Activation of the Steroid and Xenobiotic Receptor (Human Pregnane X Receptor) by Nontaxane Microtubule-Stabilizing Agents

Sridhar Mani,[1,2,4] Haiyan Huang,[1] Sumathy Sundarababu,[2] Wenjing Liu,[2] Ganjam Kalpana,[1,4] Amos B. Smith,[5] and Susan B. Horwitz[1,3]

Abstract

Purpose: Because induction of drug efflux transporters is one of the major underlying mechanisms of drug resistance in cancer chemotherapy, and human pregnane X receptor (hPXR) is one of the principal "xenobiotic" receptors whose activation induces transporter and drug-metabolizing enzyme gene transcription, it would be ideal to develop chemotherapy drugs that do not activate hPXR. This report describes studies undertaken to explore the characteristics of hPXR stimulation and mechanisms of drug-receptor interactions *in vitro* with new anti-tubulin drugs.

Experimental Design: *In vitro* transient transcription, glutathione *S*-transferase pull-down assays, and mammalian one-hybrid and two-hybrid systems were used to explore drug-receptor interactions. Loss of righting reflex was used to assess effects of drugs on PXR activity *in vivo*.

Results: The current study showed that paclitaxel, discodermolide, and an analogue of epothilone B, BMS-247550, induced CYP3A4 protein expression in HepG2 hepatoma cells. Transient transcription assays of a luciferase reporter in the presence and absence of a GAL4-steroid and xenobiotic receptor (SXR) plasmid in HepG2 cells showed that these drugs activate hPXR. This was not true for the inactive analogue of paclitaxel, baccatin III, or for an analogue of epothilone A, analogue 5, none of which stabilizes microtubules. To determine the mechanisms by which paclitaxel, discodermolide, and BMS-247550 activate hPXR, a mammalian two-hybrid assay was done using VP16SRC-1 (coactivator) and GAL4-SXR. SRC-1 preferentially augmented the effects of these drugs on hPXR. Expression of SMRT (corepressor) but not NCoR suppressed the drug-induced activation of SXR by ~50%, indicating a selectivity in corepressor interaction with hPXR. These drugs resulted in shortened duration of loss of righting reflex *in vivo*, indicating drug-induced activation of PXR in mice.

Conclusion: These findings suggest that activation of hPXR with selective displacement of corepressors is an important mechanism by which microtubule-stabilizing drugs induce drug-metabolizing enzymes both *in vitro* and *in vivo*.

Microtubule-binding agents are a structurally diverse set of antineoplastic agents (1). Prototypical drugs in this class include paclitaxel (Taxol), docetaxel (Taxotere), and the *Vinca* alkaloids that are components of both palliative and curative treatment regimens in cancer (2). Although paclitaxel and docetaxel are metabolized by the *P*450 system, it is not clear how the more recently discovered nontaxane analogues, epothilones and discodermolide, are metabolized (e.g., CYP3A4; refs. 3–6).

Cytochrome *P*450 3A4 is expressed predominantly in the liver and intestines and is involved in the biotransformation of a variety of structurally diverse xenobiotics (7). Additionally, CYP3A family members are transcriptionally regulated by structurally diverse xenobiotics that in turn affect xenobiotic drug metabolism or other *P*450-dependent drug-drug interactions (8–11). Several recent studies have shown that the orphan nuclear receptor, human pregnane X receptor [hPXR; also known as steroid and xenobiotic receptor (SXR), PAR, NR1I2, or PRR], can regulate the transcription of CYP3A4 as well as the transcription of other phase I and II enzymes involved in xenobiotic metabolism (12–14). This nuclear receptor is activated by xenobiotics that include rifampicin, hyperforin, and SR12813 (15–17); however, these ligands exhibit species-specific interaction with PXR. For example, whereas the rodent PXR is activated by pregnenolone 16 α-carbonitrile (PCN) and not by rifampicin, the human PXR is activated by rifampicin but not by PCN (8). It has been reported that paclitaxel activates hPXR and enhances P-glycoprotein–mediated drug clearance; however, docetaxel

Authors' Affiliations: [1]Albert Einstein Cancer Center and Departments of [2]Medicine, [3]Molecular Pharmacology, and [4]Molecular Genetics, Albert Einstein College of Medicine, Bronx, New York and [5]University of Pennsylvania, Philadelphia, Pennsylvania

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Requests for reprints: Sridhar Mani, Albert Einstein Cancer Center, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Chanin 302D-1, Bronx, NY 10461. Phone: 718-430-2871; Fax: 718-904-2892; E-mail: smani@montefiore.org.

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barely activates hPXR and displays a longer plasma and intracellular half-life when compared with paclitaxel (8). One mechanism by which paclitaxel stimulates hPXR transcriptional activity seems to be by altering its interaction with coactivators and corepressors. Although paclitaxel disrupts the interaction of hPXR with corepressors, it augments the interaction with coactivators, thus leading to a net stimulation of hPXR transcriptional activity. These mechanisms may also be relevant to the regulation of hPXR by other xenobiotics and drugs (8, 18).

Paclitaxel and docetaxel are effective in the treatment of breast cancer and other malignancies; however, a major limiting factor is the development of multidrug resistance in part through activation of P-glycoprotein by hPXR (8). The success of paclitaxel in the clinic has stimulated a search for new natural products that could stabilize microtubules yet not be a substrate for, or an inducer of, P-glycoprotein, an ATP-dependent drug efflux pump that results in drug resistance. Some interesting new agents include the epothilones and discodermolide (1). These agents are poor substrates for P-glycoprotein; therefore, it is hypothesized that they depend more on CYP450-mediated enzymatic action for drug clearance. Epothilones have already shown antitumor efficacy in breast and other malignancies and have entered phase III clinical trials.

Because induction of drug transporters efflux is one of the major underlying mechanisms of drug resistance during cancer chemotherapy, and hPXR is one of the principal "xenobiotic" receptors whose activation induces the production of such transporters and drug-metabolizing enzymes, it would be ideal to develop chemotherapy drugs that do not activate hPXR. This report describes studies undertaken to explore the characteristics of hPXR stimulation and mechanisms of drug-receptor interactions with these new agents. This information may be used to guide the future development of therapeutically active but hPXR-neutral microtubule-stabilizing agents.

Materials and Methods

Plasmids and reagents. The reporter plasmids Tk-(3A4)3-Luc and Tk-MH100x4-Luc as well as plasmids for hPXR and Gal4SXR-ligand-binding domain (LBD) were kindly provided by Dr. Ronald M. Evans (Salk Institute, La Jolla, CA). The full-length hPXR (hPAR-2 or hPAR) plasmid in pcDNA3 and the CYP3A4 luciferase reporter plasmid (−10,466 to +53) were provided by Dr. Jonas Uppenberg (Stockholm, Sweden). pGEX-cSMRT was provided by Dr. J. Don Chen (Piscataway, NJ). Additional plasmids, GAL4-SXR-LBD, VP16SXR-LBD, VP16SRC-1-receptor-interacting domain (RID), VP16NCoR-RID, VP16SMRT-RID, pCEP4-NCoR, and pCMX-SMRT were provided by Dr. Akira Takeshita (Toranomon Hospital, Tokyo, Japan). Docetaxel was a gift from Aventis (Strasbourg, France); Cremaphor-EL (Sigma Chemical Co., St. Louis, MO) and clinical-grade paclitaxel (Albert Einstein Pharmacy, Bronx, NY) were purchased. Baccatin III was obtained from the Drug Development Branch of the National Cancer Institute (Bethesda, MD). The epothilone A, epothilone B, BMS-247550 (Bristol-Myers Squibb, Princeton, NJ), rifampicin (Sigma Chemical), PCN, clinical-grade cisplatin (Paraplatin), eleutherobin, discodermolide, and SKBIII have been described previously (19). All drugs were dissolved in 100% DMSO and stored at −20°C. The final concentration of DMSO was ≤0.2% in all experiments.

Cell culture and cell survival (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. HepG2 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Where indicated, the medium was changed to RPMI 1640 with 10% charcoal-adsorbed fetal bovine serum and 1% penicillin/streptomycin. Aliquots of 5×10^3 HepG2 cells (passage 4) were plated in 96-well microdilution plates in triplicate. Twelve hours later, the cells were treated with serial dilutions of each drug. Following incubation for 48 hours, 20 μL 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt and 100 μL phenazine methosulfate (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit, Promega, Madison, WI) were added to each well. Four hours later, an ELISA reader at 490 nm analyzed the plates. Absorbance at this wavelength correlates with viability ($r = 0.997$). The absorbance values from the control wells on each plate were compared with ensure equal numbers of cells from plate to plate in a given experiment and from experiment to experiment. Survival curves were generated based on ratios of absorbance values (adjusted for background) of drug-treated cells to control cells.

Northern blot analysis. Total RNA was isolated from cultured HepG2 cells using TRIzol reagent (Invitrogen, Palo Alto, CA), washed with 75% ethanol, and quantitated by absorbance at 260 nm. RNA purity was assessed from the 260/280 nm absorbance ratio and by integrity of the 28S and 18S bands on 1.0% agarose gels. Total RNA (20 μg) was subjected to electrophoresis on 1% agarose-2.2 mol/L formaldehyde gels followed by transfer to a membrane using the rapid downward transfer system (Nytran; Schleicher & Schuell, Keene, NH). RNA was bound to the membranes by baking at 80°C for 2 hours. The cDNA probes for human CYP3A4 or β-actin were generated by reverse transcription-PCR using the following primers: CYP3A4 forward 5′-GCTCCTCTATCTATATGGAAC-3′ and reverse 5′-CACTGGACCAAAAG-GCCTCC-3′ and β-actin forward 5′-CAAGAGATGGCCACGGCTGCT-3′ and reverse 5′-TCCTTCTGCATCCTGTCGGCA-3′.

The cDNA probe for human CYP3A4 spanned a 263-bp region between 110 and 383 bp, and that for β-actin, which was used to normalize for RNA quantity, was 274 bp long. The membranes were first prehybridized in ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion, Inc., Austin, TX) for 30 minutes, after which the membranes were hybridized with random-primed ^{32}P-labeled cDNA probes encoding human CYP3A4 or β-actin RNA (Strip-EZ DNA kit, Ambion) for 18 hours at 42°C. Autoradiography on Kodak (Rochester, NY) BioMax MR film was done at −80°C for 12 to 48 hours.

Immunoblotting. The relative abundance of each specific protein in 25 to 50 μg whole cell lysate or nuclear extract (NucBuster Protein Extraction kit, Novagen, San Diego, CA) was determined by Western blot analysis as described previously (20). Briefly, cells were washed thrice with PBS, lysed with boiling buffer containing 1% SDS, 10 mmol/L Tris-HCl (pH 7.4), boiled for an additional 5 minutes, strained six times through a 26-gauge needle, and centrifuged for 5 minutes in a microfuge. NucBuster Protein Extraction kit instructions were followed for nuclear protein extract isolation. Proteins in the supernatants were resolved by SDS-PAGE and transferred to nitrocellulose. The following antibodies were used for immunoblot analysis: monoclonal anti-CYP3A4, WB-MAB-3A (Gentest, San Jose, CA), anti-PXR (N-16), anti-NCoR (Abcam, Cambridge, MA), and polyclonal glyceraldehyde-3-phosphate dehydrogenase as a loading control. Each blot was repeated at least twice with a minimum of three exposure times and reprobed with polyclonal antibody to guanosine dissociation inhibitor or to glyceraldehyde-3-phosphate dehydrogenase for protein content normalization. Bands on film were optically scanned (Epson Expression 1600, San Diego, CA) and quantitated by ImageQuant software (Molecular Dynamics).

Glutathione S-transferase protein interaction assay. The glutathione *S*-transferase (GST)-cSMRT fusion protein was expressed in *Escherichia coli* BL21 cells and purified using glutathione-Sepharose (Amersham Biosciences, Piscataway, NJ) as described previously (21). Verification of intact protein synthesis was obtained on 4% to 20% SDS-PAGE. Full-length human PXR in pcDNA3.1 was translated *in vitro* in the presence of [^{35}S]methionine using the TNT-coupled reticulocyte lysate system

(Promega) according to the manufacturer's instructions. Purified GST fusion protein (~5 μg) was incubated with 5 μL *in vitro* translated ^{35}S-labeled protein with moderate shaking at 4°C overnight in NETN [20 mmol/L Tris (pH 8.0), 100 mmol/L NaCl, 1.0 mmol/L EDTA, 0.5% NP40] and in the presence of 0.2% DMSO, 10 μmol/L rifampicin, or 5 μmol/L each of paclitaxel, PCN, docetaxel, or discodermolide. GST was used as a negative control. The bound protein was washed thrice with NETN, and the beads were collected by centrifugation at 3,000 rpm for 5 minutes. The bound protein was eluted into SDS sample buffer and subjected to 10% SDS-PAGE, and the gel exposed to Hyperfilm MP (Amersham Biosciences, Buckinghamshire, United Kingdom) at −80°C for 5 days.

Transient cotransfection experiments. HepG2 cells (passage 4) were transiently transfected using Lipofectin reagent (Invitrogen) in 10 mm plastic dishes. Each 10 mm dish was seeded with ~3 × 10^5 cells 24 hours before transfection with 1.5 μg reporter plasmid, Tk-MH100x4-Luc, with expression vectors as indicated in the figure legends. pSV-β-galactosidase (0.1 μg) control vector was used as an internal control. As a positive control, cells were cotransfected with Tk-MH100x4-Luc and expression vectors and subsequently treated with known activating ligands like rifampicin. As negative controls, empty expression vectors were used in some samples; in others, drugs, such as PCN, which do not activate hPXR, were used. In all experiments, cells were treated with ligands or microtubule-interacting drugs for 48 hours before harvesting. Where required, an equivalent quantity of empty vector (pcDNA3) was cotransfected to keep the total quantity of DNA constant. Cells were transfected for 8 to 12 hours, rinsed with PBS, incubated with drug, and diluted in medium containing 0.3% serum for the indicated times. Cells were lysed in 500 μL extraction buffer [40 mmol/L Tricine (pH 7.8), 50 mmol/L NaCl, 2 mmol/L EDTA (pH 7.8), 1 mmol/L $MgSO_4$, 5 mmol/L DTT, 1% Triton X-100]. Lysate (50 μL) was used to measure luciferase and β-galactosidase activities. All luciferase values were normalized to β-galactoside and expressed relative to basal control levels, which were assigned a value of 1.

Loss and regain of righting reflex in C57BL/6 mice. To show *in vivo* significance of the effects of drugs on the metabolism of tribromoethanol anesthesia (a substrate for enzymes induced by hPXR activation), 6- to 8-week-old C57BL/6 mice received tail vein injections in the morning on days 1 and 2 (24 hours after the first injection) of vehicle (0.1% DMSO in 0.9% saline and 0.05% Tween) or either rifampicin (0.025 mg/g/d) or paclitaxel (0.08 mg/g/d), BMS-247550 (0.0063 mg/g/d), or PCN (0.1 mg/g/d). Forty-eight hours after the first drug or vehicle injection, the mice were anesthetized with tribromoethanol (0.23 mg/g) given by i.p. injection. After the mice lost their righting reflex, they were put on their backs in a V-shaped bed. The duration of loss of righting reflex (LORR) was defined as the time from LORR to that at which it was regained. Recovery was determined when mice could right themselves twice in 1 minute after being placed on their backs. All animals recovered the righting reflex. The behavioral room was illuminated with a soft light and external noise was attenuated. Statistical analysis was done with Analyze-It software using the nonparametric Mann-Whitney test (22, 23).

Results

Effect of microtubule-stabilizing drugs on CYP3A4 protein expression in HepG2 cells. HepG2 cells were treated with a panel of microtubule-stabilizing drugs, and relative levels of

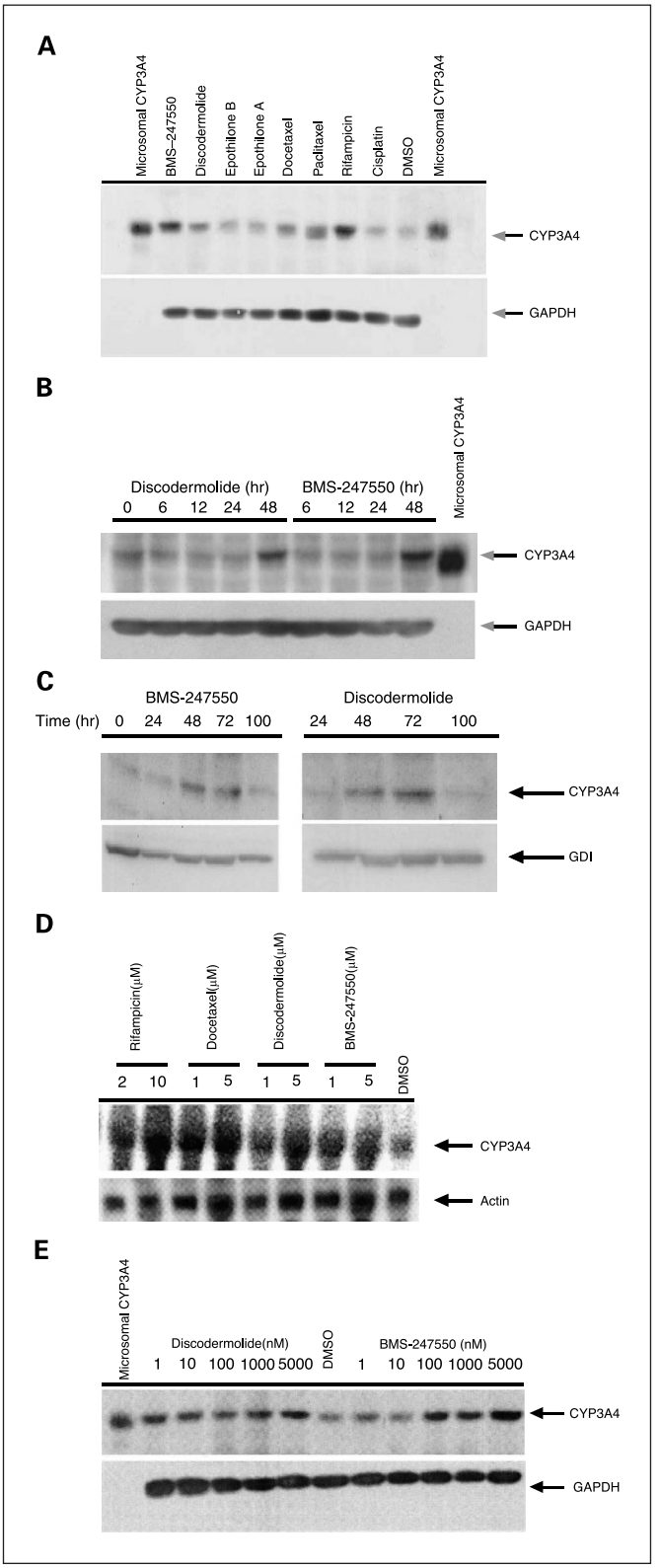

Fig. 1. Microtubule-stabilizing drugs induce CYP3A4 protein expression in HepG2 cells. *A,* Western blot showing CYP3A4 protein expression after exposure to 5 μmol/L of BMS-247550, discodermolide, epothilone B, epothilone A, docetaxel, paclitaxel, 10 μmol/L rifampicin, and 5 μg/mL cisplatin for 48 hours. *B,* Western blot analysis showing CYP3A4 protein expression at 6, 12, 24, and 48 hours after 5 μmol/L drug treatment. *C,* Western blot analysis showing CYP3A4 protein expression at 48, 72, and 100 hours after 5 μmol/L drug treatment. *D,* Northern blot analysis of CYP3A4 mRNA expression after exposure to rifampicin, docetaxel, discodermolide, and BMS-247550 at the specified concentrations for 18 hours. *E,* Western blot analysis showing a CYP3A4 protein expression profile after exposure to varying concentrations (1-5,000 nmol/L) of BMS-247550 and discodermolide for 48 hours. Human CYP3A4 plus *P*450 reductase supersomes were used as positive controls for all blots. All experiments were done thrice.

CYP3A proteins in treated and untreated controls were assessed by immunoblot analysis. Several of these drugs increased CY3A4 expression. Discodermolide (IC_{50}, 10 μmol/L) treatment of HepG2 cells and BMS-247550 (IC_{50}, 10 μmol/L) at a concentration of 5 μmol/L resulted in a 3.7- and 5.8-fold increase in CYP3A4 levels, respectively, compared with DMSO-treated controls. Epothilone A and epothilone B had only a minimal effect on CYP3A expression. Docetaxel (IC_{50}, >10 μmol/L) at 5 μmol/L resulted in a 3.5-fold induction of CYP3A4; however, paclitaxel showed a 4.3-fold induction compared with DMSO-treated control cells. As expected, rifampicin, a known inducer of CYP3A4 in mammalian cells, increased the level of CYP3A4 protein, whereas cisplatin as expected had no effect on CYP3A4 abundance when compared with DMSO-treated control cells (Fig. 1A).

The time course of induction of CYP3A4 by BMS-247550 and discodermolide was assessed. HepG2 cells were exposed to either 5 μmol/L discodermolide or BMS-247550 and lysates were harvested at 6, 12, 24, or 48 hours after treatment. As shown in Fig. 1B, there was a marked increase in the abundance of CYP3A4 at 48 hours of exposure to both drugs, which did not increase further at 100 hours (data not shown). However, as shown in Fig. 1C, when cells were grown in charcoal-adsorbed serum, CYP3A levels decreased between 72 and 100 hours after treatment with drug. Northern blot analysis of HepG2 cells treated with either rifampicin, discodermolide, or BMS-247550 shows induction of CYP3A4 mRNA as early as 18 hours after drug treatment (Fig. 1D).

To determine concentration dependence, HepG2 cells were treated with discodermolide or BMS-247550 at concentrations ranging between 1 nmol/L and 5 μmol/L (Fig. 1E) for 48 hours. Both drugs exhibited a dose-dependent increase in CYP3A levels. For discodermolide at 1, 10, 100, 1,000, and 5,000 nmol/L drug exposures, there was a 1.7-, 2.9-, 3.2-, 3.3-, and 3.7-fold increase in CYP3A4 levels over DMSO-treated cells, respectively. For BMS-247550, under the same conditions, there was a 1.6-, 2.9-, 3.3-, 3.3-, and 5.8-fold increase in CYP3A4 protein level over DMSO-treated cells, respectively.

Effect of microtubule-stabilizing agents and their analogues on steroid and xenobiotic receptor–mediated transcription. Paclitaxel increases CYP3A levels by enhancing hPXR-mediated transcription of the CYP3A promoter. hPXR activates transcription by binding to specific sequences in the CYP3A promoter and by recruiting various coactivators and corepressors. A fusion of hPXR to the GAL4 DNA-binding domain (GAL4-SXR) would mediate the activation from a GAL4-dependent promoter while recruiting the same coactivators and corepressors. The GAL4-SXR fusion protein was used to monitor transcription from a GAL4-dependent promoter in the presence and absence of various microtubule-binding drugs in HepG2 cells. HepG2 cells were transfected with the GAL4-SXR expression vector and the reporter construct, Tk-MH100x4-luc, and were exposed to drugs for 48 hours at which time the luciferase activity was assayed. Induction of the luciferase activity was observed with all drug(s) that induced microtubule stabilization and cell cytotoxicity (Fig. 2). Essentially, all drugs that activated microtubule stabilization and inhibited cell proliferation also

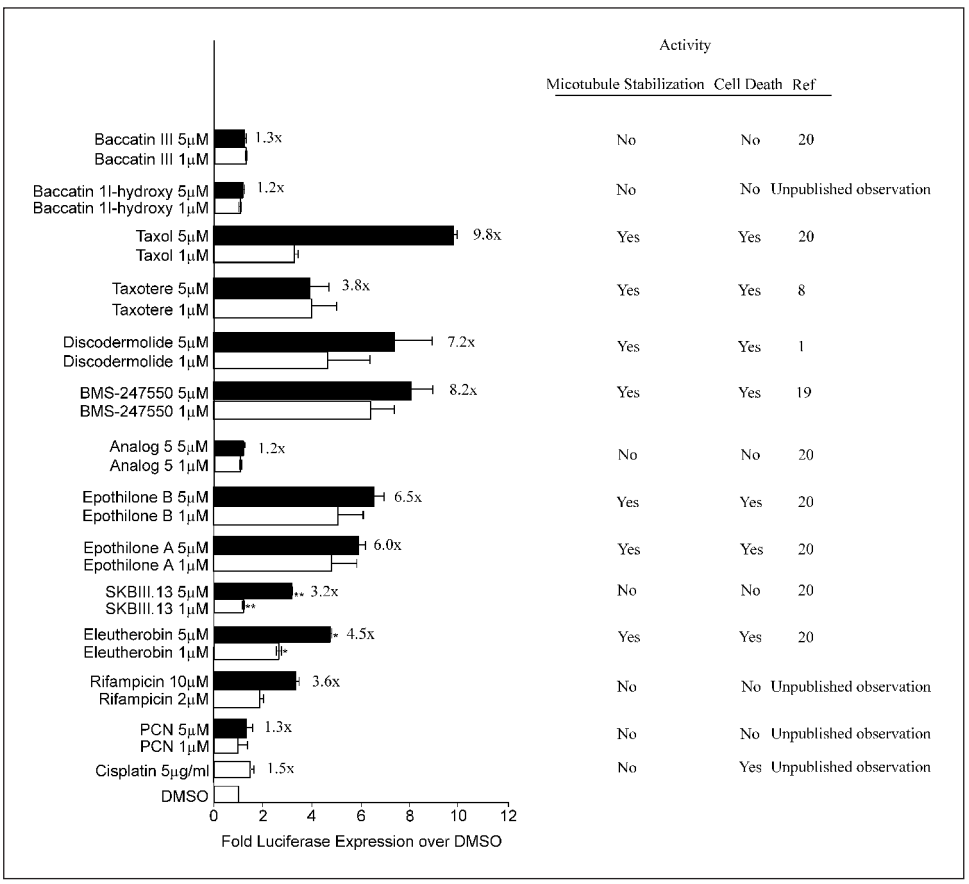

Fig. 2. Effect of microtubule-stabilizing agents on the transcriptional activity of GAL4-SXR. HepG2 cells were cotransfected with Tk-MH100x4-luc expression (GAL4-SXR reporter) vector (1.5 μg), Gal4-SXR/LBD plasmid (0.1 μg), and pSV-β-galactosidase control vector (0.1 μg) for 18 hours. Subsequently, the transfected HepG2 cells were treated as indicated for 48 hours. The cells were harvested in equal aliquots at 48 hours for luciferase and galactosidase assays (for details, see Materials and Methods). *Columns,* mean; *bars,* SD. All transfection studies were done at least thrice in triplicate.

stimulated GAL4-SXR–dependent transcription. As expected, cisplatin (a drug that does not affect *CYP450* gene expression or CYP450 metabolism) and PCN (a drug that only activates rodent PXR) did not induce reporter activity. In HepG2 cells, 10 μmol/L rifampicin induced a 3.6 ± 0.4–fold expression of Tk-MH100x4-luc as described previously (24, 25). Microtubule-stabilizing drugs induced luciferase activity in the following order: paclitaxel (9.8-fold) > BMS-247550 (8.2-fold) > discodermolide (7.2-fold) > epothilone A and epothilone B (6.0-6.5-fold) > docetaxel. Inactive analogues, such as baccatin and analogue 5, which do not stabilize microtubules *in vitro* or do not inhibit cell proliferation, did not significantly induce luciferase activity. Eleutherobin, a novel natural product isolated from a marine soft coral, which induces microtubule polymerization (19), resulted in a 4.5 ± 0.1–fold increase in activation of Tk-MH100x4-luc; however, its noncytotoxic analogue, SKBIII, at 5 μmol/L exhibited a 3.2 ± 0.1–fold activation of the reporter in HepG2 cells (Fig. 2). A similar pattern of activation was observed with HepG2 cells were maintained and treated in medium containing charcoal-adsorbed sera. In these experiments, however, the fold induction of luciferase at 1 and 5 μmol/L rifampicin, paclitaxel, discodermolide, and BMS-247550 was 8 and 15.5, 6 and 27.8, 2.4 and 4.92, and 6.0 and 7.4, respectively. These results clearly indicate that the microtubule-binding class of drugs can stimulate the transcriptional activity of hPXR in HepG2 cells.

Effect of the dose of discodermolide and BMS-247550 on the activation of human pregnane X receptor. To further explain dose dependency of hPXR activation, HepG2 cells were transfected with the GAL4-SXR construct and Tk-MH100x4-luc reporter and treated with increasing concentrations of BMS-247550 and discodermolide. Analysis of luciferase activity 48 hours after drug treatment indicated that there was a dose-dependent activation of the Tk-MH100x4-luc reporter construct. For discodermolide, at 10 nmol/L, there was a 1.2-fold activation of luciferase expression, which increased to 7.2-fold activation of Tk-MH100x4-luc at a concentration of 1 μmol/L (Fig. 3). In comparison, for BMS-247550 at 10 nmol/L, there was a 2.1-fold activation, which increased to 8.2-fold activation of Tk-MH100x4-luc at a concentration of 1 μmol/L (Figs. 2 and 3). At concentrations of 5 μmol/L of either drug, ~30% of the cells were inhibited from proliferating as assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown), suggesting that for HepG2 cells the IC_{50}s for these drugs were >5 μmol/L. Concentrations above 20 μmol/L resulted in significant cell death and the reliability of readings for luciferase activity, even when adjusted to cell number, was erroneous. Hence, there was a dose-dependent increase in the GAL4-SXR–mediated transcription, which saturated at drug concentrations higher than 1 μmol/L. However, inhibition of cell proliferation required much higher drug concentrations (i.e., IC_{50}s, >5 μmol/L).

SMRT suppresses and SRC-1 augments drug-induced human pregnane X receptor–mediated transcription. Further experiments were done to determine whether selective corepressors or coactivators were involved in drug-induced transcriptional activation of CYP3A4. Specifically, the effect of overexpression of NCoR or SMRT on drug-mediated enhancement of hPXR activity was tested. An assay was done by using pCEP4-NCoR, pCMX-SMRT (corepressors), or a mammalian two-hybrid assay using VP16SRC-1-RID (coactivator) and GAL4-SXR (see Fig. 4; see Materials and Methods). NCoR or SMRT expression constructs were transfected into HepG2 cells along with GAL4-SXR and the reporter construct. It was also shown that cells transfected with NCoR expression construct along with GAL4-SXR had minimal change in the abundance of hPXR or NCoR protein at 48 hours after transfection when treated with

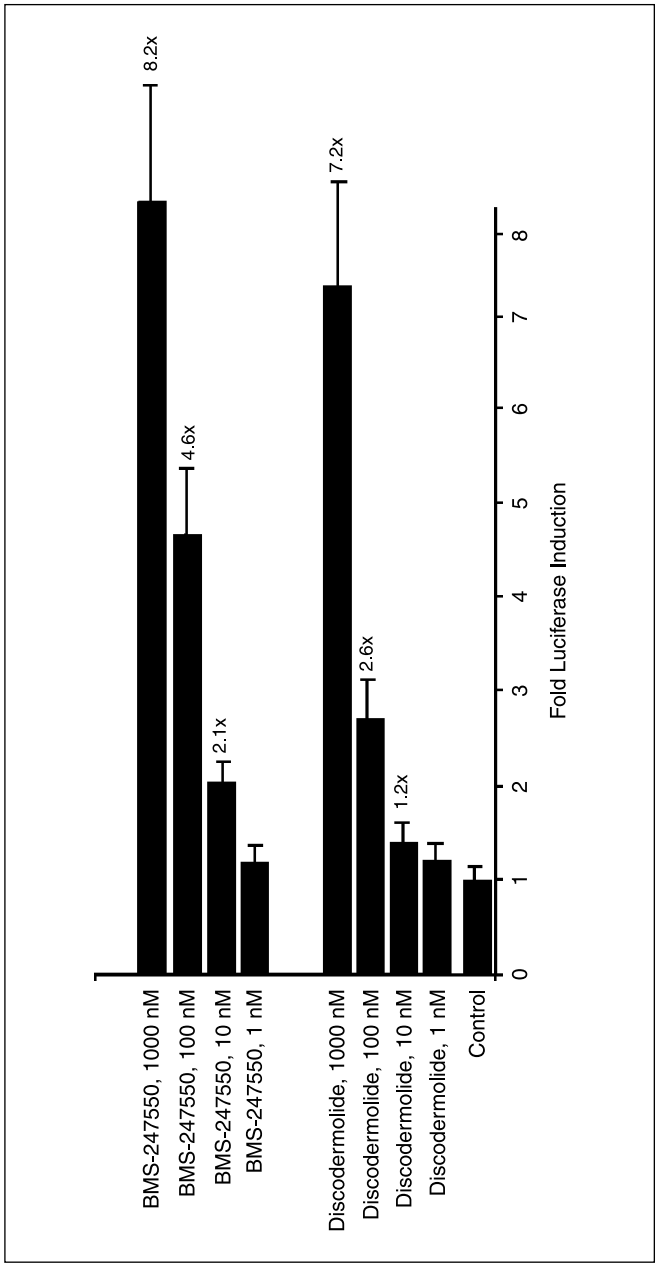

Fig. 3. Dose-dependent stimulation of GAL4-SXR transcriptional activity by discodermolide and BMS-247550. HepG2 cells were cotransfected with Tk-MH100x4-luc expression vector, Gal4-SXR/LBD plasmid, and pSV-β-galactosidase control vector for 18 hours. Subsequently, these cells were treated with either discodermolide or BMS-247550 at concentrations ranging between 1 and 1,000 nmol/L for 48 hours (for details, see Materials and Methods). Columns, mean; bars, SD. All transfection studies were done at least thrice in triplicate. Data on microtubule stabilization and cell death have been published previously (19, 45, 55). All unpublished experiments were done in our laboratory. Microtubule stabilization was assessed by immunofluorescence staining for α-tubulin, and cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (for details, see Materials and Methods).

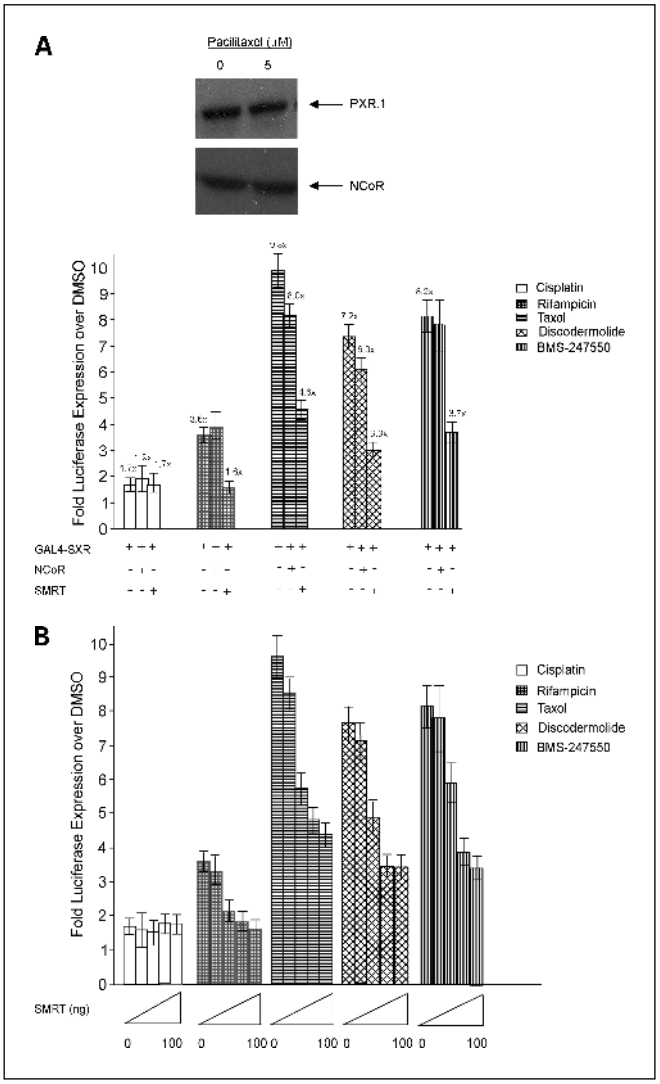

Fig. 4. Effect of microtubule-binding drugs on the interaction of SXR with coactivators and corepressors. Inset, Western analysis of PXR and NCoR in HepG2 cells cotransfected with expression construct of hPXR, pSV-β-galactosidase control vector, and pCEP4-NCoR. These cells were treated with paclitaxel or vehicle for 48 hours at the specified concentration. A and B, SMRT suppresses drug-mediated hPXR-induced transcription of the CYP3A4 promoter. *A,* HepG2 cells were cotransfected with Tk-MH100x4-luc expression vector, Gal4-SXR/LBD plasmid, pSV-β-galactosidase control vector, and pCEP4-NCoR or pCMX-SMRT (100 ng) for 18 hours. *B,* HepG2 cells were cotransfected with Tk-MH100x4-luc expression vector, Gal4-SXR/LBD plasmid, pSV-β-galactosidase control vector, and pCMX or pCMX-SMRT (in amounts ranging from 12.5, 25, 50, and 100 ng) for 18 hours. Subsequently, cells in A and B were treated with the indicated drugs for 48 hours (for details, see Materials and Methods). *Columns*, mean; *bars*, SD. All transfection studies were done at least thrice in triplicate.

5 μmol/L paclitaxel (see Fig. 4A, *inset*). Altogether, the results of these experiments indicate that, although overexpression of either NCoR or SMRT had very little effect on basal level GAL4-SXR activity, expression of SMRT, but not NCoR, antagonized the increase in GAL4-SXR activity by drugs, such as rifampicin, paclitaxel, discodermolide, and BMS-247550 (Fig. 4A). Dose titration of the SMRT expression construct showed that SMRT was able to overcome drug-induced activation of GAL4-SXR, provided sufficient amounts of the corepressor were available. Specifically, the inhibitory effect of SMRT was reached when 50 ng of the expression vector were used in combination with rifampicin, paclitaxel, discodermolide and BMS-247550; however, no suppressive effects on basal level hPXR activity was observed in cisplatin-treated cells (Fig. 4B). NCoR had minimal effects on drug-induced activation of hPXR even when exposed to high concentrations (data not shown).

Because one-hybrid systems do not replicate the natural interactions at the CYP3A4 promoter, a separate system was used to test for xenobiotic-mediated activation of hPXR. In this system, a reporter plasmid containing the CYP3A4 promoter (−10,466 to +53) upstream of a luciferase gene was transfected with a plasmid expressing full-length hPXR (also denoted as hPAR) in the presence or absence of drug(s). Both rifampicin (1.72-fold) and paclitaxel (3.6-fold) activated hPAR when compared with 0.2% DMSO-treated HepG2 cells. Expression of increasing concentrations of SMRT resulted in decreasing luciferase activity, indicating a dose-dependent inhibition of

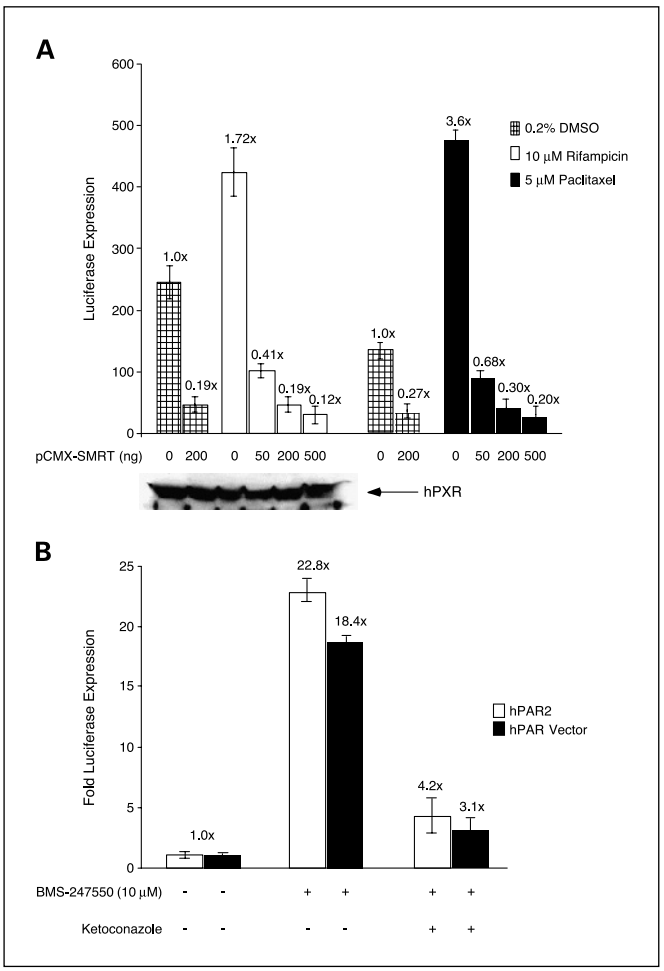

Fig. 5. Effect of microtubule-stabilizing agents on the transcriptional activity of hPXR. *A,* HepG2 cells were cotransfected with 10-kb CYP3A promoter luciferase expression vector (0.2 μg), hPAR plasmid (0.2 μg), pCMX-SMRT (0-500 ng), and pSV-β-galactosidase control vector (0.2 μg) for 18 hours. Inset, effect of pCMX-SMRT transfection on hPXR abundance in HepG2 nuclear extracts in the presence or absence of 10 μmol/L rifampicin (*A*). This immunoblot was repeated twice. *B,* HepG2 cells were cotransfected with 10-kb CYP3A promoter luciferase expression (hPAR reporter) vector (0.4 μg), hPAR plasmid (0.1 μg), or pcDNA3 (hPAR) vector (0.1 μg) and pSV-β-galactosidase control vector (0.1 μg) for 18 hours. Subsequently, the transfected HepG2 cells were treated as indicated for 48 hours. The cells were harvested in equal aliquots at 48 hours for luciferase and galactosidase assays (for details, see Materials and Methods). *Columns*, mean; *bars*, SE. All transfection studies were done thrice in duplicate.

hPXR activity (Fig. 5A). In Fig. 5B, using the same transient transfection assay as described in Fig. 5A, it was shown that BMS-247550 stimulated the activities of hPXR transiently expressed from the hPAR plasmid (22.8-fold) as well as endogenous hPXR (18.4-fold) in HepG2 cells. Ketoconazole, a known CYP3A4 inhibitor as well as an inhibitor of corticosterone-mediated hPXR activation, also represses hPAR and endogenous hPXR activation by BMS-247550 (18). Together, all these results suggest that the microtubule-stabilizing drugs activate hPXR by possibly affecting its interaction with specific corepressors.

To determine if microtubule-stabilizing drugs affect the interaction of hPXR with corepressors or coactivators, we used a mammalian two-hybrid system using GAL4-SXR as one partner and either VP16NCoR-RID, VP16SMRT-RID, or VP16SRC-1-RID as the other interacting partner. The results show that both VP16NCoR-RID and VP16SMRT-RID can interact with hPXR in cisplatin-treated cells. The addition of drugs (i.e., discodermolide, paclitaxel, and BMS-247550) disrupted GAL4-SXR–mediated transcription resulting in suppressed luciferase activity of the reporter. The suppressive effects of the drugs on GAL4-SXR–mediated transcription was more pronounced in cells transfected with VP16SMRT-RID compared with those with VP16NCoR-RID (Fig. 6A). A similar pattern of suppression with corepressors was observed when HepG2 cells were propagated in medium containing charcoal-adsorbed sera (Fig. 6B). These results are consistent with the overexpression studies and indicate that microtubule-stabilizing drugs disrupt the interaction of hPXR with selective corepressors.

To determine if microtubule-binding drugs affect the interaction of hPXR with coactivator, SRC-1, a mammalian two-hybrid assay using GAL4-SXR and VP16SRC-1-RID in the presence and absence of drugs was done. Cotransfections with GAL4-SXR and VP16SRC-1-RID, when normalized for β-galactosidase activity, in the absence of drug (but presence of DMSO) did not activate Tk-MH100x4-luc compared with VP16, the empty vector. However, in the presence of paclitaxel, discodermolide, or BMS-247550, VP16 resulted in a 12-, 9-, and 11-fold increase in luciferase activity, respectively. However, in experiments with VP16SRC-1-RID, the fold activation of Tk-MH100x4-luc in the presence of paclitaxel, discodermolide and BMS-247550 was 45-, 35-, and 42.5-fold, respectively (Fig. 6C). These results suggest that the microtubule drugs augment coactivator interaction with hPXR.

Paclitaxel and discodermolide disrupt SMRT interactions with human pregnane X receptor. To directly show that the drugs disrupted hPXR-corepressor interactions, an *in vitro* GST pull-down assays using ^{35}S-labeled full-length hPXR and GST-SMRT was carried out. Expression of intact GST-SMRT was shown on SDS-PAGE gel by Coomassie blue staining. The results of these *in vitro* experiments showed basal level interaction in the absence of drugs (0.2% DMSO; Fig. 7). In the presence of 10 μmol/L rifampicin or 5 μmol/L each of either paclitaxel or discodermolide, there was a decrease in the intensity of the signal on the autoradiograph compared with that of 0.2%

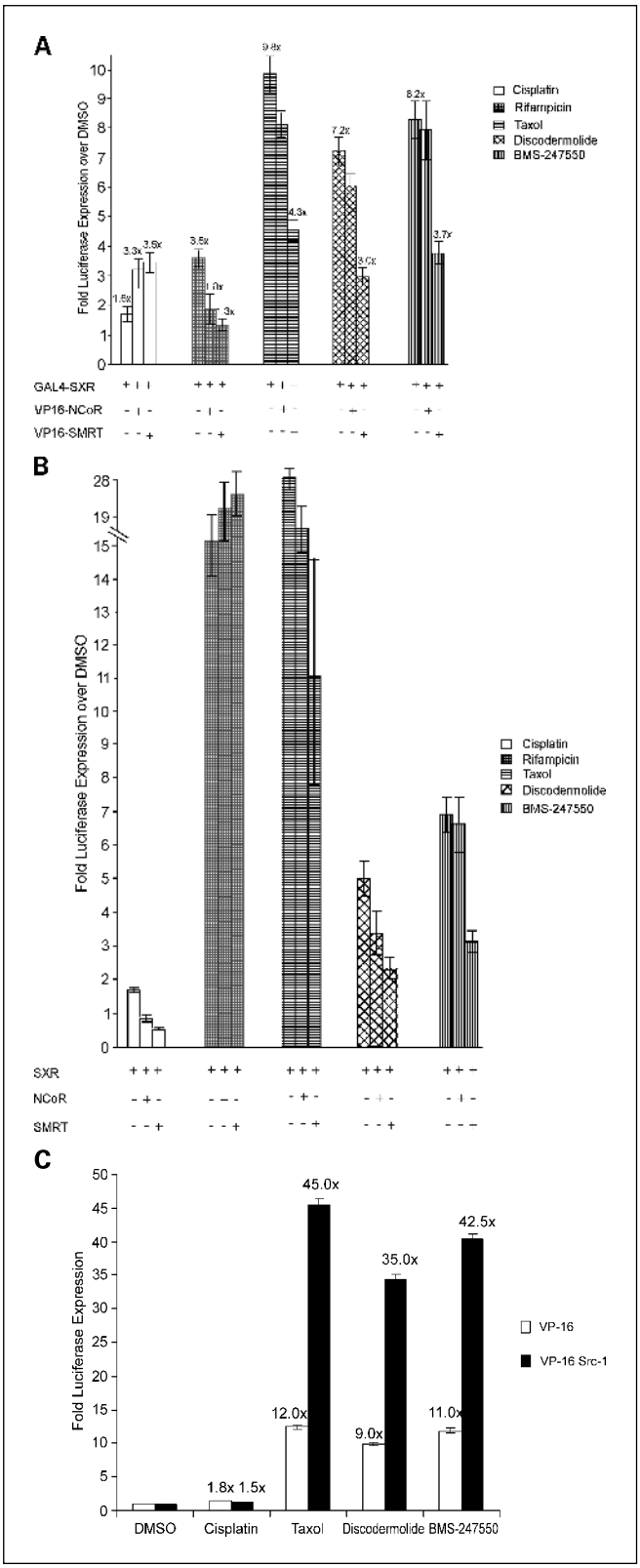

Fig. 6. Effect of microtubule-binding drugs on the interaction of SXR with corepressors using the mammalian two-hybrid system. *A,* HepG2 cells were cotransfected with Tk-MH100x4-luc expression vector, Gal4-SXR/LBD plasmid, pSV-β-galactosidase control vector, and VP16NCoR-RID or VP16SMRT-RID for 18 hours. *B,* identical experiment as in *A.* However, transfections were done in charcoal-adsorbed sera with medium. *C,* identical experiment as in *A.* However, VP16SRC-1-RID was used instead of the corepressor constructs. Subsequently, cells in *A* to *C* were treated with the indicated drugs for 48 hours (for details, see Materials and Methods). *Columns*, mean; *bars*, SD. All transfection studies were done at least thrice in triplicate.

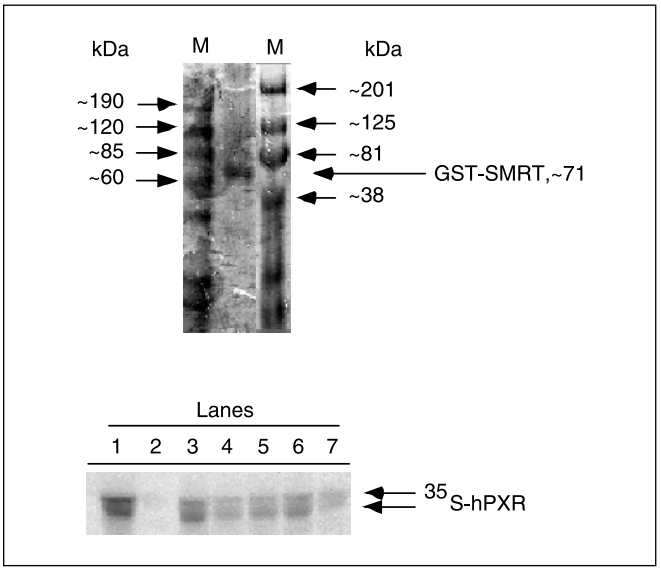

Fig. 7. GST interaction assay of SMRT. *In vitro* translated ^{35}S-labeled full-length hPXR (5 μL) was incubated with GST-SMRT, washed extensively, and analyzed by SDS-PAGE. Lane 1, 25% input; lane 2, GST beads; lane 3, 0.2% DMSO; lane 4, 10 μmol/L rifampicin; lane 5, 5 μmol/L paclitaxel; lane 6, 5 μmol/L PCN; lane 7, 5 μmol/L discodermolide. Coomassie blue – stained gel of GST-SMRT fusion protein, with arrow indicating the intact fusion protein, is shown on the top 4% to 20% SDS-PAGE gel (56). Two bands for hPXR are seen in the autoradiograph and reflect the use of two ATG codons in the *hPXR* gene (21). Gels were run thrice and subjected to autoradiography.

DMSO lane (see *lane 3*). However, in the presence of PCN (which should not displace corepressors from hPXR), there was no significant change in the band density when compared with the 0.2% DMSO lane. These results suggest that microtubule-stabilizing drugs directly displace SMRT interactions with hPXR *in vitro*.

Paclitaxel and BMS-247550 shortens duration of loss of righting reflex in B57BL/6 mice. Because PXR is a master regulator of genes involved in drug metabolism (e.g., CYP450s and uridine glucuronosyl transferases), the *in vivo* physiologic significance of PXR activation can be studied using mice challenged with tribromoethanol anesthesia (26). In this assay, the drug-induced change in the duration required for the LORR acts as a phenotypic measure of the *PXR* target gene activity and xenobiotic metabolism. The mean ± SE duration of LORR (a phenotypic measure of xenobiotic metabolism) was significantly attenuated with paclitaxel (46.6 ± 5.2 minutes) compared with control animals injected with 0.1% DMSO vehicle (86.67 ± 9.2 minutes; $P < 0.01$). Rifampicin had only a slight effect on the duration of LORR (65.1 ± 35 minutes) compared with vehicle-treated controls (86.67 ± 9.2 minutes; $P <$ NS). Notably, in the rifampicin group, three animals had prolonged sleep times greater than 90 minutes. BMS-247550 also had a significant effect on the mean ± SE duration of LORR (40.6 ± 4.2 minutes) compared with vehicle-treated controls ($P < 0.01$). PCN, as expected had a mean ± SE duration of LORR of 50.02 ± 4.0 minutes, which was also significantly lower than vehicle-treated control animals ($P < 0.01$; see Fig. 8). These results indicate that the microtubule drugs affect the activity of *PXR* target gene *in vivo* possibly by affecting its interaction with corepressors/coactivators. As expected, PCN is a known activator of mouse PXR and significantly shortened the duration of LORR; however, in contrast, rifampicin, a known effector of hPXR and not mouse PXR, failed to significantly shorten LORR duration in mice.

Discussion

Paclitaxel is the prototypical microtubule-stabilizing drug that inhibits the dynamic qualities of the polymer that are essential for its normal physiologic functions. The drug is metabolized by CYP3A4 to 6α-hydroxy paclitaxel and induces expression of CYP3A4 in HepG2 cells as well as in rat and human hepatocytes in culture. Paclitaxel stimulates hPXR-mediated transcriptional activation of CYP3A by disrupting binding of corepressors to hPXR (8). Hence, in cell culture, paclitaxel may induce its own metabolism. Docetaxel, a paclitaxel analogue, is also metabolized by CYP3A4 (6). However, in comparison with paclitaxel, it is a weaker inducer of CYP3A4 in cell culture systems (8). These observations are consistent with *in vivo* observations demonstrating that these drugs have different pharmacokinetic properties (5, 8, 27). Because taxanes are generally given in combination with other antineoplastics, some of which induce CYP450s (e.g., estramustine induces CYP3A4), the clinical relevance of these findings is that CYP3A4 induction may be significant enough to alter drug (e.g., paclitaxel) pharmacokinetics in patients receiving combination drug therapies (28). There is evidence for activation of CYP3A4 by taxanes in humans (29); however, the study of clinically relevant induction of CYP3A4 is hampered by the necessary coadministration of agents used for hypersensitivity prophylaxis (i.e., H2-blockers and dexamethasone; ref. 30). Furthermore, some microtubule-stabilizing drugs (e.g., epothilone analogues and discodermolide) act as weak to moderate inhibitors of CYP3A4 enzyme activity and this may mask any true effect of CYP3A4 induction *in vivo*.

It is important to note that CYP3A4 is an inducible enzyme and its activity varies markedly (up to 40-fold) across the population largely due to drug-mediated variation in CYP transcription (31, 32). Therefore, any activation or suppression of hPXR activity would affect CYP3A4 levels, and depending on liver extraction ratios of drugs, plasma drug concentrations would have very little predictive effect on the degree of hPXR activation. Hepatic extraction of microtubule-binding drugs are extensive; therefore, it is conceivable that much higher liver tissue concentrations could be attained even when plasma concentrations fall below that predicted to activate hPXR (33). Furthermore, because taxanes are given in a dose-intense schedule for the objective of attaining a cure in certain malignancies (e.g., breast cancer), even higher doses are used more frequently with growth factor support (34). This should result in even higher tissue levels of parent drug. Finally, taxanes or its formulations that are modified to improve selective drug delivery to tumor tissue [i.e., poly(L-glutamic acid) paclitaxel and nanoparticle formulations of paclitaxel] have the propensity to accumulate to much higher levels in both liver and tumor tissue when compared with conventional formulations of taxanes (33, 35). Therefore, there is a possibility that these drugs could act as ligands to hPXR at therapeutic doses given to patients with cancer. This may be clinically relevant as these drugs have already entered late phase II to III testing and have shown promise as therapeutic agents that may be used for paclitaxel-resistant tumors (35, 36).

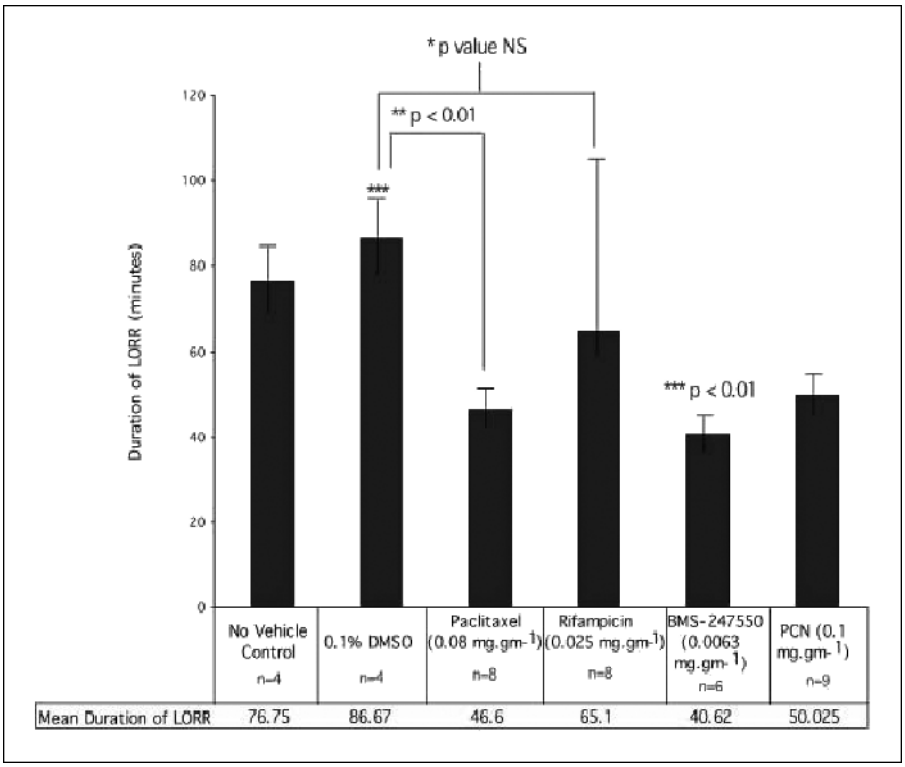

Fig. 8. Effect of paclitaxel and BMS-247550 on duration of LORR in B57BL/6 mice. Six- to 8-week-old B57BL/6 mice were randomized in separate cages and received tail vein injections on days 1 and 2 of vehicle (0.1% DMSO; $n = 4$), no injections (no vehicle; $n = 4$), paclitaxel (0.08 mg/g/d, $n = 8$), rifampicin (0.025 mg/g/d, $n = 8$), BMS-247550 (0.0063 mg/g/d, $n = 6$), and PCN (0.1 mg/g/d, $n = 9$). On day 3 (48 hours after first injection), all animals received tribromoethanol anesthesia (0.23 mg/g i.p). The duration of righting after sleep onset was measured in minutes and recorded by two investigators as described previously (22, 23, 26).

The current studies show that, after 48 hours of exposure to a variety of microtubule-stabilizing drugs, at concentrations that are clinically achievable in humans, there is an increase in the level of CYP3A4. At equimolar concentrations, BMS-247550 and paclitaxel were the strongest inducers of CYP3A4. BMS-247550 and discodermolide induction of CYP3A4 was clearly dose dependent. However, whereas BMS-247550 induced CYP3A4 at doses as low as 100 nmol/L, a concentration of 5 μmol/L discodermolide was required to induce CYP3A4. For both drugs, there was a sharp increase in CYP3A protein levels after 48 hours of exposure to HepG2 cells, which persisted in nonadsorbed serum conditions for 100 hours. In cells exposed to charcoal-adsorbed sera, there was a reduction in CYP3A content by 100 hours. Therefore, there is a need to create a standard procedure for the analysis of CYP protein expression in HepG2 cells.

It has been established that CYP3A4 expression is largely regulated at the level of gene transcription and that this in turn is regulated by nuclear receptor activation. An orphan nuclear receptor, hPXR, heterodimerizes with retinoid X receptors and mediates ligand-dependent CYP450 (CYP3A4, CYP3A11, and CYP2C8) transcription (12, 13). Unliganded GAL4-SXR exhibits basal repression of a GAL4-dependent promoter. On the addition of microtubule-binding drugs, there is an ~6- to 10-fold induction of GAL4-SXR–mediated transcription, indicating activation of hPXR. These effects are generally more prominent under conditions in which the cells have been exposed to charcoal-adsorbed sera. In these conditions, paclitaxel, for example, results in a 27.8-fold induction of the reporter compared with 9.8-fold in non-adsorbed serum conditions. However, this is not a common phenomenon, as discodermolide shows reduced hPXR activation in cells exposed to charcoal-adsorbed sera compared with nonadsorbed sera. These data suggest that in the evaluation of novel agents, at least in HepG2 cells, effects on CYP3A expression should be assessed under both serum conditions. The basis for this observation is unclear but may be related to the presence of cytokines and chemokines expressed in the serum by HepG2 cells that interfere with signaling and/or activation of genes (18, 37). As with the protein expression data for BMS-247550 and discodermolide, there is a dose dependency in the activation of hPXR tested at the transcriptional level in both sera conditions.

hPXR has been shown to specifically interact with SMRT but not with NCoR in both HepG2 and CV-1 cells (8, 18). Although NCoR and SMRT share a similar structure in their COOH-terminal nuclear RID, recent studies have shown specificity in terms of nuclear receptor recruitment of corepressors (38, 39). For example, retinoic acid receptor-α and the vitamin D receptors preferentially interact with SMRT, whereas TRβ1 preferentially interacts with NCoR (38, 39). The (I/L)*XX*II motifs and adjacent α-helical structures in the interacting domains are critical for interacting with the nuclear receptors. The subtle differences between NCoR, which contains three IDs, and SMRT, which has two IDs, may account for specificities in drug-nuclear receptor interactions (38–42). Consistent with these observations, our transient transfection studies indicate a differential effect of these two corepressors on the drug-mediated activation of hPXR. Whereas overexpression of SMRT exhibited greater inhibitory effects on hPXR activation in the presence of drug, overexpression of NCoR had a minimal effect. Such an interaction is in contrast to that shown with tocotrienols, in that NCoR as opposed to SMRT plays an important role in tissue-specific activation of hPXR (43).

To further evaluate the specificity with which the drugs affect the interactions of hPXR with corepressors and coactivators, we

did mammalian two-hybrid system assays in the presence and absence of drugs. Specifically, we tested the effect of drugs on the interaction of GAL4-SXR with corepressors or a coactivator fused to VP16. In this system, interaction of GAL4-SXR with coactivator-VP16 or corepressor-VP16 fusion proteins leads to the activation of a luciferase reporter gene and any disruption of the interaction by the drug would lead to repression of GAL4-SXR–mediated transcription. Consistent with the specificity of the interaction of hPXR with SMRT, we found that rifampicin, paclitaxel, discodermolide, and BMS-247550 specifically inhibited the hPXR-SMRT interaction. These results were further corroborated by direct evidence demonstrating that microtubule-binding drugs indeed disrupt hPXR-SMRT interactions in the GST pull-down assays.

We also tested the effect of the interaction of hPXR with a coactivator. In the basal state, GAL4-SXR was bound to cellular corepressors and even in the presence of SRC-1, there was no activation due to the absence of the appropriate hPXR ligand. Interestingly, we found that, whereas the hPXR-corepressor interaction was disrupted by these drugs, the presence of the coactivator, SRC-1, when drug was bound to hPXR, resulted in an augmented reporter response. This suggests that either the drug facilitates the active recruitment of SRC-1 to hPXR or the hPXR/drug complex may be stabilized by SRC-1. This effect has been shown in the crystal structure of the PXR/SR12813/SRC-1 complex (42). These data support the observations by Synold et al. that paclitaxel activates hPXR through displacement of corepressors and/or recruitment of coactivators (8). Our data now extend this observation to a diverse set of microtubule-stabilizing drugs, which have diverse origins as well as distinct chemical structures. In this regard, although the molecular weights and structures of endogenous ligands of nuclear receptors vary widely, their van der Waals volumes are conserved within a narrow range (<350 Å; ref. 44), suggesting that the microtubule-stabilizing drugs may share this trait.

Mechanistically, these drugs may interact with nuclear receptors independent of their action on microtubules. In immunofluorescence studies done in our laboratory using α-tubulin antibodies (45), HepG2 cells showed marked tubulin bundle and aster formation within 3 hours of drug exposure even at concentrations as low as 10 nmol/L. At a concentration of 5 μmol/L, >90% of the cells in culture have asters or microtubule bundles (data not shown). This is in marked contrast to the activation profile of hPXR, which is evident by our reporter assay at drug concentrations of 100 nmol/L (for BMS-247550 and discodermolide) but requiring up to 48 hours of drug exposure. Therefore, these events may not be related, although there still exists the possibility that nuclear import/export of these receptors (46) may be aided by microtubules as has been shown for other transcription factors like p/CIP, glucocorticoid receptor, or p53 (47–50). Because these nuclear receptors are also present and regulated in cancer cells (51–53), they may represent novel targets for classic drugs, such as the microtubule-binding drugs. Therefore, these drugs may affect not only their own metabolism but also gene expression in cancer tissue through activation of tissue-specific nuclear receptors.

In our limited evaluation of inactive analogues of paclitaxel, epothilone, and eleutherobin, there is clear evidence that the analogues are less effective in activating hPXR compared with the parent drug. We have assessed activity of these drugs in their ability to inhibit cell proliferation as well as in their ability to induce stable microtubule polymers. Although there does not seem to be a clear and direct link between microtubule stabilization and activation of hPXR, the ability of hPXR to translocate from the cytoplasm to the nucleus may be influenced by altered signaling cascades emanating from the formation of stable tubulin polymers (50, 54).

Activation of hPXR and induction of drug-metabolizing enzymes *in vitro* may not imply that there is relevant induction of CYP3A in rodents or humans. To test whether microtubule-stabilizing drugs induce drug-metabolizing enzyme activity *in vivo*, we used an established *in vivo* mouse assay, duration of LORR. In this assay, it has been shown previously that ligands activating PXR induce liver enzymes that inactivate tribromoethanol anesthesia (22, 23, 26). The latter phenotype can be compared across several ligands in whole animals and serves to provide a gross measure of enzyme induction capacity. It has been shown that paclitaxel and BMS-247550 significantly reduce the duration of LORR compared with control animals, suggesting that both drugs induce drug metabolism enzyme activity *in vivo*.

In summary, the novelty of our findings is that we show for the first time that a single class of drugs (i.e., microtubule stabilizers) activate hPXR and induce CYP3A4. This would not have been predicted based on their diverse origins and structures. Furthermore, we show that only SMRT corepressor is significant in the interaction of these drugs with hPXR. This is novel because PXR-mediated gene transcription is highly tissue specific and ligand specific and both NCoR and SMRT are known to corepress hPXR. Our studies lay the basis for understanding and exploring why some paclitaxel analogues do not activate hPXR and this information may be used to guide the future development of therapeutically active but hPXR-neutral microtubule-stabilizing agents. Finally, we show that there is *in vivo* relevance to drug-mediated PXR activation as shown by our righting reflex studies with mice.

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