Microsomal cytochrome P450 mediated metabolism of protopanaxatriol ginsenosides: metabolites profile, reaction phenotyping, and structure metabolism relationship

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Running title: CYP3A mediated metabolism of PPT type ginsenosides

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Abbreviations: CYP, cytochrome P450; PPT, protopanaxatriol; PPD, protopanaxadiol; LC/MS-IT-TOF, high performance liquid chromatography hybrid ion trap and time-of-flight mass spectrometry; CL\text{int}, intrinsic clearance; Ket, ketoconazole; Naph, α-naphthoflavone; Sul, sulfaphenazole; Qui, quinidine; DDC, diethyl dithiocarbamate; TP, ticlopidine hydrochloride; Orph, orphenadrine citrate salt
Abstract

Although the biotransformation of ginsenosides in gastrointestinal tract has been extensively studied, very less is known about the hepatic cytochrome P450 catalyzed metabolism. The major aims of this study were to clarify the metabolic pathway and involved CYP isoforms, and to explore the structure metabolism relationship of, protopanaxatriol (PPT) type ginsenosides in hepatic microsomes. Efficient depletion of ginsenoside Rh1, Rg2, Rf, and PPT was found, whereas the elimination of Re and Rg1, characterized with a glucose substitution at C20 hydroxy group, was negligible, in microsomal incubation systems. Based on a LC/MS-IT-TOF analysis, the oxygenation metabolism on the C20 aliphatic branch chain was identified as the predominant metabolic pathway of PPT ginsenosides in both human and rat hepatic microsomes. By comparing with authentic standards, the C24-25 double bond was identified as one of the oxygenation site to produce the metabolites of C20-24 epoxide (ocotillol-type ginsenosides). Both chemical inhibition and human recombinant CYP isoforms assay indicated that CYP3A4 was the predominant isozyme responsible for the oxygenation metabolism of PPT ginsenosides. Enzyme kinetic evaluations in rat and human hepatic microsomes and human recombinant CYP3A4 isozyme incubation systems reached a generally consistent result in that the intrinsic clearance ranked as Rf≤Rg2<Rh1<PPT, closely correlating with their Log P values and the number of glycosyl substitutions. Results obtained from this study suggest that CYP3A4 catalyzed oxygenation metabolism plays an important role on the hepatic disposition of ginsenosides, and that the glycosyl substitution, especially at C20 hydroxy group, determines their intrinsic clearances by CYP3A4.
Introduction

Ginseng is one of the most popular herbal medicines used in oriental counties (*Panax ginseng* C.A. Meyer, Asian ginseng) to treat many diseases over 2000 years, and also came to known in the West (*Panax quinquefolius* L., American ginseng) by the 18th century (Gillis, 1997; Attele et al., 1999). More than 100 kinds of protopanaxadiol (PPD) and protopanaxatriol (PPT) type ginsenosides have been identified and claimed to be responsible for the wide therapeutic effects of ginseng and its various preparations (Christensen, 2009). A large body of references has been found in the current literature contributing to the pharmacological activities, underlying mechanisms, pharmacokinetic behaviors, and biotransformation of ginsenosides (Jia and Zhao, 2009; Jia et al., 2009). One of the important findings is that both PPD and PPT type ginsenosides always undergo sequential deglycosylation metabolism catalyzed by gastric acid and intestinal bacteria in the biological conditions, to form secondary metabolites and finally aglycones with enhancing biological activities and better pharmacokinetic characteristics (Liu et al., 2009). For example, ginsenoside Rg3 sequentially degraded in rat gastrointestinal tract to Rh2 and PPD, both of which were absorbed into the circulation system and characterized with higher plasma exposure levels (Xie et al., 2005), and stronger antitumor activities than ginsenoside Rg3 itself (Bae et al., 2004). Such important findings strongly indicate that the biotransformation and metabolic studies are of great significance on disclosing the therapeutic mysteries of ginsenosides.

Despite the intensive research on ginsenosides biotransformation in previous decades, most of them are largely limited to the sugar cleavage reactions that catalyzed by gastric acid and/or
intestinal bacteria. To date, very less is known about the further metabolic fate of such ginsenosides and their degradation products absorbed into the circulation system, where the hepatic microsomal drug metabolizing enzymes play an important role on many synthesized drugs and natural products. Recently, we have identified two mono-oxygenated metabolites from the bile and urine samples of rats treated intravenously with ginsenoside Rh1, and also from the in vitro hepatic microsomal incubation media (Lai et al., 2009a). Oxygenated metabolites had been also identified for Rg3 (Qian et al., 2005b), Rh2 (Qian et al., 2005a), and Rd (Yang et al., 2007) in rats, and for PPT in rat hepatic microsomes (Kasai et al., 2000). Moreover, some previous reports demonstrated that various ginsenosides and especially their degradation products were capable of modulating the cytochrome P450 enzymes activities (Liu et al., 2006a; Liu et al., 2006b; Etheridge et al., 2007; Wang et al., 2008). Such evidences strongly suggest that the microsomal cytochrome P450 enzymes may play an important role on the systematic dispositions of ginsenosides that finally reached the systemic circulation after oral ingestion. In view that it is exactly the intact ginsenosides and their degradation products reaching the circulation system to be of therapeutic benefits in most cases, the detailed hepatic microsomal mediated metabolism of ginsenosides proposed in this study will be of great significance for better understanding of their systematic dispositions, ginseng-drug interactions, and involved mechanisms.

The present study was thus designed to make a comprehensive research on the microsomal mediated metabolism of PPT type ginsenosides. Five major PPT type ginsenosides including 20(S)-ginsenoside Re, Rg1, Rg2, Rf, and Rh1, and their aglycone PPT (Fig.1) were included
in this study to evaluate the microsomal mediated metabolism, including metabolic profiling in both human and rat microsomal incubation systems through a LC/MS-IT-TOF analysis, reaction phenotyping using human recombinant CYP450 isoforms and specific chemical inhibitors, and structure-metabolism relationship assessment based on enzyme kinetics analysis. Such components were selected for the consideration of their high contents in various ginseng preparations, well proven pharmacological activities, and the coverage of structure characteristics of PPT type ginsenosides (attached with 0-3 glycosyl groups).
Materials and Methods

Chemicals and reagents

Ginsenoside Re, Rg1, Rg2, Rf, Rh1, Pseudo Ginsenoside RF11 and RT5, and PPT (purity >98%) were from the College of Chemistry in Jilin University. Chemical inhibitors, ketoconazole (Ket), α-naphthoflavone (Naph), sulfaphenazole (Sul), quinidine (Qui), diethyl dithiocarbamate (DDC), ticlopidine hydrochloride (TP), and orphenadrine citrate salt (Orph) (purity >99%) were purchased from Sigma Chemical Company (Shanghai, China). Pooled human hepatic microsomes (20 mg/ml, liver sources were from ten male donors of trauma death, with the age ranged from 24 to 38 y) were purchased from Research Institute for Liver Diseases Co. Ltd (Shanghai, China). cDNA expressed human CYP450 isoforms (CYP3A4, CYP3A5, CYP1A2, CYP2C9*1, CYP2C19, CYP2D6*1, CYP2B6, CYP2E1, CYP1A1, and CYP1B2) from a baculovirus-infected cell system co-expressing human P450 reductase were purchased from BD Biosciences (Woburn, MA). The P450 content ranged from 1000 to 2000 pmol/ml. Glucose-6-phosphate (6-P-G) (purity 98-100%), β-nicotinamide adenine dinucleotide phosphate (NADP⁺) (purity 97%), and glucose-6-phosphate dehydrogenase (PDH) (200-400 units/mg protein) were purchased from Sigma Chemical Company (Shanghai, China). Digoxin (internal standard) was obtained from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetonitrile and methanol were obtained from Merck (Damstadt, Germany). Deionized water was purified using a Milli-Q system (Millipore, Milford, MA, USA). Magnesium chloride (MgCl₂), n-butanol, and other regents were all of analytical grade. Pooled human hepatic microsomes and human recombinant CYP450 isoforms were stored at -80°C until use.
Rat hepatic microsomes preparation

All rats used in our experiments were male Sprague-Dawley rats (body weight 200-220 g), from Academy of Military Medical Sciences, China. All animal studies were approved by the Animal Ethics Committee of China Pharmaceutical University. Microsomes were prepared by using the livers of male rats through differential centrifugation (Hao et al., 2007; Jia and Liu, 2007). The protein concentrations were determined with a commercially available kit (BCA protein assay; Pierce Chemical Co.). Rat hepatic microsomes were stored at -80°C and used within 2 weeks.

Metabolic profiling of PPT ginsenosides in microsomal incubation systems

PPT ginsenosides were incubated in rat and human hepatic microsomes to examine the potential CYP450 mediated metabolism. For NADPH-dependent oxidative metabolism study, the reaction media contained microsomal protein (1 mg/ml for rat hepatic microsomes and 0.25 mg/ml for human hepatic microsomes), 10 mM 6-P-G, 10 mM MgCl2, 1 U/ml PDH, 8 µM of each substrates (Re, Rg1, Rg2, Rf, Rh1, or PPT) and 0.5 mM NADP+ in 100 mM potassium phosphate (pH 7.4). Negative control incubations containing no NADPH regenerating system were conducted. All incubations were performed at 37°C in a shake water bath for 30 min.

All samples were extracted by n-butanol and analyzed using LC/MS-IT-TOF (Shimadzu, Japan). A Shim-Pack 250 mm × 2.0 mm column packed with 5 µm VP-ODS C18 (Shimadzu, Japan) was used for chromatographic separation. The mobile phase consisted of water (A) and
acetonitrile (B). The gradient program was set as follows: 20% B to 70% B from 0-20 min, 70% B to 20% B from 20-25 min, then holding 20% B for another 5 min. The flow rate was set at 0.2 ml/min with the column temperature at 35°C. Mass spectrometry was operated under negative-ion scan mode with the scan range set at m/z 100-1200. The nebulizing gas flow rate was set at 1.5 l/min, and the drying gas pressure was 0.1 MPa. The applied voltage was -3.5 kV. The curved desolvation line (CDL) temperature was 200°C.

**Enzyme kinetics in rat and human hepatic microsomes**

Enzyme kinetics were determined in rat hepatic microsomes (1 mg/ml) and human hepatic microsomes (0.25 mg/ml) for four PPT ginsenosides including Rg2 (1.56-50 µM), Rf (2.5-80 µM), Rh1 (0.31-50 µM), and PPT (0.31-50 µM), for which the CYP450 catalyzed metabolism had been confirmed in the metabolic profiling study. Each assay tube contained the same NADPH-regenerating system described above. All reactions were conducted at 37°C for 15 min. Preliminary experiments were carried out to ensure that the depletion of parent compounds was in the linear range of both reaction time and the protein concentration of microsomes.

**Chemical inhibition study in rat and human hepatic microsomes**

Chemical inhibition study was performed by adding each of the specific chemical inhibitors of CYP450 isoforms into the incubations of Rg2 (8 µM), Rf (8 µM), Rh1 (8 µM), and PPT (4µM) in the rat and human microsomal incubation systems containing NADPH-regenerating system as described above. The selective inhibitors and their concentrations were selected
based on previous reports and listed as follows: Ket (1 µM) for CYP3A, Naph (10 µM) for CYP1A2, Sul (10 µM) for CYP2C, Qui (10 µM) for CYP2D, DDC (20 µM) for CYP2E1, TP (5 µM) for CYP2C19, and Orph (20 µM) for CYP2B (Rodrigues, 1999; Tucker et al., 2001; Bjornsson et al., 2003; Liu et al., 2007; Meyer et al., 2009). All reactions were initiated by adding the substrate (ginsenosides). For the mechanism-based inhibitors, TP, DDC, and Orph were pre-incubated with all incubation constitutes at 37°C for 15 min before initiating the reaction by adding substrates.

Metabolism of PPT ginsenosides in recombinant human P450 isoforms

The capacity of selected major human CYP450 isoforms to metabolize Rg2 (5, 50 µM), Rf (5, 50 µM), Rh1 (5, 50 µM), and PPT (2, 20 µM) was screened using human recombinant isozymes. In brief, each ginsenoside was added into a 0.2 ml incubation mixture containing CYP450 isoform (4 pmol for CYP3A4, CYP3A5, CYP1A2 and CYP2C9*1; 8 pmol for CYP2C19 and CYP2B6; 0.25 pmol for CYP1A1; 1 pmol for CYP1B1; 20 pmol for CYP2E1; 2 pmol for CYP2D6*1), 1.3 mM NADP+, 3.3 mM 6-P-G, 0.4 U/ml PDH, and 3.3 mM MgCl₂ in 100 mM potassium phosphate (pH 7.4). Reactions were initiated by adding substrate and incubated at 37°C for 15 min. In a follow up study to estimate kinetic parameters, Rg2 (1.56-50 µM), Rf (2.5-80 µM), Rh1 (0.31-50 µM), and PPT (0.31-50 µM) was incubated with human recombinant CYP3A4 (20 pmol/ml) for 15 min.

Quantitative analysis

All reactions for quantitative analysis in rat and human hepatic microsomes and human
recombinant CYP450 isoforms were terminated by adding cold n-butanol with digoxin as their internal standard. The precipitate was removed by centrifugation and the supernatant was transferred to an eppendorf tube and evaporated to dryness by the Thermo Savant SPD 2010 SpeedVac System (Thermo Electron Corporation, USA). The residue was dissolved by mobile phase for analysis.

The quantifications of PPT ginsenosides and their aglycone PPT were performed by a method based on our previous report (Sun et al., 2005) with slight modifications. All samples were analyzed by high-performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI/MS) (Shimadzu Corporation, Japan). Quantitative analysis was performed in selected ion monitor (SIM) mode. Briefly, a Shim-Pack 250 mm × 2.0 mm column packed with 5 μm VP-ODS C18 (Shimadzu Corporation, Japan) was used. The column temperature was at 40°C. The nebulizing gas flow rate was set at 1.5 l/min, and the drying gas flow rate was 4.0 l/min. Mass spectrometry was operated under negative-ion mode with the detector potential at 1.60 kV. The CDL temperature was 200°C. The mobile phase consisted of 2 mM ammonium chloride (A) and acetonitrile (B) with a gradient program as follows: 20 % B to 60 % B from 0-4.5 min, 60 % B to 90 % B from 4.5-6.5 min, 90 % B to 20 % B from 6.5-8.5 min, and holding 20 % B for another 4 min, with the flow rate set at 0.2 ml/min. The ([M+Cl]-) ion, which has been found most sensitive for all analytes, was selected for the quantifications of Re at m/z 981.5, Rg1 at m/z 835.5, Rg2 at m/z 819.5, Rf at m/z 835.5, Rh1 at m/z 673.5, PPT at m/z 511.5, and the internal standard digoxin at m/z 815.4.
Data Analysis

The analysis of kinetic parameters was conducted using a method of parent drug depletion. In view that the oxygenation metabolism was the sole pathway for PPT ginsenosides in hepatic microsomes, the depletion rate of parent compounds may be indicative of the conglomeration rate of their oxygenation metabolism. The depletion profiles for all the tested ginsenosides were preliminarily confirmed exhibiting log-liner over the time course studied (15 min). The average depletion rate, expressed as pmol/min/mg protein, was then estimated from the amount differences between zero time point and after 15 min incubation. The apparent \( V_{\text{max}} \) and \( K_m \) values were estimated by non-linear regression from the Lineweaver-Burk plots based on the typical Michaelis-Menten equation, \( 1/V=1/V_{\text{m}}+(K_{m}/V_{\text{m}})\cdot 1/S \), where \( V \) is the rate of reaction, \( V_{\text{max}} \) is the maximum velocity, \( K_m \) is the Michaelis constant (substrate concentration at 0.5 \( V_{\text{max}} \)), and \( [S] \) is the substrate concentration. The intrinsic clearance (\( \text{CL}_{\text{int}} \)) was calculated as \( V_{\text{max}}/K_m \). Data were expressed as the mean ± S.D of triplicate experiments.
Results

Metabolites profile

In both rat and human hepatic microsomal incubation systems, Rg2, Rh1, Rf, and PPT underwent efficient depletion, whereas the elimination of Re and Rg1 was negligible, after a 30 min incubation. Samples from Rg2, Rh1, Rf, and PPT incubations were then subjected to a powerful LC/MS-IT-TOF analysis for metabolites profiling and identification. As a result, three metabolites for each of the PPT ginsenosides were detected. The chromatographic and mass data are summarized in Table 1. With benefit from the accurate measurements of both parent and product ions, it was feasible to characterize the metabolites. For Rg2, Rf, and Rh1, three metabolites characterized with exactly same quasi-molecular ions and different retention times were observed. The metabolites were readily proposed as the mono-oxygenated products of the intact ginsenosides, from a Ca. 16 Da difference between the metabolites and their respective precursors. The major fragment patterns of such ginsenosides and M1/2 were of sequential sugar loss, and the further C-20 aliphatic branch chain dissociation from the molecular ions, to produce finally an aglycone PPT diagnostic ion at m/z 391. In contrast, fragment ions of M3 were somewhat different in that a diagnostic ion at m/z 415 instead of m/z 391 was observed. By comparing with the respective authentic standards (Fig.1 and 2, and Table 1), M3 was identified as the ocatillol-type ginsenosides and the oxygenation site was located on the C24-25 double bond. For M1/2, we could only propose the oxygenation site on the C-20 aliphatic branch chain, considering that the typical aglycone ion characterized with m/z 391 was observed. For PPT, three mono-oxygenated metabolites (M1-1/2/3) and
three di-oxygenated metabolites (M2-1/2/3) were observed. As evidenced from the diagnostic ion at m/z 415, M1-1 and M2-1 were suggested as the C24-25 double bond oxygenation metabolites.

**Enzyme kinetics in rat and human hepatic microsomes**

Considering that the oxygenation was the predominant metabolic pathway of Rg2, Rf, Rh1, and PPT in the microsomal incubation system, the elimination rate of parent compound was quantified to represent the enzyme kinetics of the oxygenation metabolism of these four ginsenosides. The concentration of microsomes protein and the incubation time were optimized to ensure the linear depletion of parent compounds. Kinetic plots shown in Fig.3 and Fig.4 indicate the metabolic eliminations of these ginsenosides fit well to the Michaelis-Menten equation. Kinetic parameters including $K_m$, $V_{max}$, and $CL_{int}$ are presented in Table 2. In human hepatic microsomes, the $K_m$ value ranged from 16.8 (PPT) to 42.8 μM (Rf), and the $V_{max}$ value ranged from 67.1 (Rg2) to 116.1 (PPT) pmol/min/mg protein. The intrinsic clearance ($CL_{int}$) calculated as $V_{max}/K_m$ for the three PPT ginsenosides and their aglycone PPT ranked as follows: $Rg2\leq Rf < Rh1 < PPT$. Enzyme kinetic parameters determined from the rat hepatic microsomes were comparable with those from human hepatic microsomes, except for slight differences between Rg2 and Rf.

**Chemical inhibition study in rat and human hepatic microsomes**

To phenotype the CYP450 isoforms involved in the oxygenation metabolism of PPT ginsenosides, various specific chemical inhibitors were used and the substrate concentration
was selected based on the estimated apparent Km. Among the chemical inhibitors used in the human hepatic microsomes incubation system, only Ket, a specific CYP3A4/5 inhibitor showed a significant inhibitory effect on the oxygenation metabolism of all the four compounds. The turnover rate in the Ket pre-incubated system decreased to be 33%, 30%, 45%, and 22% of the control for Rg2, Rf, Rh1, and PPT, respectively. All other CYP450 isoform specific inhibitors resulted in negligible effect on the oxygenation metabolism of PPT ginsenosides (Fig.5). Similar results were obtained from the rat hepatic microsomes study (Fig.6).

**Metabolism of PPT ginsenosides in human P450 isoforms**

In an initial screening procedure, 10 kinds of CYP450 isoforms were selected (Sauer et al., 2009) to determine the specific CYP isozymes involved in the oxygenation metabolism of the four PPT ginsenosides Rg2, Rf, Rh1, and PPT at two substrate concentrations. Among the 10 CYPs tested, only CYP3A4 and, to a very minor extent, CYP3A5 exerted catalytic activity towards the oxygenation metabolism of all the compounds tested, whereas the metabolic elimination of parent compounds was negligible in the incubations with all other 8 isoforms. As shown in Table 3, the PPT ginsenosides turnover rate in the CYP3A4 incubation was much higher (ranged from 12 to 48-fold) than that in the CYP3A5 incubation.

Therefore, we conducted a follow up enzyme kinetic study of these ginsenosides only in recombinant CYP3A4 isoform. Same as that observed from both human and rat hepatic liver microsomes study, PPT showed the highest $V_{\text{max}}$ value at 92.6 pmol/min/pmol protein. The
$K_m$ values of PPT and Rh1 were comparable and much lower than that for Rg2 and Rf. The intrinsic clearance ($V_{max}/K_m$) ranked as follows: Rf≤Rg2<Rh1<Ppt, which was generally consistent with that determined from both human and rat hepatic microsomes study (Table 2).
Discussion

PPT type ginsenosides is one of the important classes of ginsenosides in addition to the PPD type ginsenosides. Their powerful and wide pharmacological activities and their potential of serving as leading compounds have attracted much attention from researchers worldwide. Recently, PPT ginsenosides have been found to be effective on modulating central nervous (Wang et al., 2009), immunological (Sun et al., 2006; Sun et al., 2007), cardiovascular (Li and Liu, 2008), and metabolic system (Chang et al., 2007), and on the cancer treatment (Wang and Yuan, 2008). In view of these pharmacological merits of PPT ginsenosides, it is thus very important to explore the biological transformation and metabolic pathways for better understanding their systematic dispositions and pharmacological benefits. Various previous reports focused mainly on the presystematic degradation of PPT ginsenosides in the gastrointestinal tract, whereas very little is known about the hepatic microsomal mediated metabolism. Although oral bioavailability and plasma levels of ginsenosides are generally low because of their extensive presystematic metabolism and poor membrane permeability (Tawab et al., 2003), previous studies from our laboratory and others showed that the hepatic levels of ginsenosides after oral ingestion were much higher than that in plasma and other tissues (Gu et al., 2009; Liu et al., 2009). In addition, oxygenated metabolites have been actually identified from the bile and urine samples after oral ingestion of ginsenosides (Lai et al., 2009a; Liu et al., 2009). Such evidences suggest that hepatic cytochrome P450 may play an important role on the systematic disposition of ginsenosides. In the present study, five major PPT type ginsenosides including Re, Rg1, Rg2, Rf, and Rh1, and their aglycone PPT were
selected to explore the general principles of cytochrome P450 catalyzed metabolism of PPT type ginsenosides.

The NADPH dependent CYP450 catalyzed oxygenation metabolisms of PPT ginsenosides were confirmed in this study in both rat and human hepatic microsomal incubation systems. Based on a powerful LC/MS-IT-TOF analysis that had been well developed in our laboratory for multiple compounds identification from complex matrix (Hao et al., 2008a; Zheng et al., 2009), three mono-oxygenated metabolites for each of the PPT ginsenosides, and three mono-oxygenated and three double-oxygenated metabolites for the aglycone PPT were identified from the microsomal incubation media. As evidenced from the fragment patterns of both parent compounds and metabolites, the oxygenation metabolism site was proposed on the C20 aliphatic branch chain. Such a result obtained from the in vitro hepatic microsome incubations was consistent with our previous study in vivo of Rh1 (Lai et al., 2009a), and also with previous reports in vivo of the typical PPD ginsenosides Rh2 (Qian et al., 2005a) and Rb1 (Qian et al., 2006). By comparing with the authentic standards (RF11 and RT5), one of the oxygenation site was confirmed on the C24-25 double bond to produce C20-24 epoxides (ocotillol-type ginsenosides) after rearranging immediately from the C24, 25 epoxides.

As evidenced from this study and the previous reports for both PPD and PPT ginsenosides, the microsomal CYP450 catalyzed oxygenation metabolism plays an important role on the systematic elimination of ginsenosides. Thus, it should be of great concerns to address the specific CYP isoforms involved in the oxygenation metabolism, for better understanding of
the pharmacokinetics and dispositions of ginsenosides in different species, and previously reported ginseng-drug interactions. For this purpose, the specific CYP isoforms responsible for the oxygenation metabolism of PPT ginsenosides were determined based on the chemical inhibition study and recombinant CYP isoforms screening. All evidences obtained in the present study supported that CYP3A was the sole isoform involved in the oxygenation metabolism of ginsenosides. It has been well known that CYP3A is the most abundant human hepatic CYP isoforms and accounts for the metabolism of approximately 50% known drugs. CYP3A4 and 3A5 are the two major isoforms in the CYP3A family in human adults, and share 84% sequence homology and many substrates overlapping. In this study, it has been found that the typical PPT ginsenosides turnover rate in CYP3A4 was much higher (ranged from 12-48 fold) than that in CYP3A5, suggesting that CYP3A4 should be the predominant isoform and that CYP3A5 contributed very little to the oxygenation metabolism of ginsenosides. Such a phenomenon has been also widely found for many other typical CYP3A substrates such as buspirone (Zhu et al., 2005) and cyclosporine A.

In order to explore the structure metabolism relationship of PPT ginsenosides, the detailed enzyme kinetics of four major ginsenosides were performed in rat and human microsomal, and also in the recombinant CYP3A4 incubation systems. Among the components tested in this study, Re and Rg1 structurally characterized with a glucose substitution at the C20 hydroxy group underwent negligible metabolism, whereas other four compounds without glucose substitution at the C-20 hydroxy group exhibited efficient metabolic elimination in the microsomal incubation systems. The experimental determined intrinsic clearance for Rg2,
Rf, Rh1, and PPT was generally consistent throughout rat and human microsomal, and human recombinant CYP3A4 incubation systems, and ranked as Rf≤Rg2<Rh1<PPT. Considering that the active site of CYP3A4 had been claimed to be hydrophobic (Williams et al., 2004; Yano et al., 2004), we sought to discover whether the intrinsic clearances of PPT ginsenosides by CYP3A4 were correlated with their hydrophobicity. The typical hydrophobic parameter Log P value for Rf, Rg2, Rh1, and PPT was 2.7, 3.2, 4.3, and 5.9, respectively, which was found correlated well with their intrinsic clearance in human recombinant CYP3A4 isozyme ($r^2$, 0.99). The Log P value may be used as a useful descriptor for at least qualitatively predicting the metabolic stability of ginsenosides by CYP3A4, as that proposed for other CYP3A4 substrates (Marechal et al., 2006). Results obtained from this study reveals clearly a general structure metabolism relationship for the CYP3A4 catalyzed oxygenation metabolism of PPT ginsenosides as that the more glycosyl substitutions on the aglycone skeleton (the lower hydrophobicity), the lower binding affinity and intrinsic clearance by CYP3A4. The glycosyl substitution at the C-20 hydroxy group appears to completely prevent ginsenosides metabolism, while glycosyl substitution on the aglycone skeleton is likely to increase their metabolic resistances by CYP3A4. The same was true of the structure activity relationship for ginsenosides inhibition on CYP3A (Hao et al., 2008b). Such results strongly suggest that the previously reported CYP3A inhibition effect of ginsenosides is in a mode of substrate-competitive.

In summary, the novel data obtained from this study strongly indicates that the CYP3A4 catalyzed oxygenation metabolism plays an important role on the systematic clearance of PPT
type ginsenosides (Fig. 7). The glycosyl substitutions on the aglycone skeleton and especially at the C-20 hydroxy group are likely to constitute steric hindrances to prevent the binding of ginsenosides to the active site of CYP3A4 and thus cause metabolic resistance. PPT and Rh1, characterized with a Km value at 15.0 and 14.2 μM, respectively, are possibly to serve as new probe substrates of CYP3A4 in vitro, considering that CYP3A4 has been found to be the predominant, if not sole, CYP isoform involved in the microsome mediated metabolism. Findings from this study also provide novel explanations to the previously reported ginseng-drug interactions (Coon and Ernst, 2002; Bressler, 2005), and also to that why only PPT and Rh1, but not their precursors, exhibit competitive inhibition on CYP3A4 activity (Liu et al., 2004; Liu et al., 2006a; Liu et al., 2006b; Etheridge et al., 2007; Hao et al., 2008b). However, it should be noted that although Re and Rg1 are not the direct substrates of CYP3A4, they can be transformed to the CYP3A4 substrates Rh1 and PPT by gastric acid and intestinal bacteria, and thus may also exert competitive inhibitions of other CYP3A4 substrates. In addition, the present identification of PPT ginsenosides as CYP3A4 substrates provides novel insight into the understanding of metabolism based synergistic mechanism of herbal compound prescriptions (Shen-mai-san) (Xu et al., 2008) because we have previously found that the adjunct herb Schisandra is a strong modulator of CYP3A4 (Lai et al., 2009b).
References


Footnotes

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Figure Legends:

**Fig.1** Chemical structures of PPT type ginsenosides, aglycone Ppt, and Pseudo ginsenoside RF11 (metabolite for Rg2) and RT5 (metabolite for Rh1).

**Fig.2** Representative extracted ion chromatograms obtained from human microsomal incubation with Rg2 and Rh1. (a) microsomal incubation samples, (b) and (c) authentic compounds. In the metabolic profiling assay, ginsenosides (8 µM) were incubated in rat or human microsomes for 30 min. Samples were extracted by n-butanol and analyzed using LC/MS-IT-TOF.

**Fig.3** Representative kinetic plots for the oxygenation metabolism of Rg2 (a), Rf (b), Rh1 (c) and Ppt (d) in rat hepatic microsomes. Insert, Eadie-Hofstee plots. Ginsenoside Rg2 (1.56-50 µM), Rf (2.5-80 µM), Rh1 (0.31-50 µM), or Ppt (0.31-50 µM) was incubated in rat hepatic microsomes (1.0 mg/ml) containing NADPH regenerating system for 15 min at 37 °C. The average depletion rate (y axis, pmol/min/mg protein) was estimated from the amount difference between zero time point and after 15 min incubation. Plots provided are one representative of triplicate experiments, and the obtained kinetic parameters are summarized in Table 2.

**Fig.4** Representative kinetic plots for the oxygenation metabolism of Rg2 (a), Rf (b), Rh1 (c) and Ppt (d) in human hepatic microsomes. Insert, Eadie-Hofstee plots. Ginsenoside Rg2 (1.56-50 µM), Rf (2.5-80 µM), Rh1 (0.31-50 µM), or Ppt (0.31-50 µM) was incubated in
human hepatic microsomes (0.2mg/ml) containing NADPH regenerating system for 15 min at 37 °C. The average depletion rate (y axis, pmol/min/mg protein) was estimated from the amount difference between zero time point and after 15 min incubation. Plots provided are one representative of triplicate experiments, and the obtained kinetic parameters are summarized in Table 2.

**Fig.5** Effects of chemical inhibitors on the oxygenation metabolism of Rg2 (a), Rf (b), Rh1 (c) and Ppt (d) in rat hepatic microsomes. The substrate concentration selected was 8 μM for Rg2, Rf, and Rh1, and 4μM for Ppt.

**Fig.6** Effects of chemical inhibitors on the oxygenation metabolism of Rg2 (a), Rf (b), Rh1 (c) and Ppt (d) in human hepatic microsomes. The substrate concentration selected was 8 μM for Rg2, Rf, and Rh1, and 4 μM for Ppt.

**Fig.7** CYP3A4 mediated oxygenation metabolism pathway of PPT type ginsenosides. The oxygenation metabolism sites were confirmed on the C20 aliphatic branch chain, and C24-25 double bond was identified as one of the major oxygenation site, based on the LC/MS-IT-TOF analysis and the use of authentic standards.
Table 1 Summary of retention times, quasi-molecular ions, MS/MS product ions and predicted molecular formula for metabolites identification of PPT type ginsenosides

<table>
<thead>
<tr>
<th>Compounds</th>
<th>T&lt;sub&gt;R&lt;/sub&gt;(min)</th>
<th>[M-H]⁻</th>
<th>MS/MS fragment ions</th>
<th>Predicted molecular formula</th>
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<tbody>
<tr>
<td>Rg2</td>
<td>11.9</td>
<td>783.4893</td>
<td>637.4269, 619.4161, 475.3762, 457.3663, 391.2838</td>
<td>C&lt;sub&gt;42&lt;/sub&gt;H&lt;sub&gt;72&lt;/sub&gt;O&lt;sub&gt;13&lt;/sub&gt;</td>
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<tr>
<td>M1</td>
<td>7.2</td>
<td>799.4844</td>
<td>653.424, 635.4182, 491.3714, 391.2878</td>
<td>C&lt;sub&gt;42&lt;/sub&gt;H&lt;sub&gt;72&lt;/sub&gt;O&lt;sub&gt;14&lt;/sub&gt;</td>
</tr>
<tr>
<td>M2</td>
<td>8.4</td>
<td>799.4844</td>
<td>653.4249, 635.4108, 653.4254 (653.4245),</td>
<td>C&lt;sub&gt;42&lt;/sub&gt;H&lt;sub&gt;72&lt;/sub&gt;O&lt;sub&gt;14&lt;/sub&gt;</td>
</tr>
<tr>
<td>M3 (RF11)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.9 (10.9)</td>
<td>799.4844 (799.4836)</td>
<td>653.4241 (635.4121), 491.3677 (491.3737), 415.3156 (415.3156)</td>
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</tr>
<tr>
<td>Rf</td>
<td>10.9</td>
<td>799.4775</td>
<td>637.4201, 619.4103, 475.3714, 457.3578, 391.2792, 389.2688</td>
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<tr>
<td>M1</td>
<td>6.2</td>
<td>815.4762</td>
<td>653.4133, 491.3642, 391.2772</td>
<td>C&lt;sub&gt;42&lt;/sub&gt;H&lt;sub&gt;72&lt;/sub&gt;O&lt;sub&gt;15&lt;/sub&gt;</td>
</tr>
<tr>
<td>M2</td>
<td>7.4</td>
<td>815.4762</td>
<td>653.4157, 491.3661</td>
<td>C&lt;sub&gt;42&lt;/sub&gt;H&lt;sub&gt;72&lt;/sub&gt;O&lt;sub&gt;15&lt;/sub&gt;</td>
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<tr>
<td>M3</td>
<td>9.9</td>
<td>815.4762</td>
<td>653.4112, 491.3661</td>
<td>C&lt;sub&gt;42&lt;/sub&gt;H&lt;sub&gt;72&lt;/sub&gt;O&lt;sub&gt;15&lt;/sub&gt;</td>
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<td>Rh1</td>
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<td>475.3795, 457.3684, 391.2869</td>
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<td>491.3711, 391.2872</td>
<td>C&lt;sub&gt;36&lt;/sub&gt;H&lt;sub&gt;62&lt;/sub&gt;O&lt;sub&gt;10&lt;/sub&gt;</td>
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<tr>
<td>M2</td>
<td>8.5</td>
<td>653.4262</td>
<td>491.3761</td>
<td>C&lt;sub&gt;36&lt;/sub&gt;H&lt;sub&gt;62&lt;/sub&gt;O&lt;sub&gt;10&lt;/sub&gt;</td>
</tr>
<tr>
<td>M3 (RT5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.2 (11.2)</td>
<td>653.4262 (653.4275)</td>
<td>491.3767 (491.3754), 415.3156 (415.3156)</td>
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<tr>
<td>Ppt</td>
<td>19.8</td>
<td>475.3719</td>
<td>457.3604, 391.2788, 373.2627</td>
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<tr>
<td>M1-1</td>
<td>12.7</td>
<td>491.3675</td>
<td>415.3138, 403.3146, 397.3026, 395.2917, 391.2771</td>
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</tr>
<tr>
<td>M1-2</td>
<td>14.1</td>
<td>491.3675</td>
<td>473.3514, 455.3420, 391.2782, 373.2652, 371.2572</td>
<td>C&lt;sub&gt;30&lt;/sub&gt;H&lt;sub&gt;52&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;</td>
</tr>
<tr>
<td>M1-3</td>
<td>15.6</td>
<td>491.3675</td>
<td>473.3578, 455.3514, 391.2779</td>
<td>C&lt;sub&gt;30&lt;/sub&gt;H&lt;sub&gt;52&lt;/sub&gt;O&lt;sub&gt;5&lt;/sub&gt;</td>
</tr>
<tr>
<td>M2-1</td>
<td>9.8</td>
<td>507.3616</td>
<td>489.3486, 471.3360, 431.3022, 429.3219, 391.2768</td>
<td>C&lt;sub&gt;30&lt;/sub&gt;H&lt;sub&gt;52&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
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<tr>
<td>M2-2</td>
<td>10.3</td>
<td>507.3616</td>
<td>489.3484, 431.3055, 391.2784</td>
<td>C&lt;sub&gt;30&lt;/sub&gt;H&lt;sub&gt;52&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
</tr>
<tr>
<td>M2-3</td>
<td>12.2</td>
<td>507.3616</td>
<td>431.3022, 403.3128</td>
<td>C&lt;sub&gt;30&lt;/sub&gt;H&lt;sub&gt;52&lt;/sub&gt;O&lt;sub&gt;6&lt;/sub&gt;</td>
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</table>

<sup>a</sup>Data in the bracket was obtained from the study of authentic standards RF11 and RT5.
Table 2 Enzyme kinetic parameters for PPT type ginsenosides metabolism by rat, human hepatic microsomes and human recombinant CYP3A4 isoform

<table>
<thead>
<tr>
<th>Parameters a</th>
<th>Ppt</th>
<th>Rh1</th>
<th>Rg2</th>
<th>Rf</th>
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<tr>
<td>Rat hepatic microsomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$V_{\text{max}}$ (pmol/min/mg protein)</td>
<td>118.2±8.9</td>
<td>82.1±7.5</td>
<td>83.9±4.3</td>
<td>61.7±2.2</td>
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<tr>
<td>$K_m$ (µM)</td>
<td>17.5±0.6</td>
<td>19.3±1.0</td>
<td>35.4±1.8</td>
<td>46.0±1.2</td>
</tr>
<tr>
<td>$V_{\text{max}} / K_m$ ratio (µl/min/mg protein)</td>
<td>6.8±0.7</td>
<td>4.3±0.5</td>
<td>2.4±0.2</td>
<td>1.3±0.1</td>
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<tr>
<td>Human hepatic microsomes</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>$V_{\text{max}}$ (pmol/min/mg protein)</td>
<td>116.1±6.5</td>
<td>85.1±2.3</td>
<td>67.1±4.5</td>
<td>87.9±9.5</td>
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<tr>
<td>$K_m$ (µM)</td>
<td>16.8±1.4</td>
<td>17.9±1.0</td>
<td>34.8±2.9</td>
<td>42.8±4.5</td>
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<tr>
<td>$V_{\text{max}} / K_m$ ratio(µl/min/mg protein)</td>
<td>6.9±0.3</td>
<td>4.8±0.2</td>
<td>1.9±0.1</td>
<td>2.1±0.1</td>
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<tr>
<td>CYP3A4 isoform</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>$V_{\text{max}}$ (pmol/min/pmol protein)</td>
<td>92.6</td>
<td>59.2</td>
<td>62.1</td>
<td>59.5</td>
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<tr>
<td>$K_m$ (µM)</td>
<td>15.0</td>
<td>14.2</td>
<td>30.2</td>
<td>44.6</td>
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<tr>
<td>$V_{\text{max}} / K_m$ ratio(µl/min/pmol protein)</td>
<td>6.2</td>
<td>4.2</td>
<td>2.1</td>
<td>1.3</td>
</tr>
</tbody>
</table>

a Kinetic parameters were estimated from the Michaelis-Menten equation by non-linear regression analysis of the depletion rate of parent compounds versus substrate concentration data. Data provided were the mean±SD of triplicates for microsomes, and the mean of duplicates for recombinant enzymes.
Table 3 Metabolic rates of ginsenosides by human recombinant CYP450 isoforms

<table>
<thead>
<tr>
<th>CYP450 isoforms&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Metabolic rate (pmol/min/pmol protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ppt</td>
</tr>
<tr>
<td></td>
<td>2 µM</td>
</tr>
<tr>
<td>3A4</td>
<td>3.99</td>
</tr>
<tr>
<td>3A5</td>
<td>0.19</td>
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<sup>a</sup> A panel of 10 recombinant human P450 isoforms was screened, and only CYP3A4 and, to a very minor extent CYP3A5, were found active towards the oxygenation metabolism of PPT type ginsenosides.
Figure 1

Chemical structures of different compounds:

- Re
- Rg1
- Rg2
- Rf
- Rh1
- Ppt
- RT5
- RF11
Figure 2
Figure S1

(a) 

(b) 

(c) 

(d)
Figure 7

PPT type ginsenosides

CYP3A4

Ocotillol type ginsenosides