The Traditional Chinese Herbal Remedy Tian Xian Activates Pregnan X Receptor and Induces CYP3A Gene Expression in Hepatocytes

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ABSTRACT:
The pregnane X receptor (PXR, NR1I2) is a member of the nuclear receptor superfamily that is activated by a myriad of clinically used compounds and natural products. Activation of PXR in liver regulates the expression genes encoding proteins that are intimately involved in the hepatic uptake, metabolism, and elimination of toxic compounds from our bodies. PXR-mediated herb-drug interactions can have undesirable effects in patients receiving combination therapy. This can be especially important in cancer patients who self-administer over-the-counter herbal remedies together with conventional anticancer chemotherapeutics. Tian xian is a traditional Chinese herbal anticancer remedy that activates human PXR in cell-based reporter gene assays. Moreover, tian xian alters the strength of interaction between the human PXR protein and transcriptional cofactor proteins. A novel line of humanized PXR mice are described and used here to show that tian xian increases expression of Cyp3a11 in primary cultures of rodent hepatocytes. Tian xian also induces expression of CYP3A4 in primary cultures of human hepatocytes. Taken together, these data indicate that coadministration of tian xian is probably contraindicated in patients undergoing anticancer therapy with conventional chemotherapeutic agents. These data are of particular importance due to the fact that this herbal remedy is currently marketed as an adjunct therapy that reduces the side effects of conventional chemotherapy and is available without a prescription. Future studies should be conducted to determine the extent to which coadministration of this Chinese herbal remedy alters the pharmacokinetic and pharmacodynamic properties of conventional anticancer therapy.

Nuclear receptors comprise a large superfamily of transcription factors that are characterized by a conserved N-terminal zinc-finger type DNA-binding domain and a carboxyl-terminal ligand-binding domain. They are involved in a variety of physiological, developmental, and toxicological processes (Mangelsdorf et al., 1995). Pregnan X receptor (PXR, NR1I2) was first cloned in 1998 by a research group at GlaxoWellcome as a part of an effort to identify new members of the nuclear receptor superfamily based upon homology and the mouse genome sequencing project (Kliewer et al., 1998).

Since then, PXR has been identified in various species, including human, monkey, cow, pig, rabbit, rat, mouse, chicken, fish, and worms (Blumberg et al., 1998; Lehmann et al., 1998; Moore et al., 2002). In mammals, PXR is highly expressed in the major organs that are important in xenobiotic biotransformation including the liver and intestine (Kliewer et al., 1998). Numerous studies show that activation of PXR in the liver and intestine produces increased expression of a group of genes that encode proteins involved in the uptake, metabolism, and elimination of potentially toxic compounds (Staudinger et al., 2001a, b; Maglich et al., 2002; Rosenfeld et al., 2003).

It is well established that PXR is a key regulator of xenobiotic-inducible CYP3A gene expression (Goodwin et al., 2002; Kliewer et al., 2002). In addition, PXR regulates the inducible expression of other genes involved in the metabolism of xenobiotic compounds such as CYP2B, CYP2C, CYP24, glutathione S-transferases, sulfotransferases, and glucuronosyltransferases (Maglich et al., 2002; Sonoda et al., 2002; Wei et al., 2002; Kast et al., 2002; Staudinger et al., 2003). Therefore, PXR activation has a complex nature. Although it protects cells from toxic insults, it also represents the molecular basis for an important class of drug-drug interactions. For example, if one drug activates PXR, it can be predicted that administration of this drug will promote the elimination of other coadministered drugs that are also metabolized and eliminated by PXR-target gene products, thereby reducing the efficacy of many drug therapies in patients receiving combination therapy. Additionally, if one drug is administered as a prodrug, as is the case with certain anticancer therapeutic agents, and a PXR agonist is then coadministered, the resulting increased biotransformation of the prodrug would probably produce profound and unwanted toxic side effects. This
phenomenon is also observed with numerous herbal remedies including St. John’s wort, colexus forskohli, guggulsterone and many others that contain constituents that activate PXR (Staudinger et al., 2006).

Tian xian (also known as tien hsein and pronounced “Dianne Sean”) products are herbal dietary supplements manufactured in China by the China-Japan Feida Union Co., Ltd. (Hong Kong, Republic of China; http://www.cjfu.com/en/main.php). Tian xian products are distributed worldwide and are aggressively marketed as anticancer herbal therapy through several Web sites including http://www.tianxian.co.uk, http://www.cancer-tian-xian.com, http://www.original-tianxian.com, and http://www.tianxian.com. These products are also marketed as herbal therapies that alleviate the unpleasant side effects associated with western-style anticancer treatments (http://www.tianxian.com/products/products.asp#3). The main supportive information regarding their therapeutic efficacy as anticancer agents comes in the form of online testimonials, many of which can be found as Web links to the online distributors of these products (for an example, see http://www.cancer-central.com/).

Currently there are no published studies or clinical trials in the scientific literature establishing the efficacy of these herbal remedies as treatments for cancer or their effectiveness as agents that can reduce the side effects of conventional chemotherapy in patients. However, there are three published studies from one laboratory that were performed at the School of Dentistry in the College of Medicine at National Taiwan University in Taipei, Taiwan, on the biological effects of tian xian liquid (Sun et al., 2004, 2005a,b). The authors concluded that a liquid formulation of tian xian modulates antigen-stimulated cytokine production by T cells isolated from patients with recurrent aphthous ulcerations, inhibits cell growth, and induces apoptosis in a wide variety of human cancer cells in cell-based assays.

Multiple tian xian product lines exist on the market and include several powder formulations contained in gelatin capsules, a liquid extract, a plaster, suppositories, and an ointment (http://www.cjfu.com/en/2_products). A careful examination of the information available on the Web sites reveals that the main product marketed as a treatment for cancer patients is derived from the “original” formulation of Tien-Hsien Capsule No.1. The dosing regimen for this powdered capsule product is three to six capsules three times daily with warm water after meals. The herbal ingredients for tian xian capsule no. 1, their proportions, and the purported therapeutic effects can be found at the manufacturer’s Web site (http://www.cjfu.com/en/2_products/). The herbs and their proportions are listed here: radix trichosanthis (10%), radix clematidis (10%), radix ginseng (15%), radix astragali seu hedysari (10%), jin xing zi (10%), venenum bufonis (3%), radix gentianae (7%), caculus bovis (5%), polyporus umbellatus (10%), and radix pulsatillae (20%). The high potential for herb-drug interactions in patients undergoing conventional chemotherapy is of great concern. We therefore sought to determine the extent to which these agents could potentially alter the pharmacokinetic and pharmacodynamic properties of coadministered cytochrome P-450 substrates.

Here, we use cell-based reporter gene assays and primary cultures of rodent and human hepatocytes to determine the extent to which an extract of tian xian produces alterations in the expression of CYP3A, a clinically important anticancer drug-metabolizing enzyme in liver. We also describe the creation, validation, and use of a novel line of genetically engineered transgenic mice that express a FLAG-tagged human PXR protein selectively in the liver of mice lacking the murine Pxr gene.

Materials and Methods

Animal Care. All rodents were maintained on standard laboratory chow and allowed food and water ad libitum. The studies reported here have been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health (1996).

Compounds and Plasmids. Unless otherwise stated, all chemical compounds were purchased from Sigma-Aldrich (St. Louis, MO). The pSG5-hPXR and the pSG5-mPXR were described previously (Ding and Staudinger, 2005a). The GAL4-SCR1, GAL4-PBP, and GAL4-NCoR1 expression vectors were described previously (Ding and Staudinger, 2005a). The full-length human PXR was fused to the VP16 transcriptional activation domain as described (Ding and Staudinger, 2005a). The pPR-LUC reporter gene, which is responsive to GAL4-fusion proteins is commercially available (BD Biosciences, Palo Alto, CA). The pFLAG-hPXR vector was constructed by excising the human PXR cDNA from pSG5-hPXR using EcoRI and Sall sites and inserting it into pCMV-TAG2B vector (Stratagene, La Jolla CA).

Extraction Preparation. An extract of tian xian capsule no.1 (Green and Gold International, Manila, Philippines) was prepared using one capsule (250 mg of powder) and 1 ml of absolute ethanol. The mixture was placed in a 1.5-ml centrifuge tube and extracted overnight at 4°C on a rotating shaker. The mixture was centrifuged at 16,000g for 5 min. The ethyl alcohol supernatant was decanted and kept at −20°C until use.

Cell Culture and Transient Transfection Analysis. The XREM-LUC reporter gene assays were performed as described (Brobst et al., 2004). The mammalian two-hybrid system analysis was performed as described previously (Ding and Staudinger, 2005a).

Generation of a TTR-FLAG-Tagged hPXR Minigene. The plasmid containing the TTR minigene was digested with Stul and subsequently treated with calf intestinal alkaline phosphatase. The FLAG-tagged human PXR cDNA was excised from pFLAG-hPXR using NotI and XhoI, treated with Klenow DNA polymerase and dNTPs, and ligated together with the Stul-digested pTTR minigene.

Transgenic Mouse Production and Genotyping. The TTR-FLAG-tagged hPXR transgene was excised with HindIII. The resulting 6-kilobase fragment was gel-purified using the QIAEX II (QIAGEN, Valencia, CA) DNA purification kit. The transgene was then injected into single-cell B6C3F1 mouse zygotes. Transgene-positive mice were screened using polymerase chain reaction (PCR). Briefly, a forward primer derived from the TTR promoter (5’ cctggagacagcagctgc 3’) and a reverse primer derived from human PXR (5’ ctctgacttccatcgc 3’) were used to amplify a 424-base pair sequence that would not be present in wild-type mice. Cycling conditions used for the genotyping reactions were as follows: 95°C for 15 s, 65°C for 15 s, and 68°C for 15 s for 35 cycles.

Detection of hPXR Protein and Expression-Profiling of the hPXR Transgene. Approximately 250 mg of liver tissue was homogenized using a Dounce Teflon homogenizer in 3 ml of lysis buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 and protease inhibitors. The homogenate was placed in a centrifuge at 2500g for 10 min. The supernatant was precleared using 20 μl of protein A-agarose. The resulting supernatant was immunoprecipitated using agarose linked to the M2 monoclonal antibody that recognizes the FLAG epitope. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred to a nitrocellulose membrane that was probed with our anti-hPXR antibody.

To determine the expression profile of the FLAG-tagged human PXR transgene, RNA was isolated from the heart, lung, and liver using wild-type and transgenic mice as described (Staudinger et al., 2001a). After DNAse I treatment, 1 μg of RNA was reverse transcribed, and real-time quantitative polymerase chain reaction was performed to detect the human PXR transgene (left primer, 5’ caggaggaaatgtgcagtttt 3’; right primer, 5’ gtaagatcactctgtctgactgacagt; and fluorogenic probe, 5’ eccaatggaccacacacctatgat 3’). All values were normalized to signal from 18S (left primer, 5’ cctggaatgctggctcataa 3’; right primer, 5’ gtccacacgacaaccct 3’; and fluorogenic probe, 5’ cctggatgtggtagggccctg 3’) as described (Staudinger et al., 2003).

“Humanized” PXR Mouse Production. PXR knockout (PXR KO) mice were generated as described previously (Staudinger et al., 2001b). The transgenic mice harboring the FLAG-tagged hPXR minigene were crossed with the PXR KO mice to obtain a mouse line expressing human PXR in a PXR KO mouse background (TTR-hPXR). After successful generation of humanized mice lacking the mouse Pxr gene, the transgenic mice were backcrossed to

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human PXR from GAL4-NCoR (Fig. 2B). These data strongly suggest that the extract of tian xian contains biologically active molecules that modulate PXR-cofactor interactions in cell-based assays. Although this is useful information, activation of human PXR in a reporter gene assay does not always correlate with the ability to activate PXR in the context of hepatocytes.

Construction and in Vivo Hepatic Expression of a FLAG-Tagged Human PXR Minigene in PXR Knockout Mice. The best characterized PXR-target gene in mouse liver encodes the Cyp3a11 enzyme, the heme-containing steroid monooxygenase and functional ortholog of human CYP3A4. We have produced a novel line of transgenic mice in which expression of the FLAG-tagged human PXR cDNA is under the control of an enhancer region isolated from the transthyretin promoter. As shown in Fig. 3A, this transgene drives expression of the FLAG-tagged human PXR transgene in a liver-selective manner. This is consistent with the previous use of the same transthyretin enhancer region in other lines of transgenic mice (Ye et al., 1999). We subsequently crossed this line of mice with the previously described PXR knockout mice (Staudinger et al., 2001b) and then backcrossed these mice into the C57BL/6 line of mice to create a novel strain of mice that are homozygous for the transgene and lack the murine pr gene. Using this strategy we have created a novel line of mice that express the FLAG-tagged human PXR protein in a liver-specific manner in the absence of the murine Prx gene (Fig. 3, B and inset).

Humanized PXR Transgenic Mice Exhibit Species-Specific Responses to Known Species-Specific PXR Activators. It is well known that PXR exhibits a species-specific response to certain CYP3A inducers (Lehmann et al., 1998). Indeed, several humanized PXR transgenic mouse models that are currently being used to assess the potential for drug-drug interactions commercially and in academic laboratory settings have already been developed (Xie et al., 2000; Gonzalez, 2007). The hallmark experiment that determines the utility of these mouse models is the administration of rifampicin, a selective human PXR activator, and pregnenolone-16α-carbonitrile (PCN), a selective mouse PXR activator, to distinguish the functional difference between wild-type and humanized PXR mice.

We therefore administered 10 μM concentrations of these two compounds for 48 h to primary cultures of hepatocytes isolated from wild-type, PXR KO, and humanized PXR mice (Fig. 4A). As expected, PCN induced the expression of Cyp3a11 in wild-type hepatocytes, whereas rifampicin had only a minimal effect. Also as expected, neither rifampicin nor PCN had any effect on the expression of Cyp3a11 in hepatocytes isolated from PXR KO mice. In contrast, treatment of primary cultures of hepatocytes isolated from humanized PXR mice with rifampicin produced marked induction of Cyp3a11 gene expression, whereas treatment with PCN produced only minimal increased expression of this known PXR-target gene.

To determine the extent to which PXR activity is required for induction of Cyp3a11 gene expression we treated primary cultures of hepatocytes isolated from wild-type and PXR KO mice with 10 μM PCN and increasing concentrations of tian xian (4, 31, and 250 μg/ml). Treatment with PCN produced robust and PXR-dependent induction of Cyp3a11 gene expression, whereas treatment with tian xian also increased Cyp3a11 gene expression in a concentration- and PXR-dependent manner (Fig. 4B). Primary cultures of hepatocytes isolated from humanized PXR mice were treated with 10 μM rifampicin and increasing concentrations of tian xian (4, 31, and 250 μg/ml). Tian xian treatment produced increased levels of Cyp3a11 gene expression in a concentration-dependent manner, similar to that obtained with rifampicin, the prototypical human PXR activator (Fig. 4C). These data indicate that compounds contained within the tian
xian extract activate both mouse and human PXR and produce increased expression of a known PXR-target gene, Cyp3a11, in the context of cultured hepatocytes.

**Tian Xian Induces the Expression of CYP3A4 in Primary Cultures of Human Hepatocytes.** The relative expense and low availability of primary cultures of human hepatocytes has recently led to a large effort to find suitable alternatives to test the potential for drug-drug and herb-drug interactions. One of the more positive aspects of the PXR reporter gene assay and the use of humanized mouse models includes the genetic uniformity and technical convenience of both cell-based systems and engineered mouse models. However, the standard of drug metabolism studies required by the Food and Drug Administration in the United States still remains the use of primary cultures of human hepatocytes. We therefore sought to determine the extent to which the expression of the CYP3A4 gene was altered by administration of tian xian. Figure 5 reveals that treatment of primary cultures of human hepatocytes with increasing concentrations of tian xian produced a concentration-dependent increase in the expression of the important drug-metabolizing enzyme CYP3A4.

**Discussion**

It has been nearly 20 years since the identification of drug-inducible members of the CYP3A subfamily of drug-metabolizing enzymes (Aoyama et al., 1989; Bork et al., 1989; Schuetz et al., 1989). It is now well known that induction of CYP3A4 gene expression in liver and intestine at the level of transcription by nuclear receptor proteins produces clinically relevant elevations in enzymatic activity of this extremely important drug-metabolizing enzyme (Kliewer et al., 2002).
It is also well known that both drug-drug and herb-drug interactions can affect the clinical outcome in cancer patients receiving combination therapy (Harmsen et al., 2007). The purpose of this study was to determine the extent to which treatment with tian xian has the potential to produce alterations in the expression and activity of CYP3A4 in human patients. Moreover, we present here a novel humanized PXR mouse model that will undoubtedly be useful for future studies involved in the preclinical testing of candidate drug molecules and additional herbal remedies.

As of publication of this article, there have been two previously described transgenic humanized PXR mouse models. The first transgenic mouse model created used the albumin promoter to drive expression of the human PXR cDNA selectively in liver, but this model subsequently lacked any expression of the transgene in intestine (Xie et al., 2000). To compensate for this lack of intestinal expression, another transgene was engineered using the fatty acid-binding protein promoter, thus generating a bitransgenic mouse model with expression in both liver and intestine (Gong et al., 2006). Recently, another group took a different approach that used a bacterial artificial chromosome containing the entire human PXR gene, including the relevant promoter regulatory sequences, to drive expression of potentially all PXR splice variants in a manner that more closely recapitulates PXR expression in humans (Ma et al., 2007). In the transgenic model we present here we used the transthyretin promoter to express a FLAG-tagged human PXR cDNA selectively in liver and choroid plexus in brain (data not shown) in our PXR KO model, and backcrossed to produce a congenic line of mice containing the C57BL6 genetic background.
Humanized mouse models are becoming extremely important in the preclinical testing of novel drug candidate molecules. By knocking out the rodent pxr gene and replacing it with the human receptor, humanized PXR mouse models have been established as unique tools to dissect the drug-induced xenobiotic response and are aiding the development of safer drugs at an earlier stage of preclinical drug development. These unique mouse models all have the advantage of providing reliable, cost-effective, plentiful, convenient, and genetically uniform systems that can be used to test for potential drug-drug and herb-drug interactions. This is of particular importance in the
herbal remedy industry, as the U.S. Food and Drug Administration does not currently require companies to determine the extent to which their products are safe for coadministration with concurrently used prescription medications.

Tian xian represents only one example of potential herb-drug interactions, but we feel that the experiments presented here are particularly important because this herbal remedy is marketed on the Internet and is available without a prescription as an anticancer therapy to be used in conjunction with “Western” chemotherapeutic agents. This producer of this product line purports to validate its efficacy as an anticancer herbal remedy using on-line “testimonials.” These testimonials tend to be from patients whose cancers are not responding to their conventional chemotherapy, but testify that tian xian coadministration eased their side effects and increased the effectiveness of their chemotherapy. Cancer patients cannot test the validity of these on-line claims before using tian xian, and the experiments presented here do not specifically refute these claims. However, our data strongly suggest that coadministration of tian xian together with conventional chemotherapeutic agents, many of which are indeed substrates of the CYP3A4 enzyme, would probably increase their biotransformation. The potential danger highlighted in this study is that coadministration of tian xian and conventional chemotherapeutic agents would tend to decrease the efficacy of anticancer agents that are metabolized and excreted by PXR-dependent mechanisms. Conversely, coadministration of tian xian with a prodruk that requires bioactivation would probably increase the rate of such a conversion. As is the case with cyclophosphamide and ifosfamide, induction of CYP3A4 activity would probably promote accumulation and possible toxicity because of their narrow therapeutic index. Finally, because the tian xian extract is a complex mixture of compounds, there is possibility that tian xian could simultaneously activate PXR and inhibit cytochrome P450 enzymatic activity. Future researchers should address this issue by examining alterations in cytochrome P450 activity after administration of this herbal remedy.

It is well known that activation of PXR coordinately regulates the expression and activity of multiple drug transporter proteins as well as of numerous other drug-metabolizing enzymes. In addition to CYP3A4, PXR is involved in regulating numerous members of the UDP-glucuronosyltransferase family (Chen et al., 2003; Xie et al., 2003), sulotransferases (Sonoda et al., 2002), drug transporter proteins, and many other enzymes involved in handling oxidative stress in cells (Geick et al., 2001; Maglich et al., 2002; Wei et al., 2002; Staudinger et al., 2003). Future studies will involve determining the extent to which this Chinese herbal remedy modulates the expression and activity of these enzymes in liver using primary cultures of human hepatocytes and the line of transgenic mice described here.

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Brobst DE, Ding X, Creech KL, Goodwin B, Kelley B, and Staudinger JL (2004) Guggulsterone agents would tend to decrease the efficacy of anticancer agents that are metabolized and excreted by PXR-dependent mechanisms. Conversely, coadministration of tian xian with a prodruk that requires bioactivation would probably increase the rate of such a conversion. As is the case with cyclophosphamide and ifosfamide, induction of CYP3A4 activity would probably promote accumulation and possible toxicity because of their narrow therapeutic index. Finally, because the tian xian extract is a complex mixture of compounds, there is possibility that tian xian could simultaneously activate PXR and inhibit cytochrome P450 enzymatic activity. Future researchers should address this issue by examining alterations in cytochrome P450 activity after administration of this herbal remedy.

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