Combinatorial Metabolism Notably Affects Human Systemic Exposure to Ginsenosides from Orally Administered Extract of Panax notoginseng Roots (Sanqi)

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ABBREVIATIONS: AUC_0-t, area under concentration-time curve to the last measured time point; CL_r, renal clearance; CL_{tot,P}, total plasma clearance; C_{max}, maximum plasma concentration; Cum.Ae, cumulative amount excreted; CYP, cytochrome P450; HLM, human liver microsomes; MRT, mean residence time; ppd-type, 20(S)-protopanaxadiol type; ppt-type, 20(S)-protopanaxatriol type; PK, pharmacokinetic; T_{max}, time taken to achieve the maximum concentration
ABSTRACT

Ginsenosides are medicinal ingredients of the cardiovascular herb *Panax notoginseng* roots (Sanqi). Here, we implemented a human study (ChiCTR-ONC-09000603; www.chictr.org) to characterize pharmacokinetics and metabolism of ginsenosides from an orally ingested Sanqi extract (a 1:10 water extract of Sanqi) and the human plasma and urine samples were analyzed by liquid chromatography-mass spectrometry. Plasma and urinary compounds derived from ginsenosides included (1) intestinally absorbed ginsenosides Ra3, Rb1, Rd, F2, Rg1, and notoginsenoside R1 and (2) the deglycosylated products compound-K, 20(S)-protopanaxadiol, 20(S)-protopanaxatriol, and their oxidized metabolites. The systemic exposure levels of the first group compounds increased as the Sanqi extract dose increased, but those of the second group compounds were dose-independent. The oxidized metabolites of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol represented the major circulating forms of ginsenosides in the bloodstream, despite their large interindividual differences in exposure level. The metabolites were formed via combinatorial metabolism that consisted of a rate-limiting step of ginsenoside deglycosylation by the colonic microflora and a subsequent step of sapogenin oxidation by the enterohepatic cytochrome P450 enzymes. Significant accumulation of plasma ginsenosides and the metabolites occurred in the human subjects receiving three-week subchronic treatment with the Sanqi extract. Plasma 20(S)-protopanaxadiol and 20(S)-protopanaxatriol could be used as pharmacokinetic markers to reflect the subject’s microbial activities, as well as the timely-changes and interindividual differences in plasma levels of their respective oxidized metabolites. The information gained from the current study is relevant to pharmacology and therapeutics of Sanqi.
Introduction

In China, the tradition of herbal medicine has prospered over millennia and is still accepted by the physicians, the patients, and the regulatory authority. However, the scientific standards for accepting the value of an herbal product as a medicine fall below the existing requirements for establishing the value of synthetic drugs. China has announced an ambitious attempt to bring the practice of traditional medicine into line with modern standards (Qiu, 2007).

Phytochemists traditionally work in concert with ethnopharmacologists in herbal medicine research, where whole herb extracts, as well as the individual compounds isolated and purified from the herbs, are applied to in vivo and/or in vitro pharmacological assessments. However, the studies often fail to address which chemical ingredients present are the medicinal principles responsible for the pharmacological effects of the investigative herbal medicine. The most abundant constituents present in an herbal medicine do not necessarily produce the highest in vivo concentrations (such as the plasma concentrations, the tissue concentrations, or the like) after dosing, while in vivo measured herbal metabolites are usually absent in the dosed medicine (Lu et al., 2008; Liu et al., 2009; Li et al., 2012; Yan et al., 2012; Li, 2012). In other words, there can be significant differences in nature and relative levels between the chemical ingredients present in an herbal medicine and the circulating botanical compounds after dosing. The accurate and complete information of pharmacokinetics and metabolism is vital to the pharmacological research for identification of the medicinal principles. Accordingly, pharmacokinetic
(PK) scientists are needed to participate in the herbal medicine research by working with ethnopharmacologists and phytochemists in a manner similar to that for drug discovery. Different from the early practice that was based only on pharmacological assessment of a range of chemicals, the current drug discovery is normally achieved by combining medicinal chemistry and pharmacology together with pharmacokinetics, which has resulted in improved candidate selection for preclinical and clinical development (Pritchard et al., 2003). PK and metabolism study of an herbal medicine tells the ethnopharmacologists which herb-derived compounds are worth further evaluation.

Sanqi (the dried root of *Panax notoginseng*, family *Araliaceae*) has been commonly used in the Chinese traditional medicine to eliminate blood stasis, to arrest bleeding, to cause subsidence of swelling, and to alleviate pain (Chinese Pharmacopoeia Commission, 2010). The medicinal herb is usually administered orally in such forms as powders, aqueous extracts, and teas, in daily dose ranging from 2 g to 30 g of root but averaging 3–9 g. In addition, injections prepared from Sanqi extract have also been approved for clinical use in China. The triterpene saponins ginsenosides are believed to be the pharmacologically active ingredients of Sanqi and can be classified according to their structures as 20(S)-protopanaxadiol type (ppd-type) and 20(S)-protopanaxatriol type (ppt-type) (Christensen, 2009; Lv et al., 2009). The major saponins present in Sanqi include the ppd-type ginsenosides Ra₃, Rb₁, and Rd and the ppt-type ginsenosides Re, Rg₁, and notoginsenoside R₁. Recently, we investigated intestinal absorption and disposition of ginsenosides in
rats receiving an orally (p.o.) administered Sanqi extract and found that poor membrane permeability, rapid biliary excretion, and colonic microflora-induced deglycosylation were the key factors affecting the rat systemic exposure to ginsenosides (Liu et al., 2009). The major ginsenosides measured in rat plasma were long-circulating ppd-type ginsenosides Ra₃, Rb₁, and Rd, which can be used as pharmacokinetic markers (PK markers) to substantiate rat systemic exposure to the Sanqi extract. Meanwhile, ppt-type ginsenosides Re, Rg₁, and notoginsenoside R₁, as well as the deglycosylated products of both the types, were subject to rapid biliary excretion that limited their systemic exposure levels. Interspecies differences in biliary excretion (Mahmood and Sahajwalla, 2002; Lai, 2009) and colonic microflora-induced metabolism (Nicholson et al., 2005; Sousa et al., 2008) complicate the extrapolation of human absorption and disposition of ginsenosides from the animal data. Accordingly, we implemented a human study to characterize pharmacokinetics and metabolism of ginsenosides from an orally ingested Sanqi extract. One of our important findings is that several metabolites derived from ginsenosides were measured as major circulating compounds after dosing. These metabolites, absent in the administered herbal extract, were formed via combinatorial metabolism that consisted of a rate-limiting step of ginsenoside deglycosylation induced by the colonic microflora and a subsequent step of sapogenin oxidation mediated by the enterohepatic cytochrome P450 enzymes (CYP enzymes).
Materials and Methods

A detailed description of materials and experimental procedures is provided in the Supplemental Materials and Methods.

**Materials.** Sanqi-extract, for p.o. administration to human subjects, was a 1:10 water extract, i.e., 2 kg of dried roots of *Panax notoginseng* (Sanqi) was used to make 20 l of the final extract. The prepared Sanqi-extract contained ginsenosides Rg₁ (2680 μM), Rb₁ (1377 μM), Rd (366 μM), Re (254 μM), Rh₁ (103 μM), Ra₃ (76 μM), Rg₂ (60 μM), F₁ (48 μM), F₂ (18 μM), Rg₃ (17 μM), Rb₂ (14 μM), Rf (13 μM), Rc (3 μM), and Rh₂ (1 μM), as well as notoginsenosides R₁ (360 μM), R₅/R₆ (69 μM), and 20-gluco-ginsenoside Rf (33 μM). Only one batch of Sanqi-extract was prepared and used for the whole study. The content levels of ginsenosides in Sanqi-extract were measured immediately after preparation and after storage at 4°C for 1 and 3 months. Chemical stability was defined as the retention of ≥85% of the initial levels. The herb extract was used after shaking well.

Purified ginsenosides, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol were obtained from the National Institutes for Food and Drug Control (Beijing, China) or other commercial sources, the purity of which exceeded 98%. Pooled human liver microsomes (HLM) of Chinese origin were obtained from the Research Institute for Liver Diseases (Shanghai, China). Single cDNA-expressed human cytochrome P450 (CYP) enzymes were obtained from BD Gentest (Woburn, MA).

**Human Study.** The protocol for human study was approved by the Ethics Committee of Clinical Investigation at the Second Affiliated Hospital of Tianjin
University of Traditional Chinese Medicine (Tianjin, China). The study was registered on Chinese Clinical Trials Registry (www.chictr.org) with a registration number of ChiCTR-ONC-09000603. The informed consent was obtained from each of twelve male and twelve female healthy volunteers (22–26 yr).

A two-period, open-label, single center study was performed at the National Clinical Research Center of the hospital (Tianjin, China; Fig. 1). In period 1, all the subjects were randomly assigned to one of three dosage groups, with four male and four female subjects per group. Each subject was asked to fast overnight before receiving a single p.o. dose of Sanqi-extract (90, 180, or 270 ml/subject) in the next morning. The low dose level was derived from the dose level recommended by the Pharmacopoeia of the People’s Republic of China. The intermediate and high dose levels were also used clinically. Serial blood samples (~1 ml collected in heparinized tubes) were taken from an antecubital vein catheter at 0, 0.5, 1.5, 3, 4.5, 8, 12, 15, 24, 30, 38, 48, and 56 h after dosing. The blood samples were then centrifuged at 3000×g for 5 min, and the resulting plasma fractions were frozen at −70°C until analysis. Meanwhile, serial urine samples were also collected predose and at 0–3, 3–6, 6–10, 10–14, 14–24, 24–32, 32–40, 40–48, and 48–72 h postdose, which were weighed prior to storage at −70°C without use of any preservative. In period 2 (after three-day wash-out period following the first acute p.o. treatment with Sanqi-extract), the four male subjects of the low-dose group continued to receive a subchronic p.o. treatment with Sanqi-extract at 90 ml/subject/day for three weeks. On days 11, 18, and 24 of period 2, the blood and urine samples were collected according to the preceding time
schedules, except for the sampling only performed 0–24 h after the dosing on days 11 and 18. The hepatic and renal function of subjects was monitored before period 1 and 72 h after a single dose of Sanqi-extract, as well as on days 4, 12, 15, 19, 20, 21, 22, 23, and 25 (before the daily dose of Sanqi-extract) of period 2. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin/globulin (A/G) ratio, total bilirubin (TBiL), and direct bilirubin (DBiL) were monitored as liver function markers for the human subjects, while serum creatinine (CRE) and blood urea nitrogen (BUN) were also assessed to monitor their renal function. The reference ranges, indicating normal liver or kidney function, of ALT, AST, TP, A/G ratio, TBiL, DBiL, CRE, and BUN were 0–40 U/l, 0–40 U/l, 64–87 g/l, 1–2.5, 0–17.1 μM, 0–8.0 μM, 22–106 μM, and 1.7–8.3 mM, respectively.

**In Vitro Metabolism Studies.** Pure 20(S)-protopanaxadiol, 20(S)-protopanaxatriol, and compound-K at 2 μM was incubated with HLM in presence of the cofactor(s) NADPH, UDPGA, or the both to assess metabolic stability and to support metabolite characterization. A variety of cDNA-expressed human CYP enzymes (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5) were used for the enzyme identification. To determine the enzyme kinetics, 20(S)-protopanaxadiol (1.56–200 μM) or 20(S)-protopanaxatriol (0.08–200 μM) was incubated with NADPH-fortified HLM under linear metabolite formation conditions, which were performed in duplicate in 96-well plates.

**Liquid Chromatography-Mass Spectrometry Analyses.** An AB Sciex API
4000 Q Trap mass spectrometer (Toronto, Canada) interfaced via a Turbo V ion source with a Waters Acquity UPLC separation module (Milford, MA, USA) was used for metabolite profiling and quantification for study purposes. The human plasma and urine samples (50 μl) were precipitated with 150 μl of methanol before measurement. Meanwhile, in vitro metabolism study samples (100 μl) were treated with an equal volume of methanol to stop the reaction before analysis. Validation of the quantification assays with the reference standards available were implemented according to the US Food and Drug Administration guide on bioanalytical method validation (http://www.fda.gov/cder/guidance/index.htm) to demonstrate that their performance characteristics were reliable for the intended use.

**Data Analysis.** PK parameters were determined by non-compartmental method using the Kinetica 5.0 (Thermo Fisher Scientific; Philadelphia, PA), which were shown as means ± standard deviation. Dose proportionality assessment was conducted by the regression of log-transformed data (the Power Model) with the criterion that was calculated according to the method by Smith et al. (2000). Michaelis constant (K_m) and maximum velocity (V_max) values were determined by nonlinear regression analysis using the Michaelis-Menten equation (rate of metabolite formation as a function of substrate concentration) using GraFit software (version 5; Erithacus Software Ltd., Surrey, UK). The ratio of V_max and K_m was used to calculate in vitro intrinsic clearance (CL_int). The Shapiro-Wilk test of normality was used to check the distribution shape of systemic exposure levels of ginsenoside-derived compounds in AUC_0-t. The Kendall’s tau-b coefficients were calculated using PASW statistics 18
software (SPSS Inc., Chicago, IL) to estimate whether the systemic exposure levels of a certain ginsenoside-derived compound were positively or negatively correlated to those of another ginsenoside-derived compound. Correlation was statistically significant at $P < 0.01$ level (two-tailed).
Results

Safety of Sanqi-extract. No serious adverse event was observed during the human study. After a p.o. dose of Sanqi-extract at 90, 180, or 270 ml/subject, none of liver or kidney function indicators exceeded the upper normal levels. During the subsequent multiple dose treatment, the four male subjects (m1–m4; receiving Sanqi-extract at 90 ml/subject/day) had the hepatic or renal function indicators within the normal ranges during the study period, except for some transient elevation of alanine aminotransferase (ALT) for subjects m2–m4. Subject m3 had elevated ALT (54–79 U/l) on days 15–22. This subject withdrew from the study after day 18 to let his elevated ALT return to the normal level (<40 U/l).

Appearance of Plasma and Urine Ginsenosides and Metabolites after p.o. Administration of Sanqi-extract. Ginsenosides and their deglycosylated products occurred in human plasma and urine after a p.o. dose of Sanqi-extract (270 ml/subject), which were compared with the profiles of the control plasma and urine samples before dosing. As shown in Fig. 2B, Ppd-type ginsenoside Rb1 was the most abundant plasma Sanqi saponin absorbed, while other saponins measured in plasma were ppd-type ginsenosides Ra3, Rd, F2, Rg3, and Rh2, as well as ppt-type ginsenosides Re, Rg1, F1, Rh1, and notoginsenoside R1. These plasma ginsenosides were also excreted into urine (Fig. 2C), except ginsenosides Rg3 and Rh2. Meanwhile, 20-gluco-ginsenoside Rf and notoginsenoside R3/6 were measured in urine but not in plasma. Although ppt-type ginsenoside Rg1 had a higher content level in the administered Sanqi-extract than ginsenoside Rb1, its plasma abundance
was significantly lower than that of the ppd-type ginsenoside (Fig. 2B). However, urinary ginsenoside Rg₁ was the most abundant of all the ginsenosides excreted into urine (Fig. 2C). Notably, the deglycosylated products compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol, absent in the ingested Sanqi-extract (Fig. 2A), also occurred substantially in plasma with 20(S)-protopanaxatriol being ~3-times more abundant than plasma ginsenoside Rb₁ (Fig. 2B).

Although 20(S)-protopanaxadiol and 20(S)-protopanaxatriol circulated significantly in the bloodstream, they are not measured in the urine samples. To understand the elimination of the deglycosylated products, their metabolism was further assessed. A total of 29 metabolites of the deglycosylated products were detected in the plasma samples (Fig. 2D). Plasma M₈, M₁₁, and M₁₂ had their abundances 1.2–11 times higher than 20(S)-protopanaxatriol, while the abundances of plasma M₃–M₇, M₉, M₁₀, and M₁₃–M₂₂ were 7–72% of that of the sapogenin. Meanwhile, a total of 29 urinary metabolites were measured (Fig. 2E). The plasma metabolites M₁₇–M₂₂ were absent in urine, while the urinary metabolites M₃₀ and M₃₁–M₃₅ were not measured in plasma. The most abundant metabolites in urine were M₈ and M₆, i.e., 131% and 90% of urinary ginsenoside Rg₁, respectively, while urinary M₃, M₅, M₇, M₁₁–M₁₃, M₂₃–M₂₅, M₂₇, M₂₉, and M₃₅ were only 10%–43%. It is worth mentioning that the preceding plasma and urine metabolites (M₁–M₃₅) were absent in the administered Sanqi-extract. As to the ginsenosides present in Sanqi-extract, their oxidized or conjugated metabolites were not measured.
In the human samples.

**In Vitro Metabolism of 20(S)-Protopanaxadiol, 20(S)-Protopanaxatriol, and Compound-K.** To help characterize the preceding in vivo metabolites (M1–M35), in vitro metabolism studies were implemented with HLM. 20(S)-Protopanaxadiol or 20(S)-protopanaxatriol was labile when incubating with NADPH-fortified HLM (in vitro t½, 6 and 4 min, respectively; Supplemental Table 1). Furthermore, both the sapogenins were quite stable with UDPGA-fortified HLM (>120 min). These data suggested that the oxidation of the sapogenins was rapid in the liver, while the glucuronidation was relatively slow. Compound-K was slowly metabolized with the human microsomal enzymes (>120 min). According to the in vitro data (Supplemental Table 1), M16–M22, M34, and M35 were characterized as the oxidized metabolites of 20(S)-protopanaxadiol, while M3–M15, M23–M29, and M31–M33 were derived from 20(S)-protopanaxatriol. M1, M2, and M30 were the metabolites of compound-K. Figure 3 depicts the proposed metabolic pathways of the deglycosylated products in the humans.

Human CYP3A4 and CYP3A5 were the major enzymes responsible for the oxidation of 20(S)-protopanaxadiol or 20(S)-protopanaxatriol (Fig. 4). In addition, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 could also mediate the oxidation of 20(S)-protopanaxadiol. The Michaelis constants of HLM towards the formation of M20/M21/M22 from 20(S)-protopanaxadiol and those for M8/M10/M11/M12 from 20(S)-protopanaxatriol were 4.0–6.6 and 0.2–1.1 μM, respectively (Supplemental Table 2).
Plasma Pharmacokinetics and Urinary Excretion of Ginsenosides and their
Major Metabolites after p.o. Administration of Sanqi-extract. Figure 5 shows
plasma level-time profiles of the major ginsenosides after a p.o. dose of
Sanqi-extract to the human subjects, as well as those of the deglycosylated products
[compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol] and the oxidized
metabolites of the sapogenins, while the plasma PK parameters are summarized in
Supplemental Tables 3–5. After dosing at 270 ml/subject, the plasma concentrations
of ppd-type ginsenosides Ra3, Rb1, and Rd increased up to $C_{max}$ (3.1, 27.9, and 9.3
nM, respectively) with $T_{max}$ of 8–12 h and then slowly decreased with $t_{1/2}$ of 33–57 h.
Their MRT values were 50–82 h. The $T_{max}$ and MRT of ppd-type ginsenoside F 2
were 13 h and 22–29 h, respectively. The renal excretion of ginsenosides Ra3, Rb1,
Rd, and F 2 was slow with $CL_R$ of 0.01–0.04 l/h and their urinary
Cum.$A_{e(0→72h)}$ accounted for only ~0.01% of the compound doses from the p.o. ingested
Sanqi-extract. The AUC$_{0-56h}$ of ginsenosides Ra3, Rb1, Rd, and F 2 increased in
Sanqi-extract dose-related manners (90–270 ml/subject), but the dose proportionality
was inconclusive (Fig. 6). Compound-K exhibited a significantly higher mean
AUC$_{0-56h}$ than ginsenoside Rb1, demonstrating 4749 versus 749 nM·h after dosing at
270 ml/subject, while the former’s interindividual difference in AUC$_{0-56h}$ was
significantly larger than that of the latter. Meanwhile, compound-K had a $T_{max}$ of
10–30 h and an MRT of 23–26 h. The average $CL_R$ value of compound-K was very
low (0.01 l/h). Significant interindividual difference in plasma occurrence was also
observed with 20(S)-protopanaxadiol. The AUC$_{0-56h}$ value of compound-K or
20(S)-protopanaxadiol correlated poorly with the dose of Sanqi-extract (Fig. 6). In addition, compound-K or 20(S)-protopanaxadiol was correlated poorly with ginsenosides Ra3, Rb1, Rd, and F2 in AUC0-56h for the individual subjects (Fig. 7). Compound-K was also correlated poorly with 20(S)-protopanaxadiol.

At the Sanqi-extract dose 270 ml/subject, ppt-type ginsenoside Rg1 and notoginsenoside R1 had Tmax of 6–7 h and Cmax values of 10.2 and 1.4 nM, respectively. Their MRT values were 7–13 h. The renal excretion of these ppt-type ginsenosides was rapid with CLR of 5.6 and 4.0 l/h, respectively, while their urinary Cum.Ae(0-24h) accounted for ~0.06% of the compound doses from Sanqi-extract. The AUC0-38h values of ginsenoside Rg1 and notoginsenoside R1 increased in Sanqi-extract dose-related manners, but lack of proportionality was concluded (Fig. 6). Ppt-type ginsenoside F1 had a Tmax of 14–19 h and a Cmax of 4.3 nM. Its CLR was 0.7 l/h. Although 20(S)-protopanaxatriol had higher systemic exposure as compared with the major Sanqi ginsenosides, the interindividual difference was larger. The AUC0-56h of 20(S)-protopanaxatriol was independent of the Sanqi-extract dose (Fig. 6), which was also not correlated with AUC0-38h of ginsenosides Rg1, F1, or notoginsenoside R1 for the individual subjects (Fig. 7). There appeared to be correlation between 20(S)-protopanaxatriol and 20(S)-protopanaxadiol in AUC0-56h (P < 0.01).

As shown in Fig. 5, the plasma concentrations of M16, M17, and M19–M22 increased and then decreased concurrently as the concentration of their precursor 20(S)-protopanaxadiol changed with time. In addition, there were significantly positive correlations in AUC0-56h between 20(S)-protopanaxadiol and these
metabolites for the individual subjects ($P < 0.01$; Fig. 7). Meanwhile, the plasma concentrations of $M4-M8$ and $M10-M15$ also changed concomitantly with that of 20(S)-protopanaxatriol and significant correlations existed between the sapogenin and its oxidized metabolites ($P < 0.01$). Similar to the situations of their parent sapogenins, the $AUC_{0-56h}$ value of the preceding oxidized metabolites appeared to be Sanqi-extract dose-independent (Fig. 6). With respect to the renal excretion, the ppd-derived metabolites were not or negligibly recovered in the human urine after oral administration of Sanqi-extract. In contrast, many of the ppt-derived metabolites (in the ranking order of $M6>M7>M5=M13>M8$) were notably excreted into urine with $CL_R$ of 0.1–1.3 l/h.

Figure 8 depicts $AUC_{0-24h}$ changes of ginsenosides and the metabolites for the subjects m1, m2, and m4 who received Sanqi-extract for additional 21 days. The $AUC_{0-24h}$ of ginsenosides $Ra_3$, $Rb_1$, and $Rd$ increased significantly during the multiple-dose treatment, indicative of 1.9–11.3-fold increases on day 11 as compared with the data on day 1 and 1.3–10.3-fold increases on day 18. However, the $AUC_{0-24h}$ values on day 24 tended to decrease for these subjects. The $AUC_{0-24h}$ values of compound-K and ginsenosides $F_1$ and $F_2$ did not exhibit a clear initial increase and eventual decrease. Subjects m1 and m2 had notable increases in $AUC_{0-24h}$ values of ginsenoside $Rg_1$ and notoginsenoside $R_1$ on day 18 (3.5–7.4-fold of the day 1 data) and decreases on day 24 (22%–55% of the day 18 data). 20(S)-Protopanaxadiol and 20(S)-protopanaxatriol had significantly increased $AUC_{0-24h}$ values on days 11, 18, and 24 as compared with the data on day 1. Notably, the $AUC_{0-24h}$ values of
ppd-derived M16, M17, and M19–M22 increased and decreased as the AUC$_{0-24h}$ value of 20(S)-protopanaxadiol changed with time (Fig. 8). Similar situation occurred with 20(S)-protopanaxatriol and its metabolites M4–M8 and M10–M15 (Fig. 8). In addition, there were significantly positive correlations in AUC$_{0-24h}$ between 20(S)-protopanaxadiol and its metabolites M16, M17, and M19–M22 and between 20(S)-protopanaxatriol and the metabolites M4–M8 and M10–M15 ($P < 0.01$; Fig. 9).
Discussion

Unlike synthetic drugs, herbal medicines are complex chemical mixtures. Benefits of herb therapy are normally based on a range of activities of compounds working together. Accordingly, ADME/PK study of an herb medicine involves investigation of multiple botanical compounds to link the medicine administration and the medicinal benefits. An earlier systematic ADME/PK study of a Sanqi extract was performed in rats (Liu et al., 2009), which provided the indispensable guidance for us to implement strategically this follow-up multi-compound human PK study.

Similar to the situation in rats, the ppd-type ginsenosides Ra3, Rb1, and Rd from p.o. dosed Sanqi-extract had long elimination half-lives (>30 h) in the human subjects and significant accumulation in AUC\(_{0-24h}\) during the multiple-dose treatment. Their systemic exposure levels increased in a Sanqi-extract dose-dependent manner (90–270 ml/subject). These plasma ginsenosides were considered to be Sanqi-extract dose-dependent PK markers to substantiate human systemic exposure to the oral herb extract. Unlike the situation in rats, the human systemic exposure levels of the ppt-type ginsenoside Rg1 and notoginsenoside R1 and the ppd-type ginsenoside F2 also increased in Sanqi-extract dose-related manners. Poorer biliary excretion in humans than in rats (Mahmood and Sahajwalla, 2002; Lai, 2009) likely accounted for the interspecies differences. Accordingly, plasma ginsenosides Rg1, F2 and notoginsenoside R1 could also be used as Sanqi-extract dose-dependent PK markers in humans.

The most important finding of this human study was the substantially measured
plasma metabolites that resulted from the combinatorial metabolism of ginsenosides (consisting of the saponin deglycosylation by the colonic microflora and the subsequent sapogenin oxidation by the enterohepatic CYP enzymes). Deglycosylation of ginsenosides via action of the colonic microflora has been demonstrated in vitro and in vivo for years (Hasegawa et al., 1996; Tawab et al., 2003; Yang et al., 2006) and the human CYP-mediated oxidation of the sapogenins has also been assessed in vitro recently (Kasai et al., 2000; Hao et al., 2010; Li et al., 2011). Unlike the situation in humans, these metabolites were poorly detected in rat plasma after p.o. dose of Sanqi extract (Liu et al., 2009). The human colon is heavily bacterial-colonized as compared with the rat colon (Sousa et al., 2008), which likely accounts for the observed metabolism interspecies difference. The amount of variation in the colonic microflora is considerable between human individuals (Nicholson et al., 2005). In this study, we observed marked interindividual differences in formation of the deglycosylated compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol. Notably, the systemic exposure levels of oxidized metabolites of the sapogenins, regardless of the sex of subject or the Sanqi-extract dose level, were well correlated with those of the sapogenins, rather than the levels of the ginsenosides absorbed (Fig. 7 and 9). The colonic microflora-mediated deglycosylation appeared to be the rate-limiting step in the combinatorial metabolism of ginsenosides. As shown in Fig. 5, although the $T_{\text{max}}$ values of compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol were delayed ~20 h as compared with those of the ginsenosides, these deglycosylated products and their oxidized metabolites were
almost identical in $T_{\text{max}}$. Even if the gastrointestinal transit time (normally 4–5 h) to colon is taken into consideration, these data still suggested that the deglycosylations were significantly slower than the enterohepatic oxidations. In addition, the deglycosylation activities of the colonic microflora appeared to be induced by the unabsorbed ginsenosides during the subchronic treatment with Sanqi-extract. Despite the structural similarity, multiple CYP enzymes mediated the oxidation of 20($S$)-protopanaxadiol, whereas only CYP3A4 and CYP3A5 were implicated in the metabolism of 20($S$)-protopanaxatriol. This finding suggests that the 20($S$)-protopanaxadiol oxidation is relatively refractory to the effects of CYP-inhibitors. The oxidized metabolites from the sapogenins were likely cleared via renal excretion and/or glucuronidation-associated biliary excretion (Supplemental Figure 1).

Besides the preceding dose-dependent PK markers, we further proposed two other concepts of dose-independent PK markers for Sanqi-extract. The colonic microflora mediating the ginsenoside deglycosylation played a key role in the combinatorial metabolism. The plasma compound-K, 20($S$)-protopanaxadiol, and 20($S$)-protopanaxatriol could be used as PK markers to reflect the interindividual differences in deglycosylation activities of the colonic microflora. This proposal is also supported by in vitro formation of these deglycosylated products from ginsenosides via action of the human fecal microflora under anaerobic conditions (Hasegawa et al., 1996). In addition, the plasma concentration of 20($S$)-protopanaxadiol and the plasma levels of oxidized metabolites (M16–M22)
concomitantly increased up to maxima and then decreased with almost the same $T_{\text{max}}$ and MRT values. The same situation also occurred with 20(S)-protopanaxatriol and its oxidized metabolites (M4–M8 and M10–M15). Significant correlations in AUC (Fig. 7 and 9) between 20(S)-protopanaxadiol and the ppd-derived metabolites and those between 20(S)-protopanaxatriol and the ppt-derived metabolites suggested that the plasma sapogenins could be used as PK markers to reflect the timely-changes and interindividual differences in plasma levels of their respective oxidized metabolites. In pharmacological studies of herbal medicines, the intensity of pharmacological response was often found to be correlated poorly with the dose. Unlike the absorbed ginsenosides, the plasma levels of the ppd-derived or ppt-derived metabolites were dependent on the human individual’s deglycosylation activities of colonic microflora rather than the Sanqi-extract dose. The information gained and the associated PK markers identified may be implicated when the dose-response relationship is assessed for Sanqi in humans.

Poor oral bioavailability of many herbal constituents is often associated with their low levels of systemic exposure, which impedes translation of many in vitro pharmacological activities of the compounds to the therapeutic effects of herbal medicines (Zhang et al., 2012). However, we found that ppd-type ginsenosides Ra3, Rb1, and Rd from a p.o. ingested Sanqi extract had considerably high plasma levels in rats despite their <1% oral bioavailability (Liu et al., 2009). These ppd-type ginsenosides were long-circulating because their elimination kinetics was associated with high metabolic stability and slow biliary and urinary excretion. In this human
study, we found that the deglycosylated products compound-K, 20(S)-protopanaxadiol, 20(S)-protopanaxatriol, and their many oxidized metabolites had high plasma levels and accumulated substantially with repeated doses of Sanqi-extract. Assessing the pharmacological activities of these herbal compounds with significant exposure levels after dosing is important for evaluation of oral Sanqi’s therapeutic values. Accordingly, additional studies are planned to make pharmacological assessments of the herbal metabolites found in this study.

Considerable hepatotoxicity and nephrotoxicity were observed in rats receiving multiple p.o. doses of “notoginseng total saponin” (containing ginsenosides Rg1, Rb1, Rd, Re, and notoginsenoside R1) at 1600 mg/day/kg for thirteen weeks (Ma and Dai, 2011). Our earlier rat Sanqi study indicated that the major ppt-type ginsenosides Rg1, Re, and notoginsenoside R1, as well as the ppd-type compound-K, were subject to rapid biliary excretion via an active transport mechanism and had high liver levels (Liu et al., 2009). In this study, no significant hepatotoxicity and nephrotoxicity were observed for the human subjects receiving oral Sanqi-extract, except for the subject m3. Although this subject had obviously increased ALT during the multiple-dose treatment, his plasma levels of ginsenosides and the metabolites were not high as compared with the other subjects. According to dose normalization by body surface area (Reagan-Shaw et al., 2007), the daily doses (800–1600 mg/kg) in the rat study by Ma and Dai (2011) were about 20–40 times greater than the daily dose for our subchronic treatment of the human subjects m1–m4 with Sanqi-extract (90 ml/subject). Although the potential effects of ginsenosides and their metabolites on
the hepatic and renal function need to be confirmed by more studies, it is nonetheless wise to exercise caution when high dose levels of Sanqi extracts or the associated pharmaceutical products are administered for a long period.

In pharmacological assessment of an herbal medicine, it is important to understand not only its chemical content but also which herb-derived compounds circulate considerably in the bloodstream after dosing. In this study, notable differences were found between the circulating compounds derived from ginsenosides after p.o. ingestion of Sanqi-extract and the ginsenosides present in the extract. Our study provides information for the ethnopharmacologists on which Sanqi-derived compounds are worth further evaluation, while it also tells the phytochemists which Sanqi-derived compounds should be prepared for the pharmacological and safety assessments. In summary, 20(S)-protopanaxadiol, 20(S)-protopanaxatriol, and their oxidized metabolites, as well as compound-K, represent major circulating forms of ginsenosides in the human bloodstream after p.o. ingestion of Sanqi-extract. However, the systemic exposure levels of these metabolites are of large interindividual variance, which depend on the deglycosylation activities of the colonic microflora of individual subjects, rather than the Sanqi-extract dosage size. Compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol measured in human plasma can be used as PK markers to reflect the microbial activities of human individuals. In addition, plasma 20(S)-protopanaxadiol and 20(S)-protopanaxatriol can also serve as PK markers to indicate the timely-changes and interindividual differences in plasma levels of their respective oxidized metabolites. The ppd-type ginsenosides Ra3, Rb1
and Rd, as well as the ppt-type ginsenosides Rg₁, F₂, and notoginsenoside R₁, can be used as Sanqi-extract dose-dependent PK markers to substantiate human systemic exposure to the orally ingested extract. Most plasma ginsenosides, as well as their metabolites, accumulate substantially in the systemic circulation during multi-dose treatment with oral Sanqi-extract.
Authorship Contributions

Participated in research design: Li, Yang, Huang, and Gao

Conducted experiments: Hu, Yang, Cheng, Du, Huang, Wang, Niu, and Xu

Performed data analysis: Li, Hu, Yang, Cheng, and Jiang

Wrote or contributed to the writing of the manuscript: Li, Hu, Yang, and Cheng
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Reagan-Shaw S, Nihal M, and Ahmad N (2007) Dose translation from animal to human studies


Footnotes

Zheyi Hu, Junling Yang, and Chen Cheng contributed equally to the study. This work was supported by the National Science Fund of China for Distinguished Young Scholars [Grant 30925044], the National Basic Research Program of China [Grant 2012CB518403], the National Science and Technology Major Project of China “Key New Drug Creation and Manufacturing Program” [Grants 2009ZX09304-002, 2012ZX09304007, and 2012ZX09303010], and the Knowledge Innovation Program of the Chinese Academy of Sciences [Grant KSCX2-YW-R-191].
Legends for Figures

Fig. 1. Flowchart for the two-period, open-label, single center human study. Twenty four subjects were randomly assigned to one of three dosage groups (four males and four females per group). After the random assignment, the subjects were renumbered before receiving a p.o. dose of Sanqi-extract at 90 (the male subjects m1–m4 and the female subjects f1–f4), 180 (m5–m8 and f5–f8), or 270 ml/subject (m9–m12 and f9–f12). In addition, the subjects m1–m4, after three-day wash-out period following the first acute p.o. treatment with Sanqi-extract, continued to receive multiple p.o. doses of the herb extract at 90 ml/subject/day for three weeks. Their blood and urine samples were collected on days 11, 18, and 24. “HF/RF” denotes hepatic function and renal function.

Fig. 2. Ginsenosides present in Sanqi-extract (panel A), as well as plasma (panels B and D) and urinary ginsenosides and their metabolites measured (panels C and E) after p.o. administration of Sanqi-extract (270 ml/subject). The blood was sampled from the human subjects 8 and 24 h after dosing, which normally had high levels of the ginsenosides absorbed and those of the metabolites formed, respectively. After centrifugation, all the plasma fractions (10 μl from each) were pooled before analysis. Meanwhile, the urine samples (0–24 h after dosing) were also collected from these subjects and the sample combining (20 μl from each) was also performed for analysis. Compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol were not present in Sanqi-extract; they were ginsenosides’ metabolites measured in plasma. The symbol “×” (in red) denotes the ginsenoside or metabolite not measured in human or urine. Blue, red, and black bars are used to show the content levels of ginsenosides in Sanqi-extract, their plasma levels, and their urine levels, respectively, while pink and grey bars exhibit the plasma and the urine levels of ginsenosides’ metabolites, respectively. The response was determined as compound peak area corrected with the intensity ratio of the fragment ion to the precursor ion. Relative abundance measures are expressed in percentile, with 100 being 20(S)-protopanaxatriol in the plasma data set and with 100 being ginsenoside Rg1 in the urinary data set.

Fig. 3. Proposed metabolism pathways of compound-K, 20(S)-protopanaxadiol, and 20(S)-protopanaxatriol in humans. The metabolites in the brackets are proposed intermediates, which were not measured in the samples of human plasma (HP) or human urine (HU). The metabolites M36,
M37 and M38 were the glucuronides of M20, M21, and M22, respectively, which were measured in the samples of rat bile (RB) after i.v. administration of 20(S)-protopanaxadiol.

**Fig. 4.** Human CYP enzymes mediating the oxidation of 20(S)-protopanaxadiol or that of 20(S)-protopanaxatriol. The substrate and enzyme concentration used were 2 μM and 50 pmol P450/ml, respectively, for all the test cDNA-expressed CYP enzymes and the incubation time was 5 min (panels A and B) or 30 min (panels C and D). The response was determined as compound peak area corrected with the intensity ratio of the fragment ion to the precursor ion. The relative abundance was expressed as percentile, with 100, in the data sets, being the most abundant M8 that was formed after incubation with CYP3A4 for 30 min.

**Fig. 5.** Mean plasma concentration-time profiles of ginsenosides and their major metabolites after a single p.o. dose of Sanqi-extract at 90 (green solid lines), 180 (blue solid lines), or 270 ml/subject (red solid lines). The subject f12 (receiving Sanqi-extract at 270 ml/subject; red dashed lines) exhibited marked extensive but delayed formation of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol, and their oxidized metabolites.

**Fig. 6.** AUC\(_{0-56h}\) values of compound-K, 20(S)-protopanaxadiol, 20(S)-protopanaxatriol, the ppd-derived metabolites (M16, M17, and M19–M22), and the ppt-derived metabolites (M4–M8 and M10–M15) being Sanqi-extract dose-independent. The exposure values were measured in human subjects (solid circles, the male subjects; open circles, the female subjects) receiving a p.o. dose of Sanqi-extract at 90 (green circles), 180 (blue circles), or 270 ml/subject (red circles). Unlike the metabolites’ situations, AUC\(_{0-56h}\) values of ginsenoside Ra3, Rb1, Rd, F2, Rg1, and notoginsenoside R1 increased in Sanqi-extract dose-dependent manners. The term “\(r\)” denotes the correlation coefficient. The ratio of dose-normalized geometric mean AUC values is given before the parentheses, while the numbers in the parentheses are the statistically derived 90% confidence interval. The critical interval was 0.797–1.203.

**Fig. 7.** Strong positive correlations existing in AUC\(_{0-56h}\) between 20(S)-protopanaxadiol and its oxidized metabolite (M16, M17, M19, M20, M21, or M22), between 20(S)-protopanaxatriol and its oxidized metabolite (M4, M5, M6, M7, M8, M10, M11, M12, M13, M14, or M15), and between 20(S)-protopanaxadiol and 20(S)-protopanaxatriol in the human subjects (solid circles, the male...
subjects; open circles, the female subjects) receiving a single p.o. dose of Sanqi-extract at 90 (green circles), 180 (blue circles) or 270 ml/subject (red circles). Meanwhile, no significant correlation occur in AUC_{0-56h} between compound K and ginsenoside Ra3, Rb1, Rd, or F2, or 20(S)-protopanaxadiol, between 20(S)-protopanaxadiol and ginsenoside Ra3, Rb1, Rd, or F2, between 20(S)-protopanaxtriol and notoginsenoside R1, ginsenoside Rg1, or F1.

**Fig. 8.** Plasma AUC_{0-24h} of ginsenosides and their major metabolites changing with time in the subjects m1–m4 who received Sanqi-extract at 90 ml/subject/day (green solid lines) for three weeks.

**Fig. 9.** Strong positive correlations existing in AUC_{0-24h} between 20(S)-protopanaxadiol and its oxidized metabolite (M16, M17, M19, M20, M21, or M22), between 20(S)-protopanaxatriol and its oxidized metabolite (M4, M5, M6, M7, M8, M10, M11, M12, M13, M14, or M15), and between 20(S)-protopanaxadiol and 20(S)-protopanaxatriol in the human subjects (m1–m4) who participated in the multiple dose study. Meanwhile, no significant correlation occurred in AUC_{0-24h} between compound K and Ra3, Rb1, Rd, or F2, between 20(S)-protopanaxadiol and ginsenoside Ra3, Rb1, Rd, or F2, between and notoginsenoside R1, ginsenoside Rg1, or F1. The exposure values were measured on days 1, 11, 18 and 24 (for m1, m2 and m4 only) after initiation of the multiple dose study.
Figure 4

CYP-mediated metabolism of 20(S)-protopanaxadiol

CYP-mediated metabolism of 20(S)-protopanaxatriol

Relative abundance (%)
Figure 5
Figure 6

The figure shows plasma AUC0-56h (µM·h) for various compounds and Sanqi extract doses. The graphs include correlations and statistical significance levels. The table below summarizes the dose levels:

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
<th>Dose</th>
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<tr>
<td>▶️</td>
<td>▶️</td>
<td>180 ml/subject</td>
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<tr>
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