Supplemental data

Title: In vivo and in vitro characterization of a first-in-class novel azole analog that targets PXR activation

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Lenti-based shRNA knock-down systems. For PCR template sequences containing specific target sequences were obtained through the RNAi Codex website (codex.cshl.edu/scripts/newmain.pl), and the chosen sequences are as follows :PXR1.0 TGCTGTTGACAGTGAGCG**CGGCTACGCTGACAATCAGTTA**TAGTGAAGCCACAGATGTAT**AACTGATTGTCAGCGTAGCCT**TGCCTACTGCCTCGGA, (targets NM_4154….4175), scrambled: tgctgttgacagtgagcgcg**gtactgccgcaatgctata** atagtgaagccacagatgtat**tatagcattgcggcagtacc**ttgcctactgcctcgga, PXR2.0 TGCTGTTGACAGTGAGCG**CGGACCAGATCTCCCTGCTGAA**TAGTGAAGCCACAGATGTA**TTCAGCAGGGAGATCTGGTCCT**TGCCTACTGCCTCGGA, (targets NM_003889 (2650...2671) scrambled: Tgctgttgacagtgagcgcg**gcacaccggtcagcttact** atagtgaagccacagatgtat **agtaagctgaccggtgtgcc**ttgcctactgcctcgga. Bold letter sequences were derived from PXR target gene. PXR 1.0 and 2.0 were chosen after testing five from fifteen possible hairpin sequences. These sequences were cloned into Lenti: CMV–GFP plasmid individually or combined as previously published (Sun et al., 2006). The best results were obtained with PXR1.0/PXR2.0 combination cloned into a single lentivector (Supplemental Fig.4). The following primers were used to amplify and clone silent PXR mutation for knockdown-rescue assays: (forward) 5'- gac CAGATTTCCCTCTTGAAGgg-3' and (reverse) 5'- CCC TTC AAG AGG GAA ATC TGG TC -3'. These primer sequences were based on hiRNA target sequence (underlined) 5' – acCAGATCTCCCTGCTGAAGgg- 3', which corresponds to 6 amino acid residues – Q272, I273, S274, L275, L276, and K277. Based on the likelihood of best hairpin (iRNA) contact, the final predicted nucleotide change (marked in red) that would provide us with at least three silent amino acid mutations was predicted to be 5'- acCAGATTTCCCTCTTGAAGgg- 3' with the corresponding amino acids – I273I, L275L, and

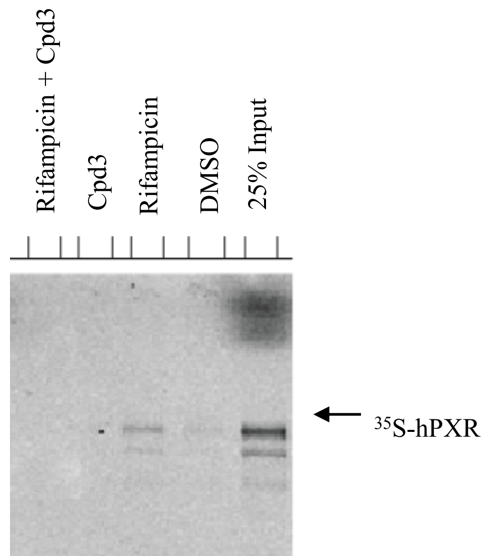
L276L being unchanged. The silent mutation was then made according to the manufacturer's instructions of the QuikChange II Site-directed Mutagenesis Kit (Cat# 200523, Stratagene).

Lenti virus assembly and transduction: 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Lentivirus were generated through co-transfection of 293T cells with lentiviral plasmids (shRNA against PXR/scrambled PXR) and packaging plasmids (pMDLg/pRRE, pRSV-REV, and pMD2-VSVG by standard calcium phosphate protocol (Sun et al., 2006). After 48 h of transfection, supernatant was collected and filtered (0.2 μm pore size). Virus titers were determined by infection of 293T cells with serial dilution of viral stock and the percentage of GFP positive cells were assessed by FACS. For transduction, LS174T cells were incubated with viral supernatants (shRNA PXR1/2 or scrambled shRNA) for 24 hours at 37°C in the presence of 8 μg/ml of polybrene (Sigma Aldrich). After 3 days, the percentage of GFP-positive cells was determined by fluorescence-activated cell sorting (FACS, BD Biosciences), as previously published (Supplemental Fig.4.) (Sun et al., 2006). GFP positive cells were assessed and sorted by fluorescence-activated cell sorting (FACS, BD Biosciences). GFP-positive cells were cultured and knockdown efficacy was assessed by real-time QPCR and immunoblot (supplemental Fig.4.). (LS174T cells in which PXR expression was knocked down using shRNA technology were validated to be PXR-specific data is presented in Wang *et al*., in review).

References

Sun D, Melegari M, Sridhar S, Rogler CE and Zhu L (2006) Multi-miRNA hairpin method that improves gene knockdown efficiency and provides linked multi-gene knockdown. *Biotechniques* **41**(1):59-63.

A



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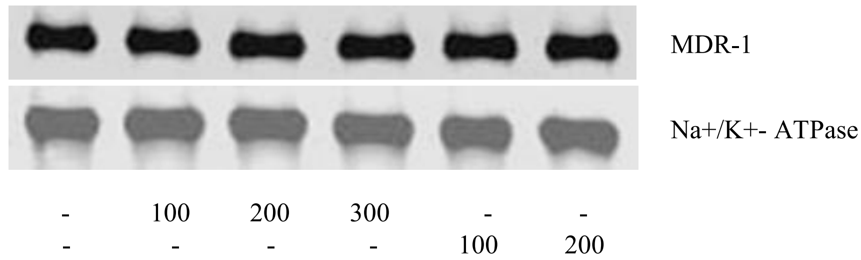
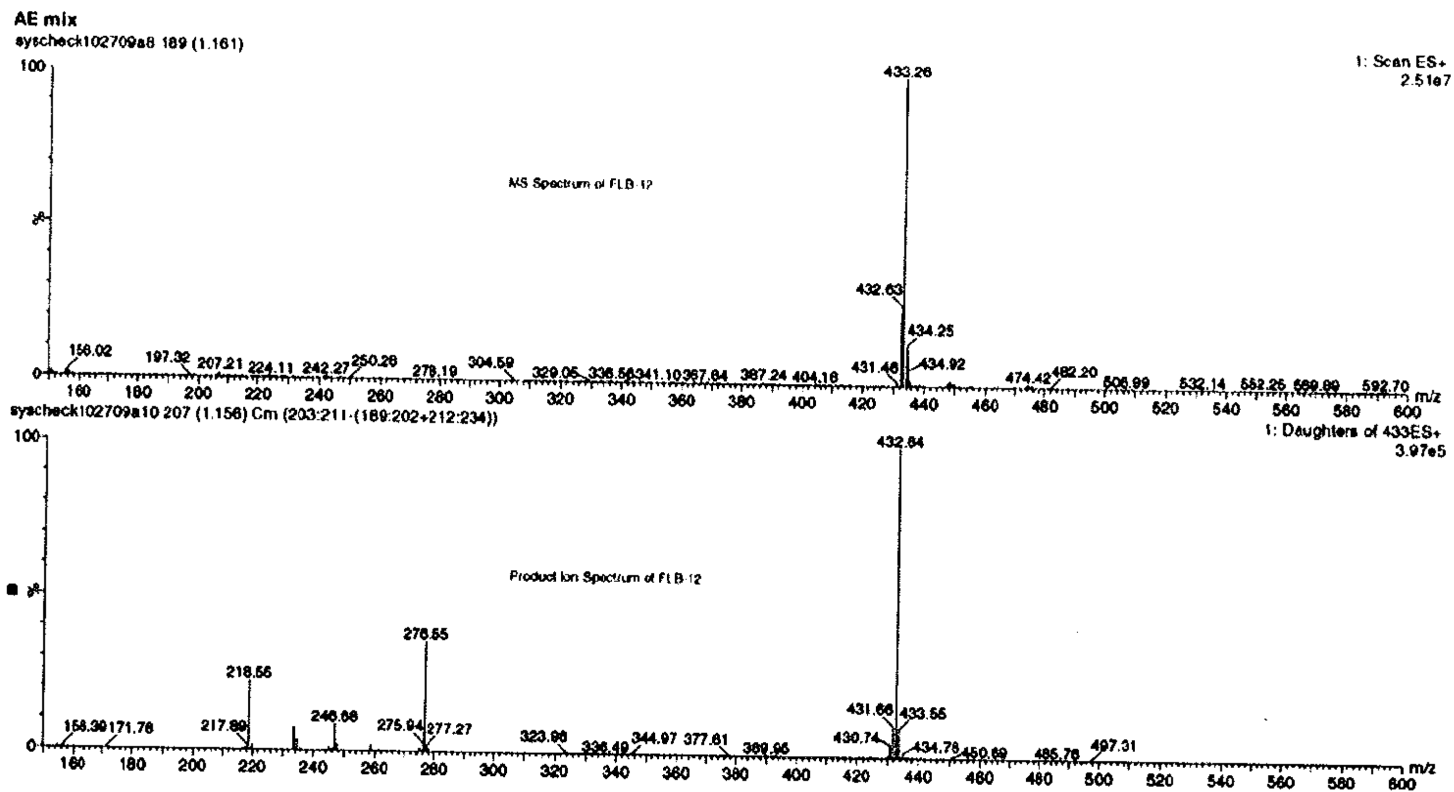
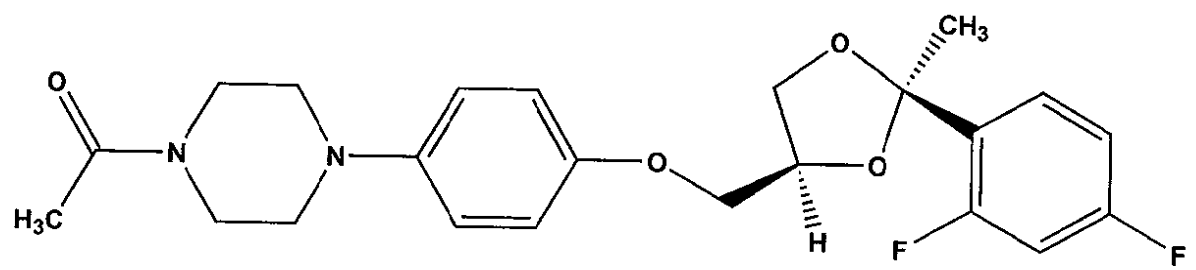


Cpd 3 (mg/kg)	-	100	200	300	-	-
KTZ (mg/kg)	-	-	-	-	100	200

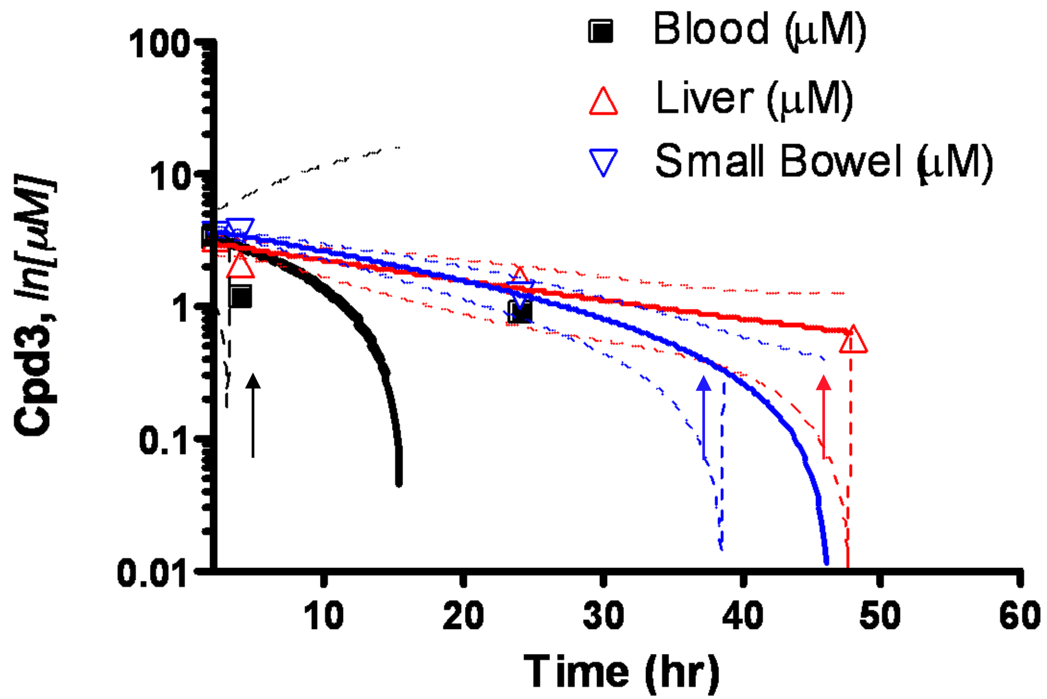
Supplemental Figure 1. hPXR LBD 35S methionine-labeled proteins pull down experiments and Immunoblot . (A) hPXR LBD 35S methionine-labeled proteins were prepared using an in vitro transcribed and translated protein kit (TNT, Promega) and co-incubated with GST-SRC-1 protein expressed in E.coli and purified using glutathione-sepharose. The mixture was then incubated with the drug(s) as shown and an aliquot volume subject to SDS-PAGE . (B) Immunoblot of MDR-1 expression in mouse livers treated with Cpd3 and KTZ, Na+/K+- ATPase was used a loading control.



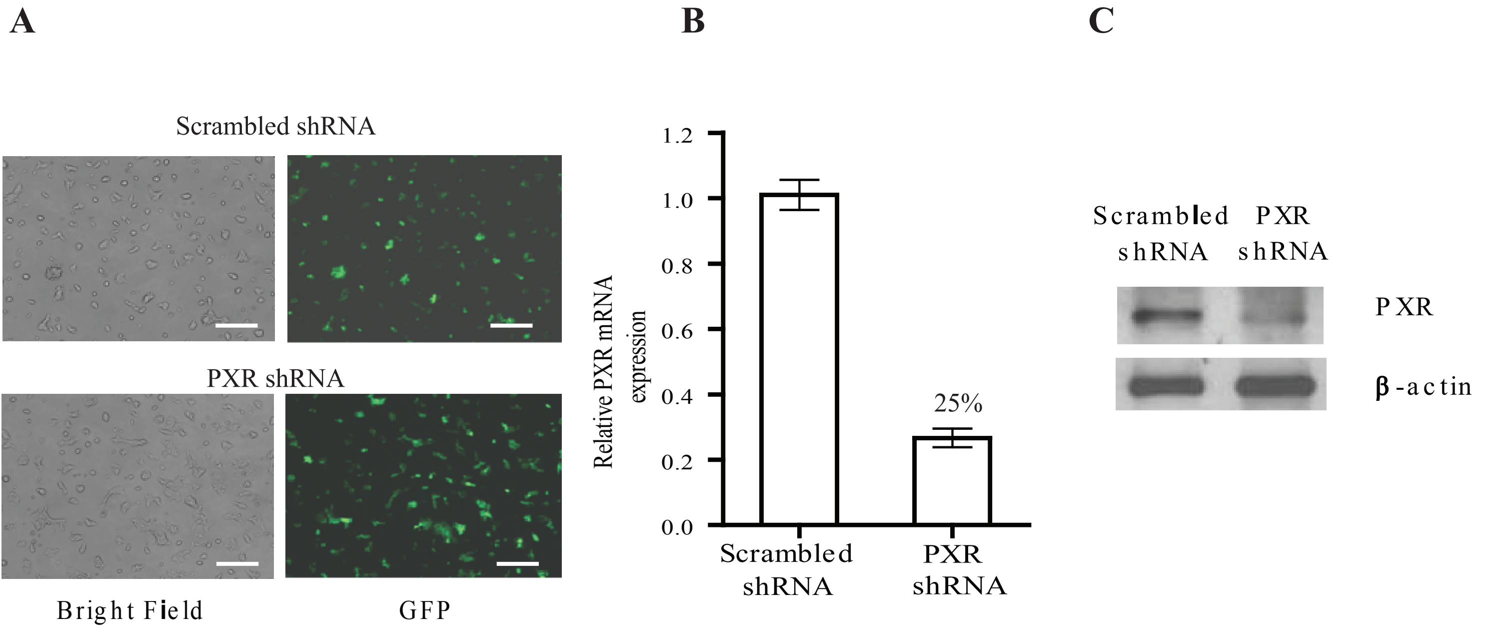


Cpd3- 1-(4-(4-(((2R,4S)-2-(2,4-difluorophenyl)-2-methyl-1,3-dioxolan-4-yl)methoxy)phenyl) piperazin-1-yl) ethane

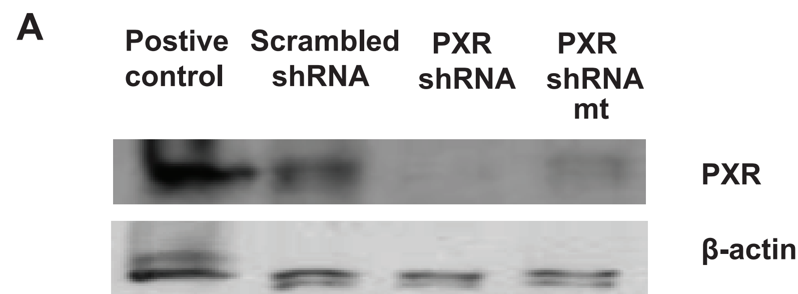
Supplemental Figure 2 MS and MS/MS spectra of Cpd 3 and Cpd3 structure

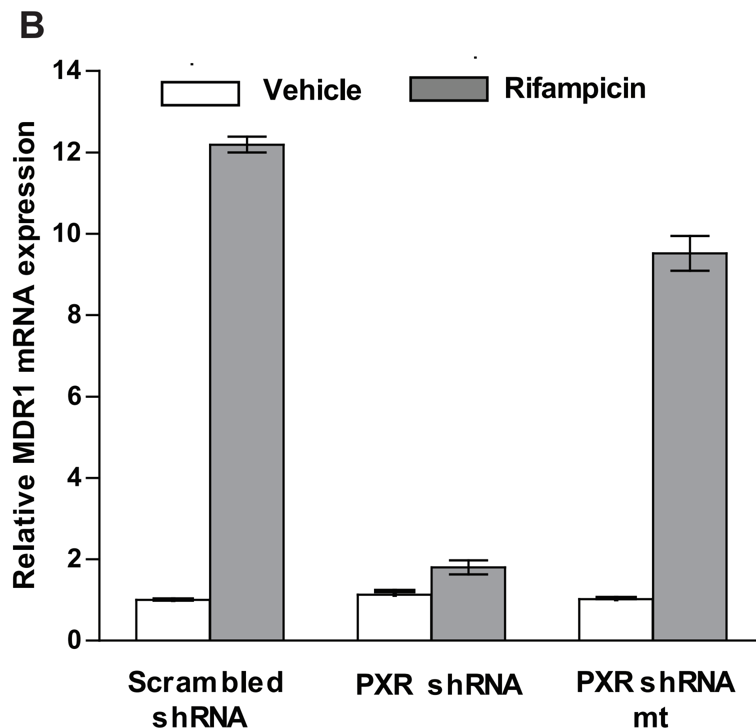


Supplemental Figure 3. Cpd 3 concentration levels in liver, small bowel and plasma extracts. At various time points Cpd 3 concentration levels in liver, small bowel and plasma extracts were determined by LC/MS/MS.

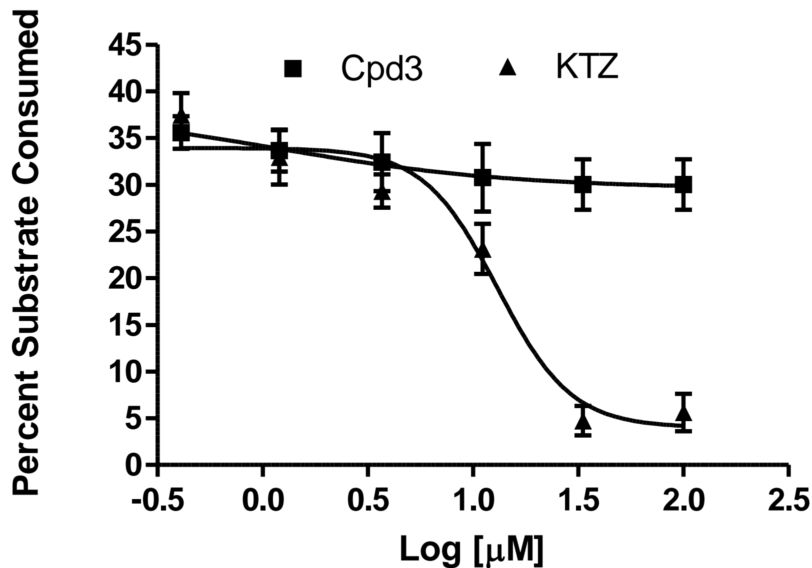


Supplemental Figure 4. PXR knockdown in LS174T cells. (A) LS174T cells transduced with scrambled and PXR shRNA with images captured under appropriate filters (GFP and phase contrast or Bright Field). Scale bar, 50 μm. (B) Real-time QPCR for PXR in cells expressing Scrambled shRNA or PXR shRNA. (C) Immunoblot of nuclear extract (100μg/lane) from cells transduced with scrambled shRNA and PXR shRNA. Data presented as mean ± SEM. Data in this panel are for review purpose only and will be published in a separate study by Wang et al.).

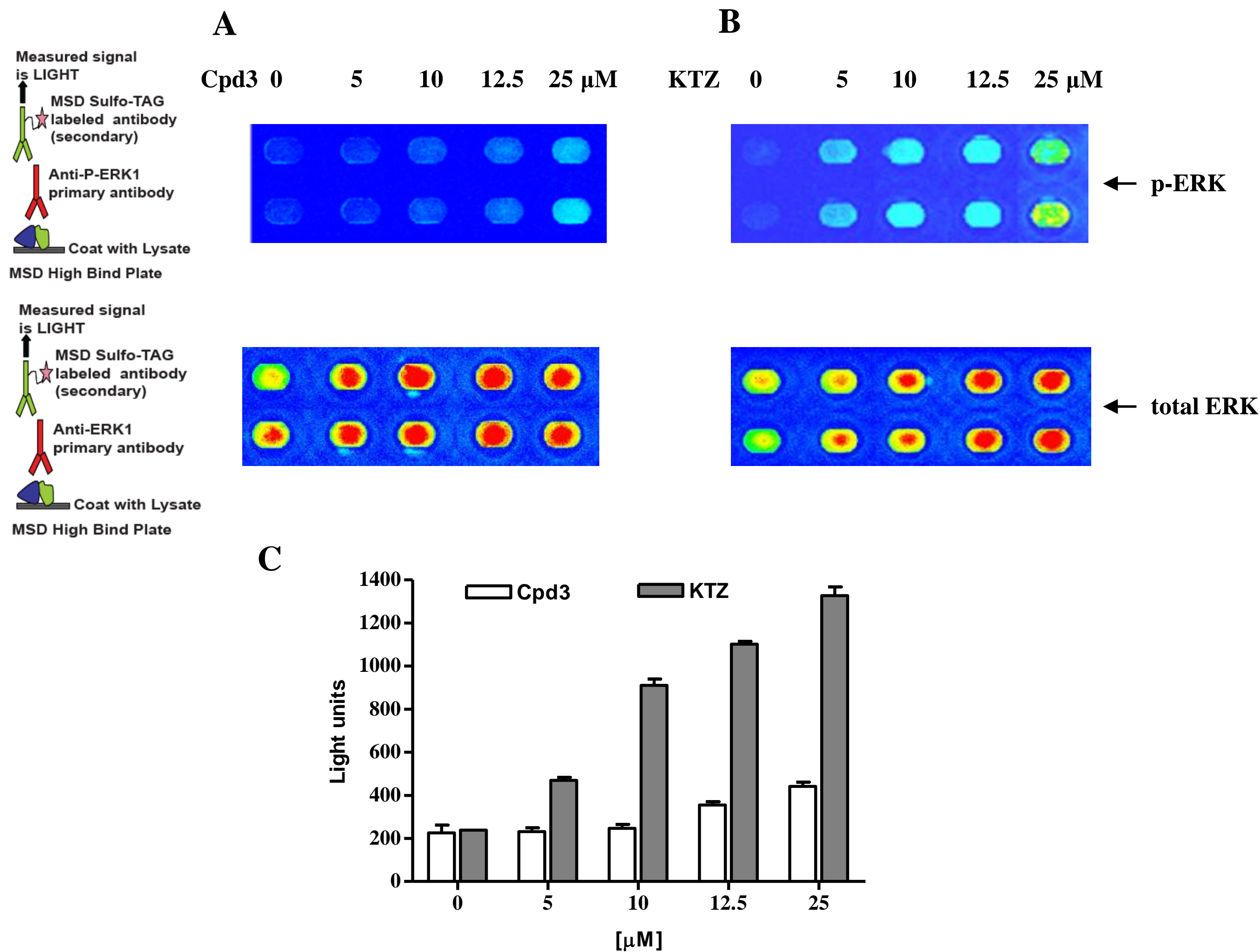




Supplemental Figure 5. Rescue of PXR phenotype in LS174T cells with PXR silent mutants. (A) Immunoblot of nuclear extract (100µg/lane) from cells transduced with Scrambled shRNA, PXR shRNA or PXR silent mutant (mt) expression plasmid. Positive control was nuclear extract (100µg/lane) from pSG5-PXR transfected 293T cells. β-actin served as loading control. (B) Real-time QPCR for MDR1 mRNA expression in cells expressing Scrambled shRNA, PXR shRNA, or PXR silent mutant (PXR shRNA mt). LS174T cells were exposed to vehicle control (0.2% DMSO) or rifampicin (10 µM) for 48h and thereafter, total RNA was isolated. Experiments were performed three separate times each in triplicate. Data presented as mean ± SD. Data in this panel are for review purpose only and will be published in a separate study by Wang et al.).



Supplemental Figure 6. Ketoconazole and Cpd 3 inhibitory activity of UGT1A1 Twenty microliter UGT-Glo reactions were performed using UGT multienzyme substrate and microsomes (0.2 mg/ml) with various concentrations of ketoconazole (KTZ) and Cpd3 (0-100 μM, respectively). RLU values were background subtracted and then converted to percent substrate consumed. All values are plotted using GraphPadTM prism. Data are expressed as the mean ± SD derived from three independent experiments



Supplemental Figure 7. Chemiluminescence method of detecting p-ERK and total ERK in LS174T cell lysate. Plates (96) wells were treated with a concentration gradient of LS174T cell lysate after the cells had been exposed with either KTZ or Cpd3 or 0.2% DMSO (vehicle) for 48 h. A and B show luminescence upon addition of secondary antibody (see cartoon of the assay). (C) Signals (light units) were plotted against the different concentration of compounds to phospho ERK expression. These assays were performed once in duplicate using total ERK antibody as loading controls.