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Human Pregnane X Receptor Activation and CYP3A4/CYP2B6 Induction by 2,3-Oxidosqualene:Lanosterol Cyclase Inhibition

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Running Title: Human PXR Activation by Squalene Metabolites

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Number of text pages: 35

Number of tables: 0

Number of figures: 7

Number of references: 33

Number of words in Abstract: 229

Number of words in Introduction: 723

Number of words in Discussion: 1408

[1]Abbreviations used are: BIBX 79, *trans*-N-(4-chlorobenzoyl)-N-methyl-(4-dimethylaminomethylphenyl)-cyclohexylamine; CAR, constitutive androstane receptor; cyclase, 2,3-oxidosqualene:lanosterol cyclase; FXR, farnesoid X receptor; GST, glutathione S-transferase; LBD, ligand-binding domain; NB-598, (E)N-ethyl-N-(6,6-dimethyl-2-hepten-4-ynyl)-3-[(3,3'-bithiophen-5-yl)methoxy]benzenemethanamine; MVA, mevalonate; PXR, pregnane X receptor; Ro 48-8071, [4'-(6-allyl-methyl-amino-hexyloxy)-2'-fluoro-phenyl]-(4-bromophenyl)-methanone fumarate; SXR, steroid and xenobiotic receptor; TBS-T, Tris-buffered saline Tween-20; TR-FRET, time-resolved

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fluorescence resonance energy transfer; U18666A, 3β-(2-diethylaminoethoxy)androst-5-

en-17-one HCl

Abstract

The effects of Ro 48-8071, an inhibitor of 2,3-oxidosqualene:lanosterol cyclase (cyclase), were evaluated on CYP3A4 and CYP2B6 mRNA content in primary cultured human hepatocytes. In seven hepatocyte culture preparations, 24 hr treatment with 3, 10 or 30 µM Ro 48-8071 produced median increases in CYP3A4 mRNA content that were 2.2-, 7.1- and 8.5-fold greater than untreated control, respectively, and produced increases in CYP2B6 mRNA content that were 3.0-, 4.6- and 3.4-fold greater than control, respectively. Increases in CYP3A4 immunoreactive protein content were also measured in Ro 48-8071-treated hepatocytes. To evaluate the effects of cyclase inhibitor treatments further, a pregnane X receptor (PXR)-responsive transactivation assay in HepG2 cells was utilized. Ro 48-8071, BIBX 79 and U18666A induced luciferase expression from a PXR-responsive reporter with EC_{50}s of 0.113, 0.916 and 0.294 µM, respectively. Treatment of the HepG2 system with NB-598, an inhibitor of squalene monooxygenase, at concentrations sufficient to achieve cholesterol biosynthesis inhibition, significantly inhibited cyclase inhibitor-mediated, but not rifampicin-mediated, reporter induction. Direct treatment of the HepG2 system with 1 to 10 µM squalene 2,3:22,23-dioxide, but not squalene 2,3-oxide, significantly activated PXR-responsive reporter expression. Also, squalene 2,3:22,23-dioxide bound to human PXR *in vitro* with an IC_{50} of 3.35 µM. These data indicate that cyclase inhibitors are capable of producing CYP3A4 and CYP2B6 induction in primary cultured human hepatocytes, and that an endogenous squalene metabolite is a conserved intracrine activator of PXR.

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Introduction

A primary mode of defense that is employed by animals against their chemical

environments involves recognition by a "xenobiotic-sensing" receptor followed by the

induction of phase I and phase II xenobiotic-metabolizing enzymes, as well as "phase III"

transporters. As the archetype of this mechanism, many xenobiotics bind to the PXR,

owing to the receptor's unusually accommodating ligand-binding pocket (Watkins, *et al.*,

2001; Watkins, *et al.*, 2003). Upon ligand binding, PXR, in partnership with the retinoic

X receptor, is transformed into an active transcription factor that increases the expression

of target genes, which include members of the CYP3A family (e.g., CYP3A23 in rat,

CYP3A11 in mouse and CYP3A4 in human). These CYP3A enzymes catalyze the phase

I metabolism of numerous xenobiotic substrates, including a large number of clinically-

used drugs (Quattrochi and Guzelian, 2001).

In addition to serving as a xenobiotic recognition and metabolizing system, PXR and

CYP3A enzymes are increasingly perceived to function in the metabolism of endogenous

molecules. For example, the cholestatic secondary bile acid, lithocholate, both activates

PXR and is a substrate for CYP3A (Xie, *et al.*, 2001; Staudinger, *et al.*, 2001). We have

utilized chemical inhibitors of various steps of the cholesterol biosynthetic pathway as an

approach for identifying endogenous modulators of hepatic cytochrome P450 expression

(Fig. 1). In this regard, we have reported that inhibitors of squalene synthase (e.g.,

squalestatin 1), the first committed step in cholesterol biosynthesis, selectively induce

CYP2B expression in primary cultured rat hepatocytes and rat liver through a mechanism

that requires the biosynthesis of one or more endogenous isoprenoids and activation of

CAR (Kocarek and Mercer-Haines, 2002). By contrast, treatment of primary cultured rat

or mouse hepatocytes with an inhibitor of cyclase (e.g., Ro 48-8071), which catalyzes the

second step downstream from squalene synthase, causes the selective induction of

CYP3A (Shenoy, *et al.*, 2004a). This latter effect is mediated by PXR, as illustrated by

the loss of cyclase inhibitor-mediated CYP3A induction in cultured hepatocytes prepared

from PXR-null mice (Shenoy, *et al.*, 2004a). In addition, cyclase inhibitor-inducible

CYP3A expression requires cyclase blockade and the ongoing synthesis of an

endogenous squalene metabolite, most likely squalene 2,3-oxide and/or squalene

2,3:22,23-dioxide, as indicated by the loss of induction when hepatocytes are co-treated

with an inhibitor of an upstream step in the cholesterol biosynthetic pathway (Shenoy, *et

al.*, 2004a). For example, cyclase inhibitor-inducible CYP3A expression was suppressed

when rat hepatocyte cultures were co-treated with NB-598, a potent inhibitor of squalene

monooxygenase, which catalyzes the step immediately upstream of cyclase (Fig. 1).

There are considerable differences among species in the regulation of xenobiotic-

metabolizing enzyme expression. A substantial portion of this variability can be

attributed to interspecies differences in the amino acid sequences of the ligand-binding

domains of PXR, with consequent differences in the dimensions of the large ligand-

binding pockets (Jones, *et al.*, 2000). As a classic example, the catatoxic steroid,

pregnenolone 16α-carbonitrile, is an efficacious activator of rodent PXR and inducer of

rodent CYP3A, while having little activity toward the rabbit or human orthologs

(Kocarek, *et al.*, 1995; Jones, *et al.*, 2000). By contrast, the macrocyclic antibiotic,

rifampicin, displays the opposite species-dependency for PXR activation and CYP3A induction (Kocarek, *et al.*, 1995; Jones, *et al.*, 2000). It therefore seems possible that interspecies differences apply not only to xenobiotics, but also to endogenous molecules. Indeed, this is clearly illustrated by recent findings involving the bile acid precursor sterol, 5β-cholestanoic acid-3α,7α,12α-triol. This sterol, which accumulates in the absence of functional CYP27, is an activator of murine PXR and a substrate for CYP3A (Furster and Wikvall, 1999; Honda, *et al.*, 2001; Goodwin, *et al.*, 2003; Dussault, *et al.*, 2003). CYP3A metabolism initiates an alternative pathway of sterol side-chain shortening, permitting the formation of cholic acid in hepatocytes lacking CYP27 activity (Honda, *et al.*, 2001; Goodwin, *et al.*, 2003). By contrast, bile acid precursor sterols do not activate human PXR, thereby explaining why humans with the genetic disease cerebrotendinous xanthomatosis, attributable to CYP27 deficiency, produce reduced levels of normal bile acids, accumulate sterols in various tissues, and exhibit a host of severe pathologies, while mice that have been genetically engineered to lack CYP27 do not (Goodwin, *et al.*, 2003; Dussault, *et al.*, 2003).

This study was undertaken to investigate whether the PXR activation and CYP3A induction that we previously reported to occur in rodent hepatocytes following cyclase inhibition is conserved in human liver cell culture systems.

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Materials and Methods

Materials. Ro 48-8071 and U18666A were purchased from BIOMOL International

(Plymouth Meeting, PA). BIBX 79 was a gift from Boehringer Ingelheim

Pharmaceuticals, Inc. (Ridgefield, CT). NB-598 was a gift from Banyu Pharmaceutical

Co., LTD (Tokyo, Japan). Rifampicin, cholesterol and squalene were purchased from

Sigma-Aldrich (St. Louis, MO). Squalene 2,3-oxide (2,3-oxidosqualene, racemic) and

squalene 2,3:22,23-dioxide (2,3,22,23-dioxidosqualene, mixture of diastereomers) were

purchased from Echelon Biosciences (Salt Lake City, UT). 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.

Primary cultured human hepatocytes. Plated primary cultures of human hepatocytes

were obtained through the Liver Tissue and Cell Distribution System (LTCDS, N01-DK-

7-0004). Following hepatocyte preparation and overnight culture at the University of

Pittsburgh, the hepatocytes, in T25 flasks, were express shipped to Wayne State

University and maintained as previously described (Duniec-Dmuchowski, *et al.*, 2007).

The day following Matrigel treatment, the hepatocytes were incubated with fresh medium

alone (untreated) or containing 3 to 30 µM Ro 48-8071, 0.1% DMSO or 50 µM

rifampicin (in DMSO). The cultures were either harvested after 24 hr incubation or re-

treated at 24 hr and harvested after a total of 48 hr incubation.

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TaqMan real-time RT-PCR analysis. Total RNA was prepared from individual T25 flasks of human hepatocytes, and levels of CYP3A4 and CYP2B6 mRNA were measured using TaqMan Gene Expression Assays Hs00430021_m1 and Hs00167937_g1, respectively (Applied Biosystems, Foster City, CA), as previously described (Duniec-Dmuchowski, *et al.*, 2007). Normalized mRNA contents were expressed relative to the untreated or DMSO-treated control group. The Wilcoxon sign-ranked test was used to compare median fold changes against the hypothetical value of 1 (GraphPad Prism, version 5.0; GraphPad Software Inc., San Diego, CA). This test requires a sample size of at least 6, and was therefore applied only to the data shown in Fig. 2.

Western blot analysis. Microsomes were isolated from primary cultures of human hepatocytes (2 pooled T25 flasks of hepatocytes per treatment group) as described previously (Kocarek and Reddy, 1996; Kocarek, *et al.*, 2002). Protein concentrations were determined by the bicinchoninic acid assay (Smith, *et al.*, 1985), using bovine serum albumin as standard. One μg of microsomal protein of each sample was resolved by SDS-PAGE on a Criterion precast 10% acrylamide Tris-HCl gel (Bio-Rad Laboratories, Hercules, CA) and electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad). Blots were blocked overnight in 5% non-fat dry milk in TBS-T in a cold room. After brief washing in TBS-T, blots were incubated with a 1:20,000 dilution of monoclonal antibody WB-MAB-3A (reported to detect CYP3A4 and CYP3A5; BD Gentest, Bedford, MA) in blocking buffer overnight in a cold room. After extensive washing in TBS-T, blots were incubated with horseradish peroxidase-

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conjugated goat anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA)

diluted 1:20,000 in blocking buffer for 1 hr at room temperature. Immunoreactive bands

were visualized using ECL Plus Western Blotting Detection Reagents (GE

Healthcare, Piscataway, NJ) according to the manufacturer's instructions.

Transient transfection analysis. HepG2 cells were cultured and transfected with the

PXR-responsive reporter plasmid, XREM-CYP3A4-Luc (provided by Dr. Bryan

Goodwin, GlaxoSmithKline, Research Triangle Park, NC), the human PXR expression

plasmid, pSG5-hPXR1 (provided by Dr. Steven Kliewer, University of Texas

Southwestern, Dallas, TX) and pRL-CMV (Promega Corporation, Madison, WI) as

previously described (Duniec-Dmuchowski, *et al.*, 2007). The following day, the cells

were treated as described in the legends to Figs. 4, 5 and 7. Twenty-four hr later, the cells

were harvested for measurement of firefly and *Renilla* luciferase activities using the

Dual-Luciferase Reporter Assay System (Promega). Luciferase data were analyzed by

one-way analysis of variance followed by the Newman-Keuls or Dunnett's multiple

comparison test and by fitting of concentration-response relationships with sigmoidal

curves (variable slopes), using Prism, version 5 (GraphPad Software, La Jolla, CA).

Thin-layer chromatographic detection of metabolically labeled lipids. Metabolic

labeling and detection of nonsaponifiable lipids was performed by modification of

methods described by Pill *et al.* (Pill, *et al.*, 1987) and Boogaard *et al.* (Boogaard, *et al.*,

1987). Briefly, 1.5 million HepG2 cells were plated into 60 mm dishes in Dulbecco's

modified Eagle's medium supplemented with non-essential amino acids, 10% fetal

bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and incubated for 48 hr.

The cells were then treated with 0.1% DMSO, rifampicin, NB-598 and/or Ro 48-8071, as

described in the legend to Fig. 6. One hr later, 2 µCi RS-[2-^{14}C]MVA (specific activity

55 mCi/mmol; GE Healthcare, Piscataway, NJ) was added to the culture medium. After

24 hr, cells were washed with and scraped into cold phosphate-buffered saline and

pelleted. The cell pellets were saponified in 15% potassium hydroxide in ethanol for 1 hr

at 80°C. Non-saponifiable lipids were extracted twice with n-hexane, and extracts were

combined and evaporated under nitrogen. The residues were dissolved in 60 µl hexane-

chloroform (70:30, v/v) and spotted onto reverse-phase thin-layer chromatography plates

(Whatman KC18), which were then developed twice with acetonitrile-chloroform (2:1,

v/v). Authentic samples of cholesterol, squalene, squalene 2,3-oxide and squalene

2,3:22,23-dioxide served as markers. These standards were spotted onto separate lanes

and detected by spraying with 10% sulfuric acid in ethanol and heating. Radiolabeled

lipids were then detected by exposing the plate to Kodak BioMax XAR film for 3 days at

room temperature.

PXR *in vitro* binding assay. The LanthaScreen TR-FRET PXR (SXR) competitive

binding assay (Invitrogen) was performed essentially according to the manufacturer's

instructions except that the assay volume was reduced to 20 µl. All steps were carried out

at room temperature. A serial dilution series of each test compound (squalene 2,3-oxide,

squalene 2,3:22,23-dioxide and T0901317) was prepared in the appropriate solvent

(ethanol for the squalene metabolites, DMSO for T0901317). The test compounds were

then diluted to 2X of their final concentrations in TR-FRET PXR (SXR) Assay Buffer

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and 10 µl of each dilution was aliquoted in triplicate into the wells of a black 384-well

assay plate (MatriCal Bioscience, Spokane, WA). Then, 5 µl of 4X Fluormone PXR

(SXR) Green (fluorescent PXR ligand) was added to each well followed by addition of 5

µl of 4X PXR-LBD (GST fusion)/dithiothreitol/terbium-labeled anti-GST antibody

solution in assay buffer. The plate was protected from light and incubated with shaking

for 1 hr, after which TR-FRET was measured using a Victor[3] multilabel reader

(PerkinElmer, Waltham, MA) at the following settings: excitation wavelength 340 nm,

emission wavelengths 520 nm and 490 nm, delay time 100 µs and integration time 200

µs. The TR-FRET ratio for each sample was calculated by dividing the emission signal at

520 nm by the emission signal at 490 nm. Binding curves with IC_{50} values were

generated by fitting emission ratio vs. log molar ligand concentration data with sigmoid

curves [log(inhibitor) vs. response - Variable slope], using Prism, version 5.

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Results

Hepatocytes isolated from seven human livers were incubated for 24 hr with the

prototypical PXR ligand, rifampicin (at 50 µM), or with the cyclase inhibitor, Ro 48-

8071, at concentrations of 3, 10 and 30 µM, and effects on CYP3A4 and CYP2B6

mRNA levels were measured (Fig. 2). Panel A shows the data plotted as fold increases

over control (i.e., Ro 48-8071 relative to untreated and rifampicin relative to DMSO-

treated) in box and whisker format. Ro 48-8071 concentrations of 3, 10 and 30 µM

produced significant median increases in CYP3A4 mRNA levels of 2.2-, 7.1- and 8.5-

fold, respectively, and increased CYP2B6 mRNA levels by 3.0-, 4.6- and 3.4-fold,

respectively (although only the effect at 10 µM was significant). For comparison,

rifampicin treatment increased CYP3A4 and CYP2B6 mRNA levels by 59- and 8.7-fold

in these hepatocyte preparations. There was considerable variability in the effects of Ro

48-8071 among hepatocyte preparations, as indicated both by the box and whisker plots

(Fig. 2A) and the individual concentration-response plots shown in Fig. 2B. In most of

the preparations, the Ro 48-8071-mediated increase in CYP3A4 mRNA content was

maximal or near-maximal at 10 µM, although in two of the preparations the increase was

at least 30% greater at 30 µM than it was at 10 µM. For CYP2B6, the Ro 48-8071-

mediated increase in mRNA content was greatest at 10 µM in all but one preparation.

In four additional human hepatocyte preparations, 48 hr treatment with Ro 48-8071

increased CYP3A4 mRNA content 3.4- to 4.8-fold and increased CYP2B6 mRNA

content 1.7- to 2.4-fold, at 3 and 10 µM, respectively (Fig. 3A). Although the sample size

was insufficient to demonstrate statistical significance, in two of these hepatocyte

preparations, microsomal CYP3A immunoreactive protein levels were also evaluated and

found to be increased in Ro 48-8071-treated hepatocytes relative to untreated controls

(Fig. 3B).

The above-described experiments demonstrated that treatment of primary cultured human

hepatocytes with a cyclase inhibitor, Ro 48-8071, was capable of increasing CYP3A4 and

CYP2B6 expression. To investigate the underlying mechanism, we used a HepG2-based

platform, in which the cells were transiently co-transfected with a plasmid expressing

human PXR and a PXR-responsive reporter plasmid (Duniec-Dmuchowski, *et al.*, 2007).

When the transfected HepG2 cells were treated with Ro 48-8071, luciferase expression

increased with an EC_{50} of 0.113 µM (Fig. 4A). Ro 48-8071-mediated induction was

maximal at 1 µM and maintained at 10 µM, but was completely attenuated at 30 µM (Fig.

4A). Treatment with either of two additional cyclase inhibitors, BIBX-79 or U18666A,

also potently increased luciferase expression (EC_{50}s of 0.916 µM and 0.294 µM,

respectively) (Fig. 4B). In these experiments, Ro 48-8071, BIBX-79 and U18666A all

produced maximal increases over control of ~7- to 8-fold (Fig. 4A and B) by comparison

to rifampicin, which increased reporter activity ~12-fold (data not shown).

To determine whether cyclase inhibitor treatments activated human PXR as a

consequence of cylase inhibition, rather than by functioning as direct PXR ligands,

experiments were performed in which the step in the cholesterol biosynthetic pathway

immediately preceding cyclase-catalyzed conversion of 2,3-oxidosqualene to lanosterol

was inhibited (Fig. 1). Thus, NB-598 was used to inhibit squalene monooxygenase, which catalyzes the conversion of squalene to 2,3-oxidosqualene. In our previous study, NB-598 treatment inhibited Ro 48-8071-inducible CYP3A expression in primary cultured rat hepatocytes, but the inhibitory effect was critically dependent on the NB-598 concentration (Shenoy, *et al.*, 2004a). Therefore, transfected HepG2 cells were treated with a range of NB-598 concentrations, alone or in combination with a cyclase inhibitor (Fig. 5A-C) or rifampicin, as a direct PXR ligand control (Fig. 5D). Treatment with NB-598 alone at concentrations of 0.1 or 0.3 µM had no significant effect on luciferase expression (Fig. 5A-D) but markedly inhibited cholesterol biosynthesis as indicated by reduction of [^{14}C]MVA incorporation into cholesterol with corresponding accumulation of [^{14}C]squalene (Fig. 6A). Higher concentrations of NB-598, which produced essentially complete blockade of cholesterol biosynthesis (Fig. 6A), caused a concentration-dependent increase in luciferase activity that attained, at 10 µM NB-598, ~45% of the increase produced by rifampicin treatment (Fig. 5D). As expected, rifampicin treatment had no effect on [^{14}C]MVA incorporation into cholesterol (Fig. 6A). When transfected HepG2 cells were treated with one of the cyclase inhibitors (at 10 µM), co-treatment with NB-598 at those concentrations that alone had no effect on PXR-responsive reporter expression (0.1 or 0.3 µM) significantly attenuated cyclase inhibitor-inducible expression, while higher NB-598 concentrations again produced concentration-dependent increases in luciferase activity (Fig. 5A-C). In correspondence with these findings, treatment with 10 µM Ro 48-8071 produced complete inhibition of cholesterol biosynthesis with accumulation of squalene 2,3-oxide and squalene 2,3:22,23-dioxide, while treatment with 0.1 µM NB-598 again produced marked inhibition of cholesterol

biosynthesis with squalene accumulation (Fig. 6B). Combination treatment with 10 µM

Ro 48-8071 and 0.1 µM NB-598 caused complete inhibition of cholesterol biosynthesis

with accumulation of squalene and a small amount of squalene 2,3-oxide attributable to

the residual squalene monooxygenase activity in the presence of 0.1 µM NB-598 (Fig.

6B). The significant reduction of cyclase inhibitor-mediated PXR activation by co-

treatment with a low concentration of NB-598 that was capable of effectively inhibiting

squalene monooxygenase but incapable of causing PXR activation strongly supports the

conclusion that cyclase inhibitor-inducible activation of human PXR is mediated through

the accumulation of squalene 2,3-oxide and/or squalene 2,3:22,23-dioxide. Co-treatment

with NB-598 did not suppress rifampicin-inducible luciferase activity at any

concentration tested (Fig. 5D). Rather, co-treatment with NB-598 concentrations of 1 µM

or greater significantly enhanced the reporter induction that was produced by rifampicin

treatment (Fig. 5D).

The abilities of squalene 2,3-oxide and squalene 2,3:22,23-dioxide to activate human

PXR directly were next evaluated. Transfected HepG2 cells were treated with 0.3 to 10

µM squalene 2,3-oxide or squalene 2,3:22,23-dioxide, either alone (Fig. 7A, top plot) or

in combination with Ro 48-8071 and NB-598, to prevent both endogenous formation and

cyclase-mediated conversion of the squalene metabolites (Fig. 7A, bottom plot). While

treatment with squalene 2,3-oxide alone did not significantly increase PXR-responsive

reporter expression (~2.5-fold increase at 10 µM), treatment with squalene 2,3:22,23-

dioxide produced a marked concentration-dependent increase in reporter expression

(~18.4-fold increase at 10 µM) (Fig. 7A, top plot). As described above, treatment with

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Ro 48-8071 increased PXR-responsive reporter expression, and this increase was significantly attenuated by NB-598 co-treatment (Fig. 7A, bottom plot). Additional co-treatment with squalene 2,3:22,23-dioxide, but not squalene 2,3-oxide, significantly increased reporter activity relative to the level measured in Ro 48-8071/NB-598 co-treated cells (Fig. 7A, bottom plot).

Finally, a TR-FRET-based *in vitro* competitive binding assay was used to assess the abilities of squalene 2,3-oxide and squalene 2,3:22,23 to bind directly to human PXR. T0901317 was used as a positive control PXR ligand in this assay, as we and others have recently reported that this compound is a potent activator of human PXR (Xue, *et al.*, 2007; Mitro, *et al.*, 2007; Duniec-Dmuchowski, *et al.*, 2007). T0901317 bound to PXR with an IC_{50} of 44.1 nM (Fig. 7B), consistent with the published PXR-activating and binding affinity of this compound (Xue, *et al.*, 2007; Mitro, *et al.*, 2007; Duniec-Dmuchowski, *et al.*, 2007). The competition curve for squalene 2,3:22,23-dioxide resembled that for T0901317, both in terms of slope and maximal displacement of the fluorescent PXR ligand (Fig. 7B, left plot). Squalene 2,3:22,23-dioxide bound to PXR with an IC_{50} of 3.35 µM (Fig. 7B, left plot), which was in accord with the concentration range that produced effective PXR transactivation in the HepG2 system. By contrast, although squalene 2,3-oxide bound to PXR with a calculated IC_{50} of 0.183 µM, the competition curve for this squalene metabolite was shallow and showed less maximal fluorescent ligand displacement relative to those for T0901317 and squalene 2,3:22,23-dioxide (Fig. 7B, left plot). For comparison, Ro 48-8071 showed little evidence of PXR binding at concentrations up to 10 µM. Higher concentrations caused fluorescent ligand

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displacement with a steep slope that permitted only estimation of an IC_{50} value (24.1 µM)

without confidence intervals (Fig. 7B, right plot).

Discussion

Treatment of primary cultures of human hepatocytes with the cyclase inhibitor, Ro 48-8071, increased the expression of CYP3A4, as well as expression of CYP2B6, which like CYP3A4 is a target gene for both PXR and CAR (Goodwin, *et al.*, 2001). Although there was considerable variability in the magnitude of Ro 48-8071-mediated CYP3A4/CYP2B6 induction among hepatocyte culture preparations, this variability was consistent with that observed with other inducers. For example, when 23 preparations of primary cultured human hepatocytes were treated with rifampicin, we observed CYP3A4 mRNA levels to increase from 1.6- to 436-fold (Fang, *et al.*, 2007). In most preparations, the magnitude of CYP3A4 mRNA induction that was produced by Ro 48-8071 treatment was lower than that produced by rifampicin treatment. For example, after 24 hr treatment with 10 μM Ro 48-8071, the median increase in CYP3A4 mRNA content was 7.1-fold, while the median increase produced by rifampicin treatment was 59-fold.

The Ro 48-8071 concentrations required to produce effective CYP3A4 induction in primary cultured human hepatocytes were in the low micromolar range (3 to 30 μM), whereas PXR-responsive reporter activation in transfected HepG2 cells was maximal at 1 μM Ro 48-8071. Thus, the Ro 48-8071 concentrations that produced CYP3A4 induction in primary cultured human hepatocytes were comparable to those we previously reported to cause CYP3A induction in primary cultured rat hepatocytes (Shenoy, *et al.*, 2004a) but were higher than those required to produce PXR activation in HepG2 cells. We speculate that these differences in Ro 48-8071 concentration-mediated effects between the primary

hepatocyte and HepG2 systems are likely attributable to differences in the xenobiotic
metabolism/transport capabilities of the cells.

One facet of cyclase inhibitor pharmacology that has been repeatedly observed is the
ability of concentrations that produce sub-maximal inhibition of cyclase activity to cause
accumulation of 24(S),25-epoxycholesterol, a potent endogenous LXR agonist, whereas
maximally effective concentrations of cyclase inhibitors prevent 24(S),25-
epoxycholesterol production (Morand, *et al.*, 1997; Mark, *et al.*, 1996; Janowski, *et al.*,
1999). We and others have reported that 24(S),25-epoxycholesterol is an effective
activator of rodent PXR (Shenoy, *et al.*, 2004b; Gnerre, *et al.*, 2005). Therefore, in
determining the mechanism by which cyclase inhibitor treatment causes PXR activation,
it is necessary to consider whether effects are due to (1) direct actions of the drugs as
PXR ligand agonists, (2) effects of endogenous oxysterols that are formed downstream of
cyclase, such as 24(S),25-epoxycholesterol or (3) effects of endogenous metabolites that
accumulate upstream of cyclase blockade. In this regard, we previously reported, through
the use of various upstream pathway inhibitors and estimation of cellular squalene/sterol
metabolite contents, that cyclase inhibition activated rodent PXR through accumulation
of one or both of the squalene metabolites, squalene 2,3-oxide and squalene 2,3:22,23-
dioxide (Shenoy, *et al.*, 2004a). The concentration-dependent effects of cyclase inhibitor
treatments on squalene/sterol metabolite production in HepG2 cells have been
investigated previously. Ro 48-8071 concentrations of 3 to 10 nM caused accumulation
of 24(S),25-epoxycholesterol, while 100 nM Ro 48-8071 produced almost complete
blockade of cholesterol biosynthesis, prevented 24(S),25-epoxycholesterol accumulation

and promoted accumulation of squalene 2,3-oxide and squalene 2,3:22,23-dioxide

(Morand, *et al.*, 1997). BIBX 79 and U18666A produced their greatest effects on PXR-

responsive reporter expression at concentrations of 10 µM and 1 µM, respectively. Short

term incubation of HepG2 cells with BIBX 79 was reported to inhibit cholesterol

biosynthesis by greater than 98% at 1 µM, a concentration that completely blocked

24(S),25-epoxycholesterol formation but caused substantial accumulation of squalene

2,3-oxide and squalene 2,3:22,23-dioxide (Mark, *et al.*, 1996). In the same study,

incubation with 0.1 µM U18666A effectively inhibited cholesterol biosynthesis, although

24(S),25-epoxycholesterol continued to accumulate at this concentration (Mark, *et al.*,

1996). At 1 µM U18666A, biosynthesis of cholesterol and 24(S),25-epoxycholesterol

was abolished, while squalene 2,3-oxide and squalene 2,3:22,23-dioxide accumulated

substantially (Mark, *et al.*, 1996). Therefore, the concentrations of the various cyclase

inhibitors that caused PXR activation in HepG2 cells were those that promote the

accumulation of squalene 2,3-oxide and squalene 2,3:22,23-dioxide, but not 24(S),25-

epoxycholesterol. Consistent with these findings, we have observed that direct incubation

of human PXR/reporter-transfected HepG2 cells with 24(S),25-epoxycholesterol does not

cause reporter induction (Duniec-Dmuchowski, *et al.*, 2007). These findings suggest that

24(S),25-epoxycholesterol, like the bile acid precursor sterol, 5β-cholestanoic acid-

3α,7α,12α-triol, activates rodent but not human PXR.

Our findings using the squalene monooxygenase inhibitor, NB-598, in combination with

the various cyclase inhibitors strongly implicate either or both of the squalene

metabolites, squalene 2,3-oxide and squalene 2,3:22,23-dioxide, as endogenous activators

of human PXR. NB-598 concentrations of 0.1 to 0.3 µM are sufficient to produce

effective inhibition of cholesterol biosynthesis in HepG2 cells [(Horie, *et al.*, 1990) and

Fig. 6A]. These NB-598 concentrations caused significant inhibition of the PXR-

responsive reporter induction that was produced by all cyclase inhibitors, while having no

inhibitory effect on rifampicin-mediated induction. Higher concentrations of NB-598

produced concentration-dependent increases in PXR-responsive reporter expression,

either when used alone or in combination with a cyclase inhibitor, an effect we speculate

is attributable to direct PXR ligand activity. However, co-incubation with NB-598, at

concentrations of 1 µM and higher, also produced significant increases in reporter

induction above that produced by a maximally effective concentration of the direct-acting

PXR ligand, rifampicin. The mechanism underlying this drug interaction is under

investigation.

Direct treatments of PXR/reporter-transfected HepG2 cells with squalene 2,3-oxide or

squalene 2,3:22,23-dioxide revealed that the latter compound was capable of producing

marked PXR activation. This effect was observed either when squalene 2,3:22,23-dioxide

was applied as the only treatment or when the squalene 2,3:22,23-dioxide was used

together with NB-598 and Ro 48-8071, the combination of which served both to prevent

the endogenous formation of squalene oxide metabolites as well as their further

conversion to lanosterol or 24(S),25-oxidolanosterol and downstream sterols. Use of an

in vitro TR-FRET-based assay confirmed the ability of squalene 2,3:22,23-dioxide to

bind directly to human PXR with an affinity that was consistent with the ability of this

metabolite to transactivate PXR in the HepG2 system. These results indicate that

squalene 2,3:22,23-dioxide is capable of activating human PXR and therefore may

mediate the PXR activation that occurs following treatment with a cyclase inhibitor.

While the HepG2 transfection data do not support a role for squalene 2,3-oxide in PXR

activation, it is not possible to conclude definitively that squalene 2,3-oxide is inactive in

this regard since the degree of cellular uptake of this metabolite following exogenous

treatment is unknown. Squalene 2,3-oxide did show some ability to bind to human PXR

in vitro although the binding curve did not reveal the same efficacy of fluorescent ligand

displacement that was seen for squalene 2,3:22,23-dioxide. It is also important to

emphasize that the squalene oxide compounds that were used for these studies were

mixtures of optical isomers rather than the pure enantiomers that are formed by

endogenous metabolism. As a final point, although NB-598 treatment significantly

inhibited cyclase-mediated PXR activation, the PXR activity was not reduced to the

control level occurring in untreated HepG2 cells. This residual PXR activation is likely

attributable to some ability of the cyclase inhibitors to function as direct PXR ligands. In

this regard, Ro 48-8071 did exhibit ability to bind to human PXR *in vitro* at high

concentrations.

In summary, our results indicate that cyclase inhibitors are capable of producing

CYP3A4 and CYP2B6 induction in primary cultures of human hepatocytes and of

activating human PXR. As in rodent systems, these effects are mediated through the

actions of endogenous squalene metabolites, the likeliest candidate being squalene

2,3:22,23-dioxide. Unlike the endogenous sterols that have been found capable of

activating rodent but not human PXR, the PXR-activating abilities of the squalene

metabolites are conserved from rodent to human. Of the other known endogenous inducers of CYP3A4, vitamin D_3 exerts its effect through the vitamin D receptor (Schmiedlin-Ren, *et al.*, 2001). In addition, although the primary bile acid, chenodeoxycholic acid, has been reported to be capable of activating PXR (Xie, *et al.*, 2001), recent findings indicate that primary bile acids can also induce CYP3A as a consequence of FXR activation, both directly through the transactivation of CYP3A4 as an FXR target gene (Gnerre, *et al.*, 2004) and indirectly through the transcriptional upregulation of PXR (Jung, *et al.*, 2006). Certain secondary bile acids, such as lithocholic acid and ursodeoxycholic acid, are known to activate murine and human PXR, consistent with a conserved role for PXR in protection from cholestasis (Uppal, *et al.*, 2007). Our results demonstrate that squalene metabolites are conserved intracrine activators of PXR and that cyclase may be considered to be a "receptor" that regulates the expression of CYP3A4 and other PXR target genes.

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Footnotes

This work was supported by National Institutes of Health Sciences grants HL50710

(T.A.K.) and ES05823 (M.R.M.). S.C.S. was supported in part by the Liver Tissue and

Cell Distribution System (LTCDS) N01-DK-7-0004/HHSN26720070004C. This work

was also supported by services provided by the Cell Culture and Gene Transfer

Technologies, Imaging and Cytometry, and Microarray and Bioinformatics Facility Cores

of National Institute of Environmental Health Sciences Center Grant P30 ES06639.

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Figure Legends

Fig. 1. Cholesterol biosynthesis pathway, abridged to highlight the metabolites (regular type), enzymes or receptors (italicized type) and drugs (boldface type) that are featured in this study. Arrows represent metabolic reactions, broken arrows indicate multiple steps, and thick shaded arrows represent receptor activation and induction processes.

Fig. 2. Effects of Ro 48-8071 treatment on CYP3A4 and CYP2B6 mRNA levels in primary cultured human hepatocytes. Seven preparations of primary cultured human hepatocytes were incubated for 24 hr with standard medium alone or containing 3, 10 or 30 µM Ro 48-8071, 0.1% DMSO or 50 µM rifampicin (Rif). After treatment, hepatocytes were harvested for preparation of total RNA, and CYP3A4 and CYP2B6 mRNA levels were measured as described in Materials and Methods. The Ro 48-8071 data are presented as fold changes relative to untreated controls, while the Rif data are presented as fold changes relative to DMSO-treated controls. A: Data are presented as box and whisker plots, in which the horizontal lines within the boxes represent the median values, the tops and bottoms of the boxes represent the 25th and 75th percentile values, and the upper and lower error bars represent the maximum and minimum values. *Significantly different from the hypothetical value of 1, $p<0.05$. B: Ro 48-8071 data from each individual hepatocyte preparation are plotted.

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Fig. 3. Effects of 48 hr Ro 48-8071 treatment on CYP3A4 and CYP2B6 expression in primary cultured human hepatocytes. A: Four preparations of primary cultured human hepatocytes were treated for 48 hr with standard medium alone or containing 3 or 10 µM Ro 48-8071, 0.1% DMSO or 50 µM rifampicin (Rif). After treatment, hepatocytes were harvested for preparation of total RNA, and CYP3A4 and CYP2B6 mRNA levels were measured as described in Materials and Methods. The Ro 48-8071 data are presented as fold changes relative to untreated controls, while the Rif data are presented as fold changes relative to DMSO-treated controls. Data are presented as box and whisker plots, in which the horizontal lines within the boxes represent the median values, the tops and bottoms of the boxes represent the 25th and 75th percentile values, and the upper and lower error bars represent the maximum and minimum values. B: Hepatocytes from two of the preparations described in A were harvested for preparation of microsomes, and CYP3A immunoreactive protein levels were measured by western blot hybridization.

Fig. 4. Concentration-dependent effects of cyclase inhibitor treatments on PXR-responsive reporter expression in HepG2 cells. HepG2 cells were transiently transfected with pSG5-hPXR1, XREM-CYP3A4-Luc and pRL-CMV and then incubated for 24 hr with medium alone or containing 10^{-8} to $3x10^{-5}$ M Ro 48-8071 (A) or with medium containing 0.1% DMSO or 10^{-8} to 10^{-5} M BIBX 79 or U18666A (B). After treatment, cells were harvested for measurement of firefly and *Renilla* luciferase activities. Normalized (firefly/*Renilla*) values are expressed as fold changes $\pm$ sd (n=3 wells per treatment group) relative to the appropriate negative control group (i.e., medium alone for Ro 48-8071; DMSO-treated for BIBX 79 and U18666A).

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Concentration-response data were fit to sigmoid relationships, and EC_{50} values with 95%

confidence intervals (CI) are shown. *$p<0.05$ and **$p<0.01$ versus negative control.

Fig. 5. Effects of NB-598 co-treatments on cyclase inhibitor-mediated induction of

PXR-responsive reporter expression in HepG2 cells. HepG2 cells were transiently

transfected with pSG5-PXR, XREM-CYP3A4-Luc and pRL-CMV and then incubated

for 24 hr with medium alone or containing 0.1% DMSO or 0.1 to 10 µM NB-598, alone

or in combination with 10 µM Ro 48-8071 (A), 10 µM BIBX 79 (B), 10 µM U18666A

(C) or 50 µM rifampicin (D). After treatment, cells were harvested for measurement of

firefly and *Renilla* luciferase activities. Data are expressed as mean normalized

(firefly/*Renilla*) luciferase values ± sd (n=3 wells per treatment group). Groups not

sharing a letter are significantly different from each other, $p<0.05$.

Fig. 6. Effects of NB-598 and Ro 48-8071 treatments on sterol biosynthesis in HepG2

cells. A and B: HepG2 cells were incubated for 24 hr with medium containing 0.1%

DMSO, 30 µM rifampicin (Rif) or 0.1 to 10 µM NB-598 (A) or with 0.1% DMSO (0, 0),

10 µM Ro 48-8071, 0.1 µM NB-598, or Ro 48-8071 and NB-598 in combination (B).

After treatment and metabolic labeling with [^{14}C]MVA, cells were harvested for

evaluation of nonsaponifiable lipid contents by thin-layer chromatography and

autoradiography as described in Materials and Methods.

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Fig. 7. Concentration-dependent abilities of squalene 2,3-oxide and squalene 2,3:22,23-dioxide treatments to activate PXR-responsive reporter expression in HepG2 cells and to bind to PXR *in vitro*. A: HepG2 cells were transiently transfected with pSG5-hPXR1, XREM-CYP3A4-Luc and pRL-CMV and then incubated for 24 hr with (top plot) medium alone (UT) or containing 0.1% DMSO (DM), 0.1% methanol (MeOH), 0.3 to 10 µM squalene 2,3-oxide (SO) or 0.3 to 10 µM squalene 2,3:22,23-dioxide (SDO) or (bottom plot) 10 µM Ro 48-8071 (Ro), 0.1 µM NB-598 (NB), Ro and DM, or Ro and NB alone (UT) or in combination with MeOH, SO or SDO. After treatment, cells were harvested for measurement of firefly and *Renilla* luciferase activities. Normalized (firefly/*Renilla*) values are expressed as mean ± sd (n=3 wells per treatment group). Groups not sharing a letter are significantly different from each other, $p < 0.05$. B: The LanthaScreen TR-FRET PXR (SXR) competitive binding assay was used to evaluate the abilities of SO and SDO (left panel) and Ro 48-8071 (right panel) to interact with PXR *in vitro*. T0901317 was included as a positive control PXR ligand (the same binding data for T0901317 are replicated in each panel). IC_{50} values and 95% confidence intervals (CI) are shown. For the Ro 48-8071 data, the IC_{50} value is an estimate and CI could not be calculated.
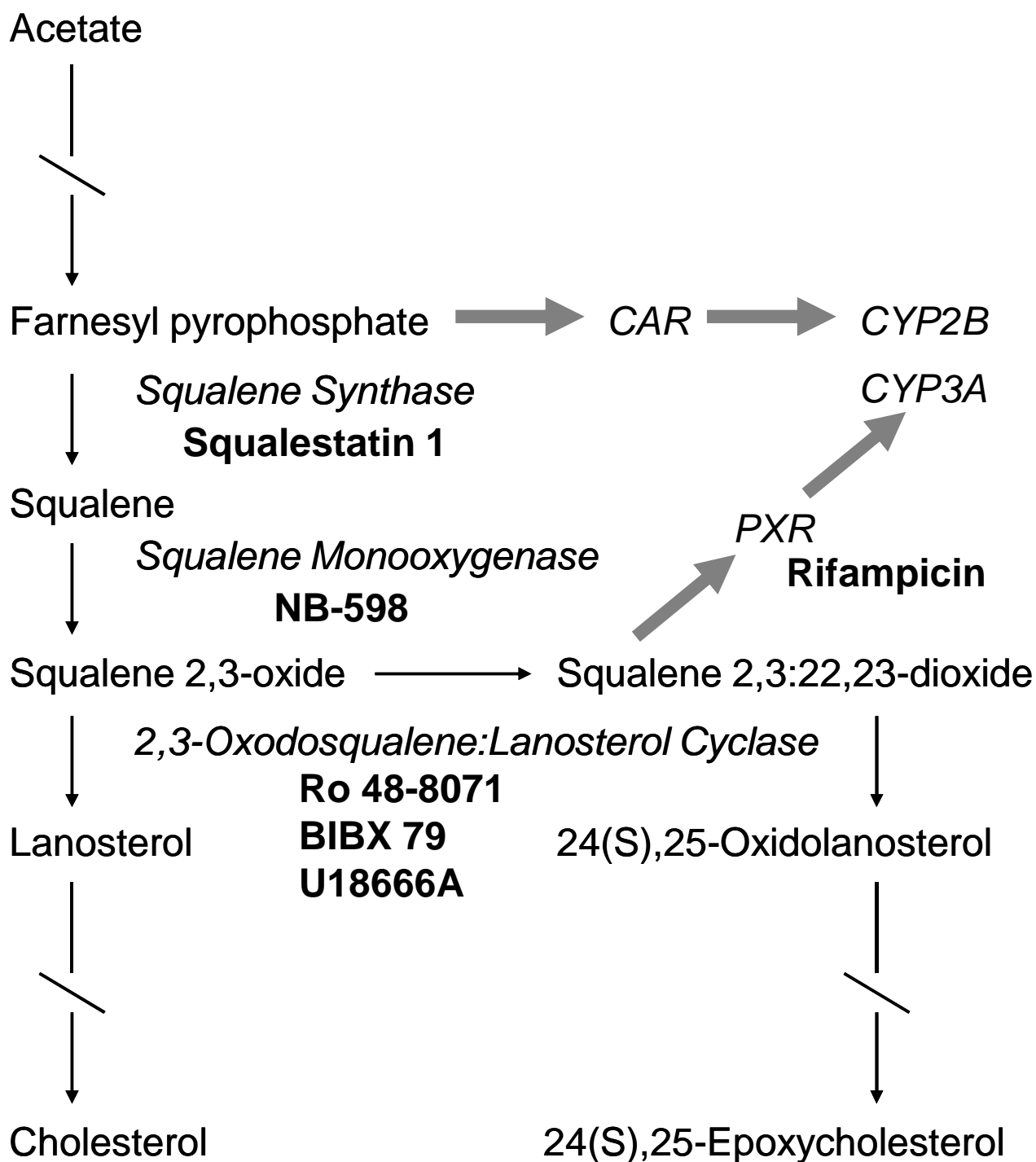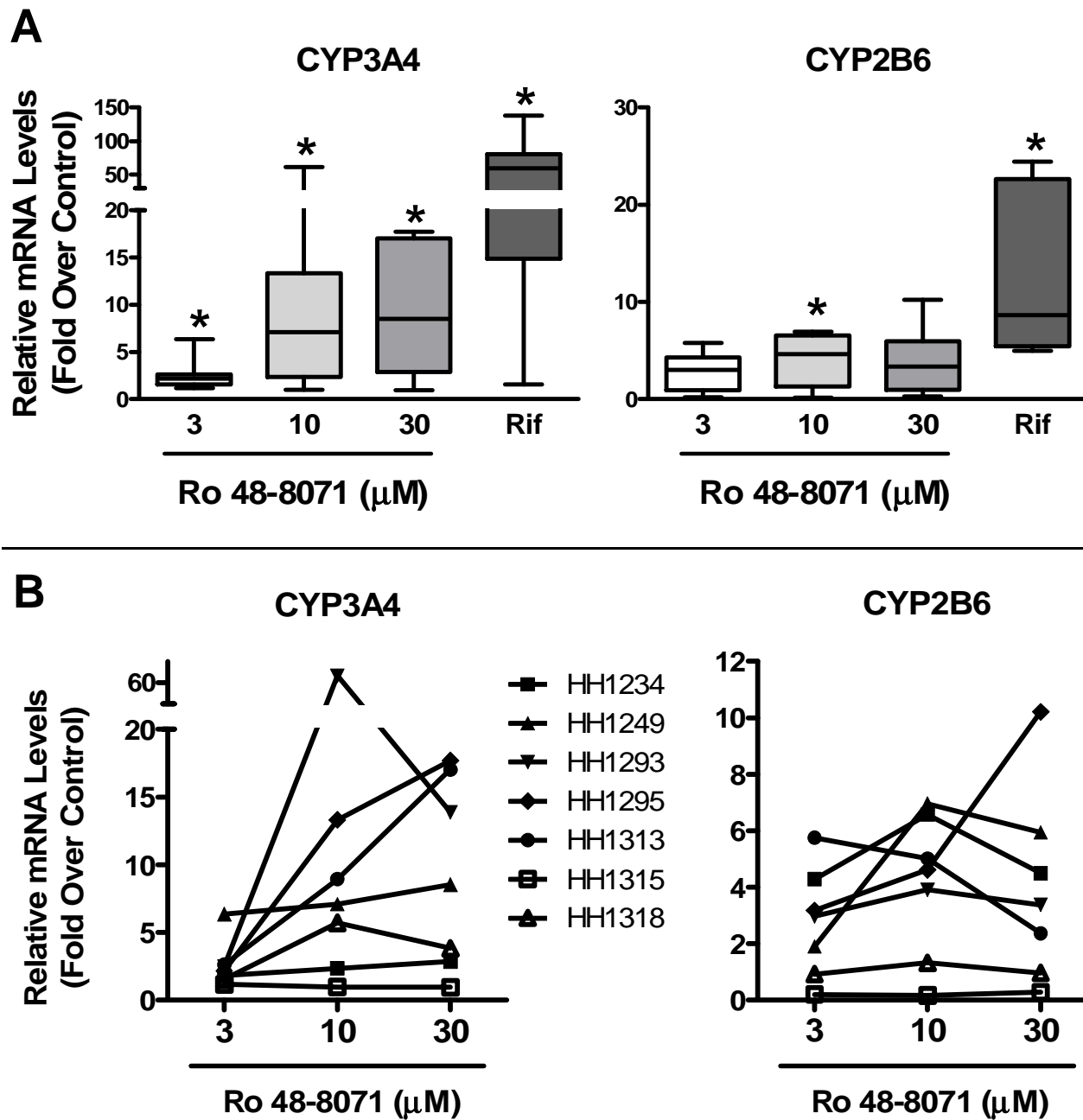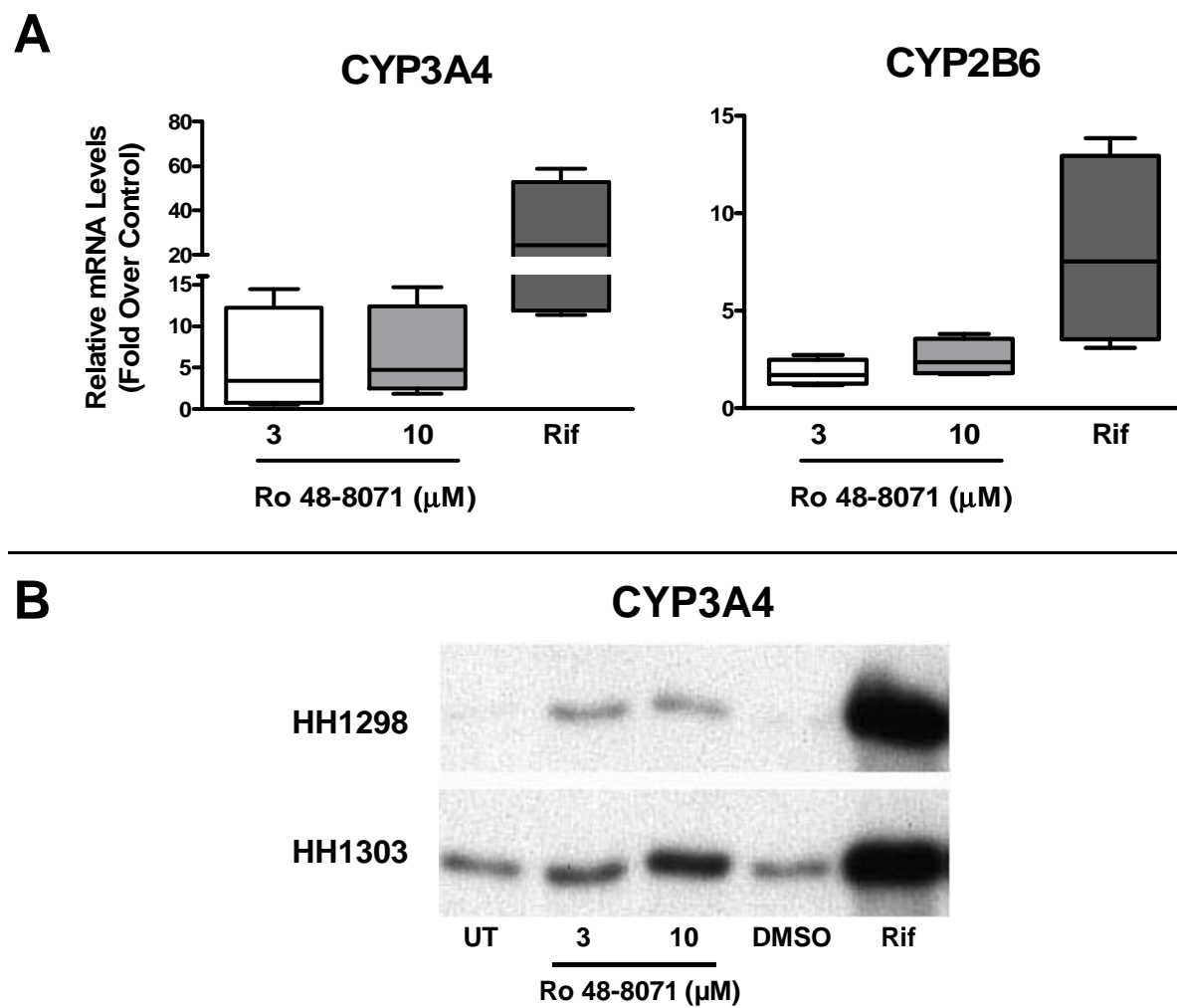
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Fig. 1

Fig. 2

A

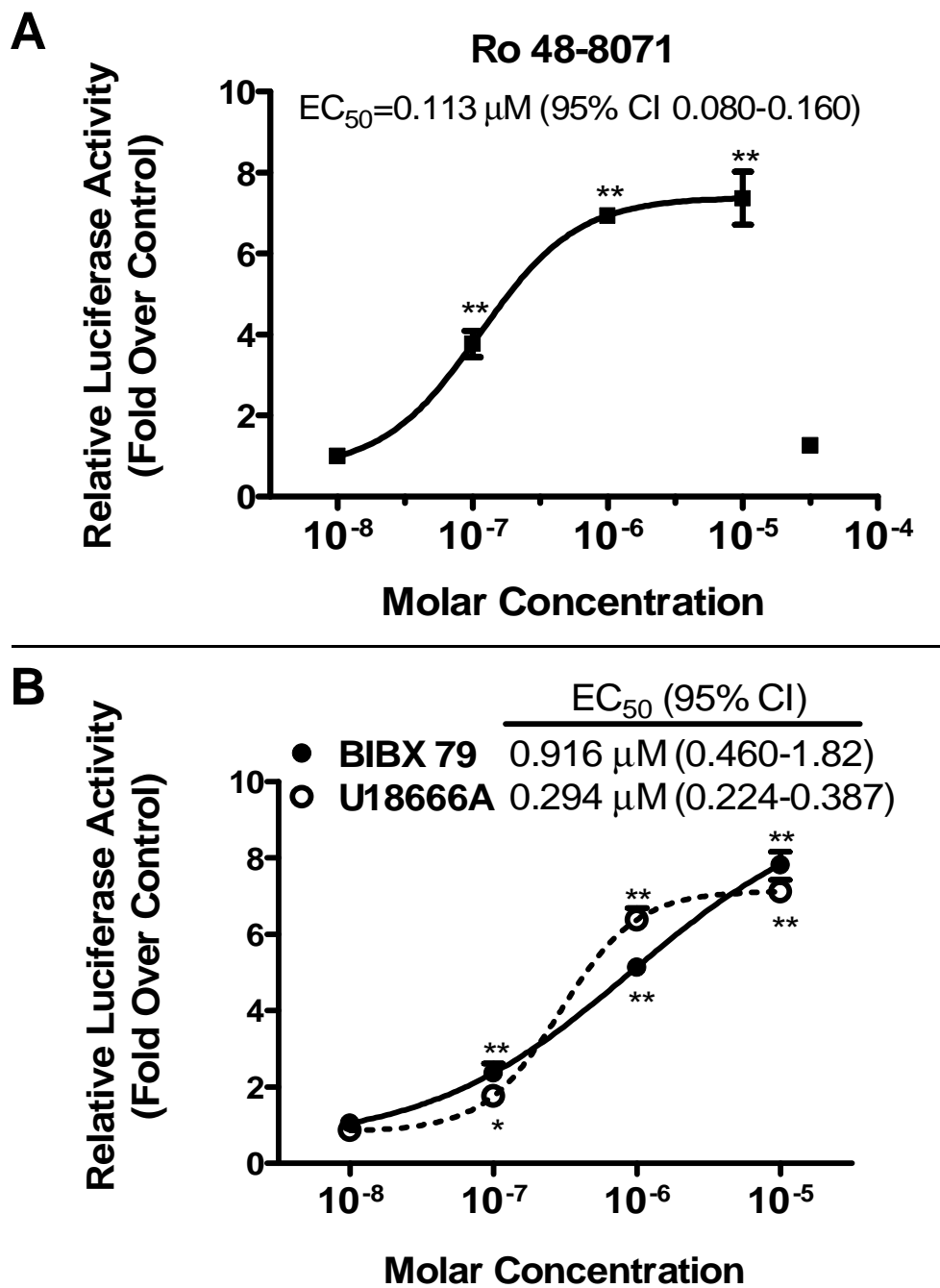**B**

Fig. 3

A

CYP3A4

CYP2B6

B

CYP3A4

Fig. 4

A

Ro 48-8071

EC_{50}=0.113 µM (95% CI 0.080-0.160)

B

EC_{50} (95% CI)

● **BIBX 79** 0.916 µM (0.460-1.82)
○ **U18666A** 0.294 µM (0.224-0.387)

Fig. 5

Fig. 6

Fig. 7

A

B