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Role of Pregnane X Receptor in Control of All-Trans Retinoic Acid (ATRA) Metabolism and Its Potential Contribution to ATRA Resistance

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Abstract

Although all-trans-retinoic acid (ATRA) is an effective treatment for acute promyelocytic leukemia and several solid tumors, its use is limited by resistance due to increased metabolism. The most studied mechanism for ATRA resistance is the autoinduced metabolism regulated by the retinoic acid receptor-CYP26 pathway. However, treatment of cancer is usually not done with a single antineoplastic agent, but with a variety of combined chemotherapy regimens, including several anticancer drugs, and other concomitantly administered supportive drugs. Pregnane X receptor (PXR), an orphan nuclear receptor that functions as a ligand-activated transcription factor, serves as an important xenobiotic sensor regulating metabolism and elimination. Many prescription drugs are PXR ligands, which can activate PXR target genes, including phase I enzyme, phase II enzyme, and transporter genes. The present study was designed to examine the role of PXR in ATRA metabolism. Due to the marked species differences in response to PXR ligands, *Pxr*-null, wild-type, and *PXR*-humanized transgenic mouse models were used. In addition to pregnenolone 16 α -carbonitrile, several clinically relevant PXR ligands (rifampicin and dexamethasone) all increased ATRA metabolism both in vitro and in vivo, which was PXR-dependent, and up-regulation of *Cyp3a* was the major contributor. Furthermore, induction of the *Mdr1a*, *Mrp3*, and *Oatp2* genes was also observed. This study suggested that coadministration of PXR ligands can increase ATRA metabolism through activation of the PXR-CYP3A pathway, which might be a mechanism for some form of ATRA resistance. Other PXR target transporters might also be involved.

All-trans retinoic acid (ATRA) is currently used as a chemotherapeutic agent against acute promyelocytic leukemia (APL), breast cancer, Kaposi's sarcoma, and glioma (Muindi et al., 1992b; Defer et al., 1997; Toma et al., 2000; Bernstein et al., 2002). ATRA modulates the transcription of a set of genes associated with cellular apoptosis, growth, and differentiation by binding to retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Incorporation of ATRA into the treatment of APL was the most significant step forward over the past 25 years, resulting in very high rates of complete remission and cure. Unfortunately, despite the general efficacy of ATRA-based therapy, a major drawback to its clinical application is the prompt development of resistance.

Several mechanisms have been proposed to explain the involved ATRA resistance arising during cancer therapy, including increased metabolism, sequestration by cellular retinoic acid-binding proteins, decreased uptake by P-glycoprotein (Pgp), and mutations in the ligand-binding domain of promyelocytic leukemia protein-RAR α fusion protein, most of which,

however, were not well elucidated and still remain controversial when comparing in vitro data with in vivo data (Gallagher, 2002). The only well recognized mechanism is the clear relationship between induction of ATRA metabolism and progressive clinical resistance to ATRA demonstrated in APL patients (Muindi et al., 1992a).

ATRA metabolism is complex and only partially understood at the present time. However, it is thought that ATRA can be oxidized by cytochromes P450 (P450s) to various metabolites, including 4-hydroxy-RA (4-OH-RA), 18-OH-RA, 5,6-epoxy-RA, and 4-oxo-RA, which may be further biotransformed into glucuronides via UDP-glucuronosyltransferase-mediated conjugation (Napoli, 1999; Samokyszyn et al., 2000). The most prominent pathway begins with a rate-limiting hydroxylation at the C-4 position of the cyclohexenyl ring leading to formation of 4-OH-RA. More recently, a major involvement of P450s CYP3A4/5/7, CYP2C8/9, CYP1A1, and CYP4A11 in the oxidation of ATRA, and the CYP3A subfamily as the most active P450s in the formation of 4-OH-RA, 4-oxo-RA, and 18-OH-RA was reported (Marill et al., 2000). A unique ATRA-inducible P450, CYP26, that specifically metabolizes ATRA was also identified, and this enzyme is considered to be an important regulator of endogenous ATRA homeostasis (Ray et al., 1997; White et al., 1997). Due to its key role in a feedback loop where ATRA levels are controlled in an auto-regulatory manner, CYP26 was supposed to be one of the mechanisms causing resistance to continuous ATRA therapy. However, ATRA resistance still occurs in patients who relapsed from regimens combining chemotherapy with ATRA, despite limited and intermittent ATRA exposure. Most importantly, treatment of cancer is usually not done with a single antineoplastic agent, but with a variety of combined chemotherapy regimens, including several anticancer drugs, and other concomitantly administered supportive drugs. Therefore, the possibility exists that another pathway is induced by concomitantly administered drugs that regulates ATRA metabolism and that may be responsible for some form of ATRA resistance.

Pregnane X receptor (PXR; NR1I2), a member of the nuclear receptor family that is activated by many prescription drugs, regulates a large number of genes involved in xenobiotic metabolism, including oxidation, conjugation, and transport; thus, it serves as a generalized xenobiotic sensor that protects the body from chemical challenge. Therefore, the present study was designed to analyze the role of PXR in ATRA metabolism. Due to the species differences in response to PXR ligands, *Pxr*-null, wild-type (mPXR), and *PXR*-humanized transgenic (hPXR) mice were used to examine the effects of various PXR ligands on ATRA metabolism and the possible mechanisms. In this way, a more effective translation of bench research to bedside is envisaged.

Materials and Methods

Chemicals

ATRA, CD437, pregnenolone 16 α -carbonitrile (PCN), rifampicin (RIF), dexamethasone (DEX), and NADPH were purchased from Sigma-Aldrich (St. Louis, MO). 4-OH-RA and 4-oxo-RA were kindly provided by Dr. Hector DeLuca.

Animals and Treatments

Pxr-null mice were generously provided by Dr. Steven Kliewer and *PXR*-humanized transgenic mice were generated as described previously (Ma et al., 2007). Mice were maintained under a standard 12-h light/12-h dark cycle with water and chow provided ad libitum. Handling was in accordance with animal study protocols approved by the National Cancer Institute Animal Care and Use Committee. Eight- to 9-week-old mice were treated with either vehicle (corn oil); PCN, a rodent-specific PXR ligand (10 mg/kg i.p. days 1–3); RIF, a human-specific PXR

ligand (10 mg/kg p.o. days 1–3); or DEX (10 mg/kg i.p. days 1–5). The mice were used in pharmacokinetics study or sacrificed for tissues 24 h after the last dose.

RNA Preparation and Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA from mouse livers was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). qPCR was performed using cDNA generated from 1 μ g of total RNA with the SuperScript II Reverse Transcriptase kit (Invitrogen). Primers were designed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA), and sequences are available upon request. qPCR reactions were carried out using SYBR Green polymerase chain reaction master mix (SuperArray, Frederick, MD) with an ABI Prism 7900HT Sequence Detection System instrument and software (Applied Biosystems). Values were quantified using the comparative CT method, and samples were normalized to β -actin mRNA.

Microsome Preparation and Western Blot Analysis

Liver microsomes were prepared as described previously (Ma et al., 2007). For Western blot analysis, microsomal protein (10 μ g) from each sample was separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membrane, and then it was incubated overnight at 4°C with a 1:1000 dilution of a primary antibody either against rat CYP3A1/2 (monoclonal antibody 2-3-2) or mouse Gapdh (Chemicon International, Temecula, CA). Secondary antibody, goat anti-mouse IgG was purchased from Jackson Immuno-Research Laboratories Inc. (West Grove, PA). Detection of immuno-reactive proteins was done by an enhanced chemiluminescence blot detection system (Pierce Chemical, Rockford, IL).

In Vitro ATRA 4-Hydroxylation

The standard assay mixture contained, in a total volume of 200 μ l, 2 mM NADPH, 100 to 150 μ g of mouse liver microsomal protein in 0.1 M sodium phosphate buffer, pH 7.4, and 2.5, 5, or 10 μ M ATRA. Reactions were initiated by the addition of NADPH at 37°C for 30 min, and they were terminated by the addition of 100 μ l of ice-cold methanol. High-performance liquid chromatography (HPLC) analysis was carried out using a ZORBAX SB-C18 column (3.5 μ m; 150 $\times$ 4.6 mm; Agilent Technologies, Santa Clara, CA). The initial mobile phase was 58% methanol in 10 mM sodium acetate, pH 7.4, at a flow rate of 1 ml/min. After 6.5 min, the methanol concentration was increased linearly to 82% over a 2.5-min time period and continued at 82% for an additional 5 min. Each analysis lasted for 20 min. The detection wavelength for 4-OH-RA was set at 343 nm with UV detector. All manipulations were performed under yellow light since retinoids are light-sensitive.

Pharmacokinetic Analysis of ATRA by LC-MS/MS

Mice were administered 10 mg/kg ATRA orally by gavage 24 h after the last dose of PXR ligands. Blood samples were collected from suborbital veins using heparinized tubes at 15, 30, 60, 120, 180, 240, 300, 360, and 420 min after the administration. Serum samples mixed with CD437 (internal standard) were extracted with ethyl acetate as described previously (Peng et al., 1987). ATRA and CD437 were separated on a Luna 3 μ m C18 50 $\times$ 2 mm column (Phenomenex, Torrance, CA) and detected by liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) using a high-performance liquid chromatography system consisting of a PerkinElmer Series 200 quaternary pump, vacuum degasser, and autosampler with a 100- μ l loop interfaced to an API2000 Sciex triple-quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). The initial mobile phase was 40% acetonitrile in 10 mM ammonium acetate, pH 7.0, at a flow rate of 0.2 ml/min. After 3 min, the acetonitrile concentration was increased linearly to 90% over a 2-min time period and continued at 90% for an additional 4 min. Each analysis lasted for 10 min. The mass spectrometer was equipped with a turbo ion spray source and run in the negative ion mode.

The turbo ion spray temperature was maintained at 300°C, and a voltage of 4.8 kV was applied to the sprayer needle. Nitrogen was used as both turbo ion spray and nebulizing gas. The detection and quantification of analytes were performed using the multiple reactions monitoring mode, with m/z 299.1/246.4 for ATRA and 397.1/347.0 for CD437. The raw data were processed using Analyst Software (Agi-lent Technologies). Pharmacokinetic parameters for ATRA were estimated from the serum concentration-time data by a noncompartmental approach using WinNonlin (Pharsight, Mountain View, CA). The maximum concentration in serum ($C_{\max}$) was obtained from the original data. The area under the serum concentration-time curve (AUC)_{0-∞} was calculated by the trapezoidal rule.

Statistical Analysis

All values are expressed as the means $\pm$ S.D., and group differences were analyzed by unpaired Student's *t* test.

Results

Role of PXR Ligands on the CYP3A Pathway of ATRA 4-Hydroxylation

ATRA is metabolized by P450s via several routes leading to a number of polar metabolites. The most prominent pathway commences with rate-limiting 4-hydroxylation. To investigate the influences of PXR ligands on ATRA metabolism in vitro, hepatic microsomal ATRA 4-hydroxylation was examined by HPLC. Mouse liver microsomes were incubated over a range of ATRA concentrations, in an attempt to represent the range of in vivo concentrations. The detection limit of 4-OH-RA was 3.5 pmol. At a low substrate concentration (2.5 μ M ATRA), 4-OH-RA was not detectable in all the three vehicle-treated mouse lines. However, ATRA 4-hydroxylation was 2.07 ± 0.61 pmol/min/mg protein ($P = 0.04$) in PCN-treated mPXR mice, 3.66 ± 0.17 pmol/min/mg protein ($P < 0.01$) in DEX-treated mPXR mice, 2.84 ± 0.11 pmol/min/mg protein ($P < 0.01$) in RIF-treated hPXR mice, and 3.87 ± 0.33 pmol/min/mg protein ($P < 0.01$) in DEX-treated hPXR mice. At a higher substrate concentration (5 μ M ATRA), ATRA 4-hydroxylation increased 234% in mPXR mice treated with PCN (9.28 ± 1.17 versus 3.96 ± 1.15 pmol/min/mg protein; $P = 0.04$), 239% in mPXR mice treated with DEX (11.32 ± 0.67 versus 4.74 ± 0.99 pmol/min/mg protein; $P = 0.02$), 198% in hPXR mice treated with RIF (8.13 ± 0.43 versus 4.11 ± 0.77 pmol/min/mg protein; $P = 0.02$), and 220% in hPXR mice treated with DEX (8.74 ± 0.38 versus 3.97 ± 1.32 pmol/min/mg protein; $P = 0.04$) compared with the vehicle-treated controls. At the highest concentration used (10 μ M ATRA), the metabolism increased 291% in mPXR mice after PCN treatment (20.30 ± 2.28 versus 6.97 ± 0.09 pmol/min/mg protein; $P < 0.01$), 216% in mPXR mice after DEX treatment (16.01 ± 1.59 versus 7.42 ± 0.50 pmol/min/mg protein; $P = 0.02$), 225% in hPXR mice following RIF administration (18.73 ± 0.63 versus 8.31 ± 0.37 pmol/min/mg protein; $P < 0.01$), and 222% in hPXR mice following DEX administration (14.95 ± 1.47 versus 6.74 ± 1.61 pmol/min/mg protein; $P = 0.03$) relative to the vehicle controls (Fig. 1). Consistent with the increased ATRA metabolism, an up-regulation of mouse Cyp3a protein was noted in mPXR and hPXR mice following PCN, RIF, or DEX treatment (Fig. 2). In agreement with these data, Cyp3a11 mRNA levels were elevated 2.7-fold ($P < 0.01$) in PCN-treated mPXR mice and 2.4-fold ($P = 0.04$) in RIF-treated hPXR mice above the control levels. There was no difference observed in the expression of other phase I enzymes, including the specific ATRA 4-hydroxylases Cyp26a1 and Cyp26b1, in PCN- or RIF-treated mice (Fig. 3). In contrast, PCN and DEX had no significant effect on *Pxr*-null mice, either on ATRA 4-hydroxylation, or on the level of Cyp3a protein.

Role of the CYP26 Pathway of ATRA 4-Hydroxylation

To replicate better the clinical regimen of ATRA administration, mPXR mice were given 10 mg/kg ATRA by gavage daily for 32 days. Liver samples were collected 8 h after the last dose.

At low concentration (2.5 μ M ATRA), ATRA 4-hydroxylation was 2.05 ± 0.36 pmol/min/mg protein in ATRA-pretreated mPXR mice compared with undetectable in the vehicle-treated controls ($P < 0.01$). At higher concentrations (5 μ M ATRA), metabolism increased 171% in mPXR mice pretreated with ATRA (5.01 ± 0.09 versus 2.93 ± 0.16 pmol/min/mg protein; $P < 0.01$) relative to controls. At the highest concentration used (10 μ M ATRA), a slight but not highly significant increase (130%) was noted in mPXR mice after ATRA pretreatment (9.02 ± 0.62 versus 6.95 ± 0.36 pmol/min/mg protein; $P = 0.06$) compared with controls (Fig. 4A). Corresponding to the increased ATRA metabolism, an up-regulation of Cyp26 mRNA expression (3.6-fold for Cyp26a1, $P < 0.01$; and 5.1-fold for Cyp26b1; $P < 0.01$) but not Cyp3a expression was observed in mPXR mice following ATRA pretreatment (Fig. 4, B and C).

Clinical PXR Ligands Decrease Serum Concentrations of ATRA

To verify the clinical implications of PXR ligand-induced ATRA metabolism, in vivo pharmacokinetic studies were performed and samples analyzed by LC-MS/MS. Following a single oral dose of ATRA (10 mg/kg), the plasma level measurable by LC-MS/MS (>0.05 μ M) was observed 15 min after drug ingestion. The $C_{\max}$ of ATRA was generally reached within 1 to 3 h, and high levels of ATRA were sustained during a plateau period lasting up to 3 h; thereafter, the plasma content of ATRA decreased rapidly, and it was below the limit of detection by 7 h. The $C_{\max}$ of ATRA decreased slightly but not significantly in PXR ligand-treated mPXR mice (20% for PCN) and hPXR mice, 36% for RIF and 27% for DEX relative to vehicle-treated controls. Moreover, pretreatment with PXR ligands reduced $AUC_{0-\infty}$ significantly in both mPXR mice (48% for PCN, $P < 0.01$; and 33% for DEX, $P = 0.04$) and hPXR mice (33% for RIF, $P = 0.04$; and 44% for DEX, $P = 0.03$) compared with controls. However, all of these PXR ligands produced no significant change in the pharmacokinetics of ATRA in *Pxr*-null mice. Detailed pharmacokinetic parameters are shown in Table 1 and Fig. 5.

PXR Ligands Induce PXR Target Drug Transporter Genes

To determine the mechanisms of PXR ligand-induced ATRA metabolism, the expression of mRNAs encoding P450s, transporters and nuclear receptors was analyzed by qPCR after inducer treatment. In addition to Cyp3a, an induction of mRNAs encoding PXR target drug transporters was observed in both PCN-treated and DEX-treated mPXR mice compared with controls, including multidrug resistance (Mdr)1a (2.6-fold for PCN, $P < 0.01$; and 2.3-fold for DEX, $P = 0.02$), multidrug resistance protein (Mrp)3 (2.4-fold for PCN, $P = 0.03$; and 1.6-fold for DEX, $P = 0.02$), and organic anion transporting polypeptide (Oatp)2 (2.1-fold for PCN; $P = 0.02$). Expression of the mouse *Pxr* mRNA was also induced (1.4-fold for PCN, $P = 0.03$; and 2.3-fold for DEX, $P < 0.01$). Meanwhile, Mdr1a (1.8-fold for DEX; $P = 0.04$), Mrp3 (2.7-fold for RIF; $P = 0.02$), and Oatp2 (2.5-fold for RIF, $P = 0.04$; and 2.4-fold for DEX, $P = 0.03$) were induced in RIF-treated and DEX-treated hPXR mice compared with controls. Messenger RNA encoding the human PXR was also induced (2.7-fold for RIF, $P = 0.02$; and 9.3-fold for DEX, $P < 0.01$). Conversely, there was no significant induction of any of these mRNAs in *Pxr*-null mice (Fig. 6). To obtain a more global view of ligand effects, other hepatic genes involved in drug metabolism and elimination were also investigated. No significant difference was detected in the mRNAs encoding Cyp1a1, Cyp1a2, Cyp1b1, Cyp2c29, Cyp2c37, Cyp2e1, Cyp4a10, Cyp4a14, Mdr1b, Mrp2, and two cellular retinoic acid-binding protein genes, Crabp1 and Crabp2, in mice treated with these ligands (data not shown).

Dual Effects of DEX on Both PXR-CYP3A and RAR-CYP26 Pathways

To estimate whether the RAR-CYP26 pathway is involved in PXR ligand-induced ATRA metabolism, expression of mRNAs encoding Rar, Rxr, and Cyp26 were also assessed; no difference were found except in DEX-treated mice (Figs. 3 and 7). As expected, DEX induced

mRNA encoding Cyp3a11 in mPXR mice (3.5-fold; $P < 0.001$) and hPXR mice (1.9-fold; $P = 0.03$). In addition, DEX also induced mRNAs encoding Rar (2.2-fold for Rar α , $P = 0.02$; and 2.7-fold for Rar γ , $P = 0.02$), Rxr (1.5-fold for Rxr α , $P < 0.01$; and 1.6-fold for Rxr γ , $P = 0.01$), and Cyp26 (3.0-fold for Cyp26a1, $P < 0.01$; and 2.0-fold for Cyp26b1, $P = 0.01$) subfamilies in mPXR mice, and Rar (1.5-fold for Rar α , $P = 0.01$; and 1.4-fold for Rar γ , $P < 0.01$), Rxr (2.0-fold for Rxr α , $P = 0.04$), and Cyp26 (2.6-fold for Cyp26a1, $P = 0.03$; and 1.7-fold for Cyp26b1, $P = 0.02$) subfamilies in hPXR mice. No induction of these mRNAs were observed in Pxr-null mice (Figs. 3 and 7).

Discussion

Efficient detoxication of harmful xenobiotics is essential to the survival of living organisms, which is also the essence of drug resistance as a self-protective response to some extent. PXR plays a crucial role in this regard, by regulating several superfamilies of genes involved in xenobiotic metabolism and elimination. Upon ligand binding, PXR forms a heterodimer with the RXR and then transactivates target genes, including oxidation enzymes, in particular, P450s and conjugation enzymes, together with transporters such as Pgp, MRPs, and OATPs (Bertilsson et al., 1998; Synold et al., 2001; Guo et al., 2002; Cheng and Klaassen, 2006). The most well known and important PXR target gene is CYP3A subfamily. A variety of PXR ligands activate the transcriptions of CYP3A genes, and, as the most abundant P450s in human liver and intestine, CYP3A enzymes are involved in the metabolism of approximately two thirds of clinically used drugs (Lewis et al., 1998), including ATRA. With this background, the present study was designed to clarify whether the PXR-CYP3A pathway participates concomitant drug-induced ATRA metabolism.

Since the peak plasma levels of ATRA detected in was around 10 μM , a little lower than reported previously, and the levels within many tissues, including the liver, may exceed this concentration (Kalin et al., 1981), the substrate concentrations for in vitro ATRA 4-hydroxylation analysis were set as 2.5, 5, and 10 μM to more accurately approximate in vivo metabolism in vitro. This study revealed that PXR ligands (PCN for mPXR mice and RIF for hPXR mice) induced both expression of Cyp3a and its associated ATRA 4-hydroxylation activity. Conversely, PXR ligands had no effect on Pxr-null mice, either on expression or on activity level of Cyp3a. In addition, in vitro incubations with a panel of recombinant human P450s confirmed the previous report (Marill et al., 2000) that the CYP3A enzymes have the highest catalytic efficiency for ATRA 4-hydroxylation (data not shown). However, the efficiency of these human P450s compared with CYP26 was not examined because there are no commercially available sources of this P450. qPCR mRNA analysis confirmed no significant change in expression of the Cyp26 family and other P450s possibly involved in ATRA 4-hydroxylation in PXR ligand-treated mPXR and hPXR mice, except for DEX-treated mice (discussed below). Also, a previous study demonstrated that pretreatment of Caco-2-T7 cells with RIF increased the metabolism of ATRA to 4-OH-RA and CYP3A expression, whereas it had no effect on CYP26 expression (Lampen et al., 2000). In this study, a chronic ATRA administration model was used to investigate the role of CYP26 in the auto-induced ATRA metabolism. As expected, ATRA treatment induced expression of mRNA encoding Cyp26 together with the up-regulation of in vitro ATRA 4-hydroxylation. However, unlike the induction of ATRA 4-hydroxylation mediated by PXR ligand-activated Cyp3a, the increase caused by ATRA-induced Cyp26 was only statistically significant with 2.5 and 5 μM substrate, not with 10 μM ATRA, suggesting P450s other than CYP26 play critical roles at high concentrations of ATRA. A possible explanation for this finding is that although CYP26 is a specific enzyme for ATRA oxidation, with pharmacologically reasonable K_m of 1 μM (White et al., 1997), it is not expressed at significant levels in a number of tissues, including liver, without ATRA induction, whereas CYP3A represents up to 30 to 60% of total P450 in human liver (Shimada et al., 1994), with a K_m value (2.6 μM) (McSorley and Daly, 2000), comparable

with that of CYP26. This huge difference in abundance means that CYP3A makes an essential contribution to ATRA metabolism in vivo, particularly in PXR ligand-induced ATRA oxidation.

The present pharmacokinetic studies demonstrated that several clinically used PXR ligands induced a hypercatabolic response that reduced serum ATRA concentration and AUC values by up to 30 to 50% compared with vehicle controls. Induction of accelerated catabolism by CYP3A could account for this phenomenon. Few reports have addressed the influences of other drugs on ATRA pharmacokinetics, and this is the first study to evaluate the effects of PXR ligands on ATRA pharmacokinetics. This issue is of potential interest since RIF is a regular prescription drug for tuberculous patients and DEX is also a first-line medicine for treatment of the ATRA syndrome. RIF is a well known PXR ligand that induces CYP3A enzymes. It was reported to increase the metabolism of many drugs in vivo and to increase ATRA metabolism in vitro (Lampen et al., 2000). In patients who experienced the ATRA syndrome and received DEX treatment, a trend was noted for a higher risk of relapse and lower complete remission rate compared with patients who had no ATRA syndrome (De Botton et al., 1998). Although other prognostic factors definitely contributed to this phenomenon, the present finding that DEX decreased serum levels of ATRA indicated that the worse prognosis may be due, at least in part, to DEX-induced ATRA metabolism. A similar drug-drug interaction was also seen in acute lymphocytic leukemia children where etoposide clearance was significantly higher in patients who had recently received a glucocorticoid (prednisone) (Kishi et al., 2004). Considering that etoposide is a substrate of CYP3A and prednisone can induce CYP3A in vitro, this finding supported the view that glucocorticoids can increase the metabolism of CYP3A substrates in vivo. A variety of xenobiotics have been demonstrated as PXR activators, including PCN, DEX, RIF, the human immunodeficiency virus protease inhibitor ritonavir, the anticancer drugs paclitaxel and tamoxifen, the antidiabetic agent troglitazone, the cholesterol-lowering drug SR12813, the sedative glutethimide, and the herbal remedy St. John's wort (Kliwer et al., 2002). Since cancer patients always take multiple drug therapies, PXR activation becomes an important consideration. Furthermore, potential PXR ligands in the nonprescription drugs, herbal or alternative medicines, and foods may also have clinical impact. When these ligands are administered concurrently with ATRA or other CYP3A-metabolized anticancer drugs, activation of PXR may decrease their bioavailability to therapeutically inefficient plasma concentrations. Strategies toward these drug combinations can be managed with appropriate dosage adjustments or with substitutions by other members of the drug class with less potential for PXR activation. One notable example of the latter is the replacement of paclitaxel, a classical PXR ligand, with docetaxel (Synold et al., 2001).

Regulation of CYP3A expression by DEX is demonstrated to occur through two distinct mechanisms (Fig. 8), first by activating PXR under physiological conditions (submicromolar) through the classical glucocorticoid receptor (GR) pathway; and second by directly activating PXR under pharmacological or stress conditions (micromolar or supramicromolar) (Pascussi et al., 2001). Interestingly, in the present study, in addition to activation of the PXR-CYP3A pathway, DEX also induced mRNAs encoding Rar, Rxr, and Cyp26, which was not observed with other ligands tested. As reported previously, submicromolar concentrations of DEX increased both RXR and PXR mRNA expression in human hepatocytes through GR-mediated activation (Pascussi et al., 2000), and CYP26 expression was dependent on RAR and RXR receptors (Sonneveld et al., 1998; Idres et al., 2005). However, the in vivo concentrations of DEX in the current study should be higher than micromolar, and the induction was not observed in *Pxr*-null mice. Promoter analysis for consensus nuclear receptor binding sites was performed using NUBIScan nuclear receptor binding database (<http://www.nubiscan.unibas.ch/>) to determine the possible existence of PXR response elements (DR3, DR4, ER6, and ER8) in the promoter regions of the *Cyp26a1*, *Cyp26b1*, *Rar* γ , *Rxr* α , and *Rxr* γ genes. Further studies are required to whether these sites are functionally relevant.

Besides the major contribution of PXR-activated CYP3A, the current data also highlight the potential roles of several PXR target transporters (*Mdr1a*, *Mrp3*, and *Oatp2*) in ATRA resistance induced by PXR ligands. Although APL cells at diagnosis do not express or express low levels of Pgp, it was reported that during the procedure of multiple relapse, levels of Pgp progressively increased (Candoni et al., 2003; Damiani et al., 2004). Moreover, the Pgp antagonist verapamil (Kizaki et al., 1996) and *MDR1* ribozymes (Matsushita et al., 1998) could restore the sensitivity to ATRA in vitro. Overexpressing the full-length *MRP1* cDNA in neuroblastoma cell line conferred ATRA resistance (Peaston et al., 2001). Meanwhile, among MRP family members, MRP3 has the highest degree of structural resemblance to MRP1. PXR-dependent up-regulation of *Oatp2* and *Cyp3a11* was found to represent an important constitutive response in the hepatic detoxication (Staudinger et al., 2001). Taken together, these data suggest that ATRA might be a substrate for these transporters and that their induction may be responsible in part for ATRA resistance.

In this study, the effects of various PXR ligands on ATRA metabolism and the possible mechanisms were examined using a combination of in vitro and in vivo studies. Overall, our data suggest that acquired resistance to ATRA might be explained in part by the concomitant administration of PXR ligands through activation of the PXR-CYP3A pathway, accelerating the catabolism of ATRA by induced CYP3A. Moreover, other induced PXR target transporter genes might also be involved. Because a variety of compounds, including prescription or nonprescription drugs, herbal or alternative medicines, and even components in foods, have been demonstrated as PXR ligands, this mechanism may have serious clinical impact. An improved understanding of the mechanisms of ATRA resistance holds considerable promise for both the prevention and attenuation of this resistance.

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ABBREVIATIONS

ATRA, all-trans-retinoic acid
 APL, acute promyelocytic leukemia
 RAR, retinoic acid receptor
 RXR, retinoid X receptor
 Pgp, P-glycoprotein
 P450, cytochrome P450
 RA, retinoic acid
 PXR, pregnane X receptor
 mPXR, wild-type mice
 hPXR, *PXR*-humanized transgenic mice
 PCN, pregnenolone 16 α -carbonitrile
 RIF, rifampicin
 DEX, dexamethasone
 qPCR, quantitative real-time polymerase chain reaction
 HPLC, high-performance liquid chromatography
 LC-MS/MS, liquid chromatography-coupled tandem mass spectrometry
 AUC, area under the serum concentration-time curve
 MDR, multidrug resistance
 MRP, multidrug resistance protein
 OATP, organic anion transporting polypeptide

GR, glucocorticoid receptor
 Gapdh, glyceraldehyde-3-phosphate dehydrogenase
 Cont, vehicle control

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Fig. 1.

In vitro hepatic ATRA 4-hydroxylation activity regulated by PXR ligands. mPXR, *Pxr*-null, and hPXR mice were treated with or without PXR ligands: Cont, vehicle control; PCN (10 mg/kg i.p. days 1–3); RIF (10 mg/kg p.o. days 1–3); and DEX (10 mg/kg i.p. days 1–5). Standards ran as follows on HPLC: ATRA, 12.25 min; 4-OH-RA, 9.56 min; and 4-oxo-RA, 8.59 min. Mouse liver microsomes were incubated with 2.5, 5, and 10 μM ATRA for 30 min. Data are expressed as means ± S.D., $n = 2$. *, $P < 0.05$ compared with control; **, $P < 0.01$ compared with control.

Fig. 2.

Hepatic protein levels of Cyp3a regulated by PXR ligands. mPXR, *Pxr*-null, and hPXR mice were treated with or without PXR ligands: Cont, vehicle control; PCN (10 mg/kg i.p. days 1–3); RIF (10 mg/kg p.o. days 1–3); and DEX, (10 mg/kg i.p. days 1–5). Gapdh protein served as a loading control for Western blot.

Fig. 3.

Hepatic mRNA levels of *Cyp3a* and *Cyp26* regulated by PXR ligands. mPXR, *Pxr*-null, and hPXR mice were treated with or without PXR ligands: Cont, vehicle control; PCN (10 mg/kg i.p. days 1–3); RIF (10 mg/kg p.o. days 1–3); and DEX (10 mg/kg i.p. days 1–5). mRNA levels of *Cyp3a11*, *Cyp26a1*, and *Cyp26b1* genes were analyzed by qPCR. Data represent -fold activation relative to vehicle control, means $\pm$ S.D., $n = 3$. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Fig. 4.

Hepatic ATRA 4-hydroxylation activity and levels of Cyp3a and Cyp26 regulated by chronic administration of ATRA. mPXR mice were treated with or without ATRA: Cont, vehicle control; and ATRA (10 mg/kg p.o. days 1–32). Mice were killed at 8 h after the last dose. A, in vitro ATRA 4-hydroxylation detected by HPLC. Mouse liver microsomes were incubated with 2.5, 5, and 10 μ M ATRA for 30 min. Data are expressed as means $\pm$ S.D., $n = 2$. **, $P < 0.01$ compared with control. B, mRNA levels of *Cyp3a11*, *Cyp26a1*, and *Cyp26b1* genes were analyzed by qPCR. Data represent -fold activation relative to vehicle control, means $\pm$ S.D., $n = 3$. **, $P < 0.01$. (C) Expression of mouse Cyp3a protein detected by Western blot. Gapdh protein served as a loading control.

Fig. 5.

Concentration-time curve of serum ATRA. mPXR, *Pxr*-null, and hPXR mice were pretreated with or without PXR ligands: Cont, vehicle control; PCN (10 mg/kg i.p. days 1–3); RIF (10 mg/kg p.o. days 1–3); and DEX (10 mg/kg i.p. days 1–5). Mice were administered 10 mg/kg ATRA orally by gavage 24 h after the last dose of ligands. Blood samples were collected at 15, 30, 60, 120, 180, 240, 300, 360, and 420 min after the administration. Serum was prepared and extracted as described under *Materials and Methods*. In vivo serum levels of ATRA were detected by LC-MS/MS. The analysis was accomplished by multiple reactions monitoring with the transitions m/z 299.1/246.4 for ATRA (retention time, 3.16 min) and 397.1/347.0 for CD437 (retention time, 1.36 min). Data are expressed as means $\pm$ S.D., $n = 3$ at each time point.

Fig. 6.

Effects of PXR ligands on drug transporter and nuclear receptor genes in livers of mPXR, *Pxr*-null, and hPXR mice. Mice were treated with or without PXR ligands: Cont, vehicle control; PCN (10 mg/kg i.p. days 1–3); RIF (10 mg/kg p.o. days 1–3); and DEX (10 mg/kg i.p. days 1–5). mRNA levels were determined by qPCR. Data represent -fold activation relative to vehicle control, means $\pm$ S.D., $n = 3$. *, $P < 0.05$; **, $P < 0.01$.

Fig. 7.

Effects of DEX on *Rar* and *Rxr* subfamilies in livers of mPXR, *Pxr*-null, and hPXR mice. Mice were treated with or without DEX: Cont, vehicle control; and DEX (10 mg/kg i.p. days 1–5). Effects of PCN (10 mg/kg i.p. days 1–3) and RIF (10 mg/kg p.o. days 1–3) were also investigated. mRNA levels were determined by qPCR. Data represent -fold activation relative to vehicle control, means $\pm$ S.D., $n = 3$. *, $P < 0.05$; **, $P < 0.01$.

Fig. 8. Mechanistic representation of the role of the PXR-CYP3A pathway in ATRA metabolism induced by the potential PXR ligands in combined therapy and supportive care.

TABLE 1

Pharmacokinetic parameters of ATRA after oral administration of 10 mg/kg to mice previously administered with PXR ligands

mPXR, *Pxr*-null, and hPXR mice were pretreated with or without PXR ligands. Cont, vehicle control; PCN (10 mg/kg i.p. days 1–3), RIF (10 mg/kg p.o. days 1–3); DEX (10 mg/kg i.p. days 1–5). Blood samples were collected at 15, 30, 60, 120, 180, 240, 300, 360, and 420 min after ATRA administration. In vivo serum levels of ATRA were detected by LC-MS/MS. Pharmacokinetic parameters were estimated from the serum concentration-time data by a noncompartmental approach using WinNonlin software. Data were expressed as means $\pm$ S.D., $n = 3$ at each time point.

Mouse Line	$C_{\max}$	$AUC_{0-\infty}$
	$\mu\text{mol/l}$	$\mu\text{mol} \cdot \text{min/l}$
mPXR		
Cont	10 ± 1.8	2490 ± 15
PCN	8 ± 0.6	$1290 \pm 82^{**}$
DEX	11 ± 1.2	$1670 \pm 22^*$
<i>Pxr</i> -null		
Cont	10 ± 1.8	1820 ± 63
PCN	12 ± 0.9	1700 ± 10
DEX	12 ± 0.3	1940 ± 12
hPXR		
Cont	11 ± 2.1	1970 ± 15
RIF	7 ± 1.1	$1320 \pm 14^*$
DEX	8 ± 2.6	$1110 \pm 13^*$

* $P < 0.05$ compared with control.

** $P < 0.01$ compared with control.