human PXR. Therefore, this drug cannot be used as a tool to investigate LXR-mediated events in biological systems, such as the human hepatocyte, that co-express human PXR.

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