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Ginsenoside Rc is a new selective UGT1A9 inhibitor in human liver microsomes and recombinant human UGT isoforms

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ABBREVIATIONS: DDI, drug-drug interaction; IC_{50}, half-maximal inhibitory concentration; LC-MS/MS, liquid chromatography-tandem mass spectrometry; PPD, protopanaxadiol; PPT, protopanaxatriol; UGT, uridine diphospho-glucuronosyltransferase

ABSTRACT

Ginseng is known to have inhibitory effects on UGT1A9 activity. However, little is known about the inhibitory effects of ginsenosides, the major active compounds in ginseng, on UGT1A9 activity. *In vitro* investigation of UGT1A9 inhibition by ginsenosides was carried out using human liver microsomes (HLMs). Among ten ginsenosides, ginsenoside Rc was the strongest inhibitor of UGT1A9-mediated mycophenolic acid glucuronidase activity. Further inhibition kinetic studies using HLMs suggested that ginsenoside Rc competitively and noncompetitively inhibited UGT1A9-mediated propofol and mycophenolic acid glucuronidation activities with K_i values of 2.83 and 3.31 μM, respectively. Next, to investigate whether the inhibitory effect of ginsenoside Rc is specific to the UGT1A9 isoform, we studied the inhibitory potency of ginsenoside Rc on nine human UGT activities using recombinant human UGT isoforms. Ginsenoside Rc exhibited a 12.9-fold selectivity (which was similar to niflumic acid at 12.5-fold) for UGT1A9 inhibition. Ginsenoside Rc at 50 μM also inhibited none of the other UGT isoform-specific activities above 12.0%, except for UGT1A9 (>91.5%) in HLMs, indicating that ginsenoside Rc might be used as a selective UGT1A9 inhibitor in reaction phenotyping studies of new chemical entities. Considering lower plasma concentrations (0.01 μM) of ginsenoside Rc in healthy subjects and no induction potential on UGT isoforms, ginsenoside Rc does not cause pharmacokinetic drug interactions with other co-administered drugs metabolized by UGT1A9.

DMD # 87965

Significance statement

Ginsenoside Rc selectively inhibited UGT1A9-mediated propofol and mycophenolic acid glucuronidation activities in human liver microsomes and recombinant UGT isoforms. It exhibited a 12.9-fold selectivity for UGT1A9 inhibition. Therefore, ginsenoside Rc might be used as a selective UGT1A9 inhibitor in reaction phenotyping studies of new chemical entities such as niflumic acid.

Introduction

Ginseng, one of the most popular medicinal herbs, has been widely used for thousands of years in Asia and has been increasingly consumed as a dietary supplement in Europe and the United States of America (Attele et al., 1999; Fang et al., 2013). A survey of herbal-based over-the-counter medicines showed that 28% of such preparations contain ginseng (Tyler, 2000). A variety of pharmacological effects have been ascribed to ginseng, including anti-aging, anti-cancer, anti-diabetic, anti-fatigue, anti-oxidant, cardioprotective, homeostatic, immunostimulatory, neuroprotective, and stress reduction effects (Chen et al., 2008; Qi et al., 2011). Many studies have demonstrated that most of the pharmacological effects of ginseng are attributable to ginsenosides (Qi et al., 2011). Co-administration of ginseng with prescribed drugs is common. Therefore, significant herb–drug interactions between ginseng and therapeutic drugs, including ginseng–warfarin (Vaes and Chyka, 2000; Cheng, 2005) and ginseng–fexofenadine interactions (Kim et al., 2018), have been reported. These herb-drug interactions can affect the pharmacokinetics of drugs in humans and alter their pharmacological effects by regulating metabolizing enzymes or transporters.

To date, there have been several reports about the effects of ginseng on cytochrome P450, a representative phase I metabolizing enzyme. For example, Kawase et al. reported that ginseng extracts significantly increased the expression levels of CYP1A1 and CYP3A11 in rat hepatocytes (Kim et al., 2018). In addition to animal studies, repeated administration of *Panax ginseng* capsules also resulted in the reduction of plasma midazolam concentration, indicating the possible induction of CYP3A in humans (Malati et al., 2012). Bilgi et al. reported that ginseng was associated with the occurrence of imatinib-induced liver toxicity after concurrent administration of ginseng in chronic myeloid leukemia patients, indicating the inhibition of CYP3A and p-glycoprotein, major imatinib-metabolizing enzyme (Bilgi et

al., 2010) and transporter (Hamada et al., 2003; Chen J and Kenneth R, 2006), respectively. Smith et al. also found ginseng to increase the plasma concentration of nifedipine, a CYP3A4 substrate drug, when co-administered (Bilgi et al., 2010). UGT inhibition is also regarded as one of the most important factors for clinical drug interactions (Sahai et al., 1994; Kiang et al., 2005; Zhang et al., 2005; Oda et al., 2015). The common modulatory effect on UGT activity is because glucuronidation accounts for more than 35% of phase II drug metabolism (Kiang et al., 2005; Mroz et al., 2018). Recently, two studies investigating UGT-based ginseng-drug interactions have been reported. Ginseng root extracts were demonstrated to have inhibitory effects on UGT1A9 and UGT1A4 (Mohamed and Frye, 2011) with IC_{50} values of 298.6 and 368.4 µg/ml, respectively. Additionally, purified dry extracts of ginseng exhibited inhibition against human UGT1A1, UGT1A9, and UGT2B7 activities with IC_{50} values ranging from 14.5 to 31.5 µg/ml (Zheng et al., 2014). These two studies indicated that ginseng had inhibitory effects on UGT1A9 activity. However, few data are available regarding the inhibitory effects of ginsenosides, major active compounds in ginseng, against UGT1A9 activity. Fang et al. reported that 100 µM of ginsenosides Rb2, Rc, and Rg3 inhibited more than 60% of recombinant UGT1A9-mediated 4-methylumbelliferone glucuronidase activity (Fang et al., 2013). Ginsenoside Rg3 inhibited human liver microsomal UGT1A9 activity with an IC_{50} value of 15.1 µM, whereas ginsenoside Rg2, Rh1, and Rh2 had negligible inhibitory effects on UGT1A9 ($IC_{50} > 100$ µM) (Kim et al., 2016).

In this study, we evaluated the inhibitory effects of ginsenoside F1, F2, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, and Rg3 on UGT1A9 using human liver microsomes (HLMs) to investigate specifically which ginsenosides in ginseng are involved in UGT1A9 inhibition. We also investigated the inhibitory potency of ginsenoside Rc (Figure 1) against the action of eight other UGT isoforms to determine the UGT1A9 selectivity of ginsenoside Rc, which most strongly inhibited UGT1A9 activity. The availability of a selective UGT1A9 inhibitor would be beneficial for assessing the extent of UGT1A9's contributions to phase II drug metabolism.

Furthermore, the inhibition mechanism and kinetic parameters (K_i) were determined for ginsenoside Rc, and compared with those of niflumic acid, a well-known selective UGT1A9 inhibitor (Miners et al., 2011).

Materials and Methods

Chemicals and Reagents. Androsterone, carvedilol, chenodeoxycholic acid, hecogenin, estrone-β-D-glucuronide sodium salt, *N*-acetylserotonin, naloxone, niflumic acid, mefenamic acid, 3-methylcholanthrene (3-MC), testosterone, trifluoperazine, and uridine 5'-diphosphoglucuronic acid ammonium salt (UDPGA) were obtained from Sigma-Aldrich (St. Louis, MO). 7-Ethyl-10-hydroxy-camptothecin (SN-38) was provided by Santa Cruz Biotechnology (Dallas, TX). Atazanavir, celastrol, mycophenolic acid, mycophenolic acid β-D-glucuronide, propofol, propofol β-D-glucuronide, and troglitazone were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Ginsenoside F1 (98.2%), ginsenoside F2 (98.4%), ginsenoside Rb1 (99.0%), ginsenoside Rb2 (100%), ginsenoside Rc (100%), ginsenoside Rd (98.9%), ginsenoside Re (100%), ginsenoside Rf (95.0%), ginsenoside Rg1 (99.7%), and ginsenoside Rg3 (98.5%) were obtained from Ambo Institute (Daejeon, Korea). All solvents were LC-MS grade (Fisher Scientific Co., Pittsburgh, PA). All the other reagents were of analytical or LC-MS grade and are commercially available. The pooled HLMs (XTreme 200) that is made up of equal numbers of male and female livers were obtained from XenoTech (Lenexa, KS). And recombinant human UGT isoforms (UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B4, UGT2B7, UGT2B15, and UGT2B17) were purchased from BD Biosciences (Woburn, MA) and were stored at –80°C until use. Cryopreserved human hepatocytes (HFC520, Lot No 321, female, age 58, Caucasian, smoker) were purchased from Corning Life Sciences (Woburn, MA) and were stored at liquid nitrogen until use.

Inhibitory effects of ten ginsenosides against UGT1A9 activity. Ten ginsenosides (0-50 μM) were screened for inhibition of human liver microsomal UGT1A9 activity. Each ginsenoside stock solution was prepared in methanol such that the final concentration of

solvent in the incubation mixtures was 1% (*v/v*), which has a negligible or minor effect on most UGT activities (Uchaipichat et al., 2004). The microsomal incubations were performed in a 100-μl reaction mixture containing 0.25 mg/ml microsomal protein, 25 μg/ml alamethicin, 10 mM $MgCl_2$, 0.1 M tris buffer (pH 7.4), and 0.2 μM mycophenolic acid, UGT1A9-specific individual substrates (Joo et al., 2014). A 5 mM UDPGA was added after pre-incubation (37°C, 5 min). Following incubation at 37°C for 1 hr in a thermo-shaker, the reactions were terminated by adding 50 μl of cold acetonitrile containing 0.25 μM estrone glucuronide (internal standard, IS) into the reaction mixture. After centrifugation (10,000 *g*, 4°C, 5 min), aliquots of the supernatant were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS, Shimadzu LCMS 8060 system, Shimadzu, Kyoto, Japan) as described previously (Joo et al., 2014).

Ginsenoside Rc inhibition of human liver microsomal UGT activity. All microsomal incubations were conducted in triplicate under linear incubation time for the formation of metabolites. Ginsenoside Rc (0, 0.5, 2, 5, 10, 20, 50, and 100 μM) was screened for inhibition of human liver microsomal UGT1A1, 1A3, 1A4, 1A6, 1A9, and 2B7 activities, and the UGT inhibitory potential of ginsenoside Rc was compared with that of niflumic acid (0, 0.2, 0.5, 1, 2, 5, and 10 μM), a known selective UGT1A9 inhibitor (Miners et al., 2011). A previously developed substrate cocktail method that enables the simultaneous incubation and measurement of compound inhibitory potential against each UGT isoform was used to obtain IC_{50} values (Joo et al., 2014). The selective UGT isoform substrates were used at concentrations approximately equal to their respective Michaelis-Menten constant (K_m) values: 0.5 μM for SN-38 (UGT1A1), 2 μM for chenodeoxycholic acid (UGT1A3), 0.5 μM for trifluoperazine (UGT1A4), 1 μM for N-acetylserotonin (UGT1A6), 0.2 μM for mycophenolic acid (UGT1A9), and 1 μM for naloxone (UGT2B7) (Joo et al., 2014). Known

inhibitors also were included as positive controls, to validate the experiments. Atazanavir (0-20 µM), celastrol (0-50 µM), hecogenin (0-50 µM), troglitazone (0-20 µM), niflumic acid (0-10 µM), and mefenamic acid (0-50 µM) were used as inhibitors of UGTs 1A1, 1A3, 1A4, 1A6, 1A9, and 2B7, respectively (Joo et al., 2014). The microsomal incubations were performed in a 100-µl reaction mixture containing 0.25 mg/ml microsomal protein (the protein concentration was in the linear range of metabolite formation), 25 µg/ml alamethicin, 10 mM $MgCl_2$, 0.1 M tris buffer (pH 7.4), and each UGT isoform-specific individual substrate. A 5 mM UDPGA was added after pre-incubation (37°C, 5 min). Following incubation at 37°C for 1 hr in a thermo-shaker, the reactions were terminated by adding 50 µl of cold acetonitrile containing 0.25 µM estrone glucuronide (internal standard, IS) into the reaction mixture. After centrifugation (10,000 g, 4°C, 5 min), aliquots of the supernatant were analyzed by a liquid chromatography-tandem mass spectrometry (LC-MS/MS, Shimadzu LCMS 8060 system, Shimadzu, Kyoto, Japan) as described previously (Joo et al., 2014). The selected reaction monitoring transitions and collision energies determined for each metabolite are listed in Table 1.

Ginsenoside Rc inhibition of recombinant UGT enzyme activity. Ginsenoside Rc was screened for inhibition of recombinant UGT1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B15, and 2B17 activities using the selective substrates as probes. The selective UGT isoform substrates were used at concentrations approximately equal to their respective Michaelis-Menten constant (K_m) values: 0.5 µM for SN-38 (UGT1A1), 2 µM for chenodeoxycholic acid (UGT1A3), 0.5 µM for trifluoperazine (UGT1A4), 1 µM for N-acetylserotonin (UGT1A6), 0.2 µM for mycophenolic acid (UGT1A9), 1 µM for carvedilol (UGT2B4) (Ohno et al., 2004), 1 µM for naloxone (UGT2B7), 50 µM for testosterone (UGT2B15) (Sten et al., 2009a) and 0.5 µM for androsterone (UGT2B17) (Sten et al., 2009b). Incubation mixtures (100 µl)

contained recombinant UGT (rUGT, final concentration: 0.25 mg/ml), 5 mM UDPGA, 10 mM MgCl$_2$, 100 mM Tris-HCl buffer (pH 7.4), and UGT isoform probe substrate in the absence or presence of ginsenoside Rc (0, 0,5, 2, 5, 10, 20, and 50 μM). After a 5-min pre-incubation period, UDPGA was added to the mixture to initiate the reaction. Following incubation at 37°C for 1 hr, the reactions were terminated by adding cold acetonitrile containing IS. After centrifugation, aliquots of the supernatant were analyzed by LC-MS/MS as described previously (You et al., 2007; Hauser et al., 2008; Sten et al., 2009b; Joo et al., 2014). The selected reaction monitoring transitions and collision energies determined for each metabolite are listed in Table 1.

Kinetic characterization of ginsenoside Rc inhibition of UGT1A9 in human liver microsomes. Mechanisms of inhibition and inhibition constants (K_i values) for ginsenoside Rc inhibition of UGT1A9 were determined with pooled HLMs and recombinant human UGT1A9 enzyme (rUGT1A9). Incubation conditions were as described in the previous section. Propofol and mycophenolic acid were used as the UGT1A9-selective probe substrates. To determine the inhibition constant of ginsenoside Rc in HLMs, different concentrations of ginsenoside Rc (0, 2, 5, 10, 20, 50 and 100 μM) were added to reaction mixtures containing different concentrations of propofol (10, 20, and 50 μM) or mycophenolic acid (0.1, 0.2, and 0.5 μM). After a 1 hr incubation period, the reactions were terminated by the addition of cold acetonitrile containing IS. After centrifugation, aliquots of the supernatant were analyzed by LC-MS/MS as described previously (Joo et al., 2014; Seo et al., 2014). The K_i values of ginsenoside Rc were compared with those of niflumic acid (0, 0.2, 0.5, 1, 2, 5, and 10 μM), a known UGT1A9 inhibitor.

Effect of ginsenoside Rc on the mRNA levels of UGT isoforms. Effects of ginsenoside Rc

on mRNA levels of UGT1A1, UGT1A4, UGT1A9, and UGT2B7 were assessed in cryopreserved human hepatocyte cultures as described previously (Seong et al., 2018). Briefly, cryopreserved human hepatocytes were thawed, and viable cells (4×10^5) were seeded in 24-well plates pre-coated with collagen type I and cultured with matrigel overlay. The cells were treated with vehicle (0.1% DMSO), 3-MC (5 μM; positive control), Ginsenoside Rc (0.5, 2.5, 25, and 50 μM) for 48 h. After 48 h, total RNA from hepatocytes was immediately isolated and the RNA concentration and purity were determined by an absorbance test at OD260/280 nm. RT-PCR was conducted using a TaqMan® RNA-to-C_T™ 1-Step Kit, TaqMan® Gene Expression Assays Kit, and respective primers for UGT1A1, 1A4, 1A9, and 2B7 (Applied Biosystems; Foster city, CA). The relative gene expression rate was calculated using the ΔCt value compared with the vehicle control. Glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous internal standard.

Determination of Rc in human hepatocytes using LC-MS/MS. Cryopreserved human hepatocytes were treated with ginsenoside Rc (0.5, 5, and 50 μM) for 48 h with identical procedure described above. After 48 h, culture medium and hepatocytes were collected for the quantitation of ginsenoside Rc. 300 μL of methanol containing 0.05 ng/mL berberine (IS) was added to 50 μL of samples and the mixtures were vortexed for 15 min. After centrifugation (10,000 g, 4°C, 5 min), aliquots of the supernatant were analyzed using an Agilent 6470 Triple Quad LC–MS/MS system (Agilent, Wilmington, DE) as described previously (Jin et al., 2019).

Data Analysis. Analytical data were processed using Shimadzu LabSolution LCMS software. The IC_{50} values were calculated using WinNonlin software (Pharsight, Mountain View, CA). The apparent kinetic parameters for inhibitory activity (K_i) were estimated by graphical

methods, such as Dixon and Lineweaver–Burk plots, and were more accurately determined by nonlinear least squares regression analysis, on the basis of the best enzyme inhibition model using the WinNonlin software (Miners et al., 2011; Phuc et al., 2017). The models tested included competitive, noncompetitive, uncompetitive, and mixed-type inhibition.

Results

Inhibition of UGT1A9 activity by ten ginsenosides. The inhibitory potential of ten ginsenosides against UGT1A9-mediated mycophenolic acid glucuronidation activity were evaluated using HLMs. Ginsenoside Rc, Rg3, Rd, Rb1, Rb2, and F2 inhibited UGT1A9 activity with IC_{50} values of 6.3, 10.2, 17.4, 21.3, 22.7, and 44.0 μM, respectively, whereas ginsenoside F1, Re, Rf, and Rg1 had no inhibitory effect on UGT1A9 ($IC_{50} > 50$ μM, Table 2).

Inhibition of UGT enzyme activities by ginsenoside Rc. The selectivity of ginsenoside Rc as an inhibitor of human UGTs were evaluated with HLMs. The IC_{50} values of known inhibitors of each UGT isoform were similar to previously reported values (Ito et al., 2001; Zhang et al., 2005; Uchaipichat et al., 2006; Choi et al., 2014; Joo et al., 2014; Zheng et al., 2014; Mendonza et al., 2016). Of the six UGT isoforms tested, ginsenoside Rc selectively inhibited UGT1A9-catalyzed mycophenolic acid glucuronidation with an IC_{50} value of 6.34 μM, whereas it had no inhibitory effect on UGT1A1, 1A3, 1A4, 1A6, and 2B7 ($IC_{50} > 200$ μM, Table 3 and Supplemental Figure 1). Ginsenoside Rc at 50 μM concentration inhibited none of the other UGT isoform-specific activities above 12.0%, except for UGT1A9 (>91.5%) (Figure 2A). Ginsenoside Rc showed slight activation for UGT1A6 and 2B7 isoform, however, it didn't show any inhibitory effects on these UGT isoforms at the

concentration range tested (0~50 μM). Niflumic acid, a known strong UGT1A9 inhibitor, inhibited UGT1A9-mediated mycophenolic acid glucuronidation and UGT1A1-mediated SN-38 glucuronidation with IC$_{50}$ values of 0.95 and 15.3 μM, respectively (Table 3 and Supplemental Figure 1). The inhibition of UGT isoform activities by ginsenoside Rc was also examined up to 200 μM using nine rUGT isoforms. Ginsenoside Rc at 50 μM inhibited none of the other rUGT isoform-specific activities above 33%, except for rUGT1A9 (>90%, Figure 2B). Ginsenoside Rc most strongly inhibited rUGT1A9-mediated mycophenolic acid glucuronidase activity, with an IC$_{50}$ value of 6.38 μM, whereas it had weak inhibitory effects on rUGT2B7-mediated naloxone-glucuronidase activity (82.2 μM) and rUGT1A1-mediated SN38-glucuronidase activity (98.9 μM) (Table 4). The IC$_{50}$ values for ginsenoside Rc inhibition of UGTs 1A3, 1A4, 1A6, 2B4, 2B15, and 2B17 exceeded 100 μM (Table 4). Niflumic acid at 50 μM inhibited rUGT1A1 and rUGT2B15 to some extent (>60%), and rUGT1A9 (>95%, Figure 2) to a greater extent.

Kinetic characterization of ginsenoside Rc inhibition against UGT1A9 in human liver microsomes. We further studied ginsenoside Rc to clarify its inhibition mechanism. Ginsenoside Rc inhibited UGT1A9-mediated mycophenolic acid glucuronidation activity with a K_i value of 3.31±0.27 μM (Table 5). The Dixon plots intersected the x-axis (Harold, 1976; Waldrop, 2009), indicating that ginsenoside Rc noncompetitively inhibited UGT1A9-mediated mycophenolic acid glucuronidase activity (Figure 3A and Supplemental Figure 2). To determine whether inhibition by ginsenoside Rc was substrate-specific, we also evaluated its inhibitory effects on UGT1A9-mediated propofol glucuronidation activity and found that ginsenoside Rc also markedly inhibited this activity, with a K_i value of 2.83±0.47 μM, in a competitive manner (Table 5 and Figure 3B). Niflumic acid inhibited UGT1A9-catalyzed mycophenolic acid glucuronidation and propofol glucuronidation activities, with K_i values of

0.72 and 1.07 µM, respectively, in a noncompetitive manner (Table 5 and Figure 3).

Effect of ginsenoside Rc on the mRNA Levels of UGT isoforms. 3-MC, a positive control

for UGT induction (Soars et al., 2004) increased the mRNA levels of UGT1A1, 1A4, 1A9,

and 2B7 in human hepatocytes by 4.77, 5.18, 7.84, and 5.86-fold, respectively, suggesting the

feasibility of this induction system. However, ginsenoside Rc treatment (0.5-50 µM) did not

significantly alter the mRNA expression of 4 isoforms of UGT (Figure 4). When measured the

ginsenoside Rc concentration in the culture medium and hepatocytes, Ginsenoside Rc

concentration in the culture medium was not significantly altered following 48 h incubation

and the hepatocytes to medium ratio of ginsenoside Rc was 0.96-1.51, suggesting ginsenoside

Rc would not accumulated in liver (Table 6).

Discussion

In the present study, the inhibitory effects of ten ginsenosides (F1, F2, Rb1, Rb2, Rc, Rd,

Re, Rf, Rg1, and Rg3) on human liver microsomal UGT1A9 activity were evaluated. Among

ten ginsenosides, ginsenoside Rc, a PPD-type ginsenoside, most strongly inhibited (IC_{50} = 6.3

µM) UGT1A9-mediated mycophenolic acid glucuronidase activity. Other PPD-type

ginsenosides weakly inhibited UGT1A9 activity, with IC_{50} values ranging from 10.2 to 44.0

µM, whereas PPT-type ginsenosides (F1, Re, Rf, and Rg1) had negligible inhibitory effects

on UGT1A9 (IC_{50} > 50 µM). These results were in concordance with earlier work, which had

reported that PPD-type ginsenosides (Rb2, Rc, and Rg3) at 100 µM inhibited more than 60%

of recombinant UGT1A9-mediated 4-methylumbelliferone glucuronidase activity, whereas

PPT-type ginsenosides (F1, Re, Rg1, and Rh1) had no inhibitory effect on UGT1A9 (Fang et

al., 2013). Kim et al. also reported that PPT-type ginsenosides, such as PPT, Rg2, and Rh1

had no inhibitory effects on human liver microsomal UGT1A9 activity ($IC_{50} > 100$ μM) (Kim et al., 2016).

Next, we evaluated the inhibitory effects of ginsenoside Rc on nine human UGT isoforms by studying the activities of the UGT isoforms' marker reactions to determine the UGT1A9 selectivity of ginsenoside Rc, which was the strongest inhibitor of UGT1A9 activity. Potent inhibition against human UGT1A9 by ginsenoside Rc was demonstrated in this study (Table 3 and Table 4). Ginsenoside Rc exhibited selective inhibition against UGT1A9 activity in HLMs and rUGT1A9 activity, with IC_{50} values of 6.34 and 6.38 μM, respectively. UGT1A9, a phase II metabolizing enzyme located in the endoplasmic reticulum, contributed to the glucuronidation reactions of endogenous compounds and xenobiotics, including arbidol (Song et al., 2013), dapagliflozin (Pattanawongsa et al., 2015), ethanol (Al Saabi et al., 2013), mefenamic acid (Gaganis et al., 2007), propofol (Court, 2005), and sorafenib (Peer et al., 2012). Therefore, new chemical entities with a similar backbone scaffold to ginsenoside Rc should be evaluated for potential UGT1A9 inhibitory effects during drug development. Some clinical drug interactions due to UGT1A9 inhibition have been reported. For example, co-administration with mefenamic acid has been shown to increase total dapagliflozin exposure (area under the concentration-time curve from time 0 to infinity [$AUC_{0\text{-inf}}$]) by 51% in healthy subjects (Kasichayanula et al., 2013).

Further inhibition kinetics studies using HLMs suggested that ginsenoside Rc competitively and noncompetitively inhibited UGT1A9-mediated propofol and mycophenolic acid glucuronidation activities, with K_i values of 2.83 and 3.31 μM, respectively (Figure 3), which are comparable to the K_i values of canagliflozin (3.0 μM) (Pattanawongsa et al., 2015) and hesperetin (3.41 μM) (Liu et al., 2016). The inhibitory potency of ginsenoside Rc was weaker than that of niflumic acid, with K_i values ranging 0.70 to 1.07 μM (Table 5 and Figure 3). It was estimated that an *in vivo* interaction potential via the inhibition of UGT would likely

occur if the ratio of inhibitor C_{max}/K_i were greater than 1 and possible if it was between 1.0 and 0.1 (Bjornsson et al., 2003; Bachmann and Lewis, 2005). Based on ginsenoside Rc's maximum concentrations (0.09 μM) in human blood after repeated administration of red ginseng extract (>60% dried ginseng, once daily for 2 weeks; content 23.0 mg ginsenoside Rb1, 11.4 mg ginsenoside Rb2, 13.0 mg ginsenoside Rc, 6.6 mg ginsenoside Rd, 6.2 mg ginsenoside Re, 2.8 mg ginsenoside Rg1, 8.0 mg ginsenoside Rh1, and 14.1 mg ginsenoside Rg3) (Choi et al., 2019), the values of C_{max}/K_i after a repeated administration of red ginseng extract in human were more than 0.032 from the data of HLMs ($K_i = 2.83$ μM), indicating that ginsenoside Rc has no drug interaction potential in human (Liu et al., 2016). The area under the plasma drug concentration-time curve of the co-administered drug may not change based on the *in vitro-in vivo* extrapolation prediction equation of Fang et al. (Fang et al., 2013) when red ginseng extract is co-administered with drugs that are mainly eliminated via UGT1A9 in human undergo 100% UGT1A9-mediated elimination in human. The ginsenoside Rc concentration in liver is important to predict in vivo drug interaction potential of ginsenoside Rc. We evaluated the accumulation potential of ginsenoside Rc in liver using human hepatocytes. The concentration of ginsenoside Rc in the hepatocytes were similar to that of medium (Table 6). In addition, we also evaluated the induction potential of ginsenoside Rc on four UGT isoforms using human hepatocytes. Ginsenoside Rc did not significantly increase the mRNA expression of four UGT isoforms (Figure 4). Taken together, these results suggest that ginsenoside Rc would not cause clinically relevant herb-drug interactions via the induction or inhibition of UGT enzymes following the usual dose of red ginseng extract (equivalent to 13.0 mg of ginsenoside Rc) in human.

Ginsenoside Rc is an active component of ginseng. The main root of *Panax ginseng* contains high amounts of ginsenoside Rb1, Rc and Rg1, whereas the leaf contains high amounts of ginsenoside Rb3 and Rh1 (Attele et al., 2002; Noh et al., 2009; Kang and Kim,

DMD # 87965

2016). In commercially available red ginseng products, ginsenoside Rc has been found to be

the second most abundant ginsenoside (Lee et al., 2015). Ginsenoside Rc has been shown to

have analgesic, antiallergic, antitumor, and sedative effects (Chu et al., 2013). Ginsenoside Rc

has also been proposed as a strong antidiabetic agent because it remarkably enhances glucose

uptake by inducing reactive oxygen species generation (Lee et al., 2010). In addition, ginseng

extract is frequently administered as a health supplement along with cancer chemotherapy

based on its ability to restore vitality and stimulate the immune system (Lee and Kim, 2014).

High dose of red ginseng extract and fermented red ginseng extract (3 g/day for more than 2

months) in combination with platinum-based chemotherapy also improved cancer related

fatigue, emotional symptoms, and quality of life scores and reduced anticancer drug-related

toxicity (Jiang et al., 2017; Kim et al., 2017). Therefore, clinical herb-drug interaction

between ginsenoside Rc and UGT1A9 following the administration of high-dose ginseng

adjuvant therapy or ginsenoside Rc single formulation need to be further evaluation. Enzyme-

selective inhibitors represent the most valuable experimental tools for the identification of

drug-metabolizing enzymes responsible for drug metabolism *in vitro*. To evaluate the

selectivity of ginsenoside Rc, we examined the inhibitory activities of ginsenoside Rc and

niflumic acid against nine UGT isoforms, including UGTs 1A1, 1A3, 1A4, 1A6, 1A9, 2B,

2B7, 2B15, and 2B17 in 0.25 mg/ml recombinant UGTs. Ginsenoside Rc inhibited UGT1A1,

UGT1A3, UGT1A9, UGT2B4, UGT2B7, and UGT2B15; however, the greatest inhibition

was observed with UGT1A9, with an IC_{50} value of 6.38 μM (Table 4). Ginsenoside Rc

showed a 12.9-fold selectivity for UGT1A9 inhibition similar to niflumic acid, a well-known

selective UGT1A9 inhibitor (Miners et al., 2011), showed a 12.5-fold selectivity (Table 4).

The selectivity of ginsenoside Rc for UGT1A9 inhibition was higher than that of previously

reported UGT1A9 inhibitors, including canagliflozin (3-fold), dapagliflozin (5-fold)

(Pattanawongsa et al., 2015), and magnolol (10-fold) (Zhu et al., 2012). At 50 μM ginsenoside

18

Rc concentration, approximately 15-fold greater than the K_i value, ginsenoside Rc was found to inhibit UGT1A9 by 90.7%, and only slightly affecting the enzyme activities of the other UGT isoforms tested (Figure 2B). Ginsenoside Rc weakly inhibited UGT1A1 and UGT2B15 activities (< 32.5%) at concentrations of 50 μM in recombinant UGT isoforms (Figure 2B). Additionally, at 50 μM of ginsenoside Rc inhibited none of the other UGT isoform-specific activities above 12.0%, except for UGT1A9 (>91.5%) in HLMs (Figure 2A), indicating that ginsenoside Rc could be used as a selective UGT1A9 inhibitor in reaction phenotyping studies. Notably, at 50 μM of niflumic acid, a well-known selective UGT1A9 inhibitor, inhibited UGT1A1 and UGT2B15 activities by 73.9% and 59.7%, respectively, in recombinant UGT isoforms.

In conclusion, we report that ginsenoside Rc is a selective UGT1A9 inhibitor. Ginsenoside Rc, when evaluated for mycophenolic acid glucuronidation inhibitory activity against UGT1A9 as well as eight other UGTs, exhibited a 13-fold selectivity for UGT1A9. Along with hecogenin, niflumic acid, and fluconazole, which are selective inhibitors of UGT1A4, UGT1A9, and UGT2B7, respectively, ginsenoside Rc could be useful as a selective UGT1A9 inhibitor in UGT reaction phenotyping studies such as niflumic acid when HLMs are used as the enzyme source. Additionally, ginsenoside Rc would not cause clinically relevant pharmacokinetic herb-drug interactions with other co-administered drugs metabolized by UGT1A9 when administered usual dose of red ginseng extract in human.

Authorship Contributions

Participated in research design: Liu

Conducted experiments: H.Lee, Heo, G.H.Lee, Park, Jang, Kim, Kwon, Song

Contributed new reagents or analytic tools: Song, Liu

Performed data analysis: H.Lee, Song, Liu

Wrote or contributed to the writing of the manuscript: H.Lee, Song, Liu

DMD # 87965

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DMD # 87965

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DMD # 87965

Footnotes

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26

Legends for Figures

Figure 1. Chemical structure of ginsenoside Rc.

Figure 2. Inhibitory effects of niflumic acid (50 μM, ■) and ginsenoside Rc (50 μM, □) on the enzymatic activities of six UGT isoforms in pooled human liver microsomes (0.25 mg/ml, Xenotech H0630) (A) and nine UGT isoforms in recombinant UGTs (0.25 mg/ml, BD Biotechnology) (B). SN-38 (0.5 μM), chenodeoxycholic acid (2.0 μM), trifluoperazine (0.5 μM), N-acetylserotonin (1.0 μM), mycophenolic acid (0.2 μM), carvedilol (1.0 μM), naloxone (1.0 μM), testosterone (50 μM), and androsterone (0.5 μM) were used as the probe substrates of UGTs 1A1, 1A3, 1A4, 1A6, 1A9, 2B4, 2B7, 2B15, and 2B17, respectively. The data are shown as means of triplicate measurements (n=3).

Figure 3. Representative Dixon plots obtained from a kinetic study of UGT1A9-mediated mycophenolic acid (A) and propofol (B) glucuronidation in the presence of different concentrations of niflumic acid and ginsenoside Rc in pooled human liver microsomes (HLMs). Increasing concentrations of mycophenolic acid [0.1 (●), 0.2 (○), and 0.5 (▼) μM] and propofol [10 (●), 20 (○), and 50 (▼) μM] were incubated with HLMs (0.25 mg/ml, Xenotech H0630) and UDPGA at 37°C for 1 hr in the presence or absence of niflumic acid (0, 0.2, 0.5, 1, 2, 5, and 10 μM) and ginsenoside Rc (0, 2, 5, 10, 20, 50, and 100 μM). The niflumic acid inhibition data fit a noncompetitive inhibition model, and the ginsenoside Rc inhibition data fit competitive and noncompetitive inhibition models. The data are means of triplicate measurements (n=3).

Figure 4. Effect of ginsenoside Rc (0.5, 2.5, 5.0, 25, and 50 μM) and 3-methylcholanthrene

(5.0 μM) on the mRNA levels of UGT1A1, 1A4, 1A9, and 2B7 after 48 h treatment in the cryopreserved human hepatocytes (HFC520). The fold changes for UGT mRNA levels of ginsenoside Rc treatment were calculated by comparing with vehicle treatment (DMSO 0.1%) and GAPDH was used as an endogenous internal standard. The data are shown as means of triplicate measurements (*n*=3).

Table 1. Substrates, their metabolites, and their LC-MS/MS conditions for human UGTs assays.

UGT enzyme	Substrate	Conc. (μM)	Metabolites	Transition (m/z)	Mode	CE (eV)
1A1	SN-38	0.5	SN-38 glucuronide	569>393	+	30
1A3	Chenodeoxycholic acid	2.0	Chenodeoxycholic acid 24-acyl glucuronide	567>391	-	20
1A4	Trifluoperazine	0.5	Trifluoperazine *N*-glucuronide	584>408	+	30
1A6	*N*-Acetylserotonin	1	*N*-Acetylserotonin glucuronide	395>219	+	10
1A9	Mycophenolic acid	0.2	Mycophenolic acid glucuronide	495>319	-	25
2B4	Carvedilol	1.0	Carvedilol glucuronide	583>407	+	25
2B7	Naloxone	1.0	Naloxone 3-glucuronide	504>310	+	30
2B15	Testosterone	50	Testosterone 17-glucuronide	465>97	+	27
2B17	Androsterone	0.5	Androsterone 3-glucuronide	465>289	-	35

Conc., concentration; CE, collision energy

29

Table 2. Inhibitory potential of ten ginsenosides against UGT1A9-mediated mycophenolic acid glucuronidase activity in human liver microsomes.

Inhibitor	Protopanaxadiol type						Protopanaxatriol type			
	F2	Rb1	Rb2	Rc	Rd	Rg3	F1	Re	Rf	Rg1
IC_{50} (μM) [a]	44.0	21.3	22.7	6.3	17.4	10.2	>50	>50	>50	>50

[a]Values represent the average of triplicate.

Table 3. IC$_{50}$ values of well-known UGT inhibitors, ginsenoside Rc, and niflumic acid against the activities of six UGT isoforms in pooled human liver microsomes.

UGTs	Substrate	IC$_{50}$ (μM)[a]		
		Well-known inhibitors	Ginsenoside Rc	Niflumic acid
UGT1A1	SN-38	Atazanavir	1.22 ± 0.51	>200	15.3 ± 2.7
UGT1A3	Chenodeoxycholic acid	Celastrol	5.88 ± 2.31	>200	>50
UGT1A4	Trifluoperazine	Hecogenin	0.97 ± 0.45	>200	>50
UGT1A6	N-Acetylserotonin	Troglitazone	11.8 ± 4.7	>200	>50
UGT1A9	Mycophenolic acid	Niflumic acid	0.95 ± 0.10	6.34 ± 1.02	0.95 ± 0.10
UGT2B7	Naloxone	Mefenamic acid	20.3 ± 2.0	>200	>50

[a]Values represent the average ± standard error of triplicate.

DMD # 87965

Table 4. Inhibitory potential of niflumic acid and ginsenoside Rc against the activities of nine UGT isoforms in human recombinant UGT isoforms. (Substrates: SN-38 (UGT1A1), chenodeoxycholic acid (UGT1A3), trifluoperazine (UGT1A4), *N*-acetylserotonin (UGT1A6), mycophenolic acid (UGT1A9), carvedilol (UGT2B4), naloxone (UGT2B7), testosterone (UGT2B15), and androsterone (UGT2B17)).

Inhibitor	IC_{50} (μM)[a]								
	rUGT1A1	rUGT1A3	rUGT1A4	rUGT1A6	rUGT1A9	rUGT2B4	rUGT2B7	rUGT2B15	rUGT2B17
Niflumic acid	17.5 ± 1.1	>50	>50	>50	1.40 ± 0.34	>50	>50	29.2 ± 4.8	>50
Ginsenoside Rc	98.9 ± 11.9	111.5 ± 12.3	>200	>200	6.38 ± 0.77	156.5 ± 3.5	82.2 ± 38.2	104.0 ± 15.1	>200

[a]Values represent the average ± standard error of triplicate

Table 5. Apparent enzyme inhibition constants on the UGT1A9 activity by ginsenoside Rc and niflumic acid in human liver microsomes. The mean reaction rates of triplicate measurements were fitted into inhibition kinetic models.

UGT1A9 Substrate	Inhibitor	Mechanism of inhibition	$K_i (\mu M)^a$
Mycophenolic acid	Ginsenoside Rc	Noncompetitive	3.31 ± 0.27
	Niflumic acid	Noncompetitive	0.72 ± 0.06
Propofol	Ginsenoside Rc	Competitive	2.83 ± 0.47
	Niflumic acid	Noncompetitive	1.07 ± 0.10

[a]Values are present as the average ± standard error of triplicate.

Table 6. Ratios of ginsenoside Rc in hepatocytes to culture medium in the cryopreserved human hepatocytes (HFC520) after 48 h treatment of ginsenoside Rc.

Hepatocytes to medium ratio	Ginsenoside Rc		
	0.5 μM	5.0 μM	25 μM
#1	1.54	0.83	1.14
#2	1.61	0.95	1.82
#3	1.38	1.11	1.25
Average	1.51	0.96	1.41
SD	0.12	0.14	0.37

34
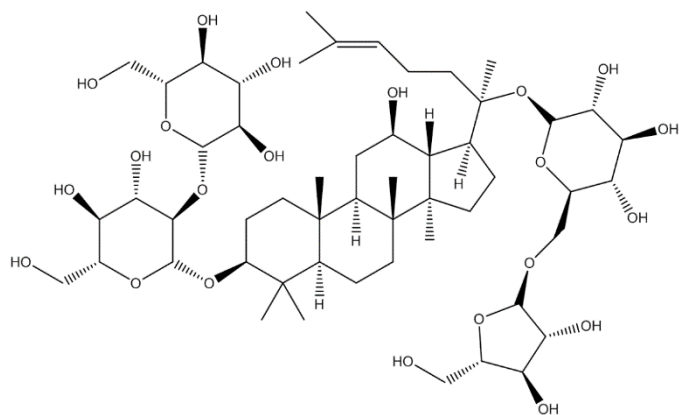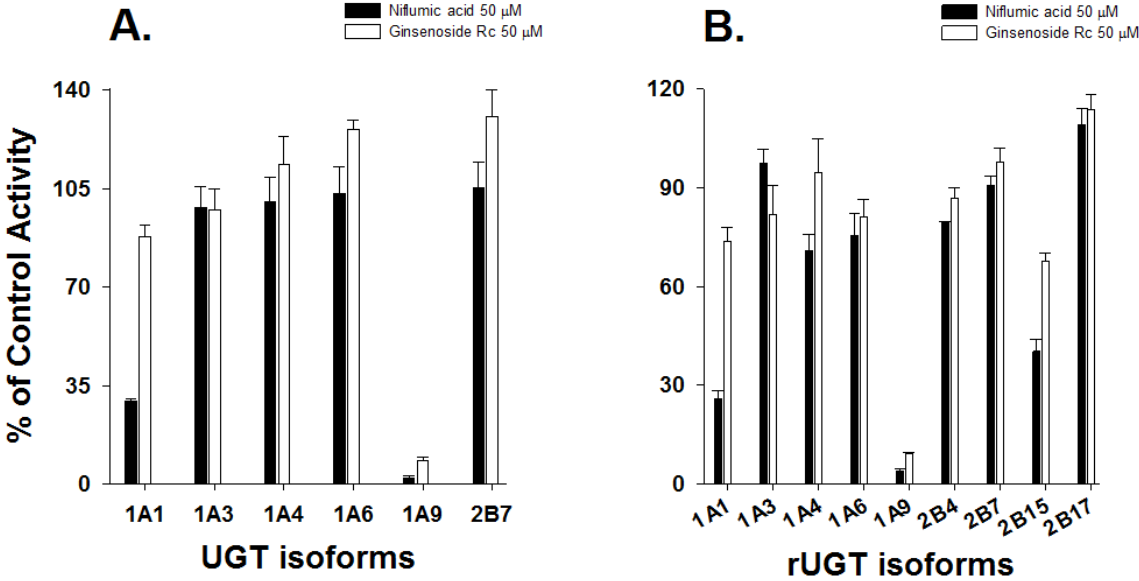
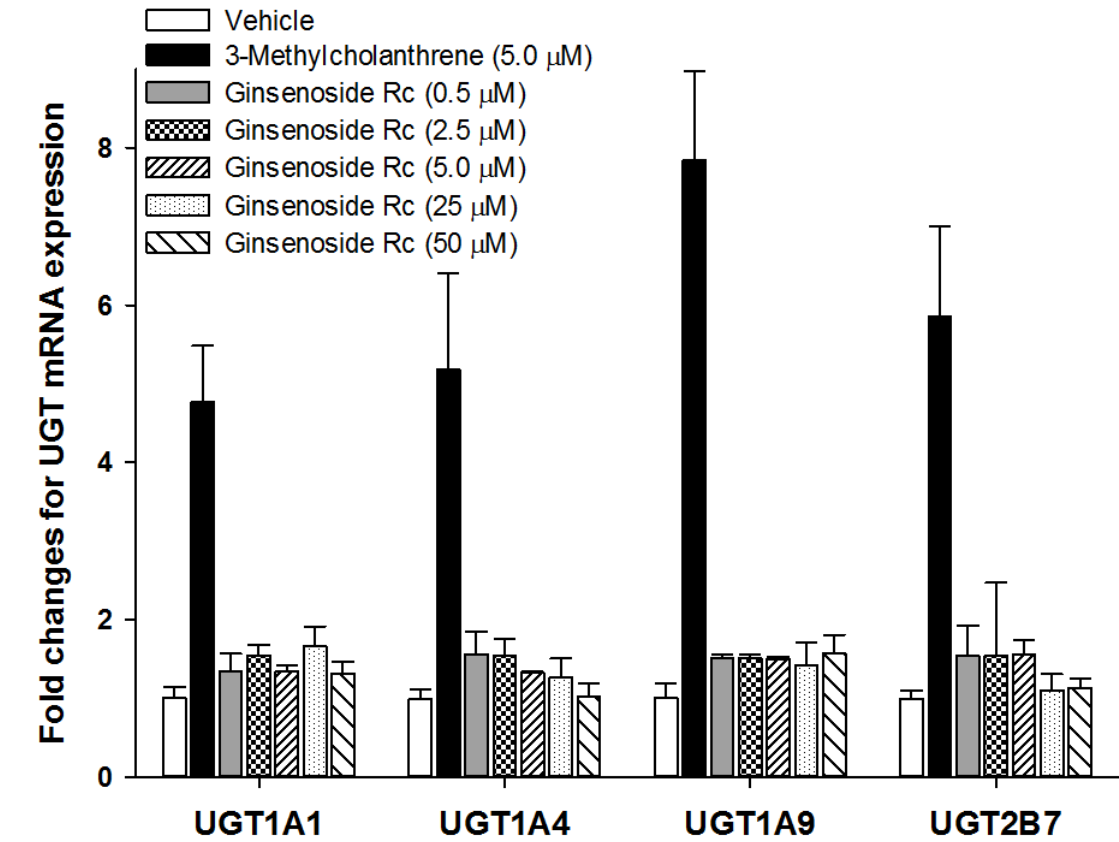**Figure 1.**

Figure 2.
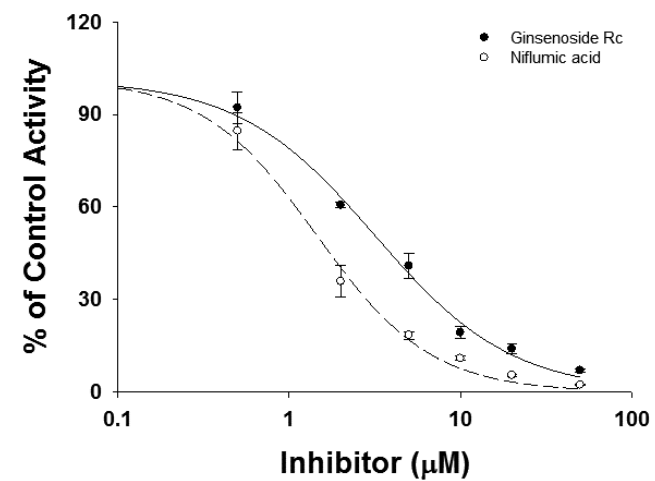
Figure 3.
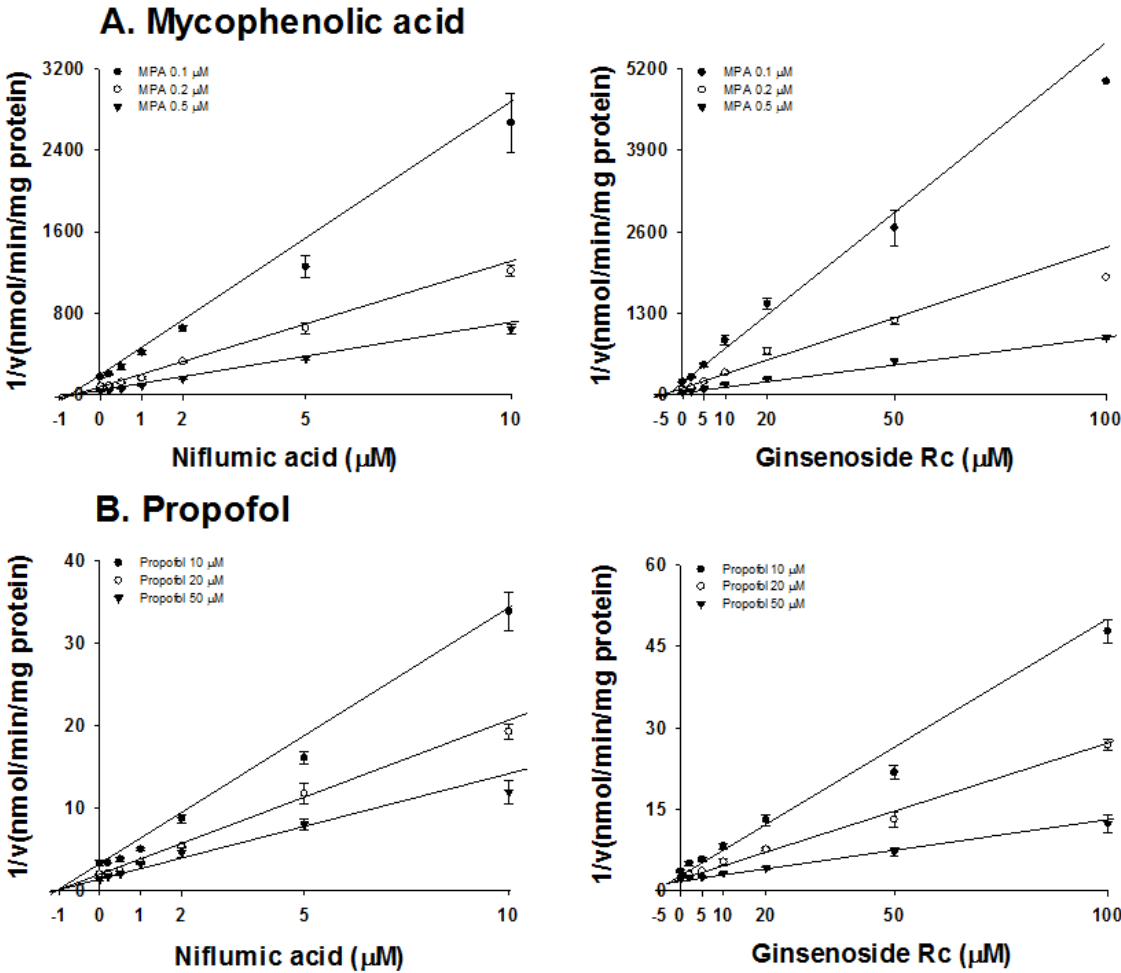
Figure 4.

Supplemental Material

Drug Metabolism and Disposition

Ginsenoside Rc is a new selective UGT1A9 inhibitor in human liver microsomes and recombinant human UGT isoforms

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Supplemental Figure 1. Inhibitory effect of ginsenoside Rc (●) and niflumic acid (○) on UGT1A9-mediated mycophenolic acid glucuronidation upon incubation with human liver microsomes.

Supplemental Figure 2. Effects of ginsenoside Rc (A) and niflumic acid (B) on rates of UGT1A9-mediated mycophenolic acid glucuronidation in pooled human liver microsomes (0.25 mg/ml, Xenotech H0630). Each symbol represents the ginsenoside Rc and niflumic acid concentration: (A) 0 (●), 0.2 (○), 0.5 (▼), 1 (△), 2 (■), 5 (□) and 10 (◆) µM; (B) 0 (●), 2 (○), 5 (▼), 10 (△), 20 (■), 50 (□) and 100 (◆) µM. The data are means of triplicate measurements (*n*=3).