Regular Article

CAR-mediated Up-regulation of CYP3A4 Expression in LS174T Cells by Chinese Herbal Compounds

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Summary: The constitutive androstane receptor (CAR) is an orphan nuclear receptor which has been shown to participate in the activation of human CYP3A4, which metabolizes more than 50% of clinically used drugs. We investigated the effects of an array of compounds isolated from herbal medicines such as Rheum palmatum (Da Huang), Peucedanum praeruptorum Dunn (Qian Hu), Cortex Mori Radicis (Sang Bai Pi), Radix Asteris (Zi Wan), Salvia miltiorrhiza (Dan Shen), Polygonum cuspidatum Sieb. et Zucc. (Hu Zhang), and Ginkgo biloba (Yin Xing) on the CAR-mediated transactivation of CYP3A4. The effect of herbal compounds on CYP3A4 expression was measured using a CYP3A4 luciferase reporter gene assay in transiently transfected human intestinal LS174T cells. The gene expression, protein expression, and catalytic activity of CYP3A4 in LS174T cells transfected with CAR were determined by using real-time PCR, Western blot analysis, and LC-MS/MS-based substrate assay. The study found that in CAR-transfected cells, praeruptorin A, C, and D significantly induced CYP3A4 luciferase activity, mRNA expression, and functional activity through the CAR-mediated pathway; conversely, induction was not found in untransfected cells. Our findings suggest that these herbal compounds can significantly up-regulate the CYP3A4 gene via the CAR-mediated pathway, which has important implications in herb–drug interactions.

Keywords: constitutive androstane receptor (CAR); cytochrome P450 3A4; induction; herb compounds; herb–drug interaction

Introduction

Herbal medicines have been widely used for the treatment of various diseases and play an important role as an established segment of the healthcare system in China. Herbal medicines have multiple pharmacological effects and are extensively used in alternative or complementary therapies. A large number of active ingredients from herbal medicines have been identified, and these naturally occurring compounds offer major opportunities for discovering novel lead structures against a wide range of therapeutic targets.1,2) On the other hand, herbal medicines are often co-administered with therapeutic drugs, dramatically increasing the potential of clinical herb–drug interactions.3) The underlying mechanisms for most reported herb–drug interactions have not been elucidated, but induction and inhibition of drug-metabolizing enzymes and drug transporters are implicated.

Cytochrome P450 (CYP) 3A4 is the most abundant and important isoform of CYP metabolizing enzymes in the human liver and gut; it metabolizes more than 50% of current clinical drugs.4) Dramatic clinically and pre-clinically relevant interactions have been reported between drugs and herbs such as St. John’s wort, pomelo, and grapefruit juice, and induction or inhibition of CYP3A4 by xenobiotics often results in such herb–drug interactions.5–7) A number of xenobiotics and endogenous substances have been identified as substrates, inducers, or inhibitors of CYP3A4.8) Induction of CYP3A4 by xenobiotics contributes to the pronounced interindividual variability of its expression and often results in clinically relevant drug–drug interactions.8,9) Enzyme induction is often governed by the activation of several nuclear receptors (NRs), such as pregnane X receptor (PXR) and constitutive androstane receptor (CAR, NR1I3), which coordinately control the drug-induced
expression of multiple drug-metabolizing enzymes and drug transporters, including the highly inducible CYP2B6 and CYP3A4.\textsuperscript{11,12} The nuclear hormone receptor CAR is a sister xenobiatic receptor of PXR and plays a pivotal role in the induction of drug metabolism and transport. The hepatic expression of phase I (e.g. CYP2B6, CYP2C9, CYP3A4) and phase II (e.g. UGT1A1, GSTA1) drug-metabolizing enzymes and of transporters (e.g. MRP2, SLC21A6) is activated by CAR in response to structurally diverse chemicals.\textsuperscript{13,14} CAR displays high constitutive activity in the absence of agonistic ligand binding\textsuperscript{15} and could be activated by either direct ligand binding, such as by 6-(4-chlorophenyl)imidazo [2,1-b][1,3] thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime (CITCO), or indirect activation, such as by phenobarbital (PB).\textsuperscript{16,17} In intact liver, CAR is localized predominantly in the cytoplasm in the absence of CAR activators, but is accumulated in the nucleus after exposure to PB-type compounds.\textsuperscript{18,19} Thus, nuclear translocation of CAR has been established as the initial step for CAR-mediated transcriptional activation. In contrast, this nuclear receptor exhibits a high level of constitutive transcriptional activity in immortalized cells and accumulates spontaneously in nuclei. On the other hand, several competitive inverse agonists of CAR have also been discovered, such as androstanol, androstenol, and clotrimazole.\textsuperscript{20,21}

A number of naturally occurring compounds from herbs such as St John’s wort,\textsuperscript{22} ginkgo (Ginkgo biloba),\textsuperscript{23,24} Gugulipid (Commiphora mukul),\textsuperscript{25} Wu Wei Zi (Schisandra chinensis), licorice (Glycyrrhiza uralensis),\textsuperscript{26} Dan Shen (Salvia miltiorrhiza),\textsuperscript{27} and Tian Xian\textsuperscript{28} have been reported to activate PXR, including ginkgolide A, cryptotanshinone, tanshinone IIA, and schisandrin A, among others. But data on the impact of herbal compounds on the expression of CAR are limited. In this regard, we investigated the hypothesis that induction of CYP3A4 at the transcriptional level was activated through the CAR pathway. We thus investigated the effects of an array of compounds isolated from commonly used herbal medicines on the activity of human CAR using a luciferase reporter gene assay in transiently transfected LS174T cells. Furthermore, the gene expression, protein expression, and catalytic activity of CYP3A4 were determined by using real-time PCR, Western blot analysis, and LC-MS/MS-based substrate assay. These findings provide new insights into potential herb–drug interactions of herbal medicines. The herbal compounds tested in this study included aloë-emodin, emodin, chrysophanol, physcion, and rhein from Rheum palmatum (Da Huang); praeeruptorin A, C, D, and E from Peucedanum praeruptorum (Qian Hu); epifriedelanol and shionone from Radix Asteris (Zi Wan); morin and mulberroside A from Cortex Mori Radicis (Sang Bai Pi); cryptotanshinone, protocatechuic aldehyde, salvianolic acid B, sodium danshensu, tanshinone I, and tanshinone IIA from Salvia miltiorrhiza (Dan Shen); bilobalide, ginkgolide A, and ginkgolide B from Ginkgo biloba (Yin Xing); and resveratrol from Polygonum cuspidatum Sieb. et Zucc (Hu Zhang), see Table 1. Da Huang, Qian Hu, Zi Wan, Sang Bai Pi, Dan Shen, Hu Zhang, and Yin Xing are used widely in Chinese medicine prescriptions, and these herbs are frequently combined against various diseases. The above compounds are the principal constituents in these herbs and, moreover, these compounds have important pharmacological effects. Therefore, discovering the effects of these compounds on CYP3A4 mRNA expression, protein expression, and enzyme activity appears particularly important to elucidate herb–drug interaction among these herbs.

### Material and Methods

**Chemicals and reagents:** 6-(4-Chlorophenyl)imidazo [2,1-b][1,3] thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl) oxime (CITCO), dimethyl sulfoxide (DMSO), nifedipine (NIF), dehydronifedipine (DNIF), and loratadine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipofectamine 2000 transfection reagent was purchased from Invitrogen (Carlsbad, CA, USA). Dual-Luciferase Reporter Assay System was purchased from Promega (Madison, WI, USA). RNAiso Plus and PrimeScript RT reagent were

### Table 1. The herbal compounds investigated in this study and their physicochemical properties

<table>
<thead>
<tr>
<th>Name of herbal compound</th>
<th>Herbal source</th>
<th>Molecular weight</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aloe-emodin</td>
<td>Rheum palmatum</td>
<td>270.24</td>
<td>ALO</td>
</tr>
<tr>
<td>Bilobalide</td>
<td>Ginkgo biloba</td>
<td>326.3</td>
<td>BB</td>
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<tr>
<td>Chrysophanol</td>
<td>Rheum palmatum</td>
<td>254.24</td>
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<tr>
<td>Cryptotanshinone</td>
<td>Salvia miltiorrhiza</td>
<td>296.36</td>
<td>CRY</td>
</tr>
<tr>
<td>Emodin</td>
<td>Rheum palmatum</td>
<td>270.24</td>
<td>EMO</td>
</tr>
<tr>
<td>Epifriedelanol</td>
<td>Radix Asteris</td>
<td>428.73</td>
<td>EPI</td>
</tr>
<tr>
<td>Ginkgolide A</td>
<td>Ginkgo biloba</td>
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<tr>
<td>Ginkgolide B</td>
<td>Ginkgo biloba</td>
<td>424.40</td>
<td>GB</td>
</tr>
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<td>Morin</td>
<td>Cortex Mori Radicis</td>
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</tr>
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<td>Mulberroside A</td>
<td>Cortex Mori Radicis</td>
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<td>Rheum palmatum</td>
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<td>PHY</td>
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<td>Praeruptorin A</td>
<td>Peucedanum praeruptorum Dunn</td>
<td>386.40</td>
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<td>Praeruptorin C</td>
<td>Peucedanum praeruptorum Dunn</td>
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<td>Praeruptorin D</td>
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<td>Praeruptorin E</td>
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<td>Protocatechuic aldehyde</td>
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<td>Rhein</td>
<td>Rheum palmatum</td>
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<td>Radix Asteris</td>
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<td>Tanshinone I</td>
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<tr>
<td>Tanshinone IIA</td>
<td>Salvia miltiorrhiza</td>
<td>294.33</td>
<td>TAN II</td>
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</tbody>
</table>

Data are from ChemSpider.
obtained from Takara (Kyoto, Japan). The primers used in real-time PCR were synthesized by Takara. Anti-cytochrome P450 enzyme CYP3A4 polyclonal antibody was purchased from Millipore Corporation (Rosemont, IL, USA). GADPH antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-rabbit IgG-HRP antibody was purchased from R&D Systems (Minneapolis, MN, USA). SDS-PAGE Gel Preparation Kit was purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). Plasmocin Anti-mpp and Omplete protease inhibitor cocktail were purchased from Invitrogen (San Diego, CA, USA) and Roche Diagnostics (Basel, Switzerland), respectively. All herbal compounds tested (see Table 1) were of the highest grade (purity >99%) available from the Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) or other commercial sources. The cytotoxic effects on LS174T cells were detected by the 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation and cytotoxicity assay; the compounds did not show cytotoxicity toward LS174T cells under the maximum dosage (40 µM).

**Plasmids:** pcD-hCAR2 expression plasmids were generously provided by Dr. Oliver Burk, Institute of Magarete Fischer-Bosch Clinical Pharmacology, Germany. The pGL3-CYP3A4-XREM luciferase reporter construct containing the basal promoter (−362/+53) with the proximal PXR response element (ER6) and the distal xenobiotic responsive enhancer module (XREM, −7836/−7208) of the CYP3A4 gene 5'-flanking region inserted to pGL3-Basic reporter vector was generously provided by Dr. Jeff Staudinger, Department of Pharmacy and Toxicology, University of Kansas, Lawrence, KS, USA. The pRL-TK Rotylenchulus reniformis control vector and pcD-empty vector were obtained from Promega (Madison, WI, USA).

**Cell culture:** LS174T cells (derived from Caucasian colon adenocarcinoma) were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone). Cell lines were cultured at 37°C under a humidified atmosphere of 5% CO2. The cytotoxicity of herbal compounds was determined by using the MTT assay as described previously.

**Transient transfection and reporter gene assays:** For transfection, LS174T cells were seeded at 70–80% confluence into 96-well plates at a density of 0.2 × 104 cells/well for 24 h. Transfection was performed in phenol red-free RPMI 1640 medium supplemented with 10% charcoal/dextran-treated FBS using Lipofectamine 2000 Transfection Reagent according to supplier's recommendations. Briefly, for each well of the 96-well plate, the transfection mix consisted of 50 ng pcD-hCAR expression vector, 100 ng CYP3A4-XREM luciferase reporter, and 15 ng pRL-TK as an internal control; 50 ng pcD-empty vectors, 100 ng CYP3A4-XREM luciferase reporter, and 15 ng pRL-TK were transfected as vector control. After 6 h of incubation, the transfected LS174T cells were exposed to CITCO at 10 µM for 24 h, which is a known prototypical CAR transactivator, and herbal compounds at 2.5, 5, 10, 20, and 40 µM. All compounds except salvianolic acid B, sodium dan Shen su, and protocatechuic aldehyde were dissolved in DMSO at a final concentration of 0.1% (v/v). Salvianolic acid B, sodium dan Shen su, and protocatechuic aldehyde were dissolved in phosphate-buffered saline (PBS). Cells were then lysed, and firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions (Promega) using a Lu Mat LB 9507 Single Tube Luminometer (Berthold Technologies, Bad Wildbad, Germany). Transfection efficiency was expressed as the fold induction of firefly to Renilla luciferase activities relative to empty or vehicle controls.

**Transient transfection and total RNA isolation:** LS174T cells (1.2 × 104 per well) were seeded into 24-well plates, cultivated for 24 h, and then transfected with CAR expression plasmids (300 ng/well). Appropriate cell samples were exposed to praeruptorin A, C, and D at a concentration of 2.5, 5, 10, 20 µM for 48 h. Incubations with 10 µM CITCO and PBS were also included as controls. Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was quantified and reverse transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara, Kyoto, Japan).

**Real-time PCR analysis of CYP3A4 mRNA and CAR mRNA:** The primers for CYP3A4 mRNA detection were designed as described by Faucette et al. For CYP3A4, the forward primer was 5'-TCAGCCTGTTCTCCTCTC-TATCTTAT-3' and the reverse primer was 5'-AAGCCCT-TATGGTAGGACAATAATTTT-3'. For CAR, the forward primer was 5'-GAAGATGGAGCCGTGTA-3' and the reverse primer was 5'-CAGGTGCCTGTGAAGATGAGG-3'. For the control gene β-actin, the forward primer was 5'-GTCATCACATTGGCAATGAG-3' and the reverse primer was 5'-CTGCTACATCCTAGGCTG-3'. All the PCR reactions were carried out using SYBR Premix Ex Taq kit (Takara, Kyoto, Japan) according to the manufacturer's instructions. Amplification was performed in PCR-Capillaries on a Light Cycler 2.0 Real Time Detection System (Roche, Hercules, CA, USA). Amplification of pre-denatured products was conducted at 94°C for 60 s; followed by 45 cycles at 95°C for 30 s, 58°C for 30 s and 72°C for 30 s; followed by 95°C for 10 s, 65°C for 45 s, and 40°C for 60 s. Fold induction values were calculated according the expression 2−ΔΔCt, where ΔCt represents the differences in cycle threshold numbers between the target gene and β-actin, and ΔΔCt represents the relative change in the differences between control and treatment groups.

**Transient transfection and Western blotting analysis:** LS174T cells (1.0 × 106 per well) were seeded into 6-well plates and cultivated for 24 h; the cells were then transfected or not transfected with CAR expression plasmids
(1.5 µg/well). Appropriate cell samples were exposed to DMSO (0.1%); CITCO at 10 µM; and praeruptorin A, C, and D at 2.5, 10, and 40 µM for 72 h. All the proteins were extracted by high-speed centrifugation and quantified using a Coomassie Protein Assay Kit (Pierce, Rockford, IL, USA). CYP3A4 protein levels were measured by Western blot analysis. Briefly, proteins were separated in SDS-polyacrylamide gel (10% acrylamide) and transferred to PVDF membranes (Millipore, Bedford MA, USA) by electrophoresis at 70 V for 30 min, 120 V for 1 h, and 200 mA for 75 min using the Mini-gel apparatus (Bio-Rad, Hercules, CA, USA). The PVDF membranes were blocked in blocking buffer for 1–2 h at room temperature. PVDF membranes were incubated with rabbit anti-human CYP3A4 polyclonal antibody for 1 h at room temperature or 4°C overnight and with anti-rabbit IgG-HRP antibody at room temperature for 1 h. The blots were detected by a SuperSignal West Pico Chemiluminescent Substrate kit purchased from Thermo (Rockford, IL, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

Transient transfection and functional analysis of CYP3A4 activity: LS174T cells (1.0 × 10⁶ per well) were seeded into 6-well plates and cultivated for 24 h. Cells were transfected or not transfected with CAR expression plasmids (1.5 µg/well) and then exposed to praeruptorin A, C, and D at a concentration of 2.5, 10, and 40 µM for 72 h. The cells were lysed using radio immunoprecipitation assay (RIPA) buffer plus phenylmethylsulfonyl fluoride (PMSF); total protein was extracted by high-speed centrifugation and quantified using a Coomassie Protein Assay Kit (Pierce, Rockford, IL, USA). For the determination of CYP3A4 activity, total protein (1 mg/mL) was pre-incubated with NIF (2.5 µM) for 5 min at 37°C. The reaction was initiated by addition of 1 mM NADPH and terminated with 1 mL chilled ethyl acetate after 20 min of incubation. A volume of 10 µL of loratadine (10 mM) was added to the final mixture as the internal standard. The metabolite was extracted by high-speed centrifugation. Subsequently, the solvent was evaporated and the sample dissolved in solvent (methanol:H₂O = 8:2, v/v). The concentration of the nifedipine metabolite was determined using an established LC-MS/MS method. 34

Statistical analysis: One-way ANOVA followed by Dunnett’s multiple comparison post hoc test or unpaired Student’s t test were used for statistical analysis of data using SPSS version 13.0 software (SPSS Inc, Chicago, IL, USA). Probability values of p < 0.05 were considered to be statistically significant.

Results

Induction of CYP3A4 luciferase activity by herbal compounds in LS174T cells: To determine the effects of herbal medicines on CYP3A4 luciferase activity, CYP3A4-XREM expression plasmids encoding pcD-hCAR2 nuclear receptors and the internal control plasmid pRL-TK were cotransfected together with appropriate promoter reporter gene plasmids into LS174T cells. For each herbal compound, we tested at concentrations of 2.5, 5, 10, 20, and 40 µM, which showed negligible cytotoxicity toward native and transiently transfected LS174T cells. As shown in Figure 1, CYP3A4 luciferase activity was significantly induced by CITCO, which acted as the positive control (7.84-fold at 10 µM). Compared to the control group, praeruptorin A at 10, 20, and 40 µM significantly induced CYP3A4 reporter gene luciferase activity to 4.31, 7.05, and 7.77-fold in transiently transfected LS174T cells, and praeruptorin C at 10, 20, and 40 µM significantly trans- activated CYP3A4 reporter gene luciferase activity to 4.53, 6.70, and 7.39-fold. Praeruptorin D had the strongest luciferase inducibility, i.e., 5.07, 6.15, 10.79, and 11.66-fold at 5, 10, 20, and 40 µM. The CYP3A4 reporter gene construct was mildly transactivated by praeruptorin E, emodin, chrysophanol, epifriedelanol, mulberroside A, physcion, salvianolic acid B, and tanoshinine IIA, although they didn’t show significant difference as compared to control group. The CYP3A4 reporter gene construct was not transactivated by other herbal compounds.

The effects of praeruptorin A, C, and D on CYP3A4 mRNA and CAR mRNA expression in LS174T cells: It was found that praeruptorin A, C, and D significantly transactivated the CYP3A4 reporter gene construct in transiently transfected LS174T cells. In order to determine whether praeruptorin A, C, and D induces CYP3A4 mRNA expression by the CAR-mediated pathway directly, LS174T cells were transfected with CAR expression plasmids and cells were exposed to praeruptorin A, C, and D at concentrations of 2.5, 5, 10, and 20 µM for 48 h; the mRNA levels of CAR and CYP3A4 were then detected by real-time PCR. As shown in Figure 2A, we found a statistically significant increase of CYP3A4 mRNA on treatment with CITCO compared with the control (3.53-fold at 10 µM, p < 0.05); thus CITCO was used as the positive control. Praeruptorin A at concentrations of 5, 10, and 20 µM significantly induced CYP3A4 mRNA expression to 3.53, 4.99, 6.19-fold, respectively (p < 0.01, p < 0.05). Praeruptorin C at concentrations of 10 and 20 µM significantly induced CYP3A4 mRNA expression to 3.66, 4.99, 6.19-fold, respectively (p < 0.01, p < 0.05). Praeruptorin D at concentrations of 2.5, 5, 10, and 20 µM significantly induced CYP3A4 mRNA expression to 3.61, 4.69, 8.75, and 6.77-fold, respectively (p < 0.01, p < 0.05). In our previous experiments, no significantly increased CYP3A4 mRNA levels were observed in untransfected LS174T cells after administration of praeruptorin A, C, or D. 31

Figure 2B illustrates the effects of PA, PC, and PD on CAR mRNA expression level in CAR-transfected LS174T cells. After treatment with PA, PC, and PD, the levels of CAR mRNA expression did not show any marked changes.

Determination of CYP3A4 protein expression in LS174T cells exposed to praeruptorin A, C, and...
After being transfected with CAR expression plasmids, cells were exposed to praeruptorin A, C, and D at concentrations of 2.5, 10, and 40 µM for 72 h. CYP3A4 protein expression was subsequently investigated employing a Western-blotting assay. The results are shown in Figure 3A and Figure 3B; cells transfected with plasmids encoding CAR yielded a statistically significant increase to 2.36-fold in CYP3A4 protein expression after 72 h exposure to CITCO at a concentration of 10 µM compared with the control group (p < 0.01). Compared with the vehicle control, the expression of CYP3A4 protein was significantly induced 1.91-fold by praeruptorin A at 10 µM (p < 0.05); 2.15 and 1.87-fold by praeruptorin C at 10 and 40 µM, respectively (p < 0.05, p < 0.01); and 1.84 and 2.50-fold by praeruptorin D at 10 and 40 µM, respectively (p < 0.05, p < 0.01). The results indicated that praeruptorin A, C, and D increased CYP3A4 protein expression. These results were generally consistent with those observed in the real-time
The experimental substance CITCO and the antimalarial artemisinin drugs have been recognized as ligands of CAR.39,40 The barbiturate drug phenobarbital activates CAR indirectly through the increase of CAR translocation from the cytoplasm to the nucleus.41 A number of CYP proteins form a superfamily of heme-containing enzymes involved in the oxidative metabolism of lipophilic compounds, including steroids, fatty acids, retinoids, bile acids, and foreign chemicals such as drugs and other xenobiotics.35 CYP3A4 is the predominant CYP expressed in human liver, accounting for up to 50% of total hepatic CYP protein.4) Traditional Chinese medicines are widely used in the treatment of various kinds of diseases or are used as complementary therapies, which dramatically increase the potential of clinical herb–drug interactions; such interactions are frequently caused by induction or inhibition of CYP3A4. Although a number of studies have addressed the effect of xenobiotics on the expression of human CYP3A4, data on the effect of herbal compounds on CYP3A4 and the underlying mechanism are still limited.

As is the case for other CYPs, nuclear receptors, in particular PXR and CAR, are significant regulators of CYP3A4 gene expression. The nuclear receptor CAR is responsible for many important xenobiotic responses.36 It functions as a heterodimeric partner of RXR and can recruit coactivators in the presence and absence of ligands.37 CAR has been reported to directly regulate the transcriptional activity of CYP3A4 and CYP2B genes both in vitro and in vivo.35 CAR is associated with a coactivator called steroid receptor coactivator 1 (SRC-1). The CAR/SRC-1 complex dimerizes with RXR before binding with the promoter region of CYP3A4. The CAR/RXR complex binds to a sequence in the 5′-untranslated region of the gene that contains two copies of the nuclear receptor organized as ER6 and controls the expression of pre-mRNA.38 Induction of CYP3A4 can occur via up-regulation of this process following an increase in the affinity of the complex for ER6, DR1, and DR3.13

The experimental substance CITCO and the antimalarial artemisinin drugs have been recognized as ligands of CAR.39,40 The barbiturate drug phenobarbital activates CAR indirectly through the increase of CAR translocation from the cytoplasm to the nucleus.41
xenobiotics or drugs have been reported to activate CAR, including retinoids, valproic acid, and buprenorphine.\textsuperscript{42-44} However, data on the effects of herbal compounds on the regulation of CYP3A4 through the CAR-mediated pathway are limited. In this regard, one of the major objectives of this study was to investigate the effects of a panel of compounds from commonly used Chinese herbal medicines on the CAR-mediated transactivation of CYP3A4.

Here we investigated the role of CAR in the inducing effects of herbal compounds on CYP3A4 using a luciferase reporter gene assay in a commonly used human LS174T cell line. The results suggest that CAR, like PXR, is a steroid receptor that is capable of recognizing structurally diverse compounds. Our study found different reporter gene transactivation effects of herbal compounds: praeruptorin A and C significantly enhanced CYP3A4 reporter luciferase activity after binding human CAR receptor, as the prototypical agonist CITCO does; the CYP3A4 inducibility of praeruptorin D even surpassed that of CITCO. However, praeruptorin E, emodin, chrysophanol, epifriedelanol, mulberroside A, physcion, and tanshinone IIA had only mild inducibility, suggesting that CAR plays no functional role in the effects of these compounds on CYP3A4.

Praeruptorin A, C, D, and E are major active ingredients in the herbal medicine Qian Hu (\textit{P. praeruptorum}). Qian Hu, which consists of the roots of \textit{Peucedanum praeruptorum} Dunn. and \textit{P. decursivum} Maxim., is used for the treatment of respiratory disease and pulmonary hypertension.\textsuperscript{45} In this sense, investigation of the activation of downstream target genes of CAR by praeruptorin A, C, and D may facilitate further understanding of the CYP3A4 induction effects of \textit{P. praeruptorum} (Qian Hu), and thus help avoid potential adverse herb–drug interactions. Based on the above results, praeruptorin A, C, and D activated CAR and up-regulated CYP3A4 reporter gene expression through interaction with specific promoter response elements.

To confirm that praeruptorin A, C, and D induce CYP3A4 directly through the CAR-mediated pathway, CYP3A4 mRNA and protein expression and catalytic activity were measured.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Effects of praeruptorin A, C, and D on the protein expression of CYP3A4 in LS174T cells}
\end{figure}
using CAR expression plasmids were measured by real-time PCR, Western blot analysis, and LC-MS/MS assay. The results indicated that praeruptorin A, C, and D significantly increased CYP3A4 mRNA expression through the CAR-mediated pathway. According to our previous results, CYP3A4 mRNA expression could not be induced by praeruptorin A, C, and D in LS174T cells that were not transfected with CAR plasmids. However, in the current experiment, in LS174T cells transfected with CAR plasmids, praeruptorin A, C, and D significantly induced the level of CYP3A4 mRNA. These results suggest that praeruptorin A, C, and D mediated transactivation of the CYP3A4 gene via interaction with the CAR nuclear receptor.

Finally, we analyzed CYP3A4 protein expression and enzymatic activity in LS174T cells exposed to praeruptorin A, C, and D for 72 h after transfection. Significant increases of CYP3A4 activity were observed in LS174T cells transfected with the CAR expression vector, which indicates that praeruptorin A, C, and D up-regulate CYP3A4 protein levels and catalytic activity through the CAR pathway. In order to further confirm the impact of praeruptorin A, C, and D on untransfected LS174T cells, such cells were treated with praeruptorin A, C, and D at a concentration of 10 µM, a dosage potent enough to induce protein expression of CYP3A4 in CAR-transfected cells. As shown in Figure 3C and Figure 3D, no increase of CYP3A4 protein expression was observed in untransfected LS174T cells; this result was consistent with the results of mRNA expression and catalytic activity (Fig. 4B). The results indicate that praeruptorin A, C, and D directly induced CYP3A4 protein expression through the CAR pathway.

As mentioned above, PXR is a critical determinant of xenobiotics-induced CYP3A4 expression; it would not be surprising if these compounds also interacted with PXR to activate CYP3A4. We recently reported that praeruptorin A, C, and D can effectively transactivate CYP3A4 luciferase activity and protein expression via the PXR-mediated pathway in human hepatic HepG2 and Huh7 cells. The results demonstrate that PXR also plays a role in the activation of CYP3A4 by praeruptorin A, C, and D. Thus, praeruptorin A, C, and D can coactivate the CAR-mediated and PXR-mediated pathways to coregulate CYP3A expression. Therefore, praeruptorin A, C, and D can regulate CYP3A4 gene expression, protein expression, and activity through PXR- and CAR-mediated pathways, and thus accelerate detoxification and metabolism of CYP3A4 substrates. Further mechanistic studies are needed to investigate how the herbal compounds interact with PXR, CAR, and their coactivators and co-repressors.

In conclusion, these results demonstrate that praeruptorin A, C, and D effectively transactivate CYP3A4 mRNA expression, protein expression, and catalytic activity via the CAR-mediated pathway in human LS174T cells. The results indicated that herb–drug interactions may possibly occur when Qian Hu is co-administered with other drugs metabolized by CYP3A4. Much more attention should be paid to ensure safety in the utilization of Qian Hu. Further studies are required to evaluate these effects in vivo.

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