Activated Pregnenolone X-Receptor Is a Target for Ketoconazole and Its Analogs

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Abstract Purpose: Variations in biotransformation and elimination of microtubule-binding drugs are a major cause of unpredictable side effects during cancer therapy. Because the orphan receptor, pregnenolone X-receptor (PXR), coordinately regulates the expression of paclitaxel metabolizing and transport enzymes, controlling this process could improve therapeutic outcome. Experimental Design: In vitro RNA-, protein-, and transcription-based assays in multiple cell lines derived from hepatocytes and PXR wild-type and null mouse studies were employed to show the effects of ketoconazole and its analogues on ligand-activated PXR-mediated gene transcription and translation. Results: The transcriptional activation of genes regulating biotransformation and transport by the liganded human nuclear xenobiotic receptor, PXR, was inhibited by the commonly used antifungal ketoconazole and related azole analogs. Mutations at the AF-2 surface of the human PXR ligand-binding domain indicate that ketoconazole may interact with specific residues outside the ligand-binding pocket. Furthermore, in contrast to that observed in PXR (+/+) mice, genetic loss of PXR results in increased (preserved) blood levels of paclitaxel. Conclusions: These studies show that some azole compounds repress the coordinated activation of genes involved in drug metabolism by blocking PXR activation. Because loss of PXR maintains blood levels of paclitaxel upon chronic dosing, ketoconazole analogues may also serve to preserve paclitaxel blood levels on chronic dosing of drugs. Our observations may facilitate new strategies to improve the clinical efficacy of drugs and to reduce therapeutic side effects.

The erratic toxicities of microtubule-binding anticancer drugs can be directly linked in part to the consequences of CYP450-directed drug biotransformation and transporter-dependent elimination (1–4). A recent randomized prospective clinical study has shown that CYP3A activity-guided docetaxel dosing results in more uniform drug pharmacokinetics and less toxicity than traditional body surface area–based dosing (5). For other drugs belonging to this class, inducing or inhibiting either CYP450 or multidrug resistance-1 (MDR1) function has resulted in altered drug metabolism, leading to distinct pharmacokinetic and pharmacodynamic sequelae (6–8).

Therefore, approaches to control or “unify” metabolism that include both biotransformation and transporter function could result in uniform pharmacokinetics and pharmacodynamics, as well as increased efficacy and predictability of drug treatment. We and others (9, 10) have previously shown that microtubule-binding drugs activate the pregnenolone X-receptor, PXR. The nuclear xenobiotic receptor PXR and, to a lesser extent, other adopted orphan nuclear receptors (e.g., constitutive androstane receptor, CAR) control genes encoding drug-metabolizing enzymes and transporters at the level of transcription (11, 12). In mice, paclitaxel activates PXR, induces expression of CYP3A4 and MDR1 gene products, and accelerates its own metabolism, resulting in lowering of blood levels with chronic dosing (13, 14). Human PXR activation mediated by rifampicin in cell culture induces CYP3A4 (responsible for 3-hydroxy-paclitaxel metabolite), CYP2C8 (responsible for 6-hydroxy-paclitaxel metabolite), MDR1, and MRP-2 (responsible for biliary excretion and intestinal cell efflux of paclitaxel and its metabolites); together, this results in altered paclitaxel pharmacokinetics through metabolic pathways (10, 15, 16). Biotransforming enzymes and transporters are regulated by PXR such that substrates and/or metabolites of CYP3A4 are coordinately exported in liver and bowel tissues by the MDR1 gene product, and overall metabolic homeostasis is achieved. Drug-drug interactions with regard to paclitaxel therapy can occur by the direct inhibition of the activities of CYP450 enzymes. Drug-drug interactions occur at the level of PXR, where xenobiotics can function as activating...
agonists (e.g., the hyperforin constituent of St. John’s wort) and induce the metabolism of paclitaxel, thereby significantly lowering its blood levels and therapeutic efficacy (17).

Consequently, we have shown that activated orphan nuclear receptors controlling drug metabolism may be inactivated by ketoconazole, a known inhibitor of CYP450 and UGT1A enzyme activity. We have shown that high doses of ketoconazole inhibit not only enzyme activity but also the transcription of genes regulated by the orphan receptors PXR, CAR, the liver X-receptor (LXR), and the farnesoid X-receptor (FXR), by limiting associations with the transcriptional coactivator steroid receptor coactivator-1 (SRC-1; ref. 18). We have proposed that at clinically achievable concentrations, less toxic analogues of ketoconazole can be employed to potently block nuclear receptor-driven transcription and, thus, can be used to modulate the coordinated activation of numerous genes involved in drug metabolism and efflux. Because the majority of anticancer drugs undergo metabolism by multiple enzymes and efflux transporters, transcriptional inhibition of nuclear receptor activity would be expected to generate relatively broad control of drug metabolism pathways, which could improve anticancer drug pharmacokinetics and pharmacodynamics.

In this study, we extend our findings of ketoconazole action on PXR-mediated gene transcription in multiple in vitro hepatocyte and colon cancer cell line models. Furthermore, we provide novel evidence that closely related analogues of ketoconazole, including enilconazole and fluconazole, provide a preliminary base to evaluate structure-activity relationships. We have also shown that human PXR (hPXR) mutants can be employed to potently block nuclear receptor-driven transcription and, thus, can be used to modulate the coordinated activation of numerous genes involved in drug metabolism and efflux. Consequently, we have shown that activated orphan nuclear receptors controlling drug metabolism may be inactivated by ketoconazole, a known inhibitor of CYP450 and UGT1A enzyme activity. We have shown that high doses of ketoconazole inhibit not only enzyme activity but also the transcription of genes regulated by the orphan receptors PXR, CAR, the liver X-receptor (LXR), and the farnesoid X-receptor (FXR), by limiting associations with the transcriptional coactivator steroid receptor coactivator-1 (SRC-1; ref. 18).

Finally, we show that inhibiting PXR-binding domain is critical for binding to ketoconazole, including enilconazole and fluconazole, suggesting that the azole compounds directly block coactivator function 2 (AF-2) region on the surface of the hPXR ligand-binding domain is critical for binding to ketoconazole, including enilconazole and fluconazole, suggesting that the azole compounds directly block coactivator binding to the AF-2 site. Finally, we show that inhibiting PXR-mediated metabolism of paclitaxel reduces variability of paclitaxel kinetics in mice. We conclude that specific inhibitors of PXR may prevent adverse drug effects and unanticipated paclitaxel kinetics in mice. We conclude that specific inhibitors of PXR may prevent adverse drug effects and unanticipated paclitaxel kinetics in mice.

**Experimental Procedures**

**Plasmids and reagents.** The CYP3A4 luciferase reporter plasmid (−10466 to +53) and PAR-2 in pCDNA3.1 was obtained from Dr. Jonas Uppenberg (Biovitrum, Stockholm, Sweden). Gal4DB–hPXR-LBD, pCMX–hPXR, and Tk-M H100x4-Luc were provided by Dr. Ronald Evans (Salk Institute, La Jolla, CA). Cremophor EL, ketoconazole, and rifampicin was obtained from Sigma Chemical Co. (St. Louis, MO). The Gal4 and VP16 plasmids were obtained from Dr. Ronald Evans and Dr. Bruce Blumberg (University of California–Irvine, Irvine, CA). Clinical-grade paclitaxel was obtained from The Albert Einstein College of Medicine Pharmacy, Bronx, NY. All drugs (except paclitaxel for in vivo use, which was formulated in Cremophor EL) were dissolved in 100% DMSO and stored at −20°C. The final concentration of DMSO was ≤0.2% in all experiments.

**Cell culture.** HepG2 cells [American Type Culture Collection (ATCC), Manassas, VA] were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, only passage 4 was used for transfection. LS174T (both from ATCC) were maintained in MEM supplemented with 10% fetal bovine serum. Where indicated, the media were supplemented with charcoal-adsorbed fetal bovine serum (Hyclone, Logan, UT). Cos-1 cells (African green monkey kidney cells; ATCC) were propagated in DMEM supplemented with 10% fetal bovine serum. Human hepatocyte(s) were purchased from In Vitro Technologies, Inc. (Baltimore, MD) and maintained in InVitroGrow HI medium. Fa2N-4 cells are SV40 large T antigen immortalized human hepatocytes and represent another in vitro model for assessment of hepatocyte function (XenoTech, Lenexa, KS). They were propagated as per manufacturer’s instructions.

**Cell survival [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.** Aliquots of 2 to 5 × 10⁴ cells were plated in 96-well plates in triplicate. Twelve hours later, the cells were treated with serial dilutions of each drug. Following incubation for 48 h, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done as previously described (9).

**Semiquantitative RT-PCR and real-time RT-PCR.** Total RNA was isolated using the Qiagen RNeasy Mini kit (Qiagen, Inc., Valencia, CA). To eliminate amplification of contaminating chromosomal DNA in the real-time quantification, the isolated total RNA was treated with RNase-free DNase (Promega, Madison, WI), following the supplier’s instructions. Reverse transcription was done using Superscript first strand synthesis system (Invitrogen, Carlsbad, CA). For semiquantitative PCR, one-tenth the reverse transcription (RT) reaction products were subjected to PCR amplification for 35 cycles in a multiplex format. Equal volumes of PCR product were loaded into each well of a 1% ethidium bromide-stained agarose gel as described previously (19). Real-time PCR for cDNA quantification was done using TaqMan universal PCR master mix and TaqMan probes, using VIC as the 5′ reporter fluorochrome and tetramethylrhodamine (TAMRA) as the 3′ quencher fluorochrome. Simultaneous quantification of the 18S RNA using a kit from ABI Systems (Foster City, CA) allowed for normalization between samples. A standard curve for CYP3A4 cDNA was constructed to ensure linearity in the concentration range studied. The CYP3A4 forward primer sequence was 5′-ctttcttcatcaactttgagat-3′, the CYP3A4 reverse primer sequence was 5′-ccgctgtgctttgtgattc-3′, and the CYP3A4 probe sequence was 5′-VIC-gagaggacaccttcctttacagac-TAMRA-3′, all spanning exon junctions, thus preventing amplification of genomic DNA. Amplification was detected and analyzed using the ABI Prism 7700 sequence detector with SDS 2.1 analysis software (Applied Biosystems, Foster City, CA). The relative fold increase in mRNA in samples compared with controls was calculated using the comparative C T method.

**Transient transcription and mammalian one-hybrid and two-hybrid assays.** Cells were split onto 24-well plates at densities of 2 to 8 × 10⁴ cells per well the day before transfection. Transfections were done using the LipofectAMINE reagent (Invitrogen) according to the manufacturer’s instructions and as previously described (9).

**Paclitaxel pharmacology studies in C57BL/6 mice.** Adult female wild-type PXR (+/+ ) and PXR-null (−/−) were maintained on standard laboratory chow and were allowed food and water ad libitum (23). To show the in vivo significance of PXR activation as it relates to paclitaxel metabolism, thirty-six 8-week-old C57BL/6 PXR (+/+ ) and PXR (−/−) female mice received...
daily morning i.v. injection with paclitaxel at a dose of 10 mg/kg for five sequential days (20–22). Clinical-grade paclitaxel was formulated as described previously (9, 14). At each time point from the start of injection of paclitaxel on days 1 and 5 (t = pretreatment, 0.25, 0.5, 2, 4, and 8 h), six mice per time point were sacrificed, and all organs, tissues, bones, and blood were isolated for further analysis of paclitaxel and its metabolites. Statistical analysis was done with Analyze-It software (Leeds LS27 7WZ, England) using the nonparametric Mann-Whitney U test.

**HPLC with tandem mass spectrometric (LC-MS-MS) method for measurement of paclitaxel and its two major metabolites (6α-hydroxy-paclitaxel and p-3-hydroxy-paclitaxel) concentrations in mouse plasma and tissue.** Total paclitaxel (bound and unbound) and its metabolites were extracted from 100 μL of mouse plasma (in this assay, the lower limit is ~50 μL) with 2 mL of a mixture of acetone/n-butyl chloride (1:4, v/v), with 20 nmol/L docetaxel used as the internal standard. Separation of paclitaxel, including the internal standard, was achieved on a Waters X-Terra C18 (50 × 2.1 mm i.d., 3.5 μmol/L) analytic column with a mobile phase consisting of acetone/n-butyl chloride (1:4, v/v), containing 0.1% formic acid (80%) and 2 mmol/L ammonium acetate containing 0.1% formic acid (20%) using isocratic flow at 0.2 mL/min and an overall run time of 5 min. The analytes of interest were monitored by tandem mass spectrometry with electrospray positive ionization at transitions 808 → 527.2, 870.4 → 286, 870.4 → 569, and 854.5 → 509.4 for paclitaxel, 6α-hydroxy-paclitaxel, p-3-hydroxy-paclitaxel, and docetaxel, respectively. The lower limit of quantitation of paclitaxel and its metabolites in mouse plasma was 10 ng/mL, corresponding to 0.0117 μmol/L (accuracy, 99.8%). For interassay precision, quality control samples containing 40, 200, and 400 ng/mL (corresponding to 0.05, 0.23, and 0.47 μmol/L) were used, resulting in coefficients of variation of 7.3%, 6.3%, and 2.2% and accuracies of 99.1%, 98.5%, and 100%, respectively. For measurement of paclitaxel and its metabolites in mouse fecs and bile, tissue concentrations were standardized by adding PBS to a final concentration of 200 mg/mL. Samples were homogenized on ice until a uniform homogenate was achieved. The tissue homogenate was diluted 1:9 in mouse plasma and 500 μL of the mixture was extracted with 5 mL of a mixture of acetone/n-butyl chloride (1:4, v/v). Paclitaxel and its metabolite concentrations in bile and fecs were determined from the calibration curve constructed in mouse plasma.

**Results**

**Donor- and cell type–independent inhibition of ligand-mediated activation of CYP3A4 and CYP2B6 gene transcription by ketoconazole.** We have previously shown that ketoconazole inhibits hPXR activation (18). To generalize this result, we determined the effect of ketoconazole on xenobiotic-mediated transcription of CYP450s in another liver-derived cell line, Fa2N-4 (Fig. 1A). Using real-time reverse transcription-PCR (RT-PCR), we found that rifampicin and paclitaxel, both hPXR agonists, significantly increased the expression of CYP3A4 mRNA when compared with DMSO-treated cells. Treatment of cells with ketoconazole resulted in only a slight increase in CYP3A4 mRNA expression relative to DMSO-treated cells. When ketoconazole was combined with either paclitaxel or rifampicin, however, the CYP3A4 expression was decreased by >90% as compared with cells treated only with paclitaxel or rifampicin, respectively.

To determine if variations in human hepatocyte donors could account for the changes induced by ketoconazole, we carried out quantitative RT-PCR to examine the effect of drugs on CYP450 levels. Human hepatocytes were isolated from three separate liver donors with no prior history of medication use or hepatitis (In Vitro Technologies). These hepatocytes were treated at the same time with either rifampicin, ketoconazole, or both in combination, and CYP3A4 and CYP2B6 mRNA expression were quantitated using RT-PCR. Ketoconazole inhibited rifampicin-mediated induction of CYP3A4 and CYP2B6 genes in a concentration-dependent manner (data not shown). Furthermore, the mean concentrations (IC50 ± SD) of ketoconazole resulting in 50% decrease in rifampicin-induced CYP3A4 and CYP2B6 expression were 13.4 ± 1.12 and 9.7 ± 4.18 μmol/L, respectively (Table 1). These results indicate that ketoconazole inhibited the ligand-mediated activation of CYP3A4 and CYP2B6 genes in primary hepatocytes in a donor-independent manner.

**Effect of ketoconazole analogues on the rifampicin-mediated activation of hPXR.** Ketoconazole belongs to the azole family of compounds, many of which have potent antifungal properties. The above findings indicated that whereas ketoconazole slightly activates the basal level transcriptional activity of hPXR in the absence of a ligand, the compound drastically inhibits hPXR transactivation in the presence of PXR agonists such as rifampicin. We used this property of ketoconazole to identify additional members of theazole family that exhibit a similar activity; such information could be used to elucidate structure-activity relationships. We did transient transcription assays in HepG2 cells using full-length hPXR and its cognate reporter in the presence or absence of various azoles to determine their effect on hPXR-mediated activation (Fig. 1B). As before, rifampicin activated hPXR 2.7-fold over control (DMSO)-treated cells. The antifungal drugs in the absence of rifampicin showed two distinct properties. Although fluconazole, enilconazole, and ketoconazole showed weak stimulatory activity (Fig. 1B and C), oxiconazole, miconazole nitrate, miconazole, and R063373 showed inhibitory effects on the basal activity of hPXR. Interestingly, only fluconazole, enilconazole, and ketoconazole inhibited the activation of hPXR to basal level in the presence of paclitaxel. The remaining drugs had profound inhibitory effects on rifampicin-activated hPXR to below the baseline. It is important to note that these drugs alone also had similar effects on the basal activity of hPXR. Collectively, these results indicated that the azole drugs tested, fluconazole and enilconazole simulated the action of ketoconazole on hPXR and can be termed “activating antagonists” because they exhibit weak agonist activities alone but clear antagonist actions in the presence of an established PXR agonist.

**Ketoconazole binds hPXR outside the ligand-binding pocket.** We have previously shown, using a scintillation-proximity assay that measures the binding of ligands to His-tagged hPXR-LBD, that the IC50 of the established PXR agonist rifampicin was 8.8 μmol/L. In contrast, the IC50 for ketoconazole was 74.4 μmol/L. Similarly, the Kd (estimated using the Cheng-Prusoff equation) for rifampicin and ketoconazole were 6.3 and 55.3 μmol/L, respectively (18). These data suggested that biologically active 25 μmol/L concentrations of ketoconazole were unlikely to compete with bona fide ligands for binding within.
the ligand-binding pocket. To firmly establish whether ketoconazole competed with rifampicin for binding to the ligand-binding pocket or bound to a distinct region on hPXR, we did transient transcription assays with full-length expression construct for hPXR and its cognate reporter. In these experiments, we used two concentrations of rifampicin (10 and 30 μmol/L) to determine the profile of inhibition by ketoconazole. The data showed a dose response for the effect of ketoconazole on the rifampicin-mediated activation of hPXR because the curves generated in the presence of the two agonist concentrations were essentially parallel (Fig. 2A). Note that we did test ketoconazole at concentrations of 100 μmol/L; however, the results were not reliable due to cytotoxicity. This behavior is compatible with noncompetitive inhibition of rifampicin.

![Fig. 1. Select ketoconazole analogues abrogate ligand-mediated activation of hPXR. A, Real-time RT-PCR analysis of mRNA isolated from immortalized Fa2N4 cells for CYP3A4 expression. The cells were treated with drugs for 48 h using the concentrations as indicated. B, Transient transcription assays in HepG2 cells to study the effect of ketoconazole analogues on xenobiotic-mediated activation and/or repression of nuclear receptors. HepG2 cells were cotransfected with pCMX-hPXR, CYP3A4 luciferase reporter plasmid (-10466 to +53), and pSV-β-galactosidase control vector for 24 h. Subsequently, the cells were treated with ketoconazole and its analogue(s) as indicated. Solid line (and ∗), the analogues that have similar potency as ketoconazole. C, These analogues are illustrated from top to bottom as fluconazole and enilconazole. Their structures are illustrated above; grey, the common chemical group (imidazole).](image-url)
activation by ketoconazole and, hence, is consistent with the idea that ketoconazole does not interact with the rifampicin-binding site. To corroborate these data, we did mammalian two-hybrid assays using both full-length and ligand-binding domain fusions of hPXR with the VP16 transactivation domain, as previously described. Both assays clearly show that ketoconazole inhibits the rifampicin-induced interaction between hPXR and SRC-1 (Fig. 2B and C). Importantly, the magnitude of the effect of ketoconazole on hPXR-SRC-1 interaction is diminished when the NH₂ terminus is truncated, which implies that the preservation of the NH₂ terminus of hPXR is important for ketoconazole action.

We hypothesized that ketoconazole impacts PXR transcriptional regulation by binding at the AF-2 surface rather than in the ligand-binding pocket. To test this hypothesis, several single and double mutations were generated in the AF-2 region of PXR (Fig. 3). Residues were replaced with corresponding amino acids from the ERα steroid receptor that is not antagonized by ketoconazole. In each case, the single mutants lead to a loss of receptor activity. The corresponding double mutations were generated in the AF-2 region of PXR for ketoconazole action.

### Table 1. Summary of the inhibitory effect of ketoconazole on rifampicin-mediated induction of CYP3A4 and CYP2B6 in human hepatocytes

<table>
<thead>
<tr>
<th>Hepatocyte donor</th>
<th>Mean IC₅₀ (CYP3A4 mRNA)</th>
<th>Mean IC₅₀ (CYP2B6 mRNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13.00</td>
<td>5.75</td>
</tr>
<tr>
<td>2</td>
<td>12.53</td>
<td>9.26</td>
</tr>
<tr>
<td>3</td>
<td>14.66</td>
<td>14.08</td>
</tr>
<tr>
<td>Mean</td>
<td>13.40</td>
<td>9.70</td>
</tr>
<tr>
<td>SD</td>
<td>1.12</td>
<td>4.18</td>
</tr>
</tbody>
</table>

NOTE: Three donor livers (as shown in Fig. 1B) were subjected to drug treatment(s) over 48 h and total RNA isolated and subjected to CYP3A4 and CYP2B6 real-time RT-PCR. This shows the mean inhibitory concentration of ketoconazole required to inhibit rifampicin-induced CYP3A4 and CYP2B6 mRNA expression by 50%.

PXR is an important determinant of paclitaxel blood levels in mice. We and others have previously shown that paclitaxel is a ligand that activates both mouse and human PXR (9, 18). In PXR (+/+) BALB/c mice, when paclitaxel is administered daily for 5 days i.v. at a dose of 20 mg/kg, the mean area under the curve (AUC) on day 5 compared with day 1 is (86.2 ± 5.5 μmol/L h versus 125.6 ± 3.8 μmol/L h), mean Cₘₐₓ (32.6 ± 6.8 μmol/L versus 79.2 ± 5.8 μmol/L), and mean t₁/₂ β (3.52 ± 0.46 h versus 3.13 ± 0.18 h; ref. 14). To determine if paclitaxel is inducing its own clearance through the activation of PXR and overexpressing CYP3A11 in mouse liver, we subjected C57BL/6 PXR (+/+ ) and PXR (−/−) mice to a 5-day course of paclitaxel injections i.v. at a dose of 10 mg/kg. The formulation and schedule of administration of paclitaxel were identical to the previously published report (9). In PXR (−/−) mice (n = 6 per time points sampled at 0.25, 0.5, 2, 4, and 8 h after paclitaxel injection), using noncompartmental analysis, the mean ± SD day 1 and day 5 paclitaxel AUC₀₋₅ were 2,121 ± 352 ng/mL h [coefficient of variation (CV), 16.6%] and 3,459 ± 297 ng/mL h (CV, 8.6%), respectively. Similarly, there was a 2.6-fold increase in paclitaxel Cᵣ₋₅ on day 5 versus day 1. In PXR (+/+ ) mice (n = 6 per time points sampled at 0.25, 0.5, 2, 4, and 8 h after paclitaxel injection), the mean ± SD day 1 and day 5 paclitaxel AUC₀₋₅ were 2,925 ± 924 (CV, 31.6%) and 1,288 ± 138 (CV, 10.7%), respectively (P < 0.05). Similarly, in contrast to PXR (−/−) mice, there was a 2.2-fold decrease in paclitaxel Cᵣ₋₁₅ on day 5 versus day 1 in PXR (+/+ ) mice (Table 2). These results suggest that PXR activation is responsible for reduced accumulation of paclitaxel in blood, which may serve as one indicator for drug resistance in mice.

### Discussion

We have previously shown that micromolar concentrations of ketoconazole antagonize PXR-dependent gene transcription (9, 14). In this study, we show that ketoconazole inhibits hPXR in several cell types, and that ketoconazole analogues are readily available to dissect structure-activity relationships to derive less toxic compounds that can inhibit PXR. At the molecular level, we reveal that ketoconazole’s effect is a direct consequence of binding to residues outside the ligand binding pocket of PXR and likely in the AF-2 region of the receptor. These are new findings and define a pharmacophore for the
interaction of small molecular inhibitors outside the ligand-binding pocket of the nuclear xenobiotic receptor PXR. Preliminary structure-activity relationship studies with ketoconazole analogues suggest that the azole ring may be central to the inhibitory activity of these compounds. Finally, we show that PXR is a key regulator of paclitaxel kinetics, and that loss of PXR activity may prevent the decrease in paclitaxel AUC observed with repetitive dosing.

The clinical implications of our findings are significant. First, this strategy of inhibiting orphan nuclear receptor activation may be used to circumvent adverse drug interactions mediated by xenobiotic activation of PXR. Many drugs in the human pharmacopeia activate hPXR, including some commonly used herbal remedies (24–29); thus, blocking the activation of PXR may prevent drug-drug interactions. Second, as we have shown, loss of PXR could be used to control drug metabolism to desired levels in an appropriate treatment setting. In addition, certain xenobiotics (e.g., paclitaxel) may activate PXR and induce its own clearance upon chronic dosing; however, proof of this phenomenon in humans is lacking (14). In such situations, CYP450 enzymatic inhibitors will only serve to enhance paclitaxel concentrations that further activate hPXR. It would be beneficial to inhibit hPXR activation, thereby preventing rising xenobiotic concentrations from activating hPXR in an autoregulatory loop. This strategy may yield more predictable steady-state levels of the drug upon chronic dosing. Although there are obvious benefits to inhibiting activated hPXR, in the context of long-term therapeutic, there are risks to this strategy that involve the effect of PXR on direct expression of many different detoxifying genes. This could result in unexpected effects of other toxins and carcinogens ingested, as well as unexpected hypersensitivities to xenobiotics. Therefore, inhibition of activated hPXR will likely have to be intermittently and cautiously approached. Third, there is growing literature on the consequences of hPXR activation as it relates to bone demineralization and the generation of osteomalacia (30, 31). Therefore, blocking unwanted PXR activation in this context, especially with other therapies like aromatase inhibitors that induce osteomalacia/osteoporosis, can be beneficial. Finally, although unproven, hPXR has been hypothesized to serve as a hormone sensitivity factor in breast cancer (32). Unwanted activation may lead to reduced effectiveness of aromatase inhibitors or estrogen receptor antagonists. In such situations, it may be beneficial to inhibit tumor PXR activation.

![Fig. 2.](image-url)
In summary, based on the data presented, we conclude that ketoconazole and selected analogues can antagonize activated forms of the nuclear xenobiotic receptor PXR. The consequence of loss of PXR activity on paclitaxel kinetics has been shown and suggests the clinical utility of PXR inhibitors. Because ketoconazole has pleiotropic effects on cellular targets and it has unpredictable kinetics (33–37), the optimal drug to inhibit PXR would depend on defining the pharmacophore on PXR, which could lead to the development of more specific drugs.

Finally, we show that the effect of ketoconazole on PXR-driven metabolism is likely due to direct binding to a region on hPXR that is distinct from the ligand-binding pocket. This observation serves as a novel paradigm for inhibitor discovery targeting nuclear receptors.

The implications of our findings, specifically in terms of the control of drug metabolism, are far reaching. In cancer medicine, targeted agents (e.g., epidermal growth factor receptor inhibitors) have surfaced as commonly used therapeutic options for cancer treatment.

### Table 2. Summary of paclitaxel concentrations in PXR (+/+ ) and PXR (−/− ) mouse plasma

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Day 1</th>
<th>Day 5</th>
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<tbody>
<tr>
<td></td>
<td>AUC0−τ</td>
<td>Cτ = 15</td>
</tr>
<tr>
<td>C57BL/6 PXR (+/+)</td>
<td>2,925 (924)</td>
<td>2,232 (629)</td>
</tr>
<tr>
<td>C57BL/6 PXR −/−</td>
<td>2,121 (352)</td>
<td>1,899 (427)</td>
</tr>
</tbody>
</table>

NOTE: Six mice per time point [predose, and 0.25 (Cτ = 15), 0.5, 2, 4, and 8 h] from the end of paclitaxel dosing (10 mg/kg) were sacrificed either on day 1 or day 5. The paclitaxel blood exposure (AUC0−τ) was calculated as described in Materials and Methods and Results. The values represent mean (SD) of plasma paclitaxel concentrations (ng/mL).
patients with a variety of malignancies. These small-molecule drugs are rapidly metabolized, and in fact, CYP3A4 is a major enzyme involved in their metabolism (e.g., Iressa, Tarceva, imatinib). Inducers of CYP3A4 (e.g., rifampicin) reduce blood exposure to the parent drug often to subtherapeutic levels. Because drug levels serve to identify both response [e.g., imatinib dose and tumor response type, chronic myelogenous leukemia (200 mg/day) versus gastrointestinal stromal tumors (400 mg/day)] and toxicity (e.g., imatinib steady-state levels and grade 3 edema), modifying drug levels from a single dose by controlling metabolism and unifying the pharmacokinetics-pharmacodynamics would be beneficial. The same principles may apply to epidermal growth factor receptor inhibitors with regard to response-toxicity relationships (e.g., rash-predicting antitumor response). Finally, for cytotoxic drugs, unifying metabolism may lead to drug exposures that normalize toward the population mean. Fewer outliers would translate to less unpredictability in terms of drug pharmacokinetics-pharmacodynamics and perhaps to better compliance and overall outcomes. For all these reasons, identifying small molecules that are themselves inert in terms of cellular toxicity but are potent in blocking PXR-driven drug metabolism would serve as a critical novel tool to apply to a wide variety of therapeutic settings.

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Ketoconazole, Nuclear Receptors, and Drug Metabolism

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