

## Title Page

### Role of human Pregnane X Receptor in tamoxifen and 4-hydroxytamoxifen mediated CYP3A4 induction in primary human hepatocytes and LS174T cells

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## Running Title Page

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**Abbreviations:** PXR, human Pregnane X Receptor; CYP, Cytochrome P450; XREM, Xenobiotic Response Element Module; GR, Glucocorticoid Receptor; ER, Estrogen Receptor; HNF-4, Hepatocyte Nuclear Factor-4; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; Tam,  
tamoxifen, 4OHT, 4-hydroxytamoxifen; NDMT, N-desmethyltamoxifen; Rif, Rifampicin

## ABSTRACT

Previously we observed that the anti-estrogens tamoxifen and 4-hydroxytamoxifen (4OHT) induce CYP3A4 in primary human hepatocytes and activate human pregnane X receptor (PXR) in cell based reporter assays. Given the complex cross-talk between nuclear receptors, tissue-specific expression of CYP3A4 and the potential for tamoxifen and 4OHT to interact with a myriad of receptors, this study was undertaken to gain mechanistic insights into the inductive effects of tamoxifen and 4OHT. Firstly, we observed that transfection of the primary cultures of human hepatocytes with PXR-specific siRNA reduced the PXR mRNA expression and the extent of CYP3A4 induction by tamoxifen and 4OHT by 50%. Secondly, in LS174T colon carcinoma cells, which were observed to have significantly lower PXR expression relative to human hepatocytes, neither tamoxifen nor 4OHT induced CYP3A4. Thirdly, N-desmethyltamoxifen (NDMT), which did not induce CYP3A4 in human hepatocytes, also did not activate PXR in LS174T cells. We then employed cell based reporter assay to evaluate the effects of other receptors such as GR $\alpha$  and ER $\alpha$  on the transcriptional activation of PXR. The co-transfection of GR $\alpha$  in LS174T cells augmented PXR activation by tamoxifen and 4OHT. The presence of ER $\alpha$  on the other hand, inhibited PXR mediated basal activation of CYP3A4 promoter, possibly via competing for common co-factors such as SRC1 and GRIP1. Collectively, our findings suggest that the CYP3A4 induction by tamoxifen and 4OHT is primarily mediated by PXR but the overall stoichiometry of other nuclear receptors and transcription cofactors also contribute to the extent of the inductive effect.

## INTRODUCTION

Tamoxifen is a prototypical selective estrogen receptor modulator (SERM), by virtue of its tissue specific estrogen/anti-estrogen properties. It is a clinically effective endocrine agent for the treatment and chemoprevention of breast cancer and despite the advent of new anti-estrogens such as the aromatase inhibitors, anastrozole and letrozole, it remains the drug of choice, especially in the pre-menopausal women. While tamoxifen is generally well tolerated, its clinical use is associated with several unresolved problems. These include inter-individual variability in its pharmacokinetics and the resulting variability in its safety and efficacy profile and drug-drug interactions. Since the systemic elimination of tamoxifen primarily entails hepatic metabolism, inter-subject variability in its metabolism is the principal cause of the overall variability in its pharmacokinetics. Tamoxifen is biotransformed to a larger number of metabolites including N-desmethyltamoxifen (NDMT) and 4-hydroxytamoxifen (4OHT) which are further converted to 4-hydroxy-N-desmethyltamoxifen (endoxifen) (Borges *et al.* 2006). Whereas, NDMT is a weak anti-estrogen, 4OHT and endoxifen are considerably more potent anti-estrogens than tamoxifen and may contribute to the overall therapeutic properties of the latter. While several members of the cytochrome P450 (CYP) superfamily of oxidative enzymes participate in tamoxifen metabolism CYP3A4 plays a prominent role. It is the major enzyme involved in the formation of NDMT, while it also contributes to the formation of 4OHT and endoxifen.

With regards to drug-drug interactions of tamoxifen, the most prominent occurrences were observed in recently completed clinical trials where tamoxifen co-administration

was associated with increased clearance of letrozole and anastrozole (Dowsett *et al.*, 1999 and 2001). To better understand the causes of inter-subject variability in tamoxifen metabolism and its apparent influence on the clearance of these aromatase inhibitors, in an earlier study, we investigated the effect of tamoxifen on the expression and activity of CYP3A4. Employing primary human hepatocytes, we observed that tamoxifen and 4OHT markedly increase the activity and expression of CYP3A4. Furthermore, we observed that both compounds activated the human Pregnane X Receptor (PXR) in cells based reporter assays (Desai *et al.*, 2002). It is well established that in addition to PXR, several other tissue-specific factors and nuclear receptors impact the transcriptional regulation of CYP3A4 (Pascussi *et al.*, 2003, Tegude *et al.* 2007). As such, the expression of PXR and other receptors such as glucocorticoid receptor (GR $\alpha$ ) and transcriptional factors such as HNF4 $\alpha$  impact the regulation of PXR target genes (Li *et al.*, 2006; Pascussi *et al.*, 2000)

This study was undertaken to gain mechanistic insights into the role of PXR and the impact of other tissue-specific transcription factors in the induction of CYP3A4 by tamoxifen and 4OHT. This investigation is particularly pertinent for these compounds since they are known to interact with various steroidal nuclear receptors, especially estrogen receptor (ER $\alpha$ ). Furthermore, in an earlier study employing Sprague-Dawley rats, oral administration of tamoxifen did not induce intestinal CYP3A4 suggesting that tissue-specific factors impact the CYP3A4 induction by tamoxifen (Cotreau *et al.*, 2001). Our overall experimental strategy included: i) assessment of tamoxifen mediated CYP3A4 induction in primary culture of human hepatocytes after down regulating PXR

in these cells by PXR specific siRNA, ii) determination of CYP3A4 induction in LS174T colon carcinoma cells and iii) evaluation of the effects of GR $\alpha$  and ER $\alpha$  on modulation of transcriptional activity of PXR by tamoxifen and 4OHT.

## MATERIALS AND METHODS

**Chemicals and Reagents.** Tamoxifen, 4OHT, rifampicin and phenobarbital were purchased from Sigma Chemical Co. (St. Louis, MO). NDMT was a gift from Dr. Frederick Beland at FDA, Bethesda, MD.

**Plasmids.** The PXR expression plasmid, pSG5-PXR- $\Delta$ ATG, the CYP3A4 promoter plasmid (CYP3A4 proximal promoter bases -362 to +53 linked to the distal XREM region) pGL3-CYP3A4 XREM- tk-luc and SRC1 expression plasmid pSG5-FL-SRC1 were obtained from Dr. Bryan Goodwin (Goodwin *et al.* 1999). GR $\alpha$  expression plasmid pSG5-hGR $\alpha$ , GR $\alpha$  response element reporter pGL3-(GRE)<sub>2</sub>- luc were kindly provided by Dr. John Cidlowski (Oakley *et al.* 1999); GRIP 1 expression plasmid pSG5-HA-GRIP 1 by Dr. Michael Stallcup (Ding *et al.* 1998) and ER $\alpha$  expression plasmid, pCMV-ER $\alpha$ , ER $\alpha$  response element reporter plasmid pGL3-(ERE)<sub>3</sub>-luc by Dr. Sohaib Khan (Singleton *et al.* 2003).

**Cell Culture.** Primary cultures of human hepatocytes as monolayers on collagen coated plates ( $2 \times 10^6$  cells/well of 6 well plate) or cell suspensions were provided by the Liver Tissue Procurement and Distribution System (Pittsburgh, PA), which was funded by NIH Contract #N01-DK-9-2310. LS174T and HepG2 cells were obtained from ATCC and maintained as recommended in Minimum Essential Medium (MEM) and supplements obtained from Invitrogen (Carlsbad, CA). The cells utilized in our study were within 7 passages after they were obtained from ATCC at passage 100.

**Transient Transfection Assays.** Transient transfection of CYP3A4 promoter reporter plasmid and nuclear receptor expression plasmids in HepG2 and LS174T cells was



performed using Lipofectamine and Plus reagents (Invitrogen, Carlsbad, CA) as described previously (Goodwin *et al.* 1999). Briefly, cells were plated in 24-well plates in MEM supplemented with delipidated fetal calf serum at a density of  $1.2 \times 10^5$  cells/well. Following 24-hours of plating the cells, overnight transfections were performed employing Lipofectamine and Plus reagent (Invitrogen, Carlsbad, CA), exactly as suggested by the manufacturer in the protocol for mammalian cells. Transfection mixes contained 75 ng nuclear receptor expression vector, 75 ng coactivators, 300 ng luciferase reporter gene construct harboring promoter sequences of interest downstream of a luciferase reporter, 300 ng pCH110 (an expression vector containing  $\beta$ -galactosidase cDNA under T7 promoter, Amersham, Piscataway, NJ). Equal quantities of DNA per transfection were maintained using appropriate amount of empty pSG5 vector plasmid. Transfections with empty pSG5 vector were performed as negative controls. Following transfections, plasmid containing medium was replaced with drug-containing medium and incubated for 24 - 48 hours. The cell layers were washed twice with ice cold phosphate buffer saline (pH 7.4) and scraped and collected in 250  $\mu$ l reporter lysis buffer provided with the  $\beta$ -galactosidase kit (Promega, WI). Cell lysates were used for determining protein content, luciferase enzyme activity using Luciferase assay system (Promega, WI) and  $\beta$ -galactosidase activity by  $\beta$ -galactosidase assay kit Promega (Madison, WI). The luciferase activity was normalized to the  $\beta$ -galactosidase activity and expressed as fold-activation with respect to the solvent (0.1 % DMSO) - treated controls.

**Transfection of siRNA in primary human hepatocytes.** Three sets of transfections (PXR siRNA, non-targeting siRNA and mock transfections with Lipofectamine 2000) were performed in parallel in 6-well plates. Lipofectamine 2000 and William's E medium supplemented with ITS was used for transfecting 10 and 50 nM quantities of siRNA SmartPool duplexes (Dharmacon, Lafayette, CO) as per manufacturer's protocol for Lipofectamine 2000. The efficiency of this duplex pool was established in preliminary experiments employing LS174T cells, wherein at concentrations ranging from 1 nM to 50 nM siRNA duplexes were transfected in LS174T cells for 24 hours. The knockdown of PXR was monitored in these transfected cells over 96 hours and was found to gradually reduce to approximately 80% of controls at all levels of duplex pool employed. Based on these results, the transfections in human hepatocytes were allowed to proceed for 24 hours after which fresh medium was added to the transfected cells. Sixty hours post transfections, drug treatments were initiated as described in the Drug Treatment section below. At the end of 72 hours of drug exposure, cells were dissolved in TriZol and processed for RNA extraction. CYP3A4 and PXR-specific mRNA levels were then determined in drug treated versus solvent treated cells. Induction of CYP3A4 mRNA by test drugs in mock transfected cells was considered maximal induction and induction observed in cells treated with PXR targeting and non-targeting siRNA sequences was determined relative to the induction observed in mock transfected hepatocytes. Cy-3 fluorescent labeled RISC-free® non-targeting siRNA, siGLO (Dharmacon Inc.) was employed in optimization of transfection process.

**Real Time PCR.** Total RNA was treated with DNase I (DNA-free kit, Ambion, Inc.) and 2 µg reverse transcribed. The resulting cDNA was amplified and analyzed by real time PCR using ABI 7000 instrument (Applied Biosystems, Inc. Foster City, CA) with SYBR green detection. Following activation of Amplitaq Gold DNA polymerase, cDNA was amplified using cycling conditions: 10 min initial denaturation at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C for primer annealing and extension. Primers: PXR (NM\_003889) Forward: 5'- GCA TCATCA GCT TTG CCA AAG - 3', Reverse: 5' - CCG CGT TGA ACA CTG TGT TG - 3'; HNF4α (NM\_178849) Forward: 5' - AGC CTG CCC TCC ATC AAT G - 3', Reverse: 5' - CTC ACA CAC ATC TGC GAT GCT - 3'; RXRα (NM\_002957) (Haugen *et al.*, 2004) Forward: 5' - GAG GCC TAC TGC AAG CAC AAG - 3', Reverse: 5' - CAG GCG GAG CAA GAG CTT AG - 3'; GRα (NM\_000176) (Raddatz *et al.*, 2004) Forward: 5' - CAA AAC TCT TGG ATT CTA TGC ATG AA- 3', Reverse: 5' - TTG GAA GCA ATA GTT AAG GAG ATT TTC- 3'; GAPDH (NM\_002046) Forward: 5' - GAA GGT GAA GGT CGG AGT C - 3', Reverse: 5' - GAA GAT GGT GAT GGG ATT TC - 3', CYP3A4 (D11131) Forward 5'- CTT CAT CCA ATG GAC TGC ATA AAT-3'; Reverse 5' - TCC CAA GTA TAA CAC TCT ACA CAG ACA A - 3' (Bowen *et al.*, 2000); ERα (NM\_000125) Forward 5'- CCA CCA ACC AGT GCA CCA TT-3'; Reverse 5'- GGT CTT TTC GTA TCC CAC CTT TC - 3' (de Cremoux *et al.* 2004).

The cDNA from HepG2 cells was serially diluted and amplified to generate relative standard curves for each mRNA under investigation. The amount of RNA in each reaction was calculated as per the starting concentration in the reverse transcription reaction (2 µg). To standardize the amount of sample cDNA added to each reaction, the

amount of the mRNA of interest was normalized to similarly calculated levels of GAPDH.

### **Drug Treatment**

Cells in culture were treated with tamoxifen (1 - 10  $\mu$ M), 4OHT (1 - 10  $\mu$ M), N-DMT (1 - 10  $\mu$ M) rifampicin (10  $\mu$ M) or phenobarbital (2 mM) for a period of 72 h. Drug-containing medium was replaced every 24 h for the 72 h drug treatment period following which cells were incubated with drug-free medium for 30 min to facilitate removal of drug from the cells. Cells were processed for the measurement of CYP3A4 activity, immunoreactive protein and mRNA levels as described below. Cell viability was assessed in cell cultures maintained in 24-well plates following drug treatment period employing the MTT assay as described previously (Carmicheal *et al.* 1987).

The choice of tamoxifen and 4OHT concentrations used here was based on the clinical pharmacokinetics of tamoxifen. With typical 20 mg bid regimen, tamoxifen plasma levels range from 0.1 to 1  $\mu$ M, whereas the maximal 4OHT levels are 0.1  $\mu$ M. However, the hepatic levels of these compounds may be ~60-fold higher than that in serum (Lien *et al.*, 1991). Plasma N-desmethyltamoxifen levels usually exceed tamoxifen levels at steady state (Kisanga *et al.* 2004).

**Determination of CYP3A4 Activity.** The rate of 6 $\beta$ -hydroxytestosterone formation by untreated control and drug-treated cells was employed as a marker for CYP3A4 activity. Following drug treatment, cells in culture were incubated with media containing testosterone (250  $\mu$ M), for 30 min. The media was collected and analyzed for

testosterone, formed metabolites and internal standard 11 $\alpha$ -hydroxyprogesterone (10  $\mu$ g/ml) by reverse phase HPLC using 60% methanol as mobile phase (Desai *et al.* 2002).

**Immunodetection of CYP3A4 and GR $\alpha$  Protein.** For CYP3A4 detection, cells were homogenized in HEPES/EDTA buffer and microsomes were prepared by differential centrifugation. For GR $\alpha$  detection, the cells were lysed in RIPA buffer (Pierce, Rockford, IL). The protein were quantitated by Lowry assay using bovine serum albumin standards. The proteins were treated uniformly and were maintained at 4°C until resolved to minimize degradation. Equal amount of proteins (3  $\mu$ g for CYP3A4, 20  $\mu$ g for GR $\alpha$ ) were resolved employing SDS-PAGE (12% acrylamide) and transferred to nitrocellulose membranes. The membranes were then blocked with 3% BSA in Tween (0.1%)-PBS (pH 7.4) for 45 min and then treated with primary anti-CYP3A4 antibody (BD Biosciences, San Jose, CA) or anti- GR $\alpha$  antibody (Santa Cruz Biotechnology, Santa Cruz CA) followed by horseradish peroxidase-conjugated anti-mouse or anti-goat secondary antibody (Sigma Chemical Co, St. Louis, MO and Santa Cruz Biotechnology, Santa Cruz CA). The protein bands were visualized using enhanced chemiluminescence detection (Amersham Biosciences Corp. Piscataway, NJ).

**Northern Blot Analysis of CYP3A4 mRNA.** Total cellular RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA), quantitated spectrophotometrically and 10  $\mu$ g fractionated using electrophoresis followed by overnight transfer on to a nylon membrane (Millipore, Bedford, MA). Isolated CYP3A4 cDNA probes (Oxford Medical

Research, MI) were labeled with [ $\alpha$ <sup>32</sup>P]-labeled dCTP (NEN, Boston, MA) using the random primer method and hybridized. The membranes were exposed to X-ray film and the developed bands were quantitated employing NucleoVision image analyzer (Nucleotech, CA). The probe used here as well as testosterone 6 $\beta$  hydroxylase activity may not be specific for CYP3A4 and may also detect CYP3A5, which is typically a minor component of the overall CYP3A pool (Westlind-Johnsson *et al.*, 2003).

**Statistical and Data Analysis.** The differences in CYP3A4 activity, immunoreactive protein content, mRNA levels and cell viability in control vs. treated groups were analyzed employing a one-factor ANOVA, followed by Tukey's test for multiple comparisons or a t-test for pair wise comparisons at  $\alpha = 0.05$ .

## RESULTS

### Role of PXR in tamoxifen and 4OHT mediated CYP3A4 induction.

We first evaluated the induction of CYP3A4 in primary cultures of human hepatocytes transfected with PXR specific siRNA. Our initial attempts entailed optimization of the conditions for PXR siRNA transfection in LS174T cells employing 10 nM to 50 nM PXR specific siRNA duplexes which was able to downregulate PXR mRNA up to 80% (data not shown). These attempts indicated that at a 10 nM concentration the siRNA sequences were non-cytotoxic, but cytotoxicity was observed at 50 nM concentration. As shown in Figure 1A, PXR expression was reduced by ~50% in cells transfected with 10 nM PXR siRNA, whereas no changes in PXR levels were apparent in mock transfected and non-targeting siRNA transfected hepatocytes. The extent of CYP3A4 induction (difference in the mRNA levels in drug-treated vs solvent-treated controls) was determined separately for mock siRNA treated and PXR siRNA treated hepatocytes. As seen in Figure 1B, cells transfected with PXR siRNA, which exhibited a 50% decrease in the PXR mRNA levels, exhibited significantly lower extent of CYP3A4 induction than mock transfected cells. The magnitude of CYP3A4 induction by tamoxifen, 4OHT and rifampicin was reduced by 53, 77 and 52%, respectively. It is important to note that the extent of CYP3A4 induction in non-targeting siRNA transfected cells was not significantly different from that in mock transfected cells. In parallel, cy-3 labeled siRNA (siGLO, Dharmacon) was transfected primary human hepatocytes on a glass slide and viewed by confocal microscopy to ensure intracellular compartmentalization of the siRNA sequence comparable in length to the silencing siRNA. In hepatocytes from the donors where we did not observe any reduction of PXR mRNA, possibly due to lack of

effective transfection in these cells, a change in CYP3A4 inducibility was not observed (data not shown). This observation corroborates that the change in extent of CYP3A4 inducibility was related to change in PXR expression.

We then determined the extent of PXR mediated activation of CYP3A4 promoter by tamoxifen and 4OHT in HepG2 and LS174T cells. These cells were transiently transfected with a luciferase reporter construct harboring PXR responsive enhancer module (Xenobiotic Response Element Module) and a PXR expression plasmid (pSG5-PXR  $\Delta$ -ATG) (Goodwin *et al.* 1999). Tamoxifen as well as 4OHT activated PXR in both LS174T and HepG2 cells (Figure 2). In HepG2 cells, at 5  $\mu$ M concentration, tamoxifen moderately activated PXR (~ 5 fold) whereas 4OHT strongly activated PXR (~10 fold), the magnitude of latter being comparable to that of the prototypical PXR activator, rifampicin (10  $\mu$ M). The overall magnitude of activation in LS174T cells, was lower compared to that observed in HepG2 cells. For instance, at 5  $\mu$ M concentration the fold activation in tamoxifen and 4OHT treated LS174T cells were 2.5 and 5-fold, respectively. NDMT did not activate PXR in HepG2 and LS174T cells.

**Effect of NDMT on hepatic CYP3A4.** In our previous study we investigated the effects of tamoxifen and 4-OHT employing four batches of human hepatocytes (Desai *et al.*, 2002). In this report we have included data from additional donors of human hepatocytes in which the effect of these agents and NDMT on the expression and activity of hepatic CYP3A4 was evaluated. First, differences in the rate of testosterone 6 $\beta$ -hydroxylase activity of cells treated with vehicle (0.1% DMSO) alone to those treated with the anti-estrogens or prototypical inducers, rifampicin and phenobarbital were



evaluated as a marker of CYP3A4 activity. Protein and mRNA levels were evaluated by western blotting and real time PCR, respectively. As indicated in Table 1, at concentrations up to 10  $\mu$ M NDMT treated hepatocytes did not exhibit an increase in CYP3A4 activity, protein or mRNA levels compared to solvent-treated cells. NDMT, although structurally very similar to tamoxifen, did not activate PXR in HepG2 and LS174T (Figure 2), which is consistent with the lack of its effect on CYP3A4 expression.

### **Expression of Nuclear Receptors in Hepatocytes and LS174T cells.**

The expression of PXR, HNF4 $\alpha$ , RXR $\alpha$ , ER $\alpha$  and GR $\alpha$  were compared in HepG2 and LS174T cell lines to primary human hepatocytes. As shown in Figure 3, while the overall levels of these transcription factors were lower in HepG2 and LS174T cells, the levels of PXR and other key CYP3A4 regulator GR $\alpha$ , was strikingly lower in the cell lines. Most notably, GR $\alpha$  was undetectable in LS174T cells, while its expression levels were easily detectable in HepG2 cells and primary hepatocytes. This observation was further confirmed by assessing the GR $\alpha$  protein levels in these cells by western blotting (Figure 4). Since tamoxifen and 4OHT are ER $\alpha$  ligands, we assessed the levels of ER $\alpha$  in these cells. In primary hepatocytes, ER $\alpha$  was expressed at levels significantly lower than those in ER $\alpha$  positive MCF-7 breast cancer cells. Its expression in HepG2 and LS174T cells was ~ 5 and 10 fold lower, respectively, compared to hepatocytes. Given this reduced expression of GR $\alpha$  and ER $\alpha$  in LS174T cells, we investigated the role of these nuclear receptors in hPXR mediated transactivation of CYP3A4 reporter by the antiestrogens.

### **GR $\alpha$ potentiated tamoxifen/4OHT mediated PXR activation.**

Transient transfection assays were performed in LS174T cells to assess the effect of GR $\alpha$  on CYP3A4-XREM-luc promoter transcription. When GR $\alpha$  was cotransfected with CYP3A4 promoter plasmid, rifampicin treatment resulted in a significant increase in luciferase activity while neither tamoxifen nor 4OHT activated transcription of CYP3A4 promoter region suggesting direct role of GR $\alpha$  in rifampicin mediated CYP3A4 transcription but not in case of tamoxifen/4OHT. However, when GR $\alpha$  was cotransfected with PXR in LS174T cells along with CYP3A4 promoter reporter plasmid, there was an increase in luciferase activity compared to transfections with PXR alone in cells treated with tamoxifen and 4OHT (Figure 5). For tamoxifen (5  $\mu$ M) there was approximately a 3-fold increase in the activation of CYP3A4 promoter when cells were co-transfected with GR $\alpha$  and PXR relative to PXR alone. Likewise there was approximately 5-fold increase due to co-transfection in cells treated with 4OHT (5  $\mu$ M) and approximately 8-fold with rifampicin (10  $\mu$ M). Concomitant promoter-reporter assay was performed by transfection with the same GR $\alpha$  expression plasmid and a reporter plasmid harboring GR $\alpha$  response elements [pGL3-(GRE) $_2$ -luc] and treated with dexamethasone (10<sup>-6</sup> M) to ensure translation of active GR $\alpha$  in these cells under experimental conditions (not shown).

### **Repressive Cross Talk between PXR and ER $\alpha$ .**

ER $\alpha$  did not activate the PXR responsive region of CYP3A4 promoter in response to either rifampicin or the antiestrogens in absence of PXR (Data not shown). However, as compared to PXR expression alone, co-transfection of PXR and ER $\alpha$  caused

repression of the basal levels of CYP3A4 promoter transcription evident from the reduced levels of luciferase expression. This repression was even more significant in the presence of estradiol (1 nM) (Figure 6). The extent of repression increased with increased expression of ER $\alpha$  and it was possible to compensate for this repression by cotransfection with transcriptional cofactors such as SRC1 or GRIP1, which are involved in signaling pathways of both ER $\alpha$  and PXR (Figure 7). It is noteworthy that despite this repression, the levels of luciferase activity in rifampicin or antiestrogen treated cells transfected with PXR alone, were not significantly different from the similarly treated cells in PXR and ER $\alpha$  cotransfections (not shown). Concomitant experiments of ER Response Element (ERE) activation by transfection of ERE upstream of a luciferase reporter by 1 nM estradiol via ER $\alpha$  expression plasmid were conducted to ensure expression of active ER $\alpha$  under experimental conditions (data not shown).

#### **Effect of tamoxifen and 4OHT on CYP3A4 expression in LS174T cells.**

We also evaluated the effects of tamoxifen and 4OHT on CYP3A4 in LS174T cells, a cell culture model previously used for assessing effects on intestinal drug metabolizing enzymes (Schuetz *et al.* 2000). Notably, neither tamoxifen nor 4OHT at concentrations ranging from 1 to 10  $\mu$ M increased testosterone 6 $\beta$ -hydroxylase activity in LS174T cells, although the prototypical inducers, rifampicin and phenobarbital, increased CYP3A4 activity in these cells by 1.97 and 5.3-fold, respectively (t-test,  $p < 0.05$ ) (Table 2).

The effect of the anti-estrogens on the amount of CYP3A4-specific mRNA and CYP3A4 immunoreactive protein levels in LS174T cells is summarized in Table 2. Neither

tamoxifen nor 4OHT had any significant influence on CYP3A4 mRNA levels in LS174T cells whereas a 1.89- and a 4.12- fold increase were observed with rifampicin and phenobarbital treatment, respectively. This observation is in contrast to our observations in human hepatocytes where we observed a marked dose-dependent increase in mRNA levels in hepatocytes treated with tamoxifen and 4OHT. We also determined the CYP3A4 mRNA levels employing more sensitive technique of real time PCR. Tamoxifen and 4OHT both exhibited a very low 2- and 3- fold increase in CYP3A4 mRNA in LS174T cells, respectively, which was in striking contrast compared to robust 17- fold increase observed upon treatment with rifampicin. Also in case of protein levels, while rifampicin and phenobarbital increased the CYP3A4 protein levels significantly (paired t-test,  $p < 0.05$ ), tamoxifen and 4OHT did not show significant change.

## DISCUSSION

We have previously shown that tamoxifen and 4OHT induce CYP3A4 in primary human hepatocytes and activate PXR in HuH7 cells (Desai *et al.* 2002). In this study we have further probed the role of PXR and other receptors that may modulate the expression of CYP3A4 or are known targets for tamoxifen and 4OHT. Firstly, we compared the extent of CYP3A4 induction in primary hepatocytes and PXR activation in HepG2 and LS174T cells by tamoxifen, 4OHT and NDMT. In general, there was a good agreement between the CYP3A4 mRNA induction in primary human hepatocytes and PXR activation in transfection assays; the order of magnitude of PXR activation i.e. 4OHT > tamoxifen > NDMT matched the order of CYP3A4 induction in human hepatocytes. The critical role of PXR in antiestrogen mediated CYP3A4 induction was further demonstrated by employing siRNA targeting PXR mRNA. Attenuation of PXR expression in primary human hepatocytes with siRNA caused a marked reduction in the overall extent of CYP3A4 induction by tamoxifen and 4OHT. For instance, with a 50% reduction in PXR mRNA levels in hepatocytes, we observed approximately 50 to 70% reduction in the magnitude of CYP3A4 induction by tamoxifen, 4OHT as well as rifampicin, a prototypical CYP3A4 inducer and PXR activator. Previously Pascussi *et al.* (2000) have shown that CYP3A4 inducibility is dependent on PXR expression as evident from decreased CYP3A4 inducibility upon cytokine mediated downregulation of PXR. Other studies assessed CYP3A4 inducibility in hepatocytes transfected with dominant negative PXR receptor (Kocarek *et al.* 2002) and in PXR null and transgenic mice (Xie *et al.* 2000). Here, we employed a novel approach that entailed attenuation of

PXR by siRNA to underscore the importance of PXR expression on CYP3A4 induction by tamoxifen and 4OHT in primary human hepatocytes.

We next assessed the potential role of other transcription factors in the CYP3A4 inductive effects of tamoxifen and 4OHT. Previous studies have suggested a role of GR $\alpha$  in CYP3A4 induction by xenobiotics. For instance, GR $\alpha$  agonist, dexamethasone, induces CYP3A4, an effect blocked by anti-glucocorticoid mifepristone (Ogg *et al.*, 1999; Matsunaga *et al.*, 2004). Rifampicin, a potent CYP3A4 inducer and PXR activator, is able to transactivate CYP3A4 promoter via GR $\alpha$  in the absence of PXR co-transfection (El-Sankary *et al.* 2000) while, co-transfection of GR $\alpha$  and PXR expression plasmids is needed for maximal CYP3A4 promoter activation (Gibson *et al.*, 2002). Furthermore, GR $\alpha$  may have a role in the expression of PXR (Pascussi *et al.* 2000, 2001). Interestingly, GR $\alpha$  ligand dexamethasone increased CYP3A1/23 levels in rat livers, but it did not do so in rat intestines (Hartley *et al.* 2004). While the role of GR $\alpha$  in CYP3A4 induction awaits further clarification, it appears that its contribution is tissue- and ligand-specific. For some ligands, presence of GR $\alpha$  is critical and their inductive effects are GR-dependent, whereas for some other ligands the inductive effects are GR-potentiated (Ogg *et al.*, 1999, Schuetz *et al.* 2000).

To evaluate the role of GR $\alpha$ , in this study we first assessed the expression of GR $\alpha$  and that of other nuclear receptors in primary human hepatocytes and HepG2 and LS174T cell lines. While the levels of several transcription factors, including PXR were lower in LS174T cells, the most striking difference was the absence of measurable GR $\alpha$  expression in LS174T cells at both mRNA and protein levels. This may account for the

observed lack of CYP3A4 induction by tamoxifen and 4OHT in LS174T cells. Further support for the role of GR $\alpha$  is derived from transient transfection assays where the effect of cotransfecting PXR and GR $\alpha$  on CYP3A4 promoter was compared with transfection of PXR or GR $\alpha$  alone. While tamoxifen and 4OHT did not activate CYP3A4 transcription when cells were transfected with GR $\alpha$  alone, co-transfection of GR $\alpha$  with PXR significantly potentiated the transcription of CYP3A4 promoter transcription by tamoxifen and 4OHT. Collectively, our findings suggest that GR $\alpha$  may be an important factor for maximal induction of CYP3A4 by tamoxifen and 4OHT. The plausible role of GR $\alpha$  in regulation of PXR expression in LS174T cells which could result in CYP3A4 induction was not explored in this study (Pascussi *et al.* 2000).

Next, we examined the role of ER $\alpha$  on the CYP3A4 promoter activation by tamoxifen and 4OHT. ER $\alpha$  regulates the expression of many genes in a complex manner resulting in either transcriptional activation or repression depending on the target gene. Given that tamoxifen and 4OHT are ER $\alpha$  ligands, a role of these receptors mediating complex downstream pathways resulting in transactivation of CYP3A4 gene can be envisioned especially since CYP3A4 promoter harbors at least partial ER response elements (Hashimoto *et al.* 1993). As in the case with GR $\alpha$ , upon tamoxifen and 4OHT treatment, ER $\alpha$  was not able to activate CYP3A4 promoter when transfected in absence of PXR. However, unlike GR $\alpha$ , ER $\alpha$  did not exhibit synergistic interaction with PXR at the CYP3A4 promoter in co-presence of PXR. In fact, when ER $\alpha$  was cotransfected alone with CYP3A4-XREM-luc reporter plasmid, it appeared to repress PXR mediated basal transcriptional activation of CYP3A4 promoter. The magnitude of the repression was considerably higher in the presence of physiologically relevant levels of estradiol. This

is in contrast to findings of Mnif *et al.* 2007 who report that estradiol is capable of activating PXR. However, in that study concentrations of estradiol were 10 to 100 fold higher than those employed in our study and the reporter employed did not harbor CYP3A4 promoter. We attributed the observed repressive effect of activated ER $\alpha$  to competition for commonly required cofactors in transcriptional machinery such as SRC1 and GRIP1. Indeed, coexpression of these transcription cofactors was able to restore the basal level of transcription even in repressive presence of activated ER $\alpha$ .

Similar examples of competitive repression have been reported earlier. Repression of ER $\alpha$  activity by CAR (Min *et al.* 2002) and thyroid receptor activity by ER $\alpha$  has also been attributed to squelching of p160 coactivators SRC1 and GRIP1 (also called SRC2) or other downstream coactivators (Lopez *et al.* 2005). Our observations with respect to PXR and ER $\alpha$  are analogous to these results. Furthermore, it is important to note that a direct involvement of ER $\alpha$  in ligand-dependent transcriptional modulation is possible as CYP3A4 promoter does harbor estrogen response element motifs (Hashimoto *et al.* 1993).

An interesting aspect of our findings is that tamoxifen and 4OHT may have the potential to induce CYP3A4 in a tissue-specific manner. An earlier study by Cotreau *et al.* (2002) suggested that tamoxifen administration may lead to induction of hepatic but not intestinal CYP3A in rats. Several other 'non classical inducers' include dexamethasone, anti-HIV agents amprenavir and nelfinavir, efavirenz and L-742694, which induce hepatic but not intestinal CYP3A (Hartley *et al.*, 2004, Mouly *et al.*, 2002, Huang *et al.*, 2001). The lack of CYP3A4 induction by tamoxifen/4OHT in LS174T cells may be partly



explained by the observed differences in the expression of nuclear receptors relative to hepatocytes. Since the presence of both hPXR and GR $\alpha$  is required for maximal induction of CYP3A4 by tamoxifen/4OHT, lower levels of hPXR and absence of GR $\alpha$  in LS174T cells may contribute to the lack of tamoxifen/4OHT mediated CYP3A4 induction in these cells. While caution is warranted in interpreting observations made employing a transformed cell line, the lack of tamoxifen/4OHT mediated CYP3A4 induction in LS174T cells may explain, at least partially, the lack of induction of intestinal CYP3A in rats. In human, colonic GR $\alpha$  is approximately 10-fold lower compared to those in liver (Pujols *et al.* 2002). A limitation of our study is that we did not explore the role of CAR and HNF4 $\alpha$  in CYP3A4 transactivation in response to tamoxifen/4-OHT. In a previous study Tirona *et al.* (2003) have suggested a critical role of HNF4 $\alpha$  in the PXR and CAR mediated transcriptional activation of CYP3A4 in Caco-2 cells. Furthermore, Tegude *et.al.* (2007) underscored the role of HNF4 $\alpha$  in directly regulating CYP3A4 in LS174T cells. Thus, it is likely that in addition to GR $\alpha$ , reduced expression of other transcription factors such as HNF4 $\alpha$  observed in LS174T cells, and/or interaction with CAR may also contribute to the apparent lack of CYP3A4 induction by tamoxifen/4-OHT in these cells.

In summary, our study shows that the induction of CYP3A4 by anti-estrogens tamoxifen and 4OHT primarily entails transactivation of CYP3A4 by PXR, which is further potentiated by GR $\alpha$ . It is noteworthy, that while prototypical CYP3A4 inducers rifampicin and phenobarbital induced CYP3A4 in the colon carcinoma cell line LS174T, tamoxifen and 4OHT did not do so. Thus, these agents may exhibit an atypical, tissue-dependent pattern of induction. Based on our study, the molecular bases for such

tissue-specific CYP3A4 upregulation may entail lower expression of PXR and GR $\alpha$  in LS174T cells compared to human hepatocytes. It is evident that CYP3A4 induction depends upon the overall stoichiometry of several key regulatory receptors, coactivators and corepressors in a given tissue which together govern the inductive effects of tamoxifen. Such findings have implications for induction of CYP3A4 and related genes by tamoxifen in non-hepatic tissues. For example, in breast carcinoma tissue, CYP3A4 induction may impact tamoxifen efficacy, whereas increased bioactivation in endometrial tissues may contribute to increased toxicity.

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## FOOTNOTES

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## FIGURE LEGENDS

Figure 1. Effect of PXR siRNA on PXR mRNA expression and CYP3A4 induction. (A) PXR mRNA expression in primary human hepatocytes transfected with PXR-specific pool of 4 siRNA duplexes (10 nM), pool of non-targeting siRNA duplexes (10 nM) and transfecting reagent alone (mock transfected) normalized to GAPDH mRNA expression. Data shown are mean  $\pm$  Std. Dev from three donors of human hepatocytes, ANOVA,  $p < 0.05$  (B) Extent of CYP3A4 induction after the primary human hepatocytes transfected with PXR-siRNA were treated for 72 hr with rifampicin (Rif, 10  $\mu$ M), tamoxifen (Tam, 5  $\mu$ M) and 4OHT (5  $\mu$ M) and expressed as percent of CYP3A4 induction achieved in mock transfected hepatocytes.

Figure 2. Activation of PXR mediated transcription at CYP3A4 promoter in HepG2 and LS174T cells. Cells were transiently transfected with the pSG5-PXR- $\Delta$ ATG expression plasmid, pGL3-XREM-CYP3A4-luciferase reporter plasmid and pCH110. Twenty four hours post transfection, cells were treated with phenobarbital, rifampicin (Rif), tamoxifen (Tam), 4OHT and NDMT for 24 hr. Fold increase in the luciferase reporter activity normalized to  $\beta$ -galactosidase activity following drug treatment is depicted. (n = 3 separate experiments, Mean  $\pm$  Std. Dev., \* Statistically significant increase ( $p < 0.05$ ) in normalized luciferase activity upon drug treatment as compared to respective untreated HepG2 or LS174T controls)

Figure 3. mRNA Expression of nuclear receptors. Reverse transcription followed by real time PCR was performed on RNA isolated from primary human hepatocytes (mean

from 3 separate donors), HepG2 and LS174T and MCF-7 cells. mRNA levels of various transcription factors, normalized to GAPDH expression are shown. (A) HNF4 $\alpha$  and PXR, (B) GR $\alpha$  and RXR $\alpha$ , and (C) ER $\alpha$ .

Figure 4. Expression of GR $\alpha$  protein. Western blot depicting immunoreactive protein levels of GR $\alpha$  protein expressed in primary human hepatocytes, HepG2 and LS174T cells. Cells lysates (20  $\mu$ g) were resolved employing SDS-PAGE and transferred to nitrocellulose membranes. The membranes were then blocked with 3% BSA in Tween (0.1%)-PBS (pH 7.4) and then treated with primary anti- GR $\alpha$  antibody followed by horseradish peroxidase-conjugated anti-rabbit secondary antibody. The protein bands were visualized using enhanced chemiluminescence detection.

Figure 5. Effect of GR $\alpha$  on transcription of CYP3A4 promoter. LS174T cells were transiently transfected with GR $\alpha$  expression plasmid and/or PXR expression plasmid, pGL3-XREM-CYP3A4-luc reporter plasmid and  $\beta$ -galactosidase expression plasmid. The transfected cells were treated for 24 hr with rifampicin (Rif, 10  $\mu$ M), tamoxifen (Tam, 5  $\mu$ M) and 4OHT (5  $\mu$ M) and luciferase activity of these cells was normalized to the  $\beta$ -galactosidase activity. Fold increase of normalized luciferase activity over untreated control cells is depicted. (n = 3 separate experiments, Mean  $\pm$  Std. Dev., paired t-test between PXR only and PXR+GR $\alpha$  expressed cells, \* p< 0.05).

Figure 6. Effect of ER $\alpha$  on repression of PXR mediated transcription of CYP3A4 promoter. LS174T cells were transiently cotransfected with pGL3-XREM-CYP3A4-luc reporter plasmid,  $\beta$ -galactosidase plasmid, PXR expression plasmid, with or without pCMV-ER $\alpha$  expression plasmid. The transfected cells were then treated with estradiol (1 nM) for 24 hr.  $\beta$ -galactosidase normalized luciferase activity in presence and absence of ER $\alpha$  /activated ER $\alpha$  were compared (n = 3 separate experiments, Mean  $\pm$  Std. Dev., paired t-test, \* p< 0.05)

Figure 7. Effect of coactivators on repression of PXR mediated transcription of CYP3A4 promoter by ER $\alpha$ . LS174T cells were transiently cotransfected with PXR expression plasmid, pGL3-XREM-CYP3A4-luc reporter plasmid and pCH110  $\beta$ -galactosidase plasmid. Additionally, in parallel, cells were also transfected with increasing concentrations of ER $\alpha$  expression plasmid (5 ng and 37.5 ng). From cells transfected with highest quantity of pCMV-ER $\alpha$  expression plasmid (37.5 ng), some were also transfected with increasing amounts of coactivators SRC1 (pSG5-FL-SRC1) and GRIP1 (pSG5-HA-GRIP1) (both 5 ng and 37.5 ng). Transfections were treated with 1 nM estradiol for 24 hr for ER $\alpha$  activation. Luciferase activity was normalized to  $\beta$ -galactosidase activity. (n = 3 separate experiments, Mean  $\pm$  Std. Dev. ANOVA, Tukey's multiple comparison test, \* p< 0.05).

**Table 1.** Fold increase in CYP3A activity, immunoreactive protein and mRNA levels compared to solvent treatment in primary human hepatocytes treated for 72 hours with rifampicin, phenobarbital, tamoxifen, 4OHT and NDMT. (Mean  $\pm$  Std.Dev., n = 3 or 4 donors, \* p < 0.05 )

	<i>Rifampicin<sup>b</sup></i>		<i>Tamoxifen</i>			<i>4OHT</i>			<i>NDMT</i>		
	<i>10 <math>\mu</math>M</i>	<i>2 mM</i>	<i>1 <math>\mu</math>M</i>	<i>5 <math>\mu</math>M</i>	<i>10 <math>\mu</math>M</i>	<i>1 <math>\mu</math>M</i>	<i>5 <math>\mu</math>M</i>	<i>10 <math>\mu</math>M</i>	<i>1 <math>\mu</math>M</i>	<i>5 <math>\mu</math>M</i>	<i>10 <math>\mu</math>M</i>
<b>CYP3A4</b>	10.54*	8.95*	1.30	2.12*	2.4*	2.05	7.51*	6.38*	0.8	0.65	0.45
<b>Activity<sup>a</sup></b>	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
	1.89	2.97	0.15	0.77	0.39	0.38	2.61	1.14	0.32	0.21	0.26
<b>CYP3A4</b>	9.43*	7.65*	1.51	4.03*	4.46*	1.60	5.79*	7.41*	0.70	0.88	0.91
<b>Protein</b>	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
<b>levels</b>	3.83	2.8	0.11	1.92	1.64	0.17	1.05	1.69	0.22	0.15	0.31
<b>CYP3A4</b>	13.46*	12.01*	1.5	3.3*	4.8*	3.3*	5.57*	9.03*	1.2	0.98	1.81
<b>mRNA</b>	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
<b>levels<sup>c</sup></b>	6.77	5.3	0.37	0.37	1.63	0.17	1.03	3.1	0.29	0.35	0.61

<sup>a</sup> measured as the rate of testosterone 6 $\beta$ -hydroxylation

<sup>b</sup> *prototypical inducers used as positive controls*  
<sup>c</sup> *measured by Northern blot analysis, normalized to 18s RNA*  
<sup>\*</sup> *significant difference at p < 0.05 by t-test*

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**Table 2.** Fold increase in CYP3A activity, immunoreactive protein and mRNA levels compared to solvent treatment in LS174T cells treated for 72 hours with rifampicin, phenobarbital, tamoxifen and 4OHT. (Mean  $\pm$  Std.Dev., n = 3, \* p < 0.05)

	<i>Rifampicin</i> <sup>b</sup>	<i>PB</i> <sup>b</sup>	<i>Tamoxifen</i>		<i>4OHT</i>			
	<b>10 <math>\mu</math>M</b>	<b>2 mM</b>	<b>1 <math>\mu</math>M</b>	<b>5 <math>\mu</math>M</b>	<b>10 <math>\mu</math>M</b>	<b>1 <math>\mu</math>M</b>	<b>5 <math>\mu</math>M</b>	<b>10 <math>\mu</math>M</b>
<b>CYP3A4</b>	1.97*	5.30*	1.35	1.05	1.10	1.49	1.01	1.01
<b>Activity<sup>a</sup></b>	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
	0.17	1.31	0.61	0.34	0.04	0.56	0.44	0.31
<b>CYP3A4</b>	2.53*	5.93*	0.40	1.20	1.30	0.47	1.34	1.27
<b>Protein</b>	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
<b>levels</b>	0.15	0.32	0.20	0.22	0.31	0.22	0.25	0.26
<b>CYP3A4</b>	1.89*	4.12*	0.79	0.78	0.65	0.61	0.65	0.83
<b>mRNA</b>	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$	$\pm$
<b>levels<sup>c</sup></b>	0.22	0.62	0.29	0.37	0.21	0.27	0.25	0.51



- <sup>a</sup> *measured as the rate of testosterone 6 $\beta$ -hydroxylation*
- <sup>b</sup> *prototypical inducers used as positive controls*
- <sup>c</sup> *measured by Northern blot analysis, normalized to 18s RNA*
- \* *significant difference at  $p < 0.05$  by t-test*

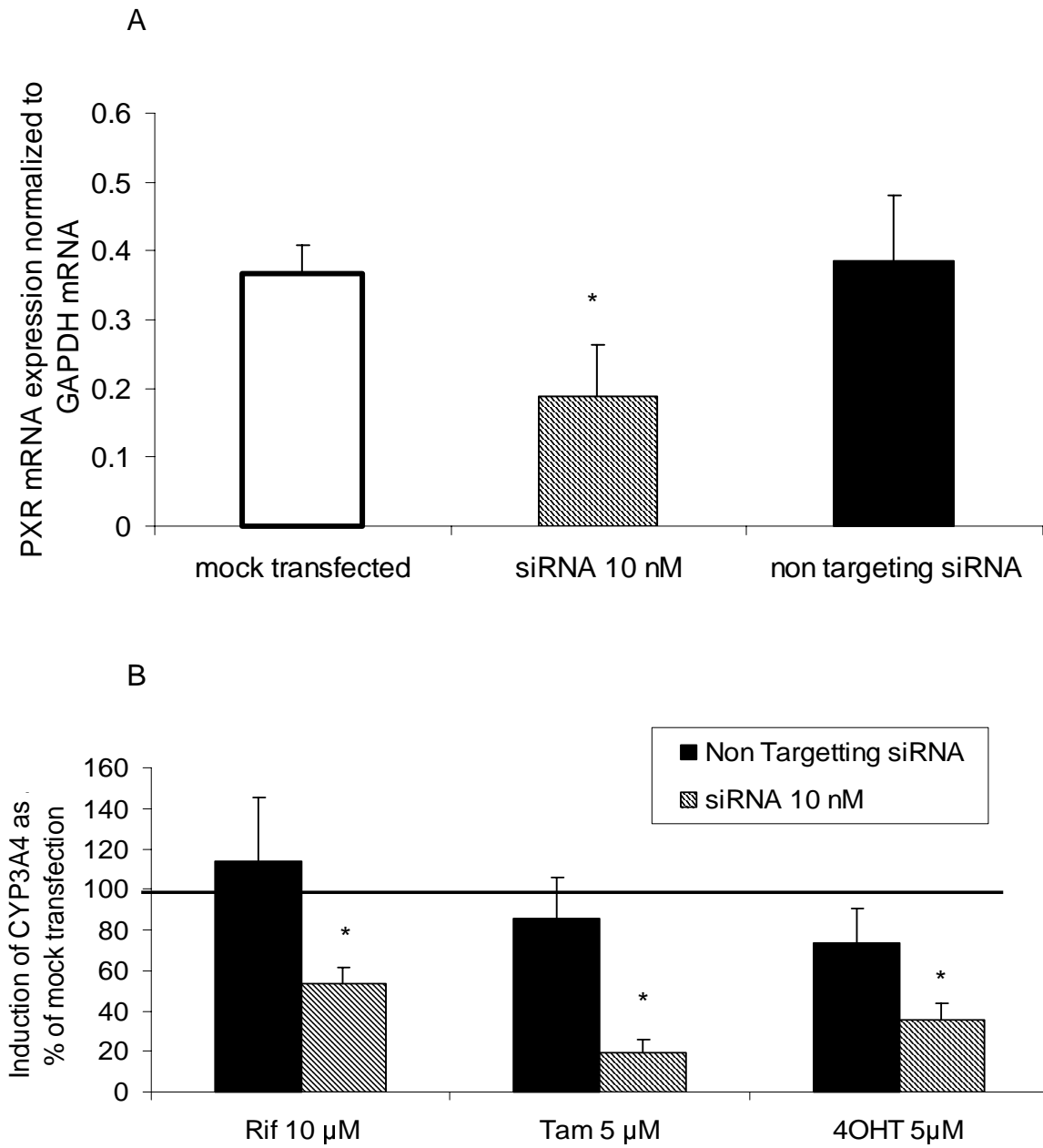


Figure 1

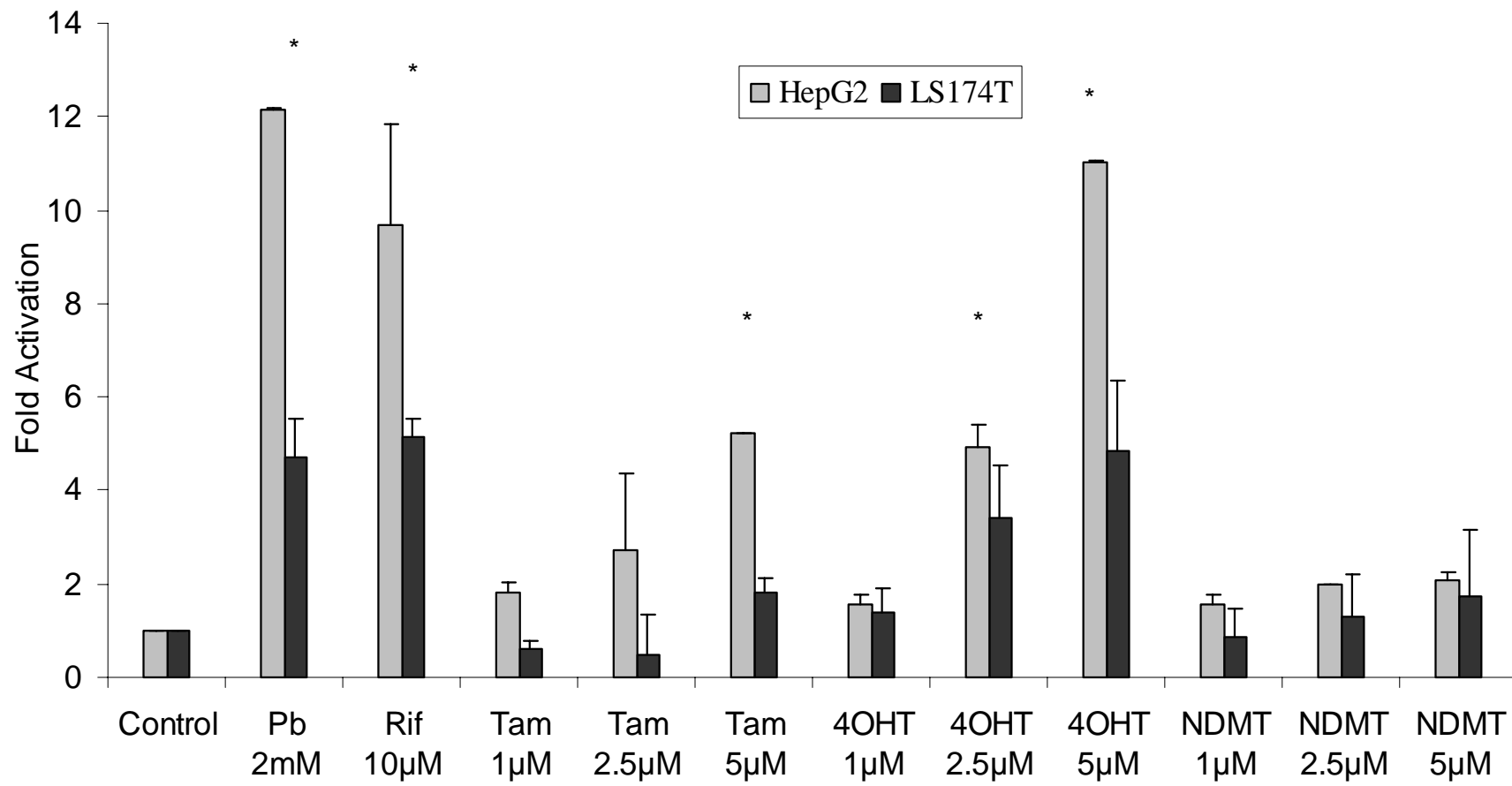


Figure 2

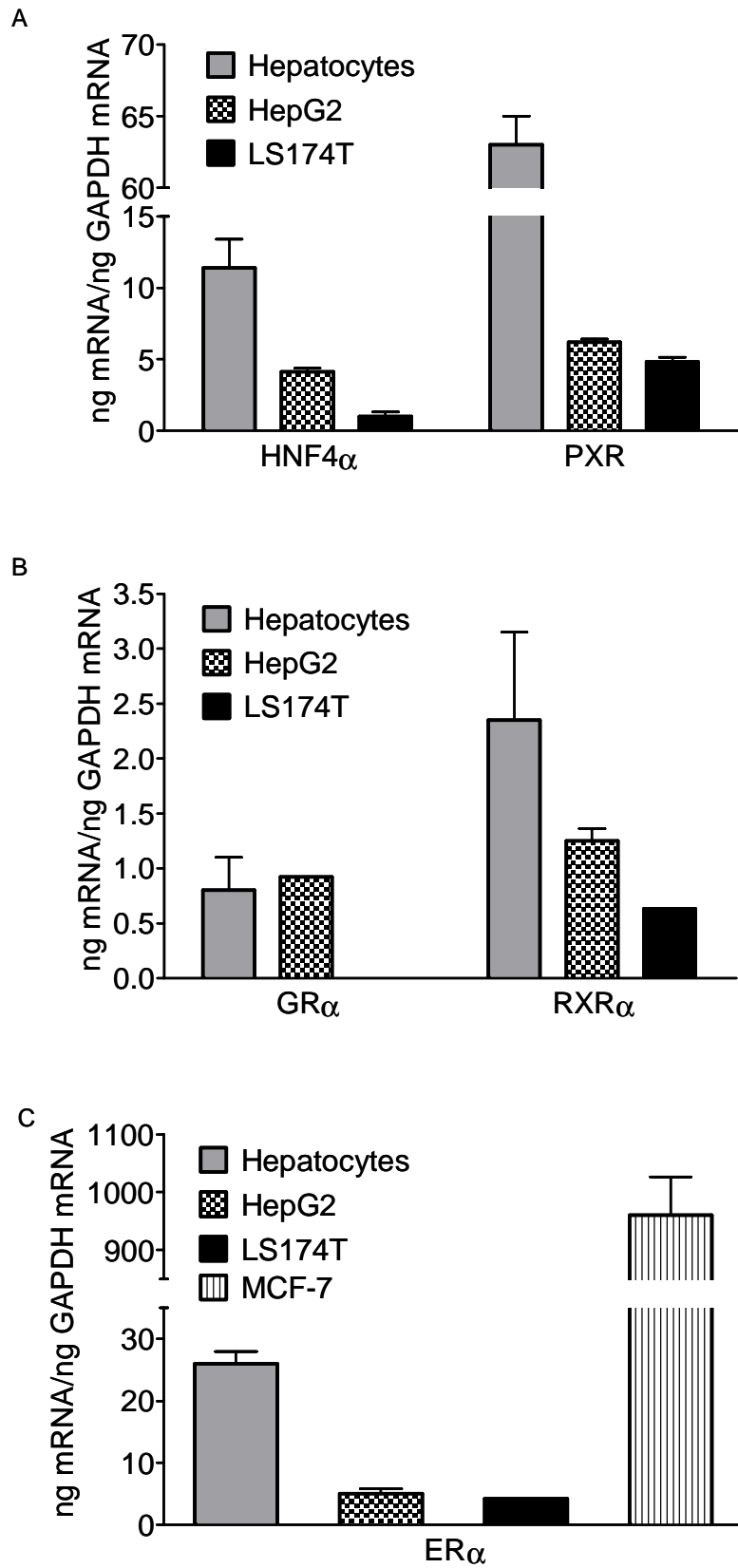


Figure 3



Figure 4

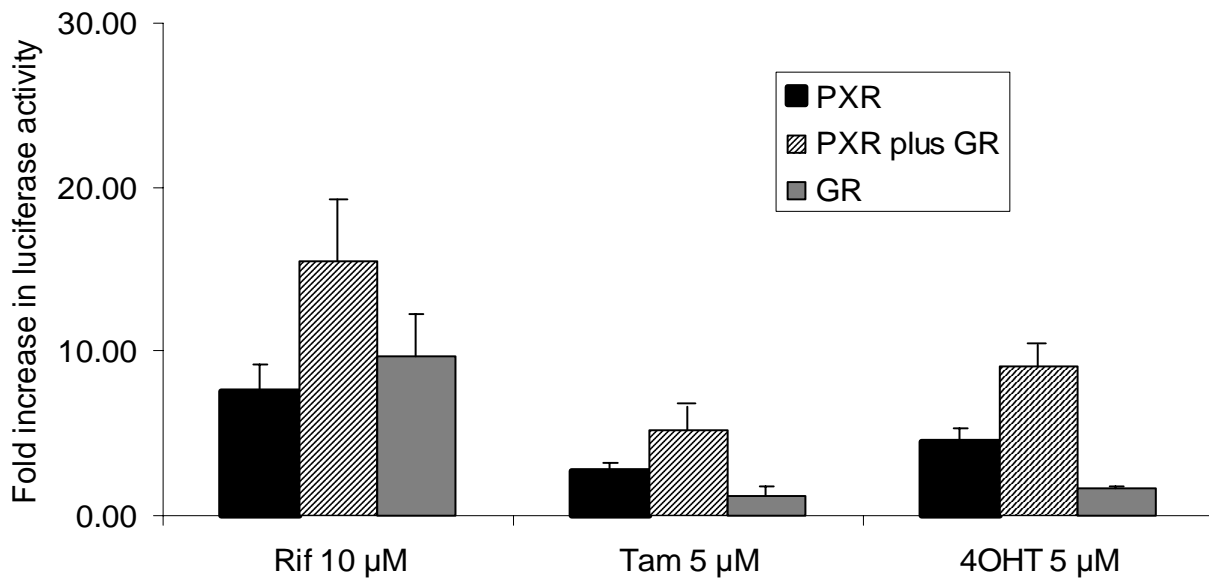


Figure 5

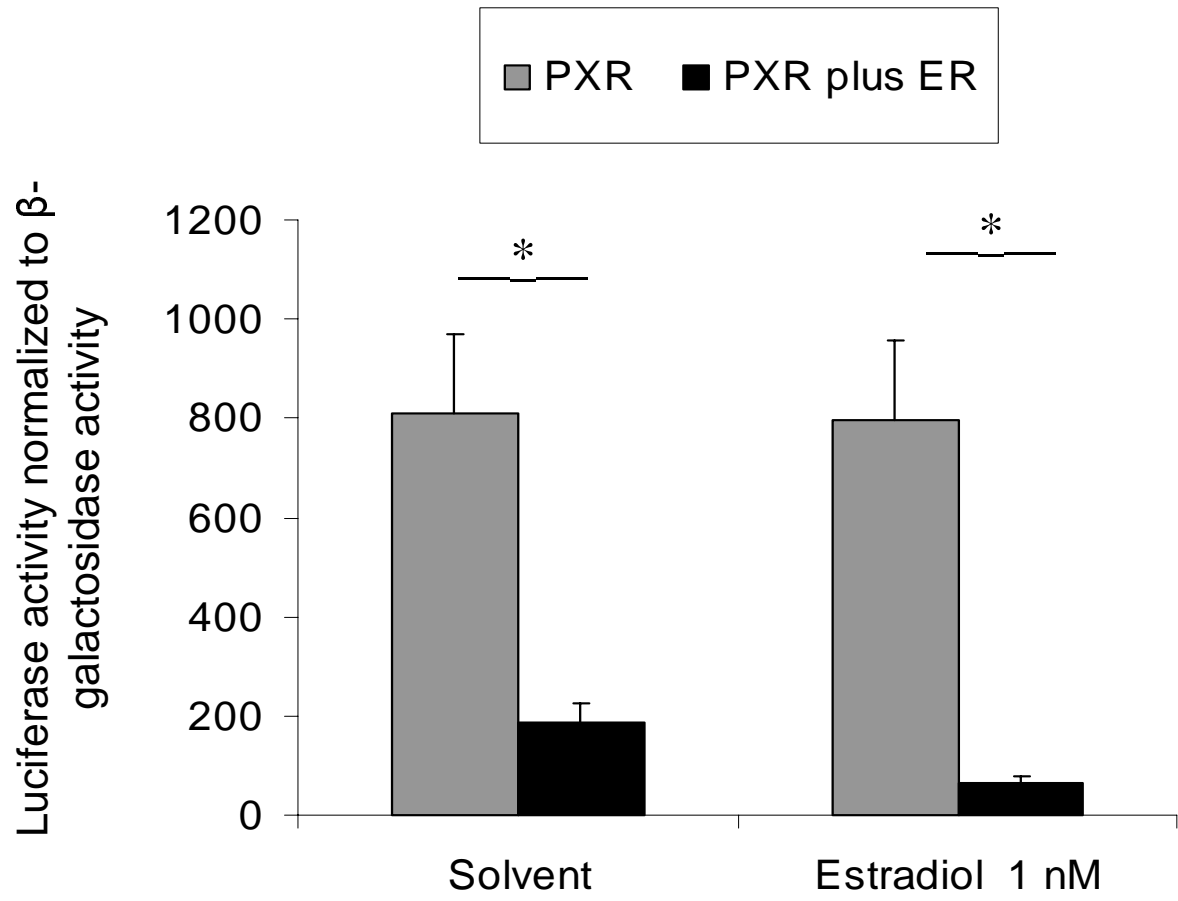


Figure 6

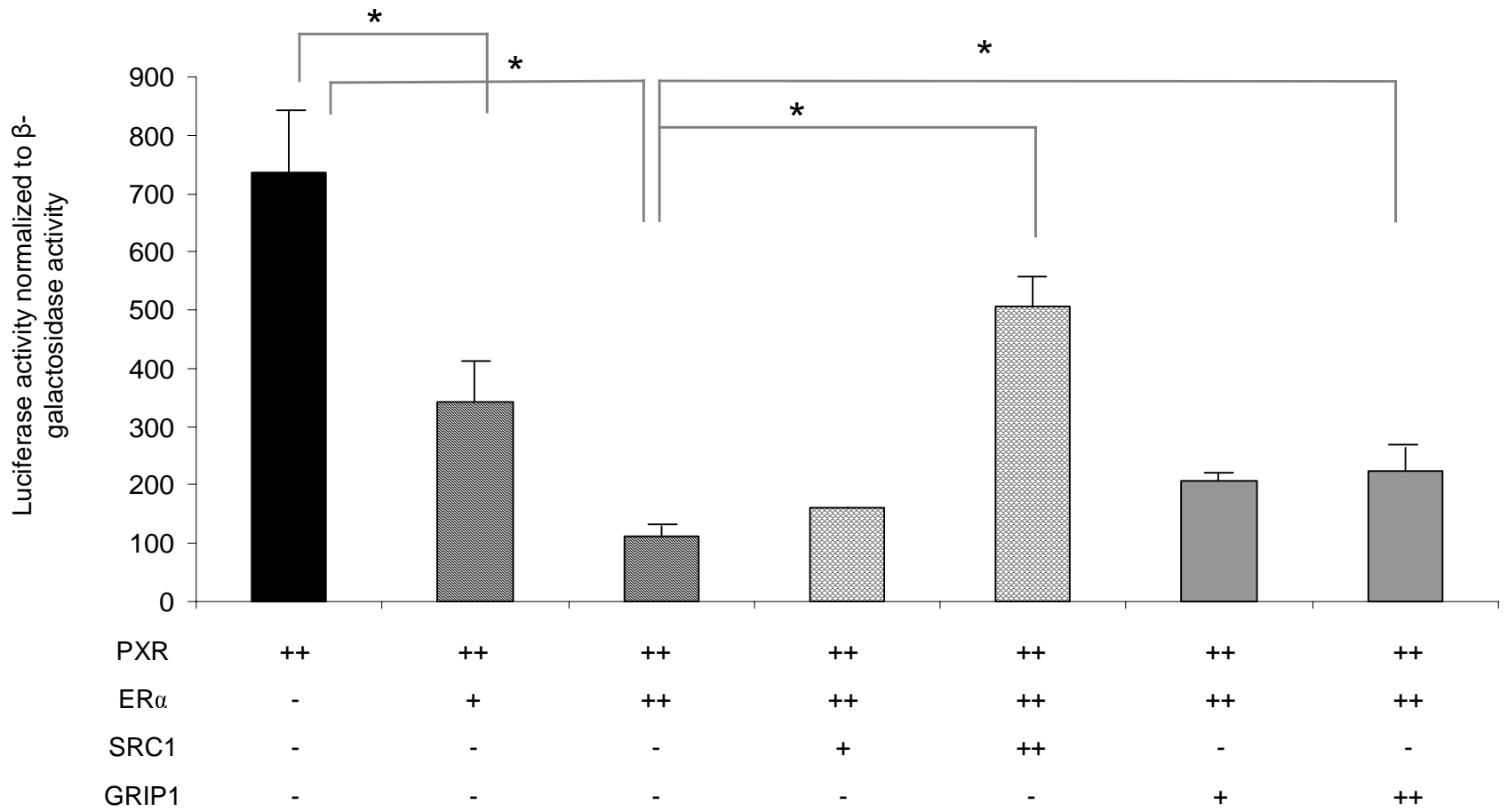


Figure 7