

# Traditional Chinese Medicines Wu Wei Zi (*Schisandra chinensis* Baill) and Gan Cao (*Glycyrrhiza uralensis* Fisch) Activate Pregnane X Receptor and Increase Warfarin Clearance in Rats

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## ABSTRACT

The traditional Chinese medicines (TCMs) are essential components of alternative medicines. Many TCMs are known to alter the expression of hepatic drug-metabolizing enzymes and transporters. The molecular mechanism by which TCMs and/or their constituents regulate enzyme and transporter expression, however, has remained largely unknown. In this report, we show that two TCMs, Wu Wei Zi (*Schisandra chinensis* Baill) and Gan Cao (*Glycyrrhiza uralensis* Fisch), and their selected constituents activate the xenobiotic orphan nuclear receptor pregnane X receptor (PXR). Treatment with TCM extracts and the Schisandrol and Schisandrin constituents of Wu Wei Zi induced the expression of drug-metabolizing enzymes and transporters in reporter gene assays and in primary hepatocyte cultures. The affected enzymes and transporters include

CYP3A and 2C isozymes and the multidrug resistance-associated protein 2. In transient transfection and reporter gene assays, the Schisandrin constituents of Wu Wei Zi had an estimated EC<sub>50</sub> of 2 and 1.25 μM on hPXR and mPXR, respectively. Interestingly, mutations that were intended to alter the pore of the ligand-binding cavity of PXR had species-specific effects on the activities of the individual Schisandrols and Schisandrins. In rats, the administration of Wu Wei Zi and Gan Cao increased the metabolism of the coadministered warfarin, reinforcing concerns involving the safe use of herbal medicines and other nutraceuticals to avoid PXR-mediated drug-drug interactions. Meanwhile, the activation of PXR and induction of detoxifying enzymes provide a molecular mechanism for the hepatoprotective effects of certain TCMs.

The traditional Chinese medicines (TCMs) are essential components of alternative medicines. However, the increased and indiscriminate use of natural products, including TCMs, has been associated with adverse effects, such as hepatotoxicity and drug-drug interactions (Kaplowitz, 1997). One important clinical implication of herbal product use is the effect

of herbal products on the metabolism of coadministered drugs. Herbal products are known to alter hepatic drug-metabolizing enzymes directly (Benson et al., 1978; Letteron et al., 1990) and also interact with several drugs (Piscitelli et al., 2000; Ruschitzka et al., 2000; Yue et al., 2000).

Many TCMs, especially those that exhibit hepatoprotective, detoxifying, and antioxidative effects, are known to alter CYP enzyme activities. For example, the general CYP content and the metabolic rate of imipramine were enhanced significantly in the liver microsomes obtained from the rats pretreated with Gan Cao (*Glycyrrhiza uralensis* Fisch) and Wu Wei Zi (*Schisandra chinensis* Baill) (Hu et al., 1999; Lin et al., 1999; Zhu et al., 2000). St. John's Wort has been known to induce CYP3A enzymes in the liver and lead to an increased clearance of several drugs, such as cyclosporine, digoxin, and theophylline (Ruschitzka et al., 2000). Although

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**ABBREVIATIONS:** TCM, traditional Chinese medicine; PXR, pregnane X receptor; RIF, rifampicin; PCN, pregnanenolone-16α-carbonitrile; RT, room temperature; HPLC, high-performance liquid chromatography; MRP2, multidrug resistance-associated protein 2; LBD, ligand-binding domain; rPXR, rat PXR; UAS, upstream activating sequence; tk, thymidine kinase; P450, cytochrome P450.

inducing CYP3A at low concentrations, hyperforin, a component in St. John's Wort, has the potential to inhibit CYP3A metabolism at high concentrations (Komoroski et al., 2004). Several studies have also suggested that hyperforin or St. John's Wort can also affect the drug transporter multidrug resistance protein 1/P-glycoprotein (Dresser et al., 2003; Weber et al., 2004). Dan-Shen (*Radix salviae Miltiorhizae*), a TCM herb, has been shown to alter the pharmacokinetics of warfarin, a CYP2C substrate (Lo et al., 1992). In addition to enzyme activation, there are also reports that certain TCM components, such as the isoflavane glabridin from Gan Cao, were able to inhibit the activities of human CYP3A4, 2B6, and 2C9 in in vitro reconstituted enzymatic assays (Kent et al., 2002). The molecular mechanism(s) by which TCMs regulate enzyme expression remains largely unknown.

The orphan nuclear receptor pregnane X receptor (PXR; NR1I2) was initially characterized as a xeno-sensor regulating the expression of the human and rodent CYP3A enzymes (Blumberg et al., 1998; Kliewer et al., 1998; Xie et al., 2000a). Combinations of molecular biology, mouse genetics, and drug metabolism studies have since established PXR as a master xenobiotic receptor in the regulation of phases I and II enzymes, as well as members of the drug transporter family. This broad regulation is due to the presence of PXR-responsive elements in the promoter regions of many xenobiotic enzyme and transporter genes (for reviews, see Sonoda et al., 2003; Xie et al., 2004; Zhou et al., 2005). Herbal medicines, such as St. John's wort, have been shown to activate PXR (Moore et al., 2000), but it remains to be seen whether TCM can activate PXR and, if so, whether the TCM-mediated PXR activation can impact drug metabolism. For example, PXR is known to regulate CYP2C9 (Chen et al., 2004), an important human CYP2C isoform responsible for the metabolism of the anticoagulant warfarin (Goldstein and de Morais, 1994). It is interesting to know whether PXR-activating herbs can increase warfarin clearance in vivo.

Xenobiotic induction of drug-metabolizing enzymes exhibits striking species specificity. For example, the antibiotic rifampicin (RIF) has been shown to be a CYP3A inducer in humans, but not in rodents, whereas pregnenolone-16 $\alpha$ -carbonitrile (PCN) is a rodent-specific CYP3A inducer (for review, see Xie et al., 2004). Accumulating evidence has established xenobiotic receptors, such as PXR, as the important cellular factors in determining this species specificity. The species specificity has been thought to be due to the divergence of amino acid sequences in the ligand-binding domains of the human and mouse PXR receptors. The hypothesis that the species origin of the receptor is the determining factor for the species specificity of the ligand response led us to create the "humanized" mice in which the mPXR was genetically replaced by its human counterpart and thus exhibiting a humanized drug response profile (Xie et al., 2000a). The identification of PXR and establishment of PXR-mediated cell culture and animal assay systems allow a direct evaluation of whether a candidate PXR agonist has a preference to the species origin of the PXR receptor (for review, see Xie et al., 2004).

In this report, using combinations of transient transfections and human hepatocyte cultures, we report the identification of the TCM herbs Wu Wei Zi and Gan Cao as PXR agonists. Both Wu Wei Zi and Gan Cao total extracts and several Wu Wei Zi constituent compounds activated PXR and

induced the expression of drug-metabolizing enzymes and transporters. Administration of Wu Wei Zi and Gan Cao extracts in rats resulted in an increased metabolism of warfarin. Our results suggest that caution should be taken when using TCM herbs to avoid unwanted excessive PXR activation and the resultant drug-drug interactions.

## Materials and Methods

**TCM Herbs, Extract Preparation, and Chemicals.** All TCM herbs were purchased from Evergreen Herbs and Medical Supplies, LLC (Carson City, NV). All herbs were imported from Taiwan with complete quality control certifications. Dry herbs were minced with a Cuisinart blender (model CBT-500W; Cuisinart, East Windsor, NJ). For the preparation of the aqueous extracts used for cell cultures, 50 g of minced dry herbs was boiled in 200 ml of endotoxin-free injectable water (NDC 03380013-04; Baxter, McGaw Park, IL) for 40 min. The supernatants were transferred to 50-ml conical tubes and spun at 3000 rpm for 30 min at room temperature (RT). The supernatants were collected in new tubes and stored at  $-20^{\circ}\text{C}$  until use. For ethanolic extract preparation, 50 g of dry herbs was mixed with 75 ml of 80% ethanol and rocked for 24 h at RT. The extracts were then spun at 3000 rpm for 30 min at RT, and the supernatants were collected and stored at  $-20^{\circ}\text{C}$  until use. For the preparation of the aqueous extracts used for in vivo studies, 10 g of herbs was boiled in 50 ml of water for 30 min and filtered. The residual herbs were added with 20 ml of water and boiled for another 15 min. The two extracts were then combined and evaporated to a concentration equivalent to 0.5 g dry herb/ml extract. The Gan Cao constituents were purchased from Sigma-Aldrich (St. Louis, MO). The Wu Wei Zi constituents were obtained from the Chinese National Center for TCM Quality Control. The quality of the Wu Wei Zi constituents was confirmed by high-performance liquid chromatography (HPLC). The Wu Wei Zi and Gan Cao constituents were also used for the quality control of their respective extracts by HPLC (data not shown).

**Plasmids and Transient Transfection.** The reporter plasmids (tk-UAS-Luc, tk-CYP3A4-Luc, tk-CYP3A23-Luc, and tk-MRP2-Luc) and the expression vectors [Gal-hPXR ligand-binding domain (LBD) and Gal-mPXR LBD] have been described previously (Saini et al., 2004; Mu et al., 2005). All reporter genes contain three copies of the corresponding response element. The tk-CYP2C9-Luc reporter contains three copies of the DR-4 type of PXR response element that was reported for the human CYP2C9 gene (Chen et al., 2004). The creation of rat PXR (rPXR) F305L and hPXR L308F mutants (gifts from Dr. Richard Kim, Vanderbilt University, Nashville, TN) was described previously (Tirona et al., 2004). Both mutant receptors have been successfully used in previous studies (Tirona et al., 2004; Mu et al., 2005). Transfections were performed on 48-well plates. CV-1 and HepG2 cells were, respectively transfected using Lipofectamine 2000 and the polyethylenimine polymer as described previously (Xie et al., 2000b; Mu et al., 2005). The transfected cells were treated with the appropriate compounds for 24 h before harvesting and assaying for luciferase activity. Luciferase activity was normalized against the cotransfected and  $\beta$ -galactosidase activities. All transfections were performed in triplicate.

**Human and Mouse Primary Hepatocyte Preparation and Treatment.** Human livers were obtained through the liver tissue procurement and distribution system, and hepatocytes were isolated via a three-step collagenase perfusion (Strom et al., 1996). Mouse hepatocytes were also prepared by collagenase perfusion. Cells were plated on a gelatin-coated T25 flask and maintained in hepatocyte maintenance medium (Cambrex Bio Science Walkersville, Walkersville, MD) supplemented with 0.1 mM dexamethasone, 0.1 mM insulin, 50  $\mu\text{g}/\text{ml}$  gentamicin, and 50 ng/ml amphotericin, and incubated overnight. Cells were treated with the appropriate drugs for 24 h before RNA harvesting and subsequent Northern blot analysis.

**Northern Blot Analysis.** Total RNA was isolated from mouse tissues or primary hepatocytes using the TRIzol reagent (Invitrogen, Carlsbad, CA). Northern blot analysis was performed as described previously (Xie et al., 2000a).

**Pharmacokinetic Study and HPLC Analysis of Warfarin.**

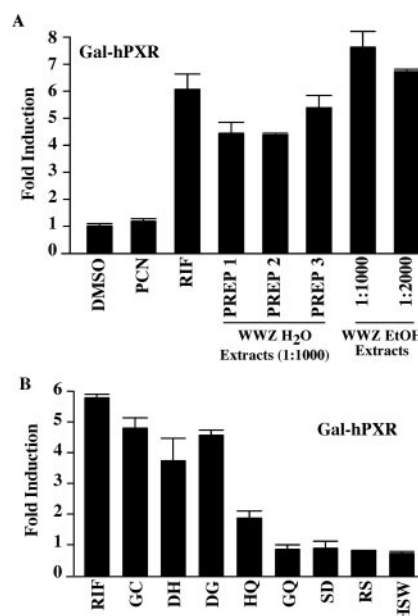
Male Sprague-Dawley rats (~230 g) were randomly divided into three groups. Two groups of rats received a daily gavage of Wu Wei Zi (500 mg/kg) or Gan Cao (900 mg/kg), and the third group received water, as a control, for 6 days. A single i.v. injection of warfarin (2 mg/kg) was given 12 h after the last dose of Wu Wei Zi or Gan Cao, and blood samples were collected 1, 2, 4, 7, 10, 15, 24, 36, 48, and 72 h following warfarin administration. Blood samples were collected by implanting a cannula into the right jugular vein the day before warfarin administration. Urine samples were collected from 0 to 24 h for estimating urinary drug recovery. Blood samples were collected in EDTA-coated tubes and centrifuged immediately for the collection of plasma. Both the plasma and urine samples were stored at -20°C before the drug assay. The warfarin concentration was measured using a modified HPLC method (Zhu et al., 2000). Phenacetin was added to the plasma as an internal standard. The plasma was extracted with chloroform twice, and the final chloroform layer was collected and evaporated to dryness at 45°C with a Concentravap Concentrator (Labconco, Kansas City, MO). The residues were reconstituted with 100  $\mu$ l of the mobile phase (25:75, acetonitrile:20 mM phosphate buffer solution in 0.3% triethylamine, pH 6.5) before injecting 80  $\mu$ l onto the HP-1050 HPLC with an HP 1050 UV detector (Hewlett Packard Analytical Direct, Wilmington, DE). Separation was achieved using an Alltech Spherisorb ODS-2 column (5, ID 4.6  $\times$  250 mm) with a Waters guard cartridge S5 ODS2 (4.6  $\times$  10 mm; Waters, Milford, MA) with a mobile phase flow rate of 0.9 ml/min. UV detection was at 308 nm for warfarin and 254 nm for phenacetin. The retention times of warfarin and phenacetin were 7.8 and 9.8 min, respectively (data not shown). The warfarin method is linear for plasma concentrations from 0.125 to 10.00  $\mu$ g/ml with a correlation coefficient of linearity ( $r^2$ ) of 0.999 (data not shown) and coefficients of variation of 5% or less. The pharmacokinetic parameter estimations were performed with WIN NONLIN software using noncompartmental analysis method. The steady-state volume of distribution equals total amount of drug in the body divided by the drug blood concentration.

## Results

**Aqueous and Ethanolic Extracts of Wu Wei Zi and Gan Cao Activate PXR.**

The effects of Wu Wei Zi and Gan Cao extracts on PXR were evaluated with a chimeric receptor in which the LBD of hPXR was fused to the DNA binding domain of the yeast transcription factor Gal4. The activity of PXR was determined using a Gal4-responsive reporter, tk-UAS-Luc, that contains three copies of the Gal4 binding site upstream-activating sequence (UAS) upstream of a minimal thymidine kinase (tk) gene promoter. In this PXR ligand assay, both the aqueous and ethanolic extracts of Wu Wei Zi activated Gal-hPXR when applied at a concentration of 1:1000. At this concentration, the ethanolic extracts were slightly more potent than the aqueous extracts but both showed potencies similar to that of RIF (10  $\mu$ M), a prototypical hPXR agonist (Fig. 1A). Three independent preparations of Wu Wei Zi aqueous extracts caused similar -fold activations of the reporter gene (Fig. 1A), consistent with the quality control HPLC results, which showed that these extracts contained similar concentrations of Wu Wei Zi signature compounds (data not shown).

We then evaluated the potential hPXR activation by an additional panel of TCM herbs, using the same Gal-hPXR transfection system. As shown in Fig. 1B, the aqueous ex-



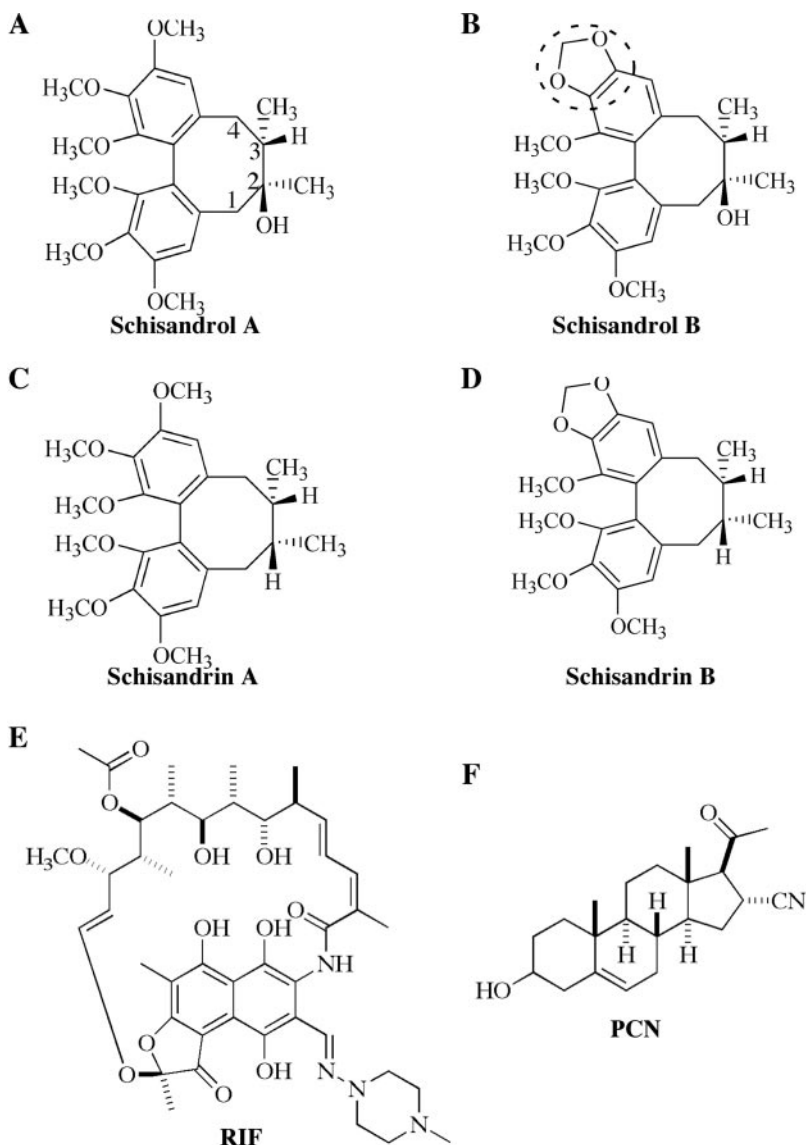
**Fig. 1.** Ethanolic and aqueous extracts of Wu Wei Zi and Gan Cao activate PXR. CV-1 cells were cotransfected with the Gal-hPXR LBD and the tk-UAS-Luc reporter and treated with the indicated drugs for 24 h before the luciferase assay. A, three independent aqueous extracts of Wu Wei Zi were evaluated for hPXR activation using the Gal-hPXR transfection system. The effect of the ethanolic extract was also evaluated. B, effects of Gan Cao and a panel of TCM herbs on hPXR activation were evaluated. Results are shown as -fold induction over vehicle control and represent the average and S.E. from triplicate assays. All herb extracts in B were applied at 1:500 dilution. WWZ, Wu Wei Zi; GC, Gan Cao; DH, Da Huang; DG, Dang Gui; HQ, Huang Qi; GQ, Gou Qi; SD, Shu Di; RS, Reng Shen; HSW, He Shou Wu. The concentration of PCN and RIF is 10  $\mu$ M.

tracts of Gan Cao, Da Huang (*Rhei rhizoma*), and Dang Gui (*Radix angelicae Sinensis*) showed 4- to 5-fold increases in reporter gene activation when applied at a 1:500 dilution. Huang Qi (*Radix astragali*) showed a modest activity, whereas Gou Qi (*Fructus lycii*), Shu Di (*Epimedium macranthum*), Reng Shen (*Radix ginseng*), and He Shou Wu (*Radix polygoni Multiflori*) were ineffective (Fig. 1B).

**Identification of PXR-Activating Constituents from Wu Wei Zi.**

Many constituent chemicals of TCMs have been purified, although whether or not these constituents are responsible for the therapeutic effects of their respective parental herbs is not clear. To gain insight into PXR activation by Wu Wei Zi, four purified Wu Wei Zi constituents, Schisandrols A and B and Schisandrins A and B, were tested for their ability to activate hPXR. The carbon scaffolds of these Wu Wei Zi constituents are distinct from the chemical structures of known PXR agonists, such as the hPXR-specific RIF and the rodent-specific PCN (Fig. 2). In addition to the Gal-hPXR transfection system, we also used the wild-type hPXR and the PXR-responsive tk-CYP3A23-Luc reporter to evaluate the PXR ligand effect. The tk-CYP3A23 reporter contains three copies of the DR-3 type of PXR response element from the rat CYP3A23 gene (Blumberg et al., 1998). Ligand-dependent PXR activation is known to have species specificity (Xie et al., 2000a). The potential species preference was evaluated by comparing the reporter activity elicited by Gal-hPXR with that elicited by Gal-mPXR.

The efficacy of Wu Wei Zi constituents as hPXR agonists was measured and compared with RIF. As shown in Fig. 3A, Schisandrol B and Schisandrins A and B activated Gal-hPXR

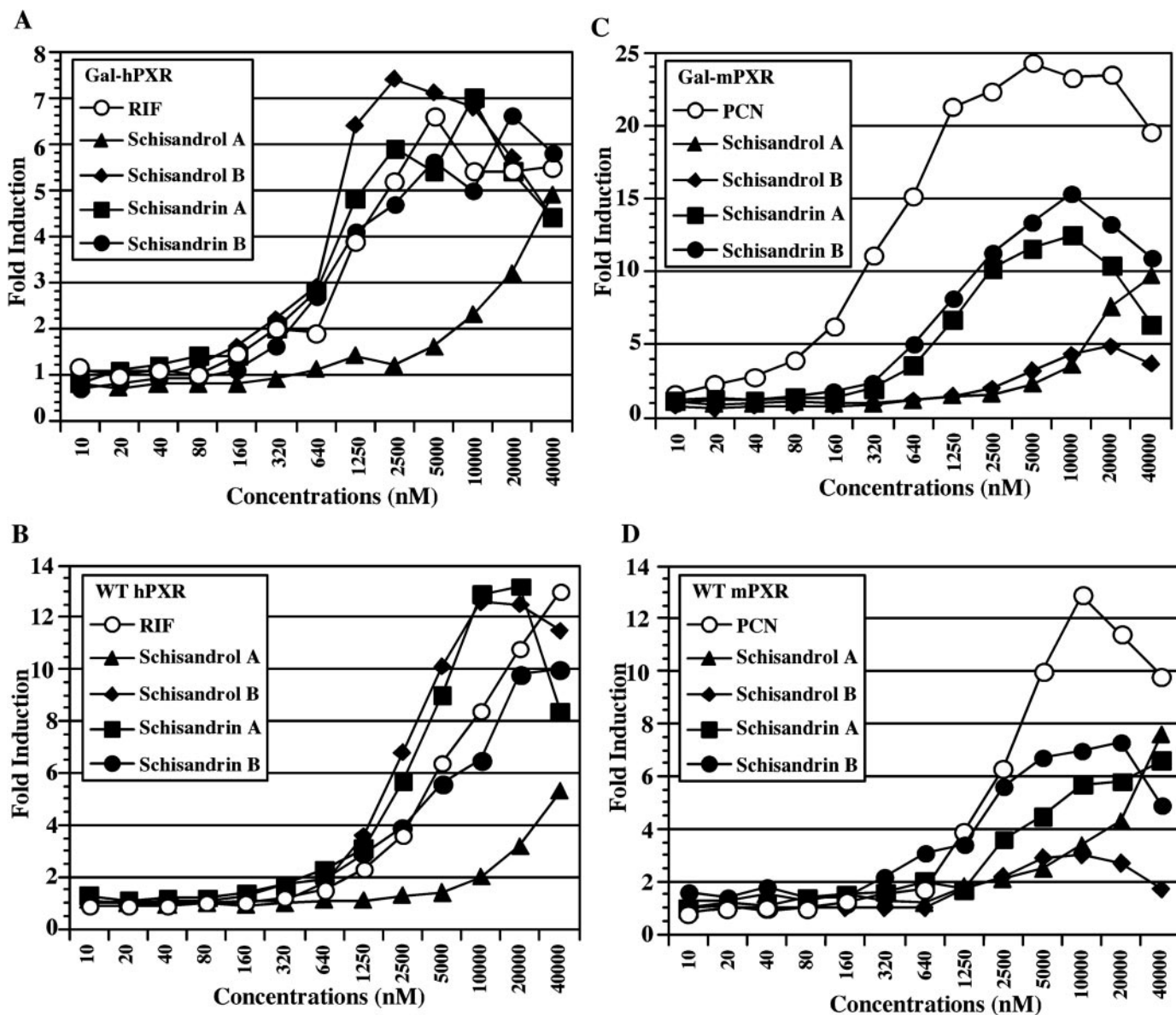


**Fig. 2.** Chemical structures of Wu Wei Zi constituents. The constituent structures (A–D) are compared with those of RIF (E) and PCN (F). The circle in B represents the dioxole structure.

in a concentration-dependent manner, with potencies and efficacies similar to those of RIF. The  $2 \mu\text{M}$   $\text{EC}_{50}$  of RIF is consistent with our previous report (Mu et al., 2005). In contrast, Schisandrol A had little activity at  $10 \mu\text{M}$  but was able to achieve 5-fold reporter gene activation at  $40 \mu\text{M}$ . A similar pattern of PXR activation was seen when the wild-type hPXR and the tk-CYP3A23-Luc reporter were used (Fig. 3B). The discrepancies in the -fold inductions of Gal-hPXR (maximal 5–7-fold) and the wild-type hPXR (maximal 10–13-fold) might be due to the differences in the sensitivity between these two reporter systems. The ability of Wu Wei Zi constituents to activate Gal-mPXR was compared with that of PCN, a prototypical mPXR-specific agonist. Schisandrins A and B were better mPXR activators compared with Schisandrols A and B, although the Schisandrins are not as potent as PCN (Fig. 3C). The  $\text{EC}_{50}$  of Schisandrins A and B was estimated to be  $1.25 \mu\text{M}$  compared with  $500 \text{ nM}$  for PCN. The superior potency and efficacy of PCN, compared with Schisandrins A and B, was also confirmed with the mPXR/tk-CYP3A23-Luc transfection system (Fig. 3D). The -fold inductions in the Gal-mPXR transfection system tend to be higher than those in the wild-type mPXR transfection sys-

tem. The Wu Wei Zi constituents' activities were PXR-specific because none of the four Wu Wei Zi constituents were effective in activating the orphan nuclear receptor peroxisome proliferator-activated receptor  $\gamma$  (data not shown). Interestingly, although the aqueous extract of Gan Cao activated Gal-hPXR, none of the four tested Gan Cao constituents,  $18\alpha$ -glycyrrhetic acid,  $18\beta$ -glycyrrhetic acid, isoliquiritigenin, and glycyrrhizin, are effective hPXR agonists (data not shown).

**TCMs Induce the Expression of Drug-Metabolizing Enzymes and Transporters in Hepatocyte Cultures and in Reporter Gene Assays.** The activation of PXR and induction of the CYP3A reporter gene (Fig. 3B) suggest that TCMS and their constituents may induce hepatic CYP3A enzyme expression. As expected, treatment of primary human hepatocytes (from donor HH1051) with the ethanolic extracts of Wu Wei Zi, but not Ling Zhi (*Ganoderma*), Jiang Huang (*Rhizoma curcumae Longae*), or Ren Shen, induced the mRNA expression of CYP3A4 as revealed by Northern blot analysis (Fig. 4A). The lack of CYP3A4 induction by Ling Zhi, Jiang Huang, and Ren Shen was consistent with the inability of these TCM herbs to activate PXR in the ligand



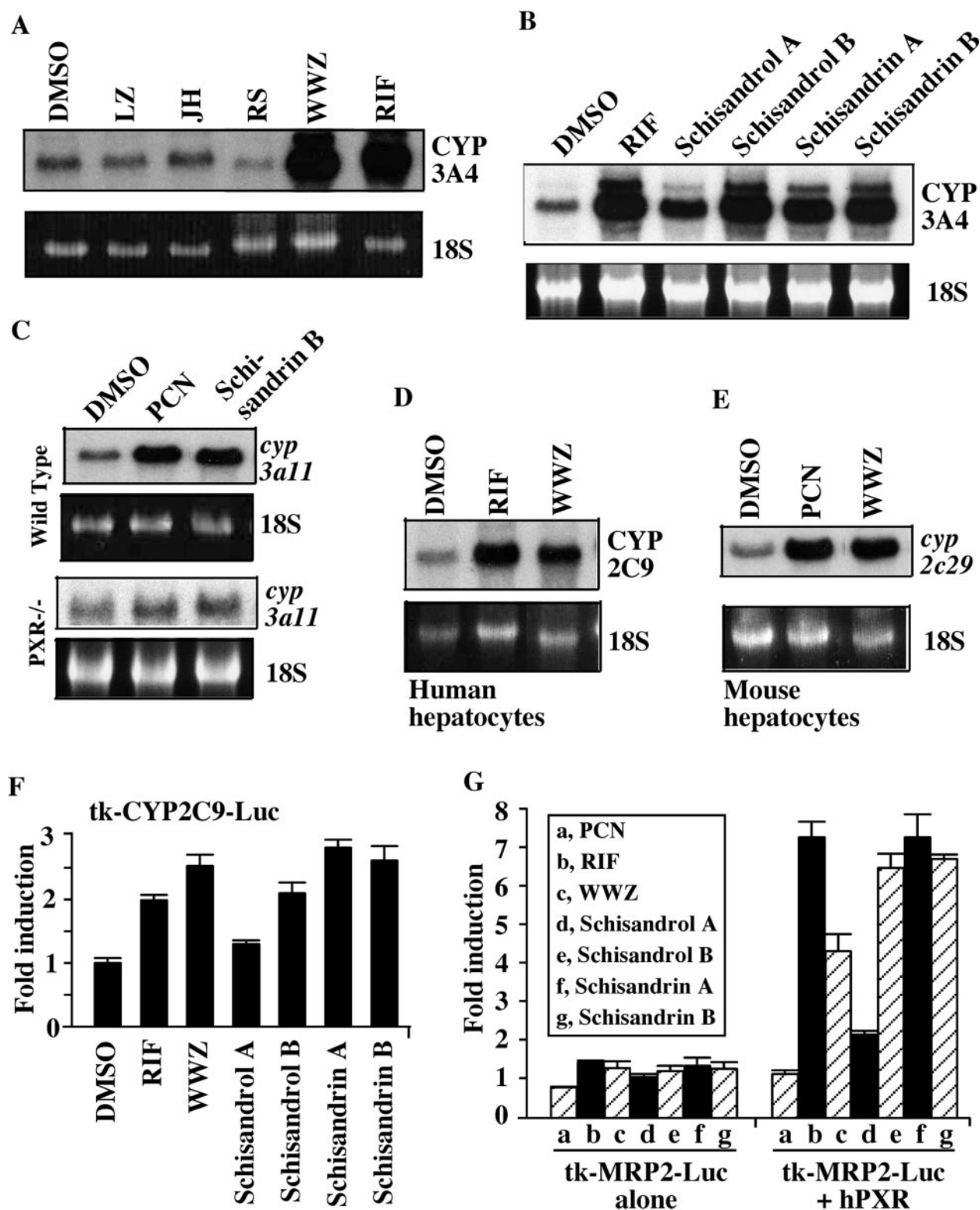
**Fig. 3.** Identification of PXR-activating constituents from Wu Wei Zi. A, Schisandrol B and Schisandrins A and B, but not Schisandrol A, are effective in activating the Gal-hPXR LBD. CV-1 cells were cotransfected with the Gal-hPXR LBD and the tk-UAS-Luc reporter before treatments with increasing concentrations of individual constituents or RIF. Results are shown as -fold induction over vehicle control and represent the average from triplicate assays. B, same pattern of hPXR activation was seen when the wild-type hPXR and a PXR-responsive tk-CYP3A23-Luc reporter gene were used to transfect CV-1 cells. C, Schisandrins A and B, but not Schisandrins A and B, are effective in activating the Gal-mPXR LBD. D, same pattern of mPXR activation was seen when the wild-type mPXR and tk-CYP3A23-Luc reporter gene were used to transfect CV-1 cells.

assays (Fig. 2B; data not shown). When the Wu Wei Zi constituents were analyzed, Schisandrol B and Schisandrins A and B were more efficient than Schisandrol A in inducing CYP3A4 mRNA expression, consistent with the transfection results (Fig. 4B).

When the mouse hepatocytes were used, Schisandrin B induced CYP3A11 expression in the wild-type mouse hepatocytes, as did the positive control PCN, as expected (Fig. 4C). In contrast, this induction was abolished in the PXR null hepatocytes (Fig. 4C), demonstrating that PXR is necessary for the induction of CYP3A by the Wu Wei Zi constituent Schisandrin B.

Wu Wei Zi extracts also induced CYP2C9 (Fig. 4D) and *cyp2c29* (Fig. 4E) mRNA expression in human and mouse hepatocytes, respectively. CYP2C9 is an important human

CYP2C isoform responsible for the metabolism of the anticoagulant warfarin (Goldstein and de Morais, 1994). The mouse *cyp2c29* is highly homologous to CYP2C9 (Matsunaga et al., 1994). Activation of PXR by Wu Wei Zi and its constituents induced the expression of tk-CYP2C9-Luc (Fig. 4F) and tk-MRP2-Luc (Fig. 4G), two reporter genes that contain the direct repeat spaced by four nucleotides (DR-4) and everted repeat spaced by eight nucleotides (ER-8) type of PXR response element derived from the human CYP2C9 (Chen et al., 2004) and rodent MRP2 gene (Kast et al., 2002), respectively. The concurrent regulation of CYP3A, 2C, and MRP2 suggests that Wu Wei Zi and Gan Cao have a broad effect on the expression of drug-metabolizing enzymes and transporters, consistent with the notion that PXR functions as a master regulator of the xenobiotic responses (Xie et al., 2004).

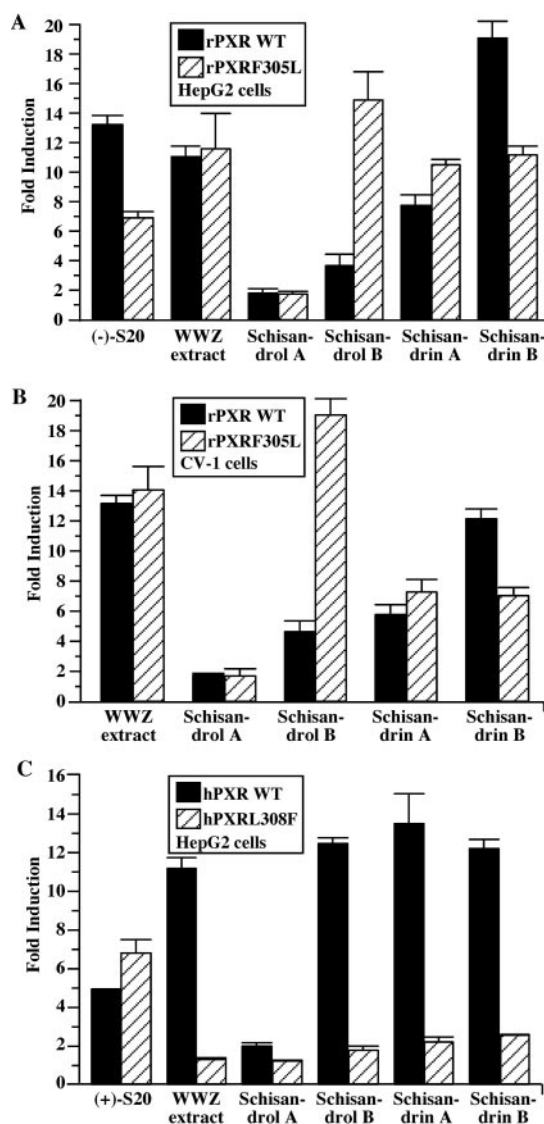


**Fig. 4.** TCMs induce the expression of drug-metabolizing enzymes and transporters in hepatocyte cultures and in reporter gene assays. **A**, treatment with extracts of Wu Wei Zi (WWZ), but not Ling Zhi (LZ), Jiang Huang (JH), and Ren Shen (RS), induced the mRNA expression of CYP3A4 in primary human hepatocytes. The cells were treated with the compounds for 24 h before harvesting for total RNA and Northern blot analysis. The ethidium bromide staining of the agarose gel (18S RNA) shows the equal loading. RIF was included as a positive control for CYP3A4 induction. **B**, Schisandrol B and Schisandrins A and B, but not the Schisandrol A, are effective in inducing CYP3A4 mRNA expression in primary human hepatocytes. **C**, induction of CYP3A11 by Schisandrin B in mouse hepatocytes is abolished in PXR null mice. PCN is included as a positive control for CYP3A11 induction in wild-type hepatocytes and loss of induction in PXR null hepatocytes. **D** and **E**, Wu Wei Zi (WWZ) extracts induce the mRNA expression of CYP2C9 (**D**) and *cyp2c29* (**E**) in the human and mouse hepatocytes, respectively. RIF and PCN were included as positive inducer controls. **F** and **G**, Wu Wei Zi (WWZ) extract and its selected constituents activate CYP2C9 and MRP2 reporter gene. CV-1 cells were cotransfected with the PXR-responsive tk-CYP2C9-Luc (**F**) or tk-MRP2-Luc (**G**) reporter and the hPXR expression vector. Transfected cells were treated with the indicated extract or individual compound. Results are shown as -fold induction over vehicle control and represent the average from triplicate assays.

### Species-Specific Structural Determinants of PXR in Its Activation by Wu Wei Zi and Its Constituents.

We used site-directed mutagenesis to investigate the structural determinants of PXR in its activation by Wu Wei Zi. It was reported that the Phe305 of rPXR and its human counterpart, Leu308, are critical in determining the species-specific ligand activation (Tirona et al., 2004). Both residues are located within, or are neighboring, the flexible loop that forms part of the pore to the ligand-binding cavity (Tirona et al., 2004). The LBD of rPXR exhibits 98% amino acid homology with that of mPXR, and the Phe305 is conserved in both species (Zhang et al., 1999). We then used rPXR F305L and hPXR L308F mutants to determine whether the same residues are also important for the activation of PXR by Wu Wei Zi and/or its constituents. In this assay, cells were cotransfected with the PXR-responsive reporter tk-CYP3A23-Luc, along with the wild type or a mutant PXR. As shown in Fig. 5A, in HepG2 cells, the rPXR F305L mutation did not alter the activation by the Wu Wei Zi extract. In contrast, the constituent activation profile has changed in that the activation by Schisandrol B increased, whereas the activity of Schisandrin B decreased. Schisandrol A remained inactive with the F305L mutation, and the activity of Schisandrin A remained largely unchanged. This change of the activation pattern may account for the intact F305L activation by the Wu Wei Zi extract. The same effect of the rPXR F305L mutation was confirmed in the kidney-originated CV-1 cells (Fig. 5B). The hPXR L308F mutation completely abolished its activation by either the Wu Wei Zi extract or its constituents (Fig. 5C). We conclude that the effect of the F305L mutation on rPXR activation is Wu Wei Zi constituent-specific, whereas L308 is indispensable for hPXR activation by Wu Wei Zi and its constituents. In these assays, (+)-S20 and (-)-S20 enantiomers were included as the control ligand for hPXR and mPXR, respectively. We have recently reported that S20, a synthetic *C*-cyclopropylalkylamide, is a PXR agonist with species stereoselectivity. The (+)-S20 enantiomer prefers to activate hPXR, and its potency of activation was increased on the hPXR L308F mutant. In contrast, the (-)-S20 enantiomer prefers to activate rPXR, and its activity reduced when the rPXR F305L mutant was used (Mu et al., 2005).

**Wu Wei Zi and Gan Cao Induce Total P450 and Increase Warfarin Metabolism in Rats.** To investigate the *in vivo* induction of P450 by TCMS, Sprague-Dawley rats were gavaged with a single dose of water or an aqueous extract of Gan Cao or Wu Wei Zi (equivalent of 3 g dry herb/kg body weight). The livers were harvested either 3 or 6 days after the gavage and subjected to microsomes preparation and total P450 measurement. As shown in Fig. 6A, the administration of either Gan Cao or Wu Wei Zi significantly induced the levels of total P450, consistent with the observation that they activated PXR and induced CYP3As and 2Cs, two of the most abundantly expressed hepatic P450 families (Fig. 4). The induction of CYP2Cs also prompted us to determine whether treatment with Wu Wei Zi and Gan Cao in rats can affect the *in vivo* metabolism of the anticoagulant warfarin, a drug known to be metabolized by CYP2C9 in humans (Goldstein, 2001). Figure 6B shows the time course of warfarin plasma concentration in rats that have been gavaged with six daily doses of the control, Wu Wei Zi aqueous extract (500 mg/kg), or Gan Cao aqueous extract (900 mg/kg). The phar-



**Fig. 5.** Species-specific structural determinants of PXR in its activation by Wu Wei Zi (WWZ) and its constituents. HepG2 (A and C) or CV-1 (B) cells were transfected with the tk-CYP3A23-Luc reporter gene, together with the wild-type and mutant rPXR (A and B) or hPXR (C). Transfected cells were treated with the vehicle or the indicated drugs for 24 h before luciferase assay. (+)-S20 and (-)-S20 enantiomers (Mu et al., 2005) are included as the control ligand for hPXR and mPXR, respectively. S20 is a synthetic *C*-cyclopropylalkylamide. Results are shown as -fold induction over vehicle control and represent the average from triplicate assays.

macokinetic parameter estimations are summarized in Table 1. The significant decreases in  $t_{1/2}$  and/or area under the curve and the corresponding increase in clearance following Wu Wei Zi and Gan Cao treatment suggest that these herbs can increase the metabolism of coadministered warfarin *in vivo*.

## Discussion

In this study, combinations of ligand screening and pharmacokinetic analysis have shown that two TCM herbs, Wu Wei Zi and Gan Cao, can activate PXR, and the resultant activation of drug-metabolizing enzymes may account for the increased warfarin metabolism in rats that were coadministered with Wu Wei Zi or Gan Cao.

The current findings have several implications for the safe

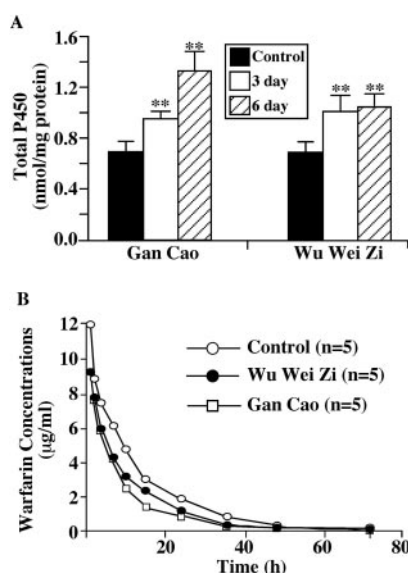
clinical use of TCMs. First, our data have provided a molecular explanation as to why some TCMs, such as Wu Wei Zi and Gan Cao, have been shown to induce total P450s in rodents (Hu et al., 1999; Lin et al., 1999; Zhu et al., 2000). Our results show that Wu Wei Zi and Gan Cao can also activate human PXR. It is conceivable that these TCMs may induce drug metabolism in humans through this hPXR activation. Because drug-induced enzyme production is the primary mechanism of drug-drug interactions, our results reinforce the concerns associated with adverse effects from the use of herbal products (Kaplowitz, 1997). Therefore, it is necessary to routinely evaluate the ability of herbal medicines to activate PXR to ensure their safe use.

The current results have also shed light on the hepatoprotective effects of TCMs. Both Wu Wei Zi and Gan Cao are known to have hepatoprotective and/or general detoxifying functions, according to traditional Chinese medicine and published biomedical literature (Lin et al., 1999; Zhu et al., 2000). Our results suggest that the activation of PXR and consequent induction of drug-metabolizing/detoxifying enzymes may be the mechanism by which these TCMs confer protection from endogenous and exogenous toxins. It is important to note that although PXR was isolated as a xenosensor that regulates drug metabolism, accumulating evidence

has suggested a potential role for this receptor in the treatment and prevention of human diseases, such as cholestasis and hyperbilirubinemia. Activation of PXR in transgenic mice is sufficient to prevent lithocholic acid-induced cholestatic liver damage (Xie et al., 2001). In contrast, mice with a combined loss of PXR and constitutive androstane receptor had a heightened lithocholic acid sensitivity (Uppal et al., 2005). PXR also plays a dual role in bilirubin detoxification in that the ligand-free PXR functions as a suppressor to inhibit the positive role of constitutive androstane receptor in bilirubin clearance (Huang et al., 2003, 2004; Xie et al., 2003). This suppression can be relieved by either a loss of PXR or an activation of PXR by genetic or pharmacological means (Xie et al., 2001; Saini et al., 2005).

Several Wu Wei Zi and Gan Cao constituents were evaluated for their ability to activate PXR. The carbon scaffold of PXR-activating Wu Wei Zi constituents is distinct from those of RIF and PCN. A comparison of chemical structures allows for some generalized structure-activity relationship analysis for Wu Wei Zi constituents as PXR agonists. Both Schisandrins A and B are efficacious activators for the human and rodent PXR. The substitution of a hydroxyl group at the C2 position abolished the activity of Schisandrol A but had little effect on Schisandrol B. It is possible that the presence of the dioxole structure in Schisandrin B (circled in Fig. 2C) allows for the tolerance of a hydroxyl group substitution in Schisandrol A. The dioxolane is a bioisostere of a furan. One possibility is that the benzofuran-type pharmacophore structure contained in Schisandrol B fits better into the hPXR binding pocket than the dimethoxy structure contained in Schisandrol A. Although a benzodioxolane structure is known to subject to metabolic conversions, it is not likely that the Schisandrol B metabolites are responsible for the PXR activity since the PXR activation profile was also seen in CV-1 cells that are known to have little drug-metabolizing activity.

Although the crystal structure of the Wu Wei Zi constituent-bound PXR LBD is not available, our mutagenesis studies have led to some intriguing observations on the species-specific structural determinants of PXR in Wu Wei Zi activation. Mutation of the rat PXR Phe305, a residue located in the flexible loop that forms part of the pore to the ligand-binding cavity (Tirona et al., 2004), did not change the activity of the Wu Wei Zi extract, but it did alter the activation profile of the individual constituents. In contrast, mutation of the human PXR Leu308, the human counterpart of rPXR Phe305, completely abolished PXR activation in response to both the Wu Wei Zi extract and its constituents. Interestingly, our recent report showed that the same mutations had a dramatic effect on the preference of rPXR, but not hPXR, for the enantiomers of S20, a synthetic *C*-cyclopropylalkyl-



**Fig. 6.** Wu Wei Zi and Gan Cao induce total P450 and increase warfarin metabolism in rats. A, total P450 levels are increased in rats treated with a single dose of an aqueous extract of Gan Cao or Wu Wei Zi. Results shown are the averages and S.D.s from the four animals in each group. \*\*,  $P < 0.01$ . B, time course of warfarin plasma concentration in rats that have been treated with the control or TCM for 5 days. Results shown are the averages from the five animals in each group.

**TABLE 1**

Pharmacokinetic parameter estimates (mean  $\pm$  S.D.) following warfarin i.v. injection. Results shown are the means  $\pm$  S.D. from the five animals in each group.

	$t_{1/2}$	AUC <sub>inf</sub>	CL	V <sub>ss</sub>
	h	mg/h/l	l/h/kg	l/kg
Control	13.1 $\pm$ 1.2	155 $\pm$ 19	0.0134 $\pm$ 0.002	0.196 $\pm$ 0.020
Wu Wei Zi	11.6 $\pm$ 0.8*	110 $\pm$ 9.8**	0.0184 $\pm$ 0.002**	0.248 $\pm$ 0.004**
Gan Cao	14.8 $\pm$ 2.3	96.8 $\pm$ 8.2**	0.0210 $\pm$ 0.002**	0.276 $\pm$ 0.006*

AUC, area under curve; CL, clearance;  $t_{1/2}$ , half-life; V<sub>ss</sub>, steady-state volume of distribution.

\*  $P < 0.05$ .

\*\*  $P < 0.01$ .



amide (Mu et al., 2005). Although the results of the functional ligand assays and in vivo pharmacokinetic studies are convincing, we have yet to perform ligand-binding studies to demonstrate the direct binding of TCMs and their constituents to PXR. Therefore, we cannot exclude the possibility that Wu Wei Zi and Gan Cao may activate PXR via indirect pathways. Again, the activity of TCMs and their constituents in CV-1 cells, a cell line that lacks the expression of the major drug-metabolizing enzymes, does suggest that this PXR activation resulted from the parental herbs or chemicals and not their metabolites.

In summary, our results show that PXR can mediate the induction of drug-metabolizing enzymes and transporters by TCMs, and this may be translated into effects on the metabolism of the coadministered drugs. On a positive note, this TCM-mediated PXR activation can be taken advantage of when PXR is used as a therapeutic target to treat cholestatic liver diseases or hyperbilirubinemia.

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