IDENTIFICATION AND CHARACTERIZATION OF PSORALEN AND ISOPSORALEN AS POTENT CYP1A2 REVERSIBLE AND TIME-DEPENDENT INHIBITORS IN HUMAN AND RAT PRECLINICAL STUDIES

Xiao-Mei Zhuang, Yu-Huan Zhong, Wei-Bin Xiao, Hua Li, and Chuang Lu

The key lab of drug metabolism and pharmacokinetics, Beijing Institute of Pharmacology and Toxicology, Beijing, 100850, China (XM Z, YH Z, WB X, H L)

Millennium Pharmaceuticals, Inc. Cambridge, Massachusetts, USA (C L)
Running title

CYP1A2 INHIBITION BY PSORALEN AND ISOPSORALEN

*Corresponding author:
(submission of this manuscript)
Chuang Lu
Millennium Pharmaceuticals, Inc.
40 Landsdowne Street
Cambridge, MA 02139
Phone: (617) 551-8952
Fax: (617) 444-1480
E-mail: chuang.lu@mpi.com

*2nd corresponding author:
(future communication with readers)
Hua Li
The key lab of drug metabolism and pharmacokinetics,
Beijing Institute of Pharmacology and Toxicology,
Beijing, 100850, China
Phone: +86 10 88270677
Fax: +86 10 68211656
E-mail address: amms_hli@126.com
List of Abbreviations

CYP, cytochrome P450; CYP3A4/5 is abbreviated as CYP3A4; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; PRN, Psoralen; IPRN, isopsoralen; RLM, rat liver microsomes; HLM, human liver microsomes;
ABSTRACT
Psoralen (PRN) and isopsoralen (IPRN), naturally occurred furanocoumarin compounds, are the bioactive constituents found in herbaceous plants. They are widely used as active ingredients in several Chinese herb medicines. In this study, the CYP1A2 inhibitory potential of PRN and IPRN was investigated in rat in vitro and in vivo, and as well as in human liver microsomes. Both compounds exhibited reversible and time-dependent inhibition toward rat microsomal cyp1a2. The IC$_{50}$, $k_{\text{inact}}$, and $K_I$ were $10.4 \pm 1.4 \, \mu\text{M}$, $0.060 \pm 0.002 \, \text{min}^{-1}$, and $1.13 \pm 0.12 \, \mu\text{M}$ for PRN, and $7.1 \pm 0.6 \, \mu\text{M}$, $0.10 \pm 0.01 \, \text{min}^{-1}$ and $1.95 \pm 0.31 \, \mu\text{M}$ for IPRN, respectively. In human liver microsomal incubations, much potent reversible CYP1A2 inhibition was observed for both compounds as the IC$_{50}$ values were $0.26 \pm 0.01$ and $0.22 \pm 0.03 \, \mu\text{M}$ for PRN and IPRN, respectively. However, time-dependent inhibition was only observed for IPRN with $k_{\text{inact}}$ and $K_I$ of $0.050 \pm 0.002 \, \text{min}^{-1}$ and $0.40 \pm 0.06 \, \mu\text{M}$, respectively. Co-administered with PRN or IPRN significantly inhibited cyp1a2 activity in rats, with AUC of phenacetin increasing more than 5-fold. Simcyp® simulation predicted that PRN would cause 1.71 and 2.12-fold increase in phenacetin AUC in healthy and smoker populations, respectively. IPRN, on the other hand, would result in 3.24 and 5.01-fold increase in phenacetin AUC in healthy and smoker populations, respectively. These findings represent the first detailed report on DDI potential comparison of PRN and IPRN, and provide useful information for balancing the safe and efficacious doses of PRN and IPRN.
Psoralen (PRN) and isopaoralen (IPRN) are active ingredients of the traditional Chinese medicines (TCMs) “buguzhi”, which is the dried seed of *Psoralea corylifolia* L. They are also found in many other TCMs, such as doubleteeth angelica root and coastal glehnia root. PRN and IPRN have been widely used clinically as the principal constituents in more than 20 clinically used herbal formulas, such as Haigou Pill, Shouwu Pill and Wenweishu Tablet. The primary clinical use of these two ingredients is for the treatment of various skin diseases, such as psoriasis, vitiligo and chronic graft-versus-host (Conforti et al., 2009). Photochemotherapy with oral PRN and ultraviolet A radiation (PUVA) is an effective treatment for many proliferative skin disorders. In 1982, the FDA approved PUVA for the treatment of psoriasis (Stern. 2007). It is estimated that more than 140,000 patients are under the PUVA treatment 2-3 times a week (Pearce et al. 2006). Results of clinical trial showed that the use of psoralen along with its chemical derivatives, supplemented with exposure to sunlight is a more effective treatment for psoriasis. In one study, 49 patients underwent 6 months of *Psoralea corylifolia* treatment. Of these patients, 14% were cured and another 19% regained pigmentation on at least two-thirds of the affected skin (Chopra et al, 2013). Recent years, extensive studies have been carried out to investigate the pharmacological activities of PRN and IPRN. Wang et al., (2011) reported an increase in tumor cell apoptosis after the treatment of PRN and IPRN.

It was reported that natural furanocoumarin compounds often show potent inhibition to cytochrome P450 (CYP). The compound 6', 7'-dihydroxybergamottin was the first...
furanocoumarin reported as a rat cyp3a inhibitor (Edwards et al., 1996). Other compounds in this family, such as bergamottin, imperatorin, isoimperatorin, and trioxsalen have been reported to inhibit CYP3A4, 2D6, 2C19, 2E1, 2B6, rat cyp1a1, hCYP1A2, or P-gp (Paine et al., 2005; Baumgart et al., 2005; Iwanaga et al., 2010). PRN and IPRN have been reported to inhibit recombinant hCYP1A2 (Peterson et al., 2006; Kang et al., 2011). The 8-methoxypsoralen was reported to be an inhibitor and inducer theophylline metabolism in rats, and an inhibitor in humans (induction was not studied, Apseloff et al., 1990). These studies suggested that the chemical structure of furanocoumarin is prone to impact of CYP enzymes and further cause drug interactions (Bendriss et al., 1996; Tantcheva-Poor et al., 2001; Guo et al., 2004).

CYP1A2 is one of the major CYP isoforms mediates biotransformation of some important clinical drugs, such as theophylline, clozapine, imipramine and paracetanol. Co-administration of CYP1A2 inhibitors with these drugs may cause severe adverse effects as some of them have a narrow therapeutic index (Sesseler and Cohen, 1990; Shad 2008; Mann et al., 1959; Dahlin et al., 1984). CYP1A2 also plays an important role in carcinogenesis, by metabolic activation of a variety of procarcinogenic compounds, such as aflatoxins, heterocyclic aromatic amines, nitrosamines, and polycyclic aromatic hydrocarbons, to form highly reactive carcinogenic intermediates (Chow et al., 2010, Shimada et al., 2005). Inhibition of CYP1A2 activity may result in the reduced formation of the reactive intermediates and consequently affect carcinogenesis. As widespread used active ingredient in Chinese medicines, PRN and IPRN are surprisingly not well investigated to date for their potential for interaction with CYP1A2 substrate drugs, it is important to understand the potential effects of PRN and IPRN might have on the inhibition of CYPs, and the consequence of risk and benefits of using these Chinese
medications.

The aim of this study was initially to evaluate the inhibitory effects of PRN and IPRN in vitro on major rat and human cytochrome P450s. The study was further focused on CYP1A2 on which PRN and IPRN was found to be inhibitors. Time-dependent inhibition was first identified using an IC₅₀ shift assay. Enzyme kinetic studies were performed to further characterize the mechanism of inhibition and to differentiate the inhibitory properties between PRN and IPRN. In the rat DDI study, α-naphthoflavone was included to compare the interaction potentials of PRN and IPRN with this known CYP1A2 inhibitor. In an attempt of predicting human DDI risk, physiologically-based pharmacokinetic (PBPK) models were investigated for both PRN and IPRN and the DDI potentials on CYP1A2 were predicted in both healthy and smoker populations.
Materials and Methods

Chemicals

Psoralen, isopsoralen and propranolol were purchased from National Institute of Food and Drug Control with the purity greater than 99%. The structures of PRN and IPRN are showed in Fig. 1. Phenacetin, acetaminophen, tolbutamide, 4-hydroxytolbutamide, S-mephenytoin, 4-hydroxymephenytoin, dextromethorphan, dextrorphan, midazolam, 1’-hydroxymidazolam were purchased from Sigma (St. Louis, MO, USA). α-naphthoflavone and β-NADPH were purchased from Roche Molecular Biochemicals. Pooled rat liver microsomes (RLM) and human liver microsomes (HLM) were provided by BD Gentest (Woburn, MA). All other reagents and solvents were of analytical grade and commercially available.

Animals

Male SD rats (200 to 240 g) were obtained from the Beijing Experimental Animal Center. Animals were housed in a temperature and humidity controlled room with a 12 h light/dark cycle. They were fed with a standard laboratory chow and had ad libitum access to water. The animal experiments were conducted in the Beijing Center for Drug Safety Evaluation and according to a protocol approved by the Institutional Animal Care and Use Committee of the Centre, which was in compliance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Quantification of PRN, IPRN, and CYP probe substrate metabolites

PRN, IPRN, phenacetin and acetaminophen were quantitatively measured using an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara,
CA) equipped with an Agilent 1290 Infinity UHPLC system and a ZORBAX SB-C18 column (50 mm × 2.1 mm, 3.5 μm I.D.). The mobile phase consisted of 5 mM ammonium formate solution containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The gradient program applied was as follows: 0-1 min 30% B, 1-1.5 min 30% B to 95% B, 1.5-3.5 min 95% B, 3.5-4 min 95% B to 30% B, followed by re-equilibration of 0.5 min. The column temperature was set at 25°C. The flow rate was 0.3 ml·min⁻¹. For quantification, the instrument was operated in the ESI positive ion multiple reaction monitoring (MRM) mode with the following optimized MS/MS conditions: transfer capillary temperature at 320°C; spray voltage of 4000 V, sheath gas of 25 psi. The selected transitions were m/z 187→131 for PRN and IPRN, m/z 152→110.1 for acetaminophen, m/z 180→110.1 for phenacetin and m/z 260→116.2 for propranolol (IS). Optimized collision energy values were 15eV, 10eV, 12eV and 16eV, respectively. The determination method was partially validated following the U.S. Food and Drug Administration guidance (FDA (2001) Guidance for Industry, Bioanalytical Method Validation, http://www.fda.gov/cder/guidance/4252fnl.pdf). The linear ranges for quantitation of PRN, IPRN, phenacetin and acetaminophen were from 2 to 2000 ng/ml and from 1 to 1000 ng/ml, respectively. The within-run precisions were not exceed 10% and accuracies were within 10% for quality control samples at low, medium and high concentrations (5 ng/ml, 90 ng/ml, and 1600 ng/ml for PRN, IPRN and phenacetin, respectively n=3; 2 ng/ml, 50 ng/ml, and 800 ng/ml for acetaminophen, respectively, n=3). The lower limit of quantitation (LLOQ) of PRN, IPRN and phenacetin was set at 2 ng/ml, and 1 ng/ml for acetaminophen because the analyte peaks were identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%. The recovery was found to be greater than 90% and the matrix effect was negligible.
detection of other CYP probe substrate metabolites (4-hydroxytolbutamide for CYP2C9, 4-hydroxymephenytoin for CYP2C19, dextrophor (for CYP2D6), and 1’-hydroxymidazolam for CYP3A4) followed a published method (Zhong et al., 2012).

IC$_{50}$ determination

To determine the IC$_{50}$ values of PRN and IPRN, CYP substrate concentrations in the incubation mixture were selected to be around their $K_m$. Seven different concentrations of PRN and IPRN were used from 0.05 µM - 100 µM in RLM, or 0.005µM - 50 µM in HLM. Incubations were carried out at 37°C in a shaking water bath. The final incubation mixtures contained RLM or HLM (0.5 mg/ml), phenacetin (50 µM), tolbutamide (120 µM), S-mephenytoin (40 µM), dextromethorphan (5 µM), midazolam (5 µM), and NADPH (1.0 mM) in 100 mM sodium phosphate buffer (MgCl$_2$ 3.3 mM, pH 7.4). The reactions were started by the addition of NADPH after pre-incubation of RLM or HLM, test drug and CYP substrate at 37°C for 5 min. All incubations were stopped at 10 min by the addition of 200 µl chilled acetonitrile containing propranolol (200 ng/ml). After centrifugation at 14,000g for 10 min, the supernatant was collected and analyzed by a UHPLC-MS/MS method described above. Positive controls, such as the α-naphthoflavone (0.025-100 µM) in RLM and (0.0004-5 µM) in HLM, were included in the study. Stock solutions of the test drugs were prepared in acetonitrile. The final concentration of acetonitrile in incubates was less than 0.2% (v/v). All measurements were performed in triplicate.

IC$_{50}$ shift assay
To evaluate the potential of time-dependent inhibition by PRN and IPRN on CYP1A2, IC$_{50}$ shift experiments were performed. Multiple concentrations of PRN or IPRN were incubated in 2 mg/ml protein concentration RLM (0.05 - 100 µM) or HLM (0.005 - 50 µM) with or without NADPH for 30 min. Vehicle controls were run to account for any decrease in enzyme activity caused by incubation under these conditions. All measurements were performed in triplicate. After the inactivation incubation, a portion of the inactivation mixture (20 µl) was added to a mixture containing 50 µM phenacetin and NADPH (1.0 mM) in 180 µl of 100 mM potassium phosphate buffer (MgCl$_2$ 3.3 mg, pH 7.4) and further incubated for 10 min. After a significant IC$_{50}$ shift was observed from the shift experiment, the $K_i$ and $k_{inact}$ values were further determined in an inactivation kinetic assay, the traditional dilution assay.

**Inactivation kinetic assay**

Inactivation kinetic experiments were conducted with six concentrations of PRN (0, 0.1, 0.3, 1, 3, 10 µM) or IPRN (0, 0.05, 0.15, 0.5, 1.5 and 5 µM) along with RLM (2 mg/ml) and NADPH (1.0 mM) in 100 mM potassium phosphate buffer (MgCl$_2$ 3.3 mM, pH 7.4). In HLM, the experiment was only performed for IPRN with the same concentration range as in RLM because IC$_{50}$ shift was only observed for IPRN. An aliquot (20 µl) of the mixture was taken at 0, 5, 10, 20 or 30 min and added to separate vials containing 180 µl mixture of 50 µM phenacetin and NADPH (1.0 mM) to initiate subsequent incubation (10 min) for CYP1A2 activity determination. All measurements were performed in triplicate.

**LogD$_{7.4}$ determination**
LogD$_{7.4}$ was measured by fully automated-shake flask method. Phosphate buffer (pH 7.4) and oil solutions (n-octanol) were used. The value was calculated by the following equation:

$$\log D = \log\left[\left(\frac{\text{Conc}_{\text{initial}} - \text{Conc}_{\text{final}}}{\text{Conc}_{\text{final}}}\right) \times \left(\frac{V_{\text{aq}}}{V_{\text{oct}}}\right)\right]$$

where Conc$_{\text{initial}}$ is the concentration of compound in the initial aqueous solution, Conc$_{\text{final}}$ is the concentration of compound in the final aqueous phase, $V_{\text{aq}}$ is the volume of aqueous solution, and $V_{\text{oct}}$ is the volume of octanol (Stopher and McClean, 1990).

**Caco-2 assay**

Caco-2 cells were seeded onto Transwell® assay plates, and the assay was performed with 21 to 28 day confluent monolayer cells. The final concentration of PRN and IPRN was 10 $\mu$M was used to determine the passive permeability (DMSO concentration <1%).

Transwell® studies were conducted at 37°C for 2 h. The compound was placed in the apical side to assess permeability in the A$\rightarrow$B (apical to basolateral) direction. Samples were analyzed by LC-MS/MS. The apparent permeability coefficient ($P_{\text{app}}$) values were calculated with the equation: $P_{\text{app}} = \frac{1}{(A \times C_0) \times (dQ/dt)}$, where A is the insert surface area, $C_0$ is initial donor drug concentration, and $dQ/dt$ is the amount of drug transported within a given time period (Walker et al., 2005).

**Plasma protein and microsomal protein binding**

Plasma protein binding of PRN and IPRN was determined at 10 $\mu$M in rat and human plasma, using rapid equilibrium dialysis devices (Thermo Fisher Scientific). In each
species, pooled plasma was obtained from at least five donor subjects with plasma from males and females pooled separately. All measurements were performed in triplicate. Plasma samples were incubated at 37°C and oscillated at 100 rpm for 4 h. At the end of dialysis, 50 μl each of post-dialysis samples from the buffer and the plasma chambers were pipetted into separate microcentrifuge tubes, and then 50 μl of plasma was added to the buffer samples while an equal volume of phosphate-buffered saline (PBS) to the collected plasma samples. For protein precipitation, 200 μl precipitating agents (acetonitrile/methanol 1:1, v/v, containing 2 ng/ml IS) was added to the diluted samples. After centrifugation at 14 000 g for 10 min, the remaining PRN and IPRN in the supernatant was analyzed by LC-MS/MS. The unbound fraction (%$F_u$) was calculated as follows: 

\[
% F_u = \left(\frac{\text{Conc}_{\text{bufferchamber}}}{\text{Conc}_{\text{plasmachamber}}}\right) \times 100\%.
\]

Microsomal protein binding assay was followed a previous method (Lu et al., 2006): microsomes (0.5 mg/ml) were mixed with 10 μM test compound in 0.1 M phosphate buffer, pH 7.4, containing 3 mM MgCl₂ (the dialysis buffer) and subject for an overnight equilibrium dialysis. All measurements were performed in triplicate. Concentrations of PRN and IPRN in receiving side (after a 1:0.5 dilution in control microsomes) and donor side (after a 1:2 dilution with dialysis buffer) were determined using the LC-MS/MS method. The free fractions were calculated as:

\[
% F_{u,mic} = \left(\frac{\text{Conc}_{\text{receivingside}} \times 1.5}{\text{Conc}_{\text{donorside}} \times 3}\right) \times 100\%.
\]

**Blood partitioning**
The blood/plasma concentration ratio of PRN and IPRN was determined after incubation of PRN and IPRN (10 μM) in rat and human blood (0.5 ml) for 3 h. After the incubation, 50 μl aliquots were removed and the remaining blood was centrifuged at 3000 g for 10 min, after that 50-μl aliquots of plasma were removed. Concentrations of PRN and IPRN in whole blood and plasma were determined using the LC-MS/MS method. All of the incubations were performed in triplicate. The blood/plasma concentration ratio (\( R_{bp} \)) was calculated by the following equation: \( R_{bp} = \frac{C_{blood}}{C_{plasma}} \).

Hepatic clearance assay

For microsomal clearance, triplicate samples of PRN and IPRN (10 μM final concentration, below or around the km values) were incubated at 37°C with rat or human liver microsomes (0.5 mg/ml) in 0.1 M potassium phosphate buffer with 3.3 mM MgCl₂, pH 7.4. NADPH (1.0 mM) was added to initiate the incubation. Aliquots were removed at 0, 10, 20, 30, 45, 60 and 90 min, and added to equal volumes of precipitating agent to stop the reaction. After centrifugation at 14 000 g for 10 min, the remaining parent drug in the supernatant was analyzed by LC-MS/MS. Negative controls were conducted by adding drug but omitting the NADPH. The clearance was calculated using the 1st order decay equation (Lu et al., 2006).

Animal experiments

Twenty male Sprague-Dawley rats were randomly divided into four groups (5 rats each) in the morning of the experiment, PRN (5 mg/kg), IPRN (5 mg/kg), α-naphthoflavone (7 mg/kg) (positive control, Bachmann et al., 1993) or vehicle was dosed intravenously for
each group, respectively. The 5 mg/kg of PRN and IPRN was selected based on maximum dose in humans (60 mg/day, PO) considering the difference of body surface area and oral bioavailability. After 15 min, all the rats received an intravenous dose of phenacetin (5 mg/kg). The dosing formulations were prepared with 40% saline and 60% PEG400. The concentration for PRN and IPRN injection solutions was 5 mg/ml, and 7 mg/ml for α-Naphthoflavone. Blood samples (0.25 ml, over disodium EDTA) were taken from the right cannulated jugular vein before dosing (0 min) and at 0.03, 0.08, 0.25, 0.5, 1, 2, 4, 6, 8, and 12 h post phenacetin dosing. Plasma was collected by centrifugation and stored at -20 °C until analysis.

**Simcyp® simulation**

Simcyp® population based simulator (version 11, Sheffield, UK) was used in this study. To build the PRN and IPRN compound profiles, the experimental data in Table 1 were used. Simulation were performed in healthy subject population (n=100, 50% male, age 40-65). A smoker population was constructed by modify the CYP1A2 abundance in healthy subject population from 52 to 94 pmol/mg microsomal protein to mimic people who smoke >20 cigarettes/day (Plowchalk and Rowland Yeo, 2012). Simcyp® default phenacetin profile was used without further modification. The maximum allowed daily doses of PRN and IPRN (60 mg, Pharmacopoeia of the People’s Republic of China, 2005) were used to predict the worst-case scenario of DDI. Simulation was extended to 10 days (steady-state) for both perpetrator and victim drugs. For parameters not listed in the Table 1, Simcyp® default or predicted values were used.
Data analysis

In the in vitro reversible inhibition assay, the LC-MS/MS peak area of acetaminophen (and dextrorphan for HLM assay only) formed at each concentration relative to the control (percentage of remaining activity) was plotted against the concentration of the inhibitor (log transformed). The IC_{50} values were calculated using nonlinear regression analysis with GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). In the in vitro TDI assay, the natural logarithm of remaining microsomal CYP1A2 activity was plotted against the pre-incubation time at each inhibitor concentration to obtain the observed rates of CYP1A2 inactivation \( k_{\text{obs}} \). Then, the \( k_{\text{inact}} \) and \( K_I \) were estimated by nonlinear regression using the following equation (Jones et al., 1999).

\[
K_{\text{obs}} = \frac{K_{\text{inact}} \cdot [I]}{K_I + [I]}
\] (3)

In the rat in vivo DDI study, pharmacokinetic parameters of phenacetin were calculated using the standard non-compartmental module of WinNonlin (Version 5.2.1, Pharsight Corp; Mountain View, CA, USA). Student’s \( t \) test was used to compare whether drug treated group is different compared to the control group with \( p < 0.05 \).
Results

Inhibition of PRN and IPRN on CYP1A2 activity

PRN and IPRN showed moderate inhibition of cyp1a1/2 with IC$_{50}$ values of 10.4 ± 1.4 and 7.1 ± 0.6 µM in RLM, respectively, but no inhibition toward human CYP substrates of CYP2C9, 2C19, 2D6, and 3A4. PRN and IPRN also showed potent inhibition of CYP1A2 in HLM with IC$_{50}$ values of 0.26 ± 0.01 and 0.22 ± 0.03 µM, respectively and moderate inhibition of CYP2D6 with IC$_{50}$ values of 3.60 ± 0.31 and 8.49 ± 1.45 µM, respectively, with no inhibition toward CYP2C9, 2C19, and 3A4 (Table 2). To investigate whether PRN and IPRN caused time-dependent inhibition on CYP1A2, the two compounds were pre-incubated at various concentrations prior to the addition of phenacetin. Significant IC$_{50}$ shifts were observed for both PRN and IPRN after 30-min pre-incubation in the presence of NADPH. The fold of IC$_{50}$ shift for PRN and IPRN were 169 and 20 in rat liver microsomes and 0.76 and 7.6 in human liver microsomes, respectively (Fig. 2). This observation suggested that PRN and IPRN could be potential time-dependent inhibitors of CYP1A2 in rats and humans (IPRN only). The compound α-naphthoflavone, a known competitive inhibitor of CYP1A1/2, was included in this study and an IC$_{50}$ value of 0.02 ± 0.002 µM was observed in HLM that is in agreement with published values (Weaver et al., 2003; Moody et al., 1999). As expected, a left shift of IC$_{50}$ was not observed for α-naphthoflavone upon 30-min pre-incubation. Instead, approximately 10-fold right shift was observed that probably due to the loss of parent compound via metabolism during the 30-min pre-incubation. Furafylline, a known CYP1A2 TDI, was also included as a positive control, a left shift from IC$_{50}$ of 5.77 ± 0.03 µM to 0.096 ± 0.000 µM after 30-min pre-incubation.

Time-dependent Inactivation of CYP1A2 by PRN and IPRN
Kinetics of CYP1A2 inactivation by both PRN and IPRN were time- and concentration-dependent. The time course data were analyzed to determine the initial rate constants of the inactivation at various concentrations for PRN and IPRN (Fig. 3). The rate constant for maximal inactivation at saturation ($k_{\text{inact}}$) and the concentration required to produce one-half the maximal rate of CYP1A2 inactivation ($K_I$) were determined to be $0.060 \pm 0.002 \text{ min}^{-1}$ and $1.13 \pm 0.12 \text{ } \mu\text{M}$ for PRN, and $0.10 \pm 0.01 \text{ min}^{-1}$ and $1.95 \pm 0.31 \text{ } \mu\text{M}$ for IPRN in rat, respectively and $0.050 \pm 0.002 \text{ min}^{-1}$ and $0.40 \pm 0.06 \text{ } \mu\text{M}$ for IPRN in human (Table 1). The result indicated the $K_I$ values of PRN and IPRN are much lower than the known CYP1A2 mechanism-based inhibitors, for example, furafylline has a $K_I$ of 6.9 $\mu\text{M}$ toward phenacetin (Tassaneeyakuo et al., 1994), dihydralazine has a $K_I$ of 42 $\mu\text{M}$ toward phenacetin (Masubuchi et al., 1999). Compounds $\alpha$-naphthoflavone was included in the study as a negative and furafylline was included as a positive control ($K_I = 1.49 \pm 0.40 \text{ } \mu\text{M}$ and $k_{\text{inact}} = 0.12 \pm 0.01 \text{ min}^{-1}$).

In vivo inhibitory effects of PRN and IPRN on phenacetin pharmacokinetics

To eliminate the influence of absorption, the effects of PRN and IPRN on the pharmacokinetics of phenacetin were assessed in the i.v. dosed rats. The known CYP1A2 inhibitor $\alpha$-naphthoflavone was tested in parallel as a positive control. The plasma concentration-time profiles of phenacetin for different dosing groups are presented in Fig. 4. The corresponding pharmacokinetic parameters are summarized in Table 3. The total plasma clearance of phenacetin obtained in the control group of this study was 54.3 ml/min/kg that is close to the rat hepatic blood flow rate (55.2 ml/min/kg, Davies and Morris, 1993). The clearance of phenacetin dramatically decreased in the groups co-administrated with PRN (10.3 ml/min/kg) or IPRN (10.2 ml/min/kg), which indicated that PRN and IPRN predominantly inhibited the phenacetin metabolism in liver.
as phenacetin is known to be mainly cleared in liver by metabolism (Raaflaub and Dubach, 1975). The $t_{1/2}$ also increased by 3.4- and 4.0-fold after treatment with PRN or IPRN. Another significant change was in the AUC value which increased more than 5-fold in both PRN and IPRN treated groups, suggesting PRN and IPRN are strong inhibitors of rat cyp1a2 at the given doses. In comparison, treatment with $\alpha$-naphthoflavone, a competitive CYP1A2 inhibitor, clearance of phenacetin also significantly decreased, as the exposure of phenacetin increased (~1.8-fold). However, at the similar dose level, the inhibition of $\alpha$-naphthoflavone was weaker than that of PRN or IPRN.

**Prediction of human DDI using Simcyp® simulation**

In vitro determined parameters of PRN and IPRN are listed in Table 1. These parameters include logD$_{7.4}$, intrinsic clearance, red blood cell partitioning, plasma and microsomal protein binding, Caca-2 permeability, reversible inhibition potential (IC$_{50}$), and time-dependent inhibition potential ($k_1$ and $k_{inact}$, IPRN only).

Simcyp® simulations were performed in healthy subject and smoker populations. Figure 5 presents the example of 10-day simulations of plasma concentration-time profile of 1500 mg QD dose of phenacetin with 60 mg QD dose of PRN or IPRN (the maximum doses in human, Pharmacopoeia of the People’s Republic of China, 2005). The DDI data, summarized in Table 4, showed that 60 mg of PRN increased the AUC of phenacetin by 1.71- and 2.12-fold in healthy and smoker populations, respectively, whereas, 60 mg of IPRN increased the AUC of phenacetin by 3.24- and 5.01-fold in healthy and smoker populations, respectively. The much potent inhibition of IPRN
compared to the PRN is attributed to both reversible and time-dependent inhibition of CYP1A2. In the smoker population, CYP1A2 activity was much higher without the inhibition of IPRN, and therefore, after addition of IPRN, the changes of clearance and AUC were more profound.
Discussion

It is commonly believed that there is a species differences in metabolism for some drugs. Therefore, selecting an appropriate animal species and probe substrate is crucial for in vitro - in vivo correlation. CYP1A2 enzyme is known to be expressed in all mammalian livers with few species differences in function or regulation, and high amino acid sequences homologys (Parkinson and Ogilvie, 2008). Phenacetin is a substrate widely used as an in vitro and in vivo probe (except for beagle dog, Whiterock et al., 2012) to measure CYP1A2 activity because of the metabolism of phenacetin to acetaminophen is thought to be a selective CYP1A2-mediated reaction (Distlerath et al., 1985; Tassaneeyakul et al., 1993). Hence, in the present study, the inhibitory effects of PRN and IPRN on CYP1A2 mediated metabolism were assessed in rat in vitro and in vivo and in human in vitro using phenacetin as the probe substrate. Recent study reported that including BSA in CYP1A2 microsomal incubation resulted in enhanced CYP1A2 activity by reducing the apparent $K_m$ values that mainly attribute to the quenching of inhibitory effect by long chain fatty acids residue in the HLM preparation by BSA (Wattanachai et al., 2012). Since including BSA in microsomal incubation has not become a standard practice in pharmaceutical industrial, addition of BSA was not applied in this study. However, should BSA be used in this study, different parameters of inhibition by PRN and IPRN would be observed, probably toward lower values of $IC_{50}$, $K_i$ and $k_{inact}$. Thus, higher DDI potential would be predicted in Simcyp® simulation.

For the first time, the results of the current study revealed and compared the reversible and time-dependent inhibitory activities of CYP1A2 by PRN and IPRN (which is the angular isomer of PRN) obtained from the in vitro and in vivo with the same substrate in the identical assay system. The inhibitory activity of PRN or IPRN on rat cyp1a2
without pre-incubation was relatively moderate, with IC$_{50}$ values of 10.4 and 7.1 µM, respectively. From the available literatures, the typical plasma concentrations of PRN and IPRN were less than 400 ng/ml after the clinical dose of herbal medications containing PRN and IPRN (Li et al., 2009; Gu et al., 2009). This concentration is lower than the IC$_{50}$ values in rats but much higher than the IC$_{50}$ values in humans. The direct inhibition of cyp1a2 by PRN and IPRN in rat would be minimal. However, much stronger inhibitory effects were observed when PRN and IPRN were pre-incubated in the presence of NADPH. The significant IC$_{50}$ shifts and much low $K_I$ values (1.13 µM for PRN and 1.95 µM for IPRN) indicated that time-dependent inhibition could be the primary contributor toward the cyp1a2 inhibition observed in rat by PRN and IPRN. In comparison, the inhibition of cyp1a2 by the selective inhibitor $\alpha$-naphthoflavone was only through reversible inhibition, as no IC$_{50}$ shift was observed after pre-incubation with NADPH. In order to validate interactions of phenacetin with PRN or IPRN further, the in vivo inhibition experiments were conducted in rats. The same dose level was used for PRN and IPRN (5mg/kg) because of the similar content of these two active ingredients present in Psoralea corylifolia L and many other commonly used Chinese herbal medications. It was confirmed that the plasma exposure of phenacetin was significantly increased by concomitant administration of either PRN or IPRN. The AUC of phenacetin in concomitant dose groups increased more than 5-fold compared to the phenacetin control group. The ratios of acetaminophen to phenacetin also decreased accordingly, suggesting that the formation of the metabolite was inhibited. When phenacetin was coadministraed with $\alpha$-naphthoflavone (7 mg/kg), a known CYP1A2 reversible inhibitor, the AUC of phenacetin increased no more than 2-fold. In earlier rat PK studies, dosing of 12.5 mg/kg of $\alpha$-naphthoflavone showed an AUC$_{0-24}$ of 526 µg*h/ml (Wang and Morris, 2008), whereas dosing of 9.12 mg/kg of PRN and IPRN
resulted in AUC_{0-24} of 278 and 425 µg*h/ml, respectively (Feng et al., 2010). Thus, the exposure in rats after 7 mg/kg dose of α-naphthoflavone was marginal higher than that after 5 mg/kg dose of PRN or IPRN. Given the fact that the K_i values of α-naphthoflavone (1.37 µM) is lower than that of PRN and IPRN (10.4 and 7.1 µM), the higher inhibition of phenacetin metabolism observed in rats when co-administrated with PRN or IPRN could be attributed to the time-dependent inhibition by PRM and IPRN.

In human microsomal study, both PRN and IPRN showed a much potent reversible inhibition compared to that from the rat microsomal study. In addition, time-dependent inhibition was only observed with IPRN. In an attempt to assess the potential human DDI risk, Simcyp® simulations were conducted to predict phenacetin AUC changes under the co-administration of PRN or IPRN by allowing perpetrators and victim dose at the same time for 10 days. Because of the combined effect of reversible inhibition and time-dependent inhibition, IPRN showed a higher DDI potential than PRN. Furthermore, in the smoker population, with induced CYP1A2 activity, co-administration of IPRN predicted to have more profound DDI. CYP1A2, which is responsible for metabolism of some drugs, is also reported to metabolize some naturally occurring chemicals and environmental contaminants, converting some of them to mutagenic and carcinogenic active metabolites along with CYP1A1 which is mostly presented in lung (Peterson et al., 2006; Chang et al., 2013). Some well known examples are the biotransformation of polyaromatic hydrocarbons into their reactive metabolites that potentially cause DNA damage, leading to cancers (Lehr and Jerina, 1977). Thus, inhibition of CYP1A2 by PRN and IPRN, as constituents of TCMs, in the smoker population may attenuate the damaging effect of CYP1A2 induction by cigarette smoking, in addition to their therapeutically beneficial effect. It is also known that smoke induces
both CYP1A1 (mostly presents in lung) and CYP1A2 (mostly presents in liver), and phenacetin is substrate for both CYP1A1 and 1A2. In the Simcyp simulation, a default phenacetin profile was used as a CYP1A2 substrate to illustrate CYP1A2 mediated DDI, since most of the DDI are mediated by CYP1A2. However, if both CYP1A1 and 1A2 in liver as well as in lung were considered, the predicted DDI potential could be different dependent on the respective inhibition potential of PRN and IPRN toward CYP1A1 and CYP1A2.

Naturally occurring furanocoumarins exist in two structural types: linear furanocoumarins and angular furanocoumarin. Previous studies reported different CYP3A4 inhibitory properties for linear and angular type of furanocoumarins and linear ones are more potent CYP3A4 reversible inhibitors (Guo et al. 2000). PRN and IPRN represent the linear and angular furanocoumarins, respectively. In the present study, no significant difference in reversible and time-dependent inhibition was found in rat in vitro and in vivo cyp1a2 inhibition between PRN and IPRN, as well as in the human liver microsomal reversible inhibition study except the inhibition was more potent in human liver microsomes. However, in human liver microsomes, the time-dependent CYP1A2 inhibition was only observed with the angular furanocoumarin, IPRN. This difference may be attributed to that difference in compounds although they are in the same class, different enzymes and probe CYP substrate specificity (Cai et al. 1993, Paine et al., 2004; Prince et al. 2006). It remains to be seen if the angular furanocoumarina are in general more prone to the formation of more reactive epoxide metabolite, similar to the well established Bay Region theory of polyaromatic hydrocarbons (Lehr and Jerina, 1977; Chang et al., 2013). The observation of time-dependent inhibition in rat for both PRN and IPRN but only IPRN in human warrants future investigation.
In summary, the present study was conducted to investigate the inhibitory potential of PRN and IPRN, the constituents of TCMs, on CYP1A2. The results obtained from the in vitro and in vivo studies showed that these two furanocoumarins are potent time-dependent inhibitors of rat cyp1a2. More potent reversible inhibition was observed in human liver microsomal incubations. Furthermore, only the angular furanocoumarin IPRN was found to be a time-dependent inhibitor in human liver microsomes. Human DDI is predicted to be around 2-3 folds in the healthy population and up to 5-fold in the smoker population. On the other hand, the profound inhibition of CYP1A2 in the smoker population by these compounds may attenuate the damaging effect of CYP1A2 induction by cigarette smoking, in addition to their therapeutically beneficial effect. These findings provide useful information for the safe and effective usage of PRN and IPRN in the clinic. It is suggested that the dosage of common clinically used drugs that are metabolized by CYP1A2, such as theophylline and clozapine should be monitored when co-administrated with Chinese medicines containing PRN or IPRN.
Authorship Contributions

Participated in research design: Zhuang, Li, and Lu.

Conducted experiments: Zhuang, Zhong, and Xiao.

Performed data analysis: Zhuang, Zhong, Li, and Lu.

Wrote or contributed to the writing of the manuscript: Zhuang, Li, and Lu.
References


DMD #53199


Footnotes

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Legend of figures

Fig. 1 The chemical structure of PRN (A) and IPRN (B).

Fig. 2 Effect of PRN (PRN) and IPRN (IPRN) on phenacetin O-deethylation (percentage of control activity) with or without NADPH. A, B: the IC_{50} shift of phenacetin inhibition curves in RLMs after 30min pre-incubation with or without NADPH; C, D: the IC_{50} shift of phenacetin inhibition curves in RLMs after 30min pre-incubation with or without NADPH. The data presented in the figure are the mean of reduplicated samples (n=3).

Fig. 3 Preincubation time and concentration dependent inhibition of phenacetin O-deethylation by PRN (0-10µM) in rat liver microsomes (A) or IPRN (0-5µM) in rat liver microsomes (B), and (C) IPRN (0-5µM) in human liver microsomes. The rate of inactivation of CYP1A2 activity by each inhibitor concentration (K_{obs}) was determined by linear regression analysis of the natural logarithm of the percentage of activity remaining versus preincubation time data (left). The K_{i} and k_{inact} were calculated by nonlinear regression analysis of the K_{obs} versus PRN or IPRN concentration [I] data according to eq. 3 (right). The data presented in the figure are the mean of reduplicated samples (n=3).

Fig. 4. Mean (±S.D.) plasma concentration-time profiles of in rats after a single iv dose phenacetin (5 mg/kg) and PRN or IPRN coadministrated with phenacetin at iv dose of 5mg/kg. (n=5).

Fig. 5. Simcyp® simulation results of phenacetin AUC_{0-24} at 1400 mg QDx10 in the presence of IPRN (60 mg QDx10) and absence of IPRN in healthy subjects (A) and
DMD #53199

smoker population (B), or presence of PRN (60 mg QDx10) and absence of PRN in healthy subjects (C) and smoker population (D). The outer curves represent phenacetin concentration in the absence of PRN or IPRN. The inner curves represent phenacetin concentration in the presence of PRN or IPRN.
Table 1  Input data of PRN and IPRN for Simcyp® simulation

<table>
<thead>
<tr>
<th></th>
<th>PRN</th>
<th>IPRN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>186.17</td>
<td>186.17</td>
</tr>
<tr>
<td>logD$_{7.4}$</td>
<td>1.63</td>
<td>1.32</td>
</tr>
<tr>
<td>Blood-plasma partition co-efficient (B/P)</td>
<td>0.82</td>
<td>0.65</td>
</tr>
<tr>
<td>Plasma protein binding ($f_o$)</td>
<td>0.283</td>
<td>0.126</td>
</tr>
<tr>
<td>Microsomal protein binding at 0.5mg/ml ($f_o$)</td>
<td>0.745</td>
<td>0.906</td>
</tr>
<tr>
<td>Apparent permeability value: $P_{app}$ ($10^{-6}$cm/s) Caco-2</td>
<td>51.6</td>
<td>44.6</td>
</tr>
<tr>
<td>(calibration compound Atenolol $P_{app}$ = $1.40 	imes 10^{-6}$cm/s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal clearance ($\mu$l/min/mg)</td>
<td>14.5</td>
<td>8.0</td>
</tr>
<tr>
<td>CYP1A2 IC$_{50}$ ($\mu$M)</td>
<td>0.26</td>
<td>0.22</td>
</tr>
<tr>
<td>CYP1A2 $K_i$($\mu$M)</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>CYP1A2 $k_{inact}$ (min$^{-1}$)</td>
<td></td>
<td>0.05</td>
</tr>
</tbody>
</table>

For reversible inhibition, $K_i$ were estimated using $IC_{50}/2$

Both compounds are in neutral condition under physiological pH, thus $pKa$ was not available

1400 mg phenacetin QDx10 and 60 mg PRN or IPRN QDx10 were applied
Table 2  CYP inhibition of PRN and IPRN in rat and human liver microsomal incubations (n=3)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>CYP1A2</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2D6</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRN in RLM</td>
<td>10.4 ±1.41</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>IPRN in RLM</td>
<td>7.1 ± 0.6</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PRN in HLM</td>
<td>0.26 ± 0.01</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>3.60 ± 0.31</td>
<td>&gt;50</td>
</tr>
<tr>
<td>IPRN in HLM</td>
<td>0.22 ± 0.03</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>8.49 ± 1.45</td>
<td>&gt;50</td>
</tr>
</tbody>
</table>

The highest concentrations of PRN and IPRN tested in rat and human liver microsomal incubations were 100 µM and 50 µM, respectively. Headings show human CYP isozymes, same substrates were used for RLM studies.
Table 3 Pharmacokinetics of phenacetin (5mg/kg, i.v.) after co-administration of α-naphthoflavone (7mg/kg, i.v.), PRN or IPRN (5 mg/kg, i.v.) (n=5)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Vehicle (Negative Control)</th>
<th>PRN</th>
<th>IPRN</th>
<th>α-naphthoflavone (Positive control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (µg*h/ml)</td>
<td>1.55±0.19</td>
<td>8.24±1.12 *##</td>
<td>8.57±2.39 **##</td>
<td>2.83±0.39 *</td>
</tr>
<tr>
<td>Vss (l/kg)</td>
<td>1.42±0.22</td>
<td>0.91±0.24 *#</td>
<td>1.05±0.18 *#</td>
<td>2.51±1.04</td>
</tr>
<tr>
<td>CL (ml/min/kg)</td>
<td>54.33±6.75</td>
<td>10.27±1.46 ***##</td>
<td>10.15±2.24 ***##</td>
<td>29.85±3.86 **</td>
</tr>
<tr>
<td>T1/2 (h)</td>
<td>0.30±0.015</td>
<td>1.02±0.17 **</td>
<td>1.21±0.11 **</td>
<td>0.97±0.40 *</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>0.31±0.02</td>
<td>2.21±0.15 ***##</td>
<td>1.81±0.22 ***##</td>
<td>0.66±0.06 **</td>
</tr>
<tr>
<td>AUC(M)/AUC(P)</td>
<td>1.39±0.13</td>
<td>0.24±0.03 ***##</td>
<td>0.19±0.04 ***##</td>
<td>0.61±0.55 **</td>
</tr>
</tbody>
</table>

Results were mean ±SD of 5 animals. *p<0.05 and **p<0.01 compared to negative control group. *p<0.05 and **p<0.01 compared to positive control group. AUC(M)/AUC(P) represents the exposure ratio of acetaminophen to phenacetin.
<table>
<thead>
<tr>
<th>Simulation group</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</th>
<th>AUC&lt;sub&gt;216-240&lt;/sub&gt; (ng*h/ml)</th>
<th>Fold of AUC change</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml)</th>
<th>AUC&lt;sub&gt;216-240&lt;/sub&gt; (ng*h/ml)</th>
<th>Fold of AUC change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacetin in healthy population</td>
<td>4800</td>
<td>28300</td>
<td></td>
<td>4800</td>
<td>28300</td>
<td></td>
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<td></td>
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<tr>
<td>Phenacetin in healthy population</td>
<td>6880</td>
<td>48300</td>
<td>1.71</td>
<td>8670</td>
<td>92300</td>
<td>3.26</td>
</tr>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenacetin in smoker population</td>
<td>3620</td>
<td>18100</td>
<td></td>
<td>3620</td>
<td>18100</td>
<td></td>
</tr>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Phenacetin in smoker population</td>
<td>6150</td>
<td>38200</td>
<td>2.12</td>
<td>8580</td>
<td>90400</td>
<td>5.01</td>
</tr>
</tbody>
</table>
| Phenacetin 1400 mg QDx10 and PRN or IPRN 60 mg QDx10 were used in simulation. The C<sub>max</sub> and AUC were taken from last dose to mimic the steady-state.
Figure 1
Figure 2
Figure 3
Figure 4
Mean Values of Systemic concentration in plasma of Sim-Phenacetin with and without Interaction over Time using PRN

A, PRN healthy

B, PRN smoker

Mean Values of Systemic concentration in plasma of Sim-Phenacetin with and without Interaction over Time using IPRN

C, IPRN healthy

D, IPRN smoker

Figure 5