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

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Drug Metabolism and Disposition

Supplemental Materials and Methods

Materials. The dried roots of *Panax notoginseng* (Sanqi) were obtained from Shanghai Huayu Chinese Herbs Co., Ltd. (Shanghai, China). For the human study, crude Sanqi was washed with water and dried before pulverization. The pulverized material (2.0 kg) was steeped in 12 l of distilled water for 30 min before 50-min sonication-enhanced extraction. The aqueous extract was separated by filtration, and the residue was re-extracted with 8 l of water. The combined extract (about 18 l) was fortified with 1.2 l of 5% (w/v) hydroxypropylmethyl cellulose solution and then added with the distilled water to 20 l. The 1:10 water extract was aliquoted and airproofed as 90 ml/bottle and was further sterilized using Co-60 γ ray to yield "Sanqi-extract". The preceding preparation of Sanqi-extract was carried out in a GMP formulation center at Tianjin University of Traditional Chinese Medicine (Tianjin, China). The prepared Sanqi-extract contained ginsenosides Rg_1 (2680 μM), Rb_1 (1377 μM), Rd (366 μM), Re (254 μM), Rh_1 (103 μM), Ra_3 (76 μM), Rg_2 (60 μM), F_1 (48 μM), F_2 (18 μM), Rg_3 (17 μM), Rb_2 (14 μM), Rf (13 μM), Rc (3 μM), and Rh_2 (1 μM), as well as notoginsenosides R_1 (360 μM), R_3/R_6 (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 4ºC 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.

Ginsenosides Rb_1, Rd, Rg_3, F_2, Rh_2, Re, Rg_1, Rf, F_1, notoginsenoside R_1, compound-K, 20(*S*)-protopanaxadiol, 20(*S*)-protopanaxatriol, midazolam and chrysin were obtained from the National Institutes for Food and Drug Control (Beijing, China). Ginsenosides Rc, Rb_2, Rh_1 and Rg_2 were purchased from Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). Ginsenosides Ra_3 and 20-gluco-ginsenoside Rf were obtained from Fengshanjian Co., Ltd. (Kunming, China). The purity of compounds exceeded 98%.

Nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate monosodium salt, glucose-6-phosphate dehydrogenase, uridine 5'-diphosphoglucuronic acid (UDPGA), and alamethicin were obtained from Sigma-Aldrich (St. Louis, MO). Pooled human liver microsomes (HLM) of Chinese origin was obtained from the Research Institute for Liver Diseases (Shanghai, China). Rat liver microsomes (RLM) and rat colon microsomes (RCM) were prepared in house by differential centrifugation. Microsomes prepared from insect cells containing baculovirus-expressing human CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 were from BD Gentest (Woburn, MA), which were fortified with NADPH-cytochrome P450 reductase. HPLC-grade organic solvents and reagents were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China).

Human Study. The protocol for human study conformed to the ethical guidelines of the Helsinki Declaration of 1975 (as revised in 1983) and 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 study details were explained to and the written informed consent was obtained from each volunteer. Twelve male and twelve female healthy volunteers between 22–26 years and within 15% of ideal body weight were enrolled in the study. The volunteers were determined healthy with regard to medical history, physical examination, hepatic and renal function tests, electrocardiogram, and routine clinical laboratory tests. The female volunteers were negative for menstruation and pregnancy. Any synthetic medicines and herbal medicines were prohibited for 2 weeks before and through the end of the study period. In addition, any alcohol-, caffeine-, tea-, or citrus-containing beverages or foods were prohibited for 2 days before and through the end of each study period. Subjects who had a history of drug or food allergy or intolerance to any *Panax* species products were excluded.

A two-period, open-label, single center study was performed at the National Clinical Research Center of the hospital (Tianjin, China; Fig. 1). There was a three-day delay for the first dosing of period 2 after the dosing of period 1 (for the four male subjects receiving 90-ml Sanqi-extract/subject only). In period 1, all the subjects were randomly assigned to one of three dosage groups, with four male and four female subjects in each 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/day/subject 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 samplings performed 0–24 h after the daily 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 hepatic function markers for each subject by the Department of Clinical Laboratory of the hospital, while serum creatinine (CRE) and blood urea nitrogen (BUN) were also assessed to monitor the 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. Several in vitro metabolism studies were performed to elucidate the precursors of the in vivo ginsenosides metabolites and to identify the human CYP enzymes that were responsible for the oxidation of 20(S)-protopanaxadiol or 20(S)-protopanaxatriol. Pure compound-K, 20(*S*)-protopanaxadiol, and 20(*S*)-protopanaxatriol at 2 μM was incubated with HLM, RLM or RCM in the presence of necessary cofactors (NADPH, UDPGA, or the both) to monitor both metabolic stability and metabolite formation. The incubations were performed in duplicate at 37°C for

0, 5, 15, 30, 60, 90, or 120 min in 96-well plates. For CYP-mediated oxidation, incubation mixtures in a total assay volume of 100 μl consisted of the tissue microsomes (0.5 mg protein/ml), substrates (2 μM), potassium phosphate buffer (100 mM; pH 7.4), and a NADPH-generating system (comprising 3.3 mM magnesium chloride, 3.3 mM glucose 6-phosphate, 0.5 U/ml glucose 6-phosphate dehydrogenase, and 1.3 mM NADP). The microsomal incubation mixtures (100 μl) for UDP-glucuronosyltransferase-mediated glucuronidation contained the microsomes (0.5 mg protein/ml), substrates (2 μM), magnesium chloride (10 mM), Tris-HCl buffer (50 mM; pH 7.5), alamethacin (25 μM), and uridine 5'-diphosphoglucuronic acid (2 mM). For reactions containing both NADPH and UDPGA, the substrates (2 μM) were incubated with the microsomes (0.5 mg protein/ml), magnesium chloride (10 mM), potassium phosphate buffer, alamethacin (25μM), the NADPH-generating system, and uridine 5'-diphosphoglucuronic acid (2 mM). In vitro metabolic stability of the substrates was assessed in terms of half-life ($t_{1/2}$) of substrates. In addition, the LC-MS/MS measurement characteristics of the in vitro formed metabolites, including the LC retention time (t_R), *m/z* of precursor ion, and CID fragmentation pattern, were also used in characterization of the in vivo measured metabolite candidates.

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. Because **M19**/**M20**/**M21**/**M22** and **M8**/**M10**/**M11**/**M12** were the predominant plasma metabolites of 20(*S*)-protopanaxadiol and 20(*S*)-protopanaxatriol, respectively, the enzymes identification was based on the formation rates of these metabolites. The incubations were performed in duplicate at the substrate concentration 2 μM and the enzyme concentration 50 pmol P450/ml for 5 or 30 min.

To determine the enzyme kinetics, including Michaelis constant (K_m), maximum velocity (V_{max}), and in vitro intrinsic clearance (CL_{int}), 20(*S*)-protopanaxadiol or 20(*S*)-protopanaxatriol was incubated with NADPH-fortified HLM under linear metabolite formation conditions. In brief, the incubations were performed in duplicate in 96-well plates. Each well contained HLM (0.5 mg protein/ml), substrate (1.56–200 μM for 20(*S*)-protopanaxadiol or 0.08–200 μM for 20(*S*)-protopanaxatriol), potassium phosphate buffer (100 mM, pH 7.4), an NADPH-generating system. Before commencement of the reaction by adding the NADPH-generating system, the incubation mixture was preincubated for 3 min at 37°C. The optimal incubation time was 15 min for 20(S)-protopanaxadiol or 5 min for 20(S)-protopanaxatriol. The reactions were terminated by adding 100 μl of ice-cold acetonitrile. The resulting samples were centrifuged at 3000*g* for 10 min to remove protein and 5 μL of the supernatants were analyzed by LC-MS/MS.

Rat Studies. Two supplementary animal studies were conducted according to protocols approved by the Review Committee of Animal Care and Use at the Shanghai Institute of Materia Medica (Shanghai, China). Male Sprague-Dawley rats (220–250 g; Shanghai SLAC Laboratory Animal Co., Shanghai, China) were housed in an environment-controlled room with a 12-h light/dark cycle and acclimatized to the facilities and environment for three days before use. The rodents received in-house surgery to become femoral artery-cannulated or bile duct-cannulated rats. In the first study, three bile duct-cannulated rats were overnight-fasted before receiving an i.v. bolus dose of the sapogenin (3 μmol/kg). The bile samples were collected at intervals of 0–4, 4–8, 8–24, 24–32 and 32–48 h after dosing and weighed. A sodium taurocholate solution (1.5 ml/h during day time; pH 7.4) was infused into the duodenum during bile collection. In the other study, surgery-free rats were sacrificed, under light ether anesthesia, by bleeding the abdominal aorta at 0, 30 min, 1, and 3 h (three rats per time point)

after i.v. dosing with the sapogenin (3 μmol/kg). The tissue samples heart, lung, brain, liver, and kidney were excised and then rinsed in ice-cold saline before gently blotting on absorbent paper. After weighing, the tissues were homogenized in 4 volumes of ice-cold saline. Meanwhile, the blood samples were also collected and centrifuged to yield the plasma fractions. All the rat samples were stored at −70°C pending analysis.

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) was used for metabolite profiling and quantification in the study. The human plasma and urine samples (50 μl), as well as the rat plasma, tissue homogenate and bile samples (50 μl), were precipitated with 150 μl of methanol. After centrifuging at 21,885g for 10 min, the supernatants 180 μl were reduced to dryness using a centrifugal concentrator under reduced pressure and the residues were reconstituted in 50% methanol (20 μl) for metabolite profiling. Meanwhile, 5 μl of the supernatant were used for quantification without any concentration treatment.

The chromatographic separation for metabolite profiling was achieved on a 3.5 μm Agilent Eclipse Plus C18 column (150 mm × 2.1 mm I.D.; Santa Clara, CA), while that for quantification was on a 5 μm Agilent ZORBAX Eclipse Plus C18 column (50 mm × 2.1 mm I.D.; Santa Clara, CA). The mobile phase consisting of methanol/water (1:99, v/v, containing 1.5 mM formic acid; A) and methanol/water (99:1, v/v, containing 1.5 mM formic acid; B) was delivered at a flow rate of 0.3 ml/min for the metabolite profiling, whereas the mobile phase consisting of methanol/water (1:99, v/v, containing 25 μM lithium acetate and 1.5 mM formic acid; A) and methanol/water (99:1, v/v, containing 25 μM lithium acetate and 1.5 mM formic acid; B) was delivered at 0.4 mL/min for the quantification. Metabolites profiling was based on a 20-min conventional gradient method, i.e., 0–1 min at 25% B, 1–14 min from 25% B to 95% B, 14–18 min at 95% B, 18–20 min at 25% B, whereas a 8-min pulse gradient method (Li et al., 2008) was used for quantification, i.e., 0–4 min at 20% B, 4–7 min at 90% B, and 7–8 min at 20% B.

Metabolite profiling was conducted in the positive ion modes according to the molecular mass gains or losses predicted for the possible metabolites compared with those of the parent compounds and based on the generation of deglycosylation fragment ion or dehydration fragment ion from the precursor protonated molecules by collision-induced disassociation (the collision energy 30 or 35 V). Only chromatographic eluent flow over a period of 4 to 18 min was introduced to the ion source for data acquisition. The metabolite prediction was carried out according to literature list of common biotransformation of drugs (Kostiainen et al., 2003), as well as the published data (Hao et al., 2010; Li et al., 2011). The metabolite candidates of 20(*S*)-protopanaxadiol or 20(*S*)-protopanaxatriol were characterized in the enhanced product ion mode using the protonated molecules.

Quantification of ginsenosides and the metabolites in biological matrices was performed using the available reference standards for calibration or using the calibration curve of an analog (also called "brother compound") that bore close structure similarity to the analyte. Using the reference standards, the matrix-matched calibration curves were constructed for the analytes (0.34 or 0.68–3000 nM) using weighted linear regressions of the analyte peak area against the corresponding nominal analyte concentrations (nM). The virtual quantification involved a correction with the intensity ratios of the fragment ion to the precursor ion of the analyte and the brother compound. For quantification, the chromatographic eluent flow over a period of 2 to 6.5 min was introduced to the ion source for data acquisition. Lithiated adduct was generated for quantification and the following precursor-to-product ion pairs were used for multiple reaction monitoring of analytes, including *m/z* 1247→349 (ginsenoside

Ra_3), *m/z* 1115→349 (ginsenoside Rb_1), *m/z* 1085→319 (ginsenoside Rb_2 and Rc), *m/z* 969→349 (20-gluco-ginsenoside Rf and notoginsenoside $R_{3/6}$), *m/z* 953→773 (ginsenosides Rd and Re), *m/z* 939→319 (notoginsenoside R_1), *m/z* 807→627 (ginsenoside Rg_1), *m/z* 807→349 (ginsenoside Rf), *m/z* 791→611 (ginsenoside F_2), *m/z* 791→349 (ginsenoside Rg_3), *m/z* 791→187 (ginsenoside Rg_2), *m/z* 691→497 (**M23**, **M24**, **M26**, **M31**, **M32**), *m/z* 675→481 (**M25**, **M27**, and **M28**), *m/z* 659→465 (**M29, M36**–**M38**), *m/z* 645→465 (ginsenosides Rh_1, F_1, and **M1**, **M2**, **M30**), *m/z* 629→449 (compound-K), *m/z* 629→187 (ginsenoside Rh_2), *m/z* 517→499 (**M3** and **M6**), *m/z* 515→497 (**M35**, **M33**, and **M11**), *m/z* 515→399 (**M4**, **M5**, and **M8**), *m/z* 513→495 (**M7**, **M9**, and **M13**), *m/z* 501→483 (**M16** and **M18**), *m/z* 499→481 (**M17**, **M19**, **M10**, and **M12**), *m/z* 497→479 (**M14** and **M15**), *m/z* 483→465 [20(*S*)-protopanaxatriol and **M20**–**M22**], and *m/z* 467→449 [20(*S*)-protopanaxadiol]. 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. The maximum plasma concentration (C_{max}) and the time taken to achieve the maximum concentration (T_{max}) were observed values without interpolation. The area under concentration-time curve up to the last measured time point (AUC_{0-t}) was calculated using the trapezoidal rule. The renal clearance (CL_R) were calculated from the cumulative amount excreted ($Cum.A_e$) in the urine divided by the plasma AUC. Dose proportionality assessment of AUC_{0-56h} data for the ginsenosides and the metabolites 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). The result of dose proportionality assessment was concluded as linear, inconclusive, or nonlinear, when the 90% CI lay entirely within, partially within, or completely out of the critical interval (0.797–1.203), respectively. 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).

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