

Research Article

Evaluation of the Inhibition of Cytochrome P450 Activity by Ginsenoside Rg₅ and Rk₁

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Abstract

Background: Heat-treated red ginseng contains pharmacologically active ginsenosides such as Rg₅ and Rk₁ and is increasingly used as a functional food. Because herbal products are frequently co-administered with conventional medications, evaluation of potential cytochrome P450 (CYP)-mediated drug–drug interactions is essential. This study examines their effects on nine cytochrome P450 isoforms to evaluate potential drug–drug interactions. **Methods:** The CYP enzyme inhibition method was assessed by determining *IC*₅₀ values using human liver microsomes and specific substrates. Various concentrations of ginsenosides Rg₅ and Rk₁ were tested, and the reactions were analyzed via UPLC-MS/MS. Positive control inhibitors were included for comparison. **Results:** Ginsenoside Rg₅ and Rk₁ exhibited potent inhibitory effects on CYP2A6, with *IC*₅₀ values of 1.04 and 0.98 μM, respectively, with moderate inhibitory effects observed for several other CYP activities and little or no inhibition observed for the remaining CYP activities. **Conclusions:** The results indicate potential adverse drug reactions with CYP2A6-metabolized drugs, highlighting the need for further *in vivo* safety evaluations. These findings provide important information regarding CYP-mediated herb–drug interaction potential associated with heat-processed ginsenosides.

Keywords: Panax; ginsenosides; cytochrome P450 enzyme system; liquid chromatography-mass spectrometry; drug interactions

1. Introduction

Ginseng has been widely used as a functional food and herbal medicine because of its diverse biological activities and favorable safety profile. When ginseng is steamed once in water vapor and subsequently dried, it is classified as red ginseng, but when it is steamed and dried nine times using the traditional method, it is classified as black ginseng [1]. Heat processing alters the chemical composition of ginseng and generates less-polar ginsenosides such as Rg₅ and Rk₁ through dehydration reactions. These structural transformations may affect biological activity and pharmacokinetic behavior, including interactions with drug-metabolizing enzymes.

The main components of heat-treated red ginseng are ginsenosides Rg₃, Rg₅, and Rk₁, which are specific saponin components [2]. Ginsenoside Rg₃ forms a double bond via dehydration at position 20 of the dammarane skeleton and is converted to the structural isomers ginsenosides Rg₅ and

Rk₁, depending on the position of the double bond (Fig. 1) [3]. Heat-treated red ginseng enriched in Rg₅ and Rk₁ has attracted considerable interest because of its diverse pharmacological properties and potential functional applications.

Taking drugs in conjunction with health functional foods can cause changes in drug action in the human body, resulting in side effects, such as toxicity and reduced drug efficacy [4]. This phenomenon is known as a drug–drug interaction (DDI), and several people who consume health functional foods and drugs are at risk of developing DDIs [5].

The liver is the backbone of drug metabolism and contains cytochrome P450 (CYP), the most important enzyme system for drug metabolism. CYP is controlled by various endogenous and exogenous substances, and CYP enzyme inhibition and induction during the combined administration of drugs are representative mechanisms that induce



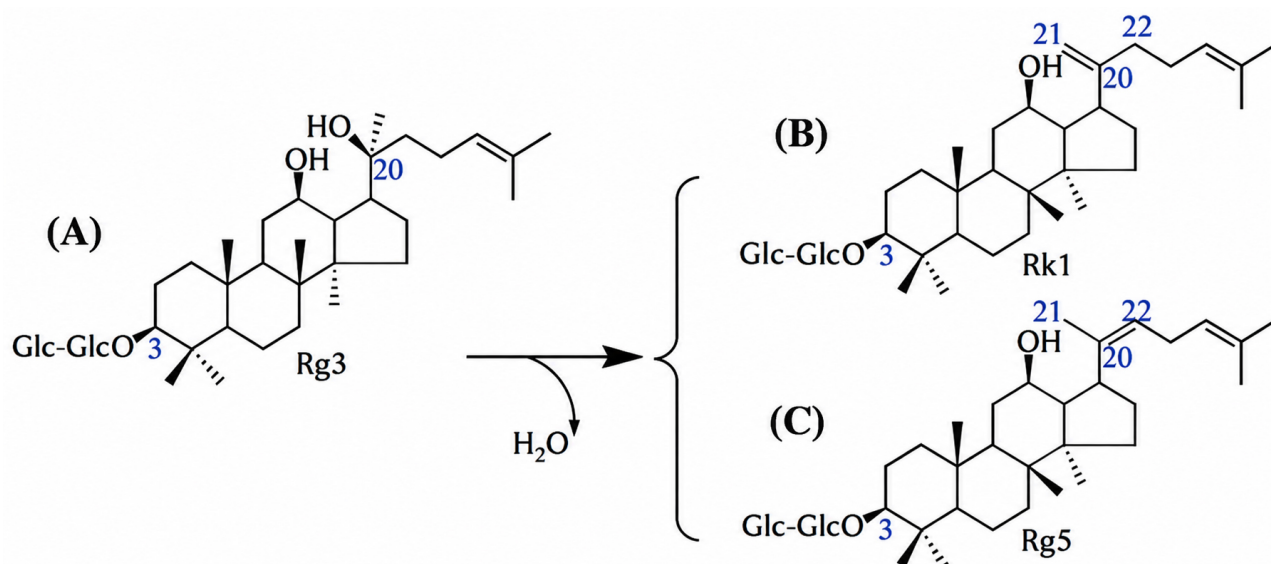


Fig. 1. Chemical structures of ginsenosides Rg₃, Rk₁, and Rg₅. (A) ginsenoside Rg₃. (B) ginsenoside Rk₁. (C) ginsenoside Rg₅.

DDIs in the clinical setting [5]. Therefore, it is important to be aware of changes in the activity of CYP enzymes to prevent drug interactions.

In this study, ginsenosides Rg₅ and Rk₁, representative components of heat-treated red ginseng, which are health functional foods, were selected as test compounds. Based on the established US FDA guidelines [6], the inhibitory effects of nine CYP isoforms were analyzed.

2. Materials and Methods

2.1 Chemicals and Reagents

Substrates (phenacetin, bupropion, amodiaquine, diclofenac, coumarin, and chlorzoxazone), metabolites (acetaminophen, 7-hydroxycoumarin, *N*-desethylamodiaquine, dextrophan, 6-hydroxybupropion, and 6-hydroxychlorzoxazone), positive control inhibitors (α -naphthoflavone, methoxsalen, montelukast, sulfaphenazole, *S*-benzylirvanol, quinidine, ketoconazole, ticlopidine, and silver diethyldithiocarbamate (DETC)), chemicals for the NADPH system (nicotinamide adenine dinucleotide phosphate, glucose 6-phosphate, MgCl₂, and glucose-6-phosphate dehydrogenase), and ginsenoside Rg₅ and Rk₁ standards were purchased from Sigma-Aldrich (St. Louis, MO, USA). Substrates (*S*-mephenytoin, dextromethorphan, and midazolam) and metabolites (4'-hydroxymephenytoin, 4'-hydroxydiclofenac, and 1'-hydroxymidazolam) were purchased from Toronto Research Chemicals (Sir Frederick Banting, North York, Canada). Pooled human liver microsomes (BD Ultra Pool™ HLM 200 donors) were purchased from BD Gentest Co. (Woburn, MA, USA).

2.2 In Vitro CYP Inhibition

The CYP enzyme inhibition evaluation method was established by determining *IC*₅₀ values and generating dose–response curves using known CYP enzyme inhibitors [7]. All samples were tested in triplicate. The reaction solution had a final volume of 100 μ L, comprising 0.2 mg/mL human liver microsomes (HLM), 100 mM phosphate buffer (pH 7.4), a reduced nicotinamide adenine dinucleotide phosphate (NADPH) system, and various CYP enzyme isoform-specific substrates (A set: phenacetin, amodiaquine, diclofenac, *S*-mephenytoin, dextromethorphan, midazolam, and coumarin, B set: bupropion and chlorzoxazone). A cocktail assay approach was employed to simultaneously evaluate the inhibitory effects on multiple CYP isoforms and assess potential drug–drug interactions. The substrate was concentrated according to each Michaelis–Menten constant (*K*_m) value as follows: phenacetin (50 μ M), bupropion (50 μ M), amodiaquine (20 μ M), diclofenac (10 μ M), *S*-mephenytoin (80 μ M), dextromethorphan (5 μ M), midazolam (2 μ M), coumarin (50 μ M), and chlorzoxazone (50 μ M). The concentrations of ginsenoside Rg₅ and Rk₁ were set to 0, 0.5, 1, 5, 10, 50, and 100 μ M.

Eight-well tube strips were used in the reaction in an 8 $\times$ 12 rack (1.2 mL; VWR, Emeryville, CA), and all substrates used in the experiment were dissolved in methanol. Inhibitors of ginsenosides Rg₅ and Rk₁ were dissolved in dimethyl sulfoxide (DMSO). The composition of the organic solvent (DMSO) used in the reaction was set to 0.5% (v/v) for both sets. The pH was adjusted with 500 mM phosphate buffer, the NADPH system for HLM activity was added for 5 min, and the HLM was incubated at 37 $^{\circ}$ C for 20 min at a final concentration of 0.2 mg/mL. The reaction was terminated by adding 300 μ L chlorpropamide

(concentration 250 nM), an internal standard dissolved in acetonitrile (ACN), and making a 1:3 protein ratio. After centrifugation at $15,000 \times g$ at 4 °C for 20 min, 200 µL of the supernatant was obtained and analyzed using UPLC-MS/MS. The positive control CYP inhibition group experiment was conducted in the same manner as described above using α -naphthoflavone, methoxsalen, montelukast, sulfaphenazole, *S*-benzylrivanol, quinidine, ketoconazole, ticlopidine, and silver DETC, a selective inhibitor.

2.3 UPLC Conditions

Under pre-established analysis conditions, the reacted drugs were analyzed using an analysis system consisting of a Nexera LC-40 series (Shimadzu, Kyoto, Japan) LC and a SCIEX Triple Quad™ 4500 (Applied Biosystems SCIEX, Foster City, CA, USA) MS/MS. A sample of 5 µL was injected and separated using Kinetex C18 (50×2.1 mm i.d., 2.6 µm; Phenomenex Cheshire, UK) and Security Guard™ C18 (2.0×4.0 mm i.d., Phenomenex, Torrance, CA, USA) guard columns at a flow rate of 0.3 mL/min. Deionized water (mobile phase A) and acetonitrile (mobile phase B) containing 0.1% formic acid were used as mobile phases. The two mobile phases were maintained at 5% B for 0.5 min using a linear gradient, increased to 80% for 1.5 min, and maintained for 1.5 min.

2.4 Tandem Mass Conditions

The ion spray voltage was set to −4500 V in the negative ion mode and 5500 V in the positive ion mode. Other parameters used for mass spectrometry were as follows: curtain gas at 30 psi, collision gas at 9 psi, nebulizer temperature at 500 °C, nebulizing gas at 35 psi, and heating gas at 35 psi. The analysis was performed using multiple reaction monitoring (MRM) scan modes. All metabolites and the internal standard were analyzed in the positive ion mode, except for 6-hydroxychlorzoxazone, which was detected in the negative ion mode. The specific MRM transitions, collision energy (CE), and ion polarities for all analytes are summarized in Table 1. The dwell time was set to 0.02 s for each channel, and the peak areas of the formed metabolites and the internal standard were used for quantification. All data acquisition and analyses were processed using SCIEX OS software (version 2.2.0.5738; AB SCIEX, Framingham, MA, USA).

2.5 Data Analysis

The activity of CYP enzymes induced by the inhibitors was converted to a percentage of the control group. All results are expressed as the mean $\pm$ standard deviation. A linear analysis to obtain the IC_{50} of CYP enzyme inhibition was performed using Sigma Plot (Version 12.0; SYSTAT Software Inc., Palo Alto, CA, USA).

3. Results

3.1 Chromatographic Separation

Nine metabolites used for CYP inhibition analysis were successfully separated using UPLC-MS/MS. The MRM chromatograms demonstrated adequate separation of all analytes for CYP inhibition analysis (Fig. 2), with retention times ranging from 2.206 to 2.819 min.

3.2 Validation of the Assay System Using Positive Controls

In vitro CYP inhibition assays were performed using nine CYP isoforms according to FDA guidelines. Positive control inhibitors were used to validate the assay system. The IC_{50} values of the positive controls were consistent with previously reported data, confirming the reliability of the experimental method (Fig. 3). The calculated IC_{50} values were as follows: α -naphthoflavone (0.02 µM), methoxsalen (0.40 µM), montelukast (0.40 µM), sulfaphenazole (1.38 µM), *S*-benzylrivanol (0.28 µM), quinidine (0.08 µM), ketoconazole (0.03 µM), ticlopidine (0.76 µM), and DETC (18.54 µM).

3.3 Inhibition Effects of Ginsenoside Rg₅ and Rk₁ on CYP Isoforms

The inhibitory effects of ginsenosides Rg₅ and Rk₁ on CYP isoforms were evaluated at concentrations ranging from 0 to 100 µM. The results are summarized in Table 2. Both ginsenosides exhibited strong inhibition against CYP2A6, while showing moderate or no significant inhibition towards other CYP activities (Figs. 4,5).

4. Discussion

This study evaluated the inhibitory effects of ginsenosides Rg₅ and Rk₁ on nine CYP isoforms using human liver microsomes according to FDA guidelines for CYP inhibition screening. Unlike previously reported major ginsenosides, including Rb₁, Rc, Rd, Re, Rg₁, and Rg₃, which generally exhibited weak or negligible CYP inhibition in previous studies [8,9], Rg₅ and Rk₁ demonstrated potent CYP2A6 inhibition with IC_{50} values below 1.1 µM. This finding suggests that dehydration-induced structural modifications generated during heat processing may substantially alter CYP interaction profiles. In *in vitro* CYP inhibition screening, IC_{50} values below 10 µM are generally considered indicative of substantial inhibitory potential [10]. Therefore, the CYP2A6 inhibition observed for Rg₅ and Rk₁ (IC_{50} = 1.04 and 0.98 µM, respectively) may be pharmacologically relevant and warrants further investigation through mechanistic inhibition studies and *in vivo* interaction assessments. These findings suggest that heat-processed ginsenosides may have distinct CYP inhibitory properties compared with conventional ginsenosides found in fresh or red ginseng.

CYP2A6 is responsible for approximately 70–80% of nicotine metabolism in humans and is also involved in the metabolism of several therapeutic agents, including tega-

Table 1. MRM conditions.

CYPs	Substrate	Metabolite	Transition (m/z)	CE (eV)	Polarity
1A2	Phenacetin	Acetaminophen	152 > 110	23	+
2A6	Coumarin	7-hydroxycoumarin	163 > 107	35	+
2C8	Amodiaquine	<i>N</i> -desethylamodiaquine	328 > 283	20	+
2C9	Diclofenac	4'-hydroxydiclofenac	312 > 230	40	+
2C19	<i>S</i> -Mephenytoin	4'-hydroxymephenytoin	235 > 150	25	+
2D6	Dextromethorphan	Dextrorphan	258 > 157	60	+
3A	Midazolam	1'-hydroxymidazolam	342 > 203	35	+
2B6	Bupropion	6-Hydroxybupropion	256 > 238	20	+
2E1	Chlorzoxazone	6-hydroxychlorzoxazone	184 > 120	20	-
IS	Chlorpropamide	-	277 > 192	19	+

MRM, multiple reaction monitoring; CYP, cytochrome P450; CE, collision energy; IS, internal standard.

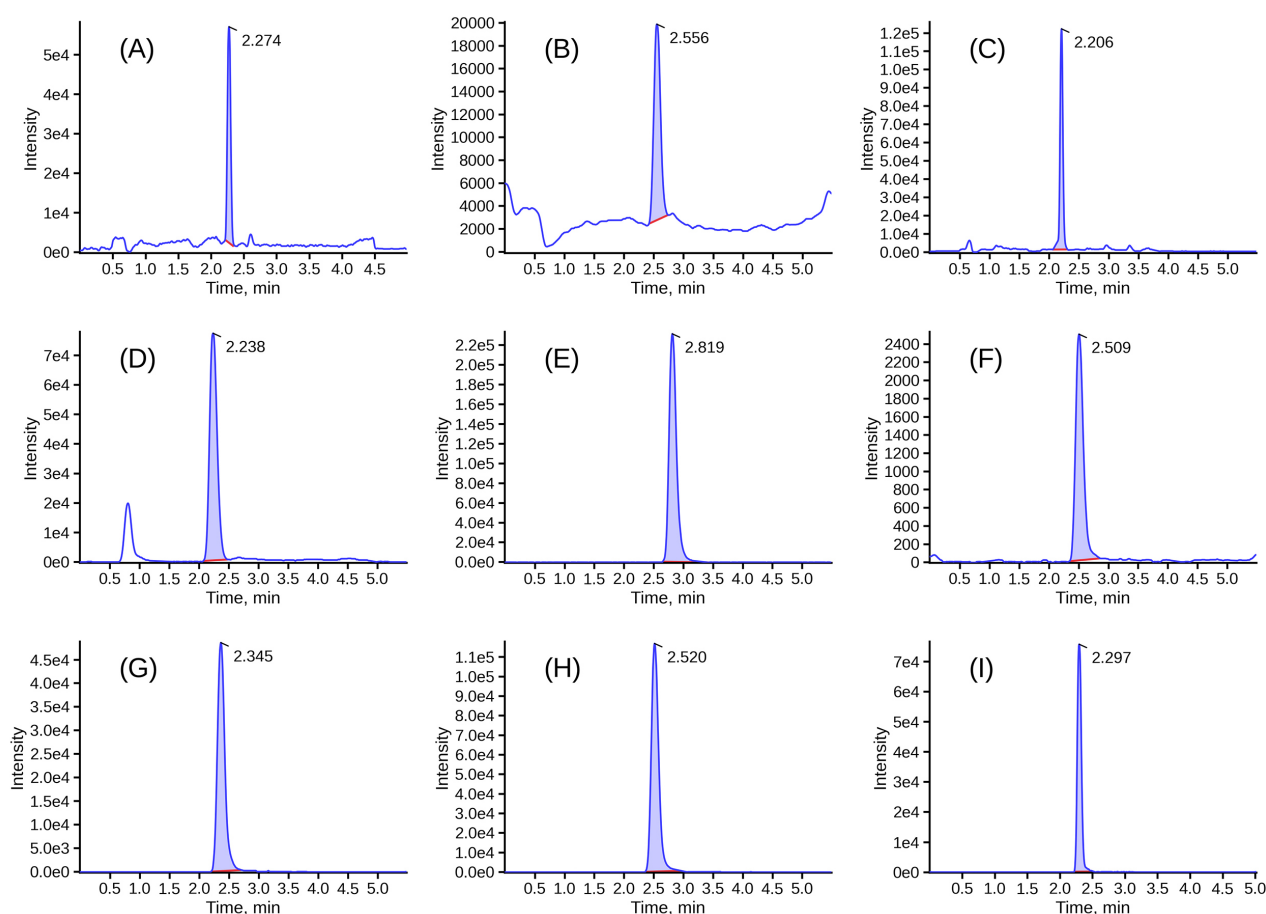


Fig. 2. MRM chromatograms of nine CYP probe metabolites analyzed using UPLC-MS/MS. (A) Acetaminophen (2.274 min). (B) 7-hydroxycoumarin (2.556 min). (C) *N*-desethylamodiaquine (2.206 min). (D) 4'-hydroxydiclofenac (2.238 min). (E) 4'-hydroxymephenytoin (2.819 min). (F) dextrorphan (2.509 min). (G) 1'-hydroxymidazolam (2.345 min). (H) 6-hydroxybupropion (2.520 min). (I) 6-hydroxychlorzoxazone (2.297 min).

fur, letrozole, valproic acid, and pilocarpine. Therefore, inhibition of CYP2A6 by Rg₅ and Rk₁ may potentially alter nicotine clearance and increase nicotine exposure in individuals consuming heat-processed ginseng products concurrently with nicotine-containing products or smoking cessation therapies.

Although both ginsenosides exhibited similar inhibitory patterns, slight differences in CYP activity may be related to structural differences between the two compounds. Rg₅ contains a double bond at the 20(22) position, whereas Rk₁ contains a double bond at the 20(21) position. Although Rg₅ and Rk₁ differ only in the position of

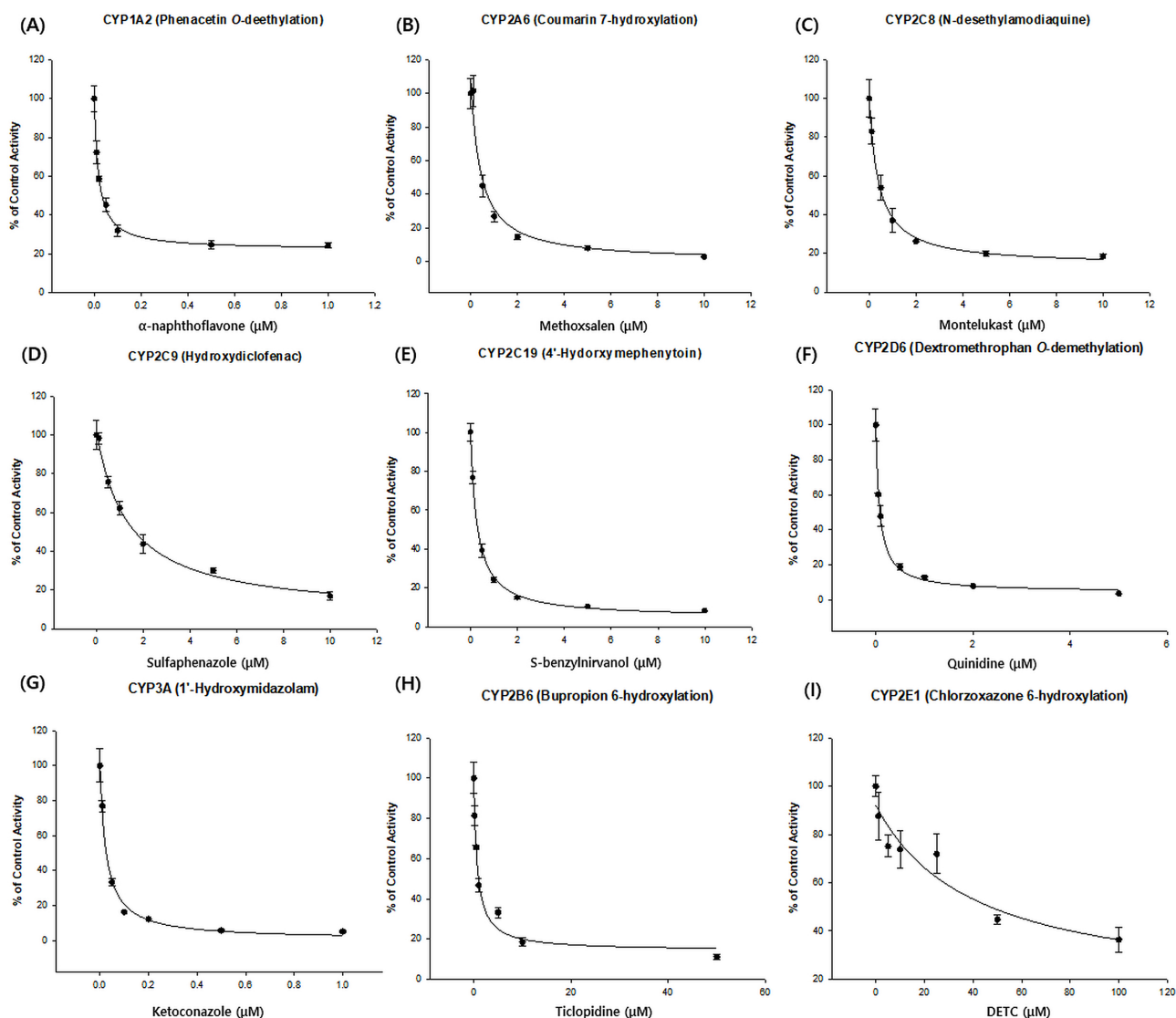


Fig. 3. Effects of positive control inhibitors on CYP isoform activities. (A) CYP1A2. (B) CYP2A6. (C) CYP2C8. (D) CYP2C9. (E) CYP2C19. (F) CYP2D6. (G) CYP3A. (H) CYP2B6. (I) CYP2E1.

Table 2. Inhibitory effects of ginsenoside Rg₅, Rk₁ on CYP isoforms.

Compound	Inhibition level	CYP isoforms (IC_{50})
Ginsenoside Rg ₅	Strong	CYP2A6 (1.04 μ M)
	Moderate	CYP2C8 (63.96 μ M), CYP3A (45.38 μ M), and CYP2B6 (53.71 μ M)
	No significant inhibition	CYP2C9, CYP2C19, CYP2D6, CYP1A2, and CYP2E1 (up to 100 μ M)
Ginsenoside Rk ₁	Strong	CYP2A6 (0.98 μ M)
	Moderate	CYP2C8 (89.71 μ M), CYP2C9 (92.78 μ M), CYP3A (63.82 μ M), CYP2B6 (58.55 μ M), and CYP2E1 (55.21 μ M)
	No significant inhibition	CYP1A2, CYP2C19, and CYP2D6 (up to 100 μ M)

Strong inhibition, $IC_{50} < 10$ μ M; moderate inhibition, $IC_{50} = 10$ –100 μ M; no significant inhibition, $IC_{50} > 100$ μ M.

the double bond, both compounds displayed nearly identical CYP2A6 inhibition profiles. This observation suggests that the dehydrated dammarane skeleton rather than the precise double-bond location may be a key structural determinant governing CYP2A6 inhibition.

Collectively, these findings suggest that heat-processed ginsenosides exhibit selective CYP inhibition profiles, with CYP2A6 appearing particularly susceptible to structural variations within dehydrated ginsenoside derivatives.

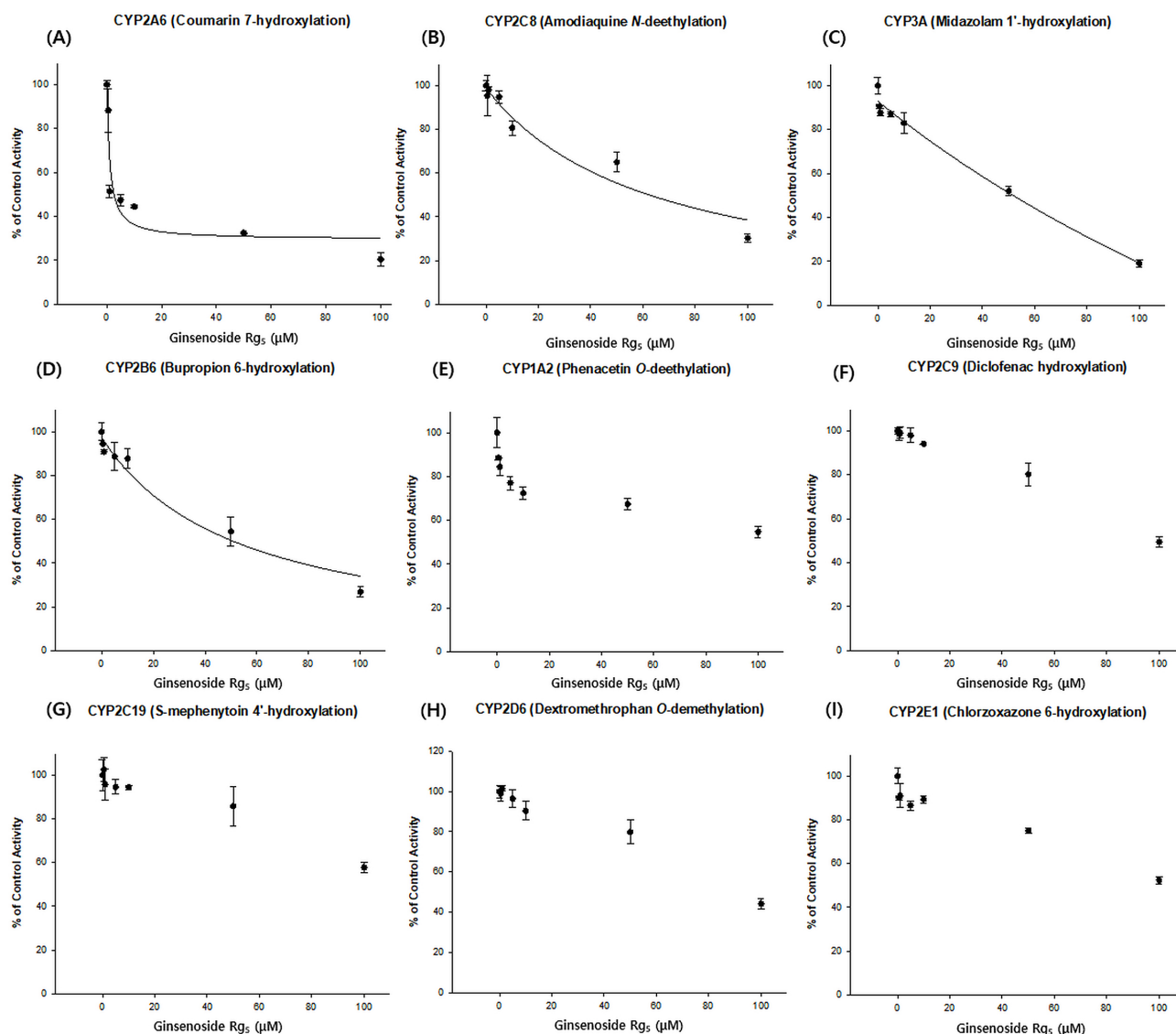


Fig. 4. Inhibitory effects of ginsenoside Rg₅ on CYP isoform activities. (A) CYP2A6. (B) CYP2C8. (C) CYP3A. (D) CYP2B6. (E) CYP1A2. (F) CYP2C9. (G) CYP2C19. (H) CYP2D6. (I) CYP2E1.

Structurally related ginsenosides such as Rh₃ and Rk₂ may warrant further investigation to determine whether similar CYP inhibition profiles are observed. This suggests that the structural characteristics of heat-processed ginsenosides are closely associated with CYP inhibition activity. Furthermore, a recent computational study predicted that most natural phytochemicals act as non-inhibitors of major CYP isoforms [11]. The discrepancy between such general ADMET predictions and our *in vitro* findings underscores the unique inhibitory properties of Rg₅ and Rk₁. This highlights the necessity of verifying general *in silico* predictions with actual *in vitro* enzyme assays in natural product research.

An additional limitation is that only reversible inhibition was evaluated in the present study. Time-dependent inhibition (TDI) and mechanism-based inactivation (MBI), which may lead to stronger *in vivo* drug interactions, were

not investigated. Future studies should therefore evaluate these mechanisms using preincubation-based CYP inhibition assays. The experiments were conducted only *in vitro* using human liver microsomes, and therefore the results may not fully reflect *in vivo* conditions in humans. In addition, only CYP inhibitory activity was evaluated, while other pharmacokinetic interactions and toxicological effects were not investigated. Further *in vivo* studies and clinical evaluations are necessary to confirm the clinical relevance and safety of Rg₅- and Rk₁-containing products.

Overall, the present findings suggest that heat-processed ginsenosides exhibit selective CYP2A6 inhibitory activity, highlighting a potential risk of herb–drug interactions during co-administration with CYP2A6 substrates. Further studies are warranted to clarify the pharmacological significance of these interactions. Furthermore, these findings provide a modern pharmacological perspec-

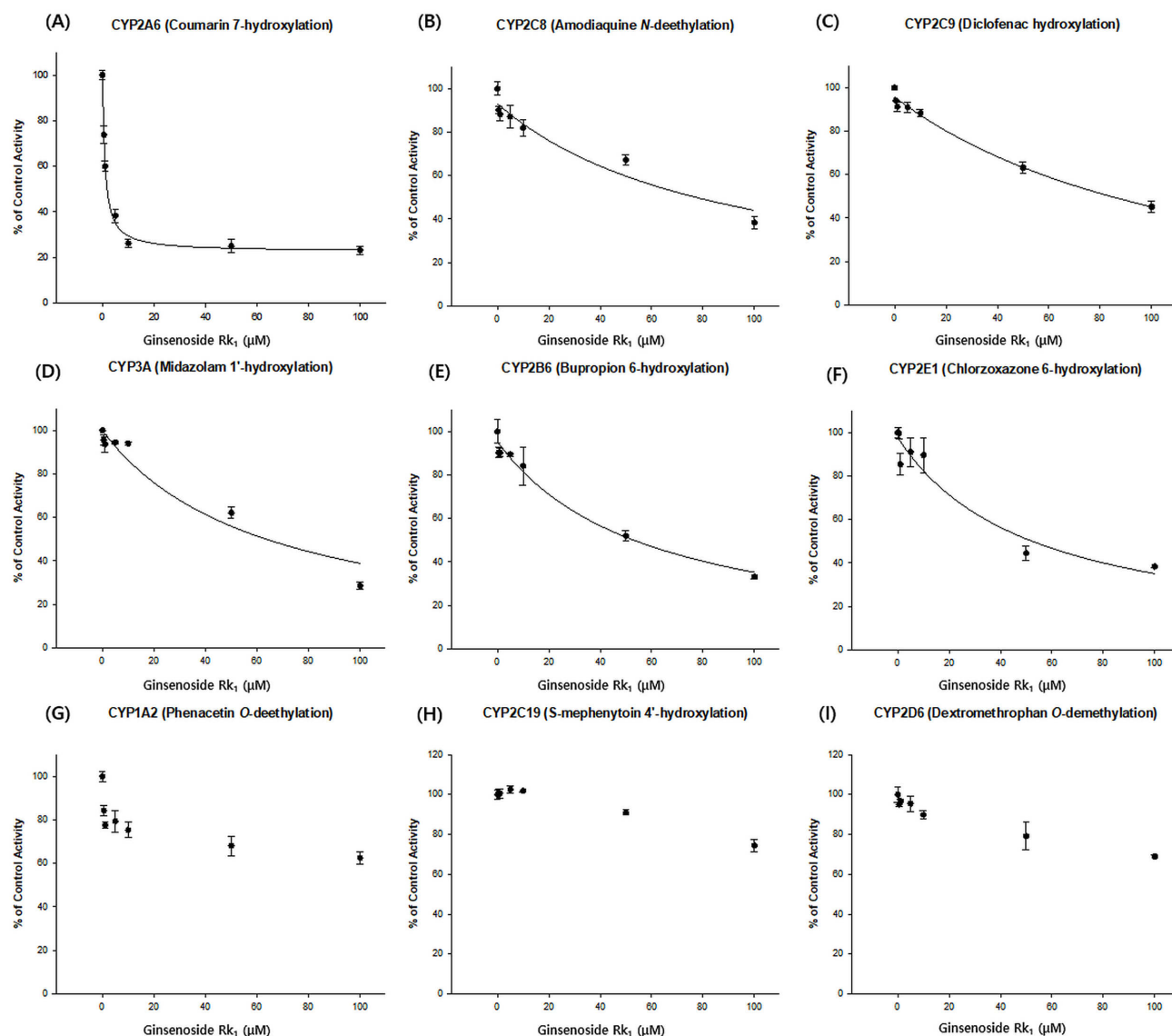


Fig. 5. Inhibitory effects of ginsenoside Rk₁ on CYP isoform activities. (A) CYP2A6. (B) CYP2C8. (C) CYP2C9. (D) CYP3A. (E) CYP2B6. (F) CYP2E1. (G) CYP1A2. (H) CYP2C19. (I) CYP2D6.

tive on the concept of contradictory combinations in traditional Chinese medicine (TCM). Traditionally, the concurrent use of ginseng with certain specific herbs is strictly contraindicated to prevent unexpected adverse effects. The potent and selective CYP inhibition by specific ginsenosides, such as Rg₅ and Rk₁, may serve as an underlying mechanism explaining not only modern herb-drug interactions but also potential traditional herb-herb incompatibilities when co-administered with substances primarily metabolized by these enzymes.

Limitation

This study has several limitations that should be considered when interpreting the results. First, in accordance with FDA guidelines, TDI assessment is recommended for compounds that demonstrate significant reversible inhibition. Because this study solely focused on reversible inhibition,

the absence of pre-incubation experiments (with and without NADPH) limits our ability to rule out or confirm TDI for CYP2A6, which may lead to an underestimation of *in vivo* interaction risks. Second, the ginsenosides were dissolved in DMSO, resulting in a final organic solvent concentration of 0.5% (v/v). Because baseline inhibition data from solvent-only controls were not explicitly evaluated in parallel, the weak inhibition observed for some isoforms (e.g., CYP2B6 and CYP2E1) may partially reflect non-specific solvent effects rather than genuine inhibitory activity. Future *in vivo* pharmacokinetic studies incorporating TDI assays and strict solvent controls are necessary to fully elucidate the clinical safety profile of these ginsenosides. While our *in vitro* data clearly demonstrate the selective inhibitory effects of Rg₅ and Rk₁ against CYP2A6, further investigations are warranted to elucidate the precise molecular mechanisms underlying this selectivity. Future stud-

ies employing *in silico* molecular docking simulations and structural biology approaches are necessary to understand the specific binding affinities and structural interactions between these ginsenosides and the CYP2A6 active site. Additionally, the coumarin concentration (50 μ M) used for the CYP2A6 inhibition assay was relatively higher than its commonly reported K_m values (0.5–2.5 μ M) in human liver microsomes. This higher concentration was selected to ensure adequate analytical sensitivity for the UPLC-MS/MS system. Consequently, as the substrate concentration exceeded the K_m value, the *in vitro* inhibitory potency (IC_{50}) of Rg₅ and Rk₁ against CYP2A6 might have been somewhat underestimated in the present study.

5. Conclusions

This study evaluated the inhibitory effects of ginsenosides Rg₅ and Rk₁, major components of heat-treated red ginseng, on nine CYP isoforms using UPLC-MS/MS-based *in vitro* assays with human liver microsomes according to FDA guidelines. Both ginsenosides showed potent inhibitory effects on CYP2A6, while moderate inhibitory effects were observed for several other CYP activities and little or no inhibition observed for the remaining CYP activities. These findings suggest that co-administration of ginsenosides Rg₅ or Rk₁ with drugs primarily metabolized by CYP2A6 may result in potential herb–drug interactions through metabolic inhibition. Therefore, further *in vivo* studies are required to evaluate the clinical significance and safety of these interactions. To our knowledge, this is the first study to systematically characterize the inhibitory effects of heat-processed ginsenosides Rg₅ and Rk₁ against nine major human CYP isoforms using a validated UPLC-MS/MS cocktail assay. These findings identify CYP2A6 as a potential target for herb–drug interactions and provide a basis for future pharmacokinetic and clinical safety evaluations. These findings may be useful for predicting metabolic interactions and guiding the safe use of heat-processed ginseng products.

Abbreviations

HLM, human liver microsomes; CYP, cytochrome P450; DDI, drug–drug interaction; FDA, Food and Drug Administration;

Availability of Data and Materials

The datasets generated and analyzed during the current study are included in this published article. Additional data related to this study are available from the corresponding authors upon reasonable request.

Author Contributions

DWL, MJO, HBY, and JSP designed the research study. DWL and MJO performed the experiments. DWL, MJO, HBY, and JSP analyzed and interpreted the data.

MJK (SPMED), SHM, MJK (Korea Food Research Institute), SHB, KSK, JB, and YSC provided scientific advice and contributed to data interpretation. DWL and MJO wrote the manuscript. HBY and JSP critically reviewed and revised the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Human liver microsomes were commercially obtained from BD Gentest (Woburn, MA, USA) and were provided in a de-identified form. The investigators had no access to donor-identifying information. Therefore, additional institutional ethical approval and informed consent were not required for the present study.

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Conflicts of Interest

The authors declare that they have no conflicts of interest. Min Jeong Kim and Dong Won Lee are employees of SPMED Co., Ltd. However, the company had no role in the study design, data collection, data analysis, interpretation of the results, manuscript preparation, or the decision to submit the manuscript for publication. The study was conducted independently, and there is no financial, commercial, or other conflict of interest related to SPMED Co., Ltd. that could have influenced the results or conclusions of this work. Therefore, the authors declare that there is no conflict of interest associated with this institution.

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