Regular Article

Evaluation of the Effects of *S*-Allyl-L-cysteine, *S*-Methyl-L-cysteine, *trans*-*S*-1-Propenyl-L-cysteine, and Their *N*-Acetylated and *S*-Oxidized Metabolites on Human CYP Activities

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Three major organosulfur compounds of aged garlic extract, *S*-allyl-L-cysteine (SAC), *S*-methyl-L-cysteine (SMC), and *trans*-*S*-1-propenyl-L-cysteine (SIPC), were examined for their effects on the activities of five major isoforms of human CYP enzymes: CYP1A2, 2C9, 2C19, 2D6, and 3A4. The metabolite formation from probe substrates for the CYP isoforms was examined in human liver microsomes in the presence of organosulfur compounds at 0.01–1 mM by using liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis. Allicin, a major component of garlic, inhibited CYP1A2 and CYP3A4 activity by 21–45% at 0.03 mM. In contrast, a CYP2C9-catalyzed reaction was enhanced by up to 1.9 times in the presence of allicin at 0.003–0.3 mM. SAC, SMC, and SIPC had no effect on the activities of the five isoforms, except that SIPC inhibited CYP3A4-catalyzed midazolam 1'-hydroxylation by 31% at 1 mM. The *N*-acetylated metabolites of the three compounds inhibited the activities of several isoforms to a varying degree at 1 mM. *N*-Acetyl-*S*-allyl-L-cysteine and *N*-acetyl-*S*-methyl-L-cysteine inhibited the reactions catalyzed by CYP2D6 and CYP1A2, by 19 and 26%, respectively, whereas *trans*-*N*-acetyl-*S*-1-propenyl-L-cysteine showed weak to moderate inhibition (19–49%) of CYP1A2, 2C19, 2D6, and 3A4 activities. On the other hand, both the *N*-acetylated and *S*-oxidized metabolites of SAC, SMC, and SIPC had little effect on the reactions catalyzed by the five isoforms. These results indicated that SAC, SMC, and SIPC have little potential to cause drug–drug interaction due to CYP inhibition or activation *in vivo*, as judged by their minimal effects ($IC_{50} > 1$ mM) on the activities of five major isoforms of human CYP *in vitro*.

Key words organosulfur compound; cytochrome P450 inhibition; drug–drug interaction; garlic

Garlic (*Allium sativum* L.) has been widely consumed since ancient times and its health benefits as a supplementary medicine have been well recognized.¹⁾ Recently, the increased use of herbal medicines has raised concerns about potential interactions with prescription medications. Of particular concern are drug–drug interactions (DDIs) caused by the inhibition or induction of drug-metabolizing enzymes, in particular, CYP enzymes.^{2–4)}

Aged garlic extract (AGE) is a unique garlic product manufactured through a long extraction process from raw garlic and its multiple pharmacologic effects have been demonstrated in several clinical studies.^{5–8)} This extraction process leads to the reduction of odorous, acidic, and irritating compounds found in fresh garlic and the enrichment of water-soluble organosulfur components in AGE, such as *S*-allyl-L-cysteine (SAC), *S*-methyl-L-cysteine (SMC), and *trans*-*S*-1-propenyl-L-cysteine (SIPC)⁹⁾ (Fig. 1). SAC is recognized as an active key component of AGE, and its favorable effects have been demonstrated, including a cholesterol-lowering effect,¹⁰⁾ an antioxidative effect,¹¹⁾ and an anticancer effect.¹²⁾ There has been limited study on the biological and pharmacologic activities of SIPC; however, its immune-enhancement effect has been recently demonstrated.¹³⁾ Additionally, several pharmacologic activities of SMC have been reported.^{14,15)}

Recent clinical studies have suggested that AGE is effective and well tolerated as an adjunct treatment when co-administered with medicines, such as blood pressure- and cholesterol-lowering agents,^{7,16)} most of which are eliminated by drug metabolism catalyzed by CYP. To our knowledge, no clinical

studies have examined the influence of AGE on the pharmacokinetics of CYP substrates. Greenblatt *et al.* examined the inhibitory activities of water-soluble garlic components, including SAC and SMC, *in vitro* on six isoforms of CYP.¹⁷⁾ However, they used only one test concentration (0.1 mM) of the components, and therefore their exact potential to inhibit the CYP isoforms was not determined. These facts prompted us to examine the potential of AGE and its components (SAC, SMC, SIPC) to cause DDIs, especially due to CYP inhibition, by employing an *in vitro* assay system. For the majority of herbal medicines, information on the pharmacokinetics of their multiple components is limited and many non-absorbable or unstable compounds may be present. Such compounds have less potential to alter the metabolism of CYP substrates *in vivo*, even when having potent CYP inhibitory activities *in vitro*, and accordingly, *in vitro* CYP inhibition data regarding herbal preparations needs to be carefully interpreted. The CYP inhibitory activity of the major oral components, but not the non-absorbable ones, should be determined *in vitro* and utilized to predict the *in vivo* DDI potential of herbal medicines. In addition, it is important to examine the CYP inhibitory potential of the major metabolites of the oral components. Our recent studies revealed that three organosulfur components of AGE (SAC, SMC, SIPC) are orally well absorbed and undergo both *N*-acetylation and *S*-oxidation metabolism.^{18,19)}

Based on these findings and considerations, we examined the effects of SAC, SMC, SIPC, along with their *N*-acetylated and *N*-acetylated/*S*-oxidized metabolites, on the activities of five major isoforms of human CYP (CYP1A2, 2C9, 2C19,

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2D6, and 3A4), by using an *in vitro* assay system with human liver microsomes (HLMs) and probe substrates for the isoforms.

MATERIALS AND METHODS

General Chemicals Formic acid of LC/MS grade, dextromethorphan, and phenacetin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). LC/MS-grade acetonitrile was obtained from Kanto Chemical (Tokyo, Japan). Dextrophan, midazolam, and propranolol were purchased from MP Biomedicals (Santa Ana, CA, U.S.A.), BD Biosciences (San Diego, CA, U.S.A.), and Tokyo Chemical Industry (Tokyo, Japan), respectively. Acetaminophen, diclofenac, furafylline, 4'-hydroxydiclofenac, 4'-hydroxymephenytoin, 1'-hydroxymidazolam, 6 β -hydroxytestosterone, ketoconazole, *S*-mephenytoin, quinidine, sulfaphenazole, testosterone, and ticlopidine were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Allicin was purchased from LKT Laboratories (St. Paul, MN, U.S.A.). β -Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), was purchased from Oriental Yeast (Tokyo, Japan). HLMs (pooled from 50 donors, mixed gender) was provided by XenoTech (Lenexa, KS, U.S.A.).

Chemicals SAC and SMC were purchased from Tokyo Chemical Industry. The authentic standards of *N*-acetyl-*S*-allyl-L-cysteine (NAc-SAC), *N*-acetyl-*S*-allyl-L-cysteine sulfoxide (NAc-SACS), *N*-acetyl-*S*-methyl-L-cysteine (NAc-SMC), *N*-acetyl-*S*-methyl-L-cysteine sulfoxide (NAc-SMCS), S1PC, *trans*-*N*-acetyl-*S*-1-propenyl-L-cysteine (NAc-S1PC), *trans*-*N*-

acetyl-*S*-1-propenyl-L-cysteine sulfoxide (NAc-S1PCS), and *S*-butenyl-L-cysteine were synthesized in the Drug Discovery Laboratory of Wakunaga Pharmaceutical (Hiroshima, Japan).

AGE AGE was manufactured under a license issued by the Ministry of Health, Labour and Welfare of Japan and formulated according to the following steps: garlic cloves (*Allium sativum* L.) were cut into slices, immersed in aqueous ethanol, and extracted over 10 months at room temperature. The specific procedure and specifications are listed in and comply with the U.S. Pharmacopeia/Natural Formula for garlic fluid extract monograph.²⁰⁾

In Vitro Evaluation of the Effects on Human CYP Activities Ten organosulfur compounds (SAC, SMC, S1PC, NAc-SAC, NAc-SMC, NAc-S1PC, NAc-SACS, NAc-SMCS, NAc-S1PCS, and allicin) and AGE were incubated with HLMs to examine their effects on the metabolism of probe substrates catalyzed by CYP1A2, 2C9, 2C19, 2D6, and 3A4. Incubation conditions, such as probe substrates for the CYP isoforms, metabolites analyzed, incubation times, and protein concentrations of HLMs, are summarized in Table 1. A typical incubation mixture (0.1 mL) contained 0.1 M phosphate buffer (pH 7.4), 5 mM magnesium chloride, 5 mM NADPH, a probe substrate at a concentration near Michaelis–Menten constant, HLMs (0.05–0.4 mg protein/mL), and a test compound at a series of concentrations. The organosulfur compounds tested were dissolved in 0.1 M phosphate buffer (pH 7.4). AGE (650 mg material/mL) and allicin (10 mg/mL) were diluted with buffer and distilled water, respectively. Probe substrates (Table 1) and CYP chemical inhibitors (Table 2) were dissolved in dimethyl sulfoxide. The final content of dimethyl sulfoxide in incubation mixtures was equal to or less than 0.2% (v/v). After preincubation at 37°C for 5 min, the reactions were initiated by the addition of NADPH. All reaction mixtures were added with 1 mL of acetonitrile to terminate the reactions and spiked with internal standard of propranolol. After centrifugation, the supernatants were evaporated to dryness. The residues were

Fig. 1. Chemical Structures of SAC, SMC, and S1PC

Table 1. Human CYP Isoforms, Probe Substrates, Metabolites Analyzed, Incubation Time, and Protein Concentrations of HLMs

Isoform CYP	Substrate	Conc. ^{a)} (μ M)	Metabolite	Incubation (min)	HLMs (mg/mL)
1A2	Phenacetin	40	Acetaminophen	40	0.2
2C9	Diclofenac	10	4'-Hydroxydiclofenac	15	0.2
2C19	<i>S</i> -Mephenytoin	50	4'-Hydroxymephenytoin	90	0.4
2D6	Dextromethorphan	2	Dextrophan	15	0.2
3A4	Midazolam	1	1'-Hydroxymidazolam	15	0.05
	Testosterone	40	6 β -Hydroxytestosterone	15	0.05

a) Conc., concentration.

Table 2. IC₅₀ Values of Known Chemical Inhibitors for Five Isoforms of Human CYP

Isoform CYP	Inhibitor	Test conc. range (μ M)	IC ₅₀ (μ M)	IC ₅₀ ^{a)} (μ M)
1A2	Furafyllin	0.1–10	2.0	1.3–5.6
2C9	Sulfaphenazole	0.03–10	0.26	0.25–0.52
2C19	Ticlopidine	0.1–30	0.70	1.2
2D6	Quinidine	0.003–1	0.021	0.058–0.11
3A4 ^{b)}	Ketoconazole	0.003–1	0.013	0.009–0.032
3A4 ^{c)}	Ketoconazole	0.003–1	0.012	0.019–0.048

a) IC₅₀ values reported in references.^{34–38)} b) Midazolam 1'-hydroxylation. c) Testosterone 6 β -hydroxylation.

dissolved in solvent A (90% water–10% acetonitrile with 0.1% formic acid) and analyzed with liquid chromatography–tandem mass spectrometry (LC–MS/MS). The IC_{50} values were calculated by nonlinear regression analysis using Kyplot software (ver. 5.0, Keyence, Osaka, Japan).

LC–MS/MS Analysis LC–MS/MS analysis was performed using a Waters Premier XE tandem mass spectrometer and an ACQUITY UPLC system (Waters Corporation, Milford, MA, U.S.A.). Chromatographic separation was achieved on an ACQUITY UPLC C18 BEH column (2.1×100 mm, 1.7 μ m; Waters Corporation) kept at 40°C, using a linear gradient at 0.3 mL/min with solvents of A and B (100% acetonitrile with 0.1% formic acid). The gradient started at 95% A and solvent B was linearly increased from 5 to 95% (0–2 min), 95% (2–2.5 min), and maintained at 5% (2.5–5 min). The mass spectrometer was operated in the positive ion mode with electrospray ionization. The ionization source parameters were capillary voltage 0.5 kV, source temperature 120°C, and desolvation gas temperature 350°C, with desolvation gas flow and cone gas flow rates of 1000 L/h and 50 L/h, respectively. The following selected ion monitoring transitions were used for analysis: m/z 152.0>110.0 for acetaminophen, 311.8>231.0 for 4'-hydroxydiclofenac, 235.0>149.8 for 4'-hydroxymephenytoin, 258.3>157.0 for dextropran, 305.2>269.1 for 6 β -hydroxytestosterone, 341.9>323.9 for 1'-hydroxymidazolam, and 260.2>182.9 for propranolol at cone and collision energies of 34 and 16 V, 22 and 22 V, 34 and 16 V, 46 and 34 V, 22 and 22 V, 40 and 22 V, and 34 and 16 V, respectively.

Assessment of Metabolic Stability against HLMs The stability of nine organosulfur compounds (SAC, SMC, SIPC, NAc-SAC, NAc-SMC, NAc-SIPC, NAc-SACS, NAc-SMCS, NAc-SIPCS) was examined in the incubation with HLMs. The incubation mixtures (0.1 mL) contained 0.1 M phosphate buffer (pH 7.4), 5 mM magnesium chloride, 5 mM NADPH, 0.01 mM organosulfur compounds, and HLMs (0.2 mg protein/mL). After preincubation at 37°C for 5 min, the reactions were initiated by the addition of the organosulfur compounds, followed by further incubation for 15–90 min. All reaction mixtures were added with 1 mL of methanol to terminate the reactions and spiked with internal standard of *S*-butenyl-L-cysteine. After centrifugation, the supernatants were evaporated to dryness. The residues were analyzed with LC–MS/MS as previously reported.^{18,19)}

RESULTS

Human CYP Inhibition by Known Chemical Inhibitors and Allicin The inhibitory effects of known chemical inhibitors on the activities of five human CYP isoforms (CYP1A2, 2C9, 2C19, 2D6, 3A4) were examined. The IC_{50} values of furafyllin (CYP1A2 inhibitor), sulfaphenazole (CYP2C9), ticlopidine (CYP2C19), quinidine (CYP2D6), and ketoconazole (CYP3A4) were 2.0, 0.26, 0.70, 0.021 and 0.012–0.013 μ M, respectively, all of which were similar to previously reported values (Table 2). Allicin is a major component of some garlic preparations and a potent inhibitor of human CYP isoforms.²¹⁾ We examined the effects of allicin on the metabolism of probe substrates for the five isoforms in HLMs (Fig. 2) and confirmed its potent inhibition of the CYP1A2- and CYP3A4-mediated metabolism with the IC_{50} values of 67 and 50–165 μ M (50 μ M for midazolam 1'-hydroxylation; 165 μ M for testosterone 6 β -hydroxylation), respectively. However, CYP2C9-catalyzed diclofenac 4'-hydroxylation was enhanced by up to 1.9 times in the presence of allicin at 3–300 μ M. Furthermore, allicin had a biphasic effect on *S*-mephenytoin 4'-hydroxylation by CYP2C19: apparent enhancement at lower concentrations (3–30 μ M) and potent inhibition at higher concentrations (100–300 μ M).

Effects of AGE, SAC, SMC, and SIPC on the Activities of Human CYP Isoforms The effects of SAC, SMC, and SIPC on the activities of the five CYP isoforms in HLMs were examined. As shown in Figs. 3A–C, the three compounds had no effect on the five isoforms, except that SIPC inhibited CYP3A4-catalyzed midazolam 1'-hydroxylation by 31% at 1 mM, the highest concentration tested. The effects of the *N*-acetylated and *N*-acetylated/*S*-oxidized metabolites of SAC, SMC, and SIPC were also examined. The *N*-acetylated metabolites of SAC, SMC, and SIPC (NAc-SAC, NAc-SMC, and NAc-SIPC, respectively) inhibited the activities of several isoforms at 1 mM (Figs. 3D–F); NAc-SAC inhibited CYP2D6 activity by 19%, whereas NAc-SMC caused a weak inhibition of CYP1A2-mediated metabolism by 26%. NAc-SIPC inhibited the activities of CYP1A2, 2C19, 2D6, and 3A4 (midazolam 1'-hydroxylation) by 27, 49, 19, and 20%, respectively. Among the three *N*-acetylated/*S*-oxidized metabolites of SAC, SMC, SIPC (NAc-SACS, NAc-SMCS, NAc-SIPCS, respectively), NAc-SIPCS inhibited the activity of only one isoform, CYP2D6, by 22% at 1 mM (Figs. 3G–I). The effect of AGE on the activity of the CYP isoforms was also examined. AGE

Fig. 2. Inhibitory and Enhancement Effects of Allicin on Five Human CYP Isoform-Catalyzed Reactions in HLMs

Probe substrates for five isoforms (CYP1A2, 2C9, 2C19, 2D6, 3A4) of human CYP were incubated with HLMs in the presence and absence of allicin at 1–300 μ M. The substrate metabolites formed were analyzed with LC–MS/MS. Data points represent the mean $\pm$ S.D. ($n=3$). a) Midazolam 1'-hydroxylation. b) Testosterone 6 β -hydroxylation.

Fig. 3. Effects of SAC, SMC, SIPC, and Their *N*-Acetylated and *N*-Acetylated/*S*-Oxidized Metabolites on Five Human CYP Isoform-Catalyzed Reactions in HLMs

Probe substrates for five isoforms (CYP1A2, 2C9, 2C19, 2D6, 3A4) of human CYP were incubated with HLMs in the presence and absence of SAC (A), SMC (B), SIPC (C), NAc-SAC (D), NAc-SMC (E), NAc-SIPC (F), NAc-SACS (G), NAc-SMCS (H), and NAc-SIPCS (I) at 0.01–1 mM. The substrate metabolites formed were analyzed with LC-MS/MS. Data points represent the mean $\pm$ S.D. ($n=3$). *a*) Midazolam 1'-hydroxylation. *b*) Testosterone 6 β -hydroxylation.

did not have any effect on the activity of the five isoforms at a concentration range of 0.01–0.3 mg material/mL (Fig. 4).

Metabolic Stability of SAC, SMC, and SIPC against HLMs The stability of SAC, SMC, SIPC, and their *N*-acetylated and *N*-acetylated/*S*-oxidized metabolites was examined in the incubation with HLMs. This study set the concentration of the organosulfur compounds at 0.01 mM, the incubation time at 15–90 min, and the concentration of HLMs at 0.2 mg protein/mL. These are common experimental conditions used in the evaluation of effects on CYP activities. SAC, SMC, SIPC, and their *N*-acetylated/*S*-oxidized metabolites (NAc-SACS, NAc-SMCS, NAc-SIPCS) were stable against metabolism by HLMs; the remaining amounts of those compounds were 99–105% of their initial concentrations after the 60-min incubation with HLMs. On the other hand, NAc-SAC, NAc-SMC, and NAc-SIPC underwent deacetylation to form

Fig. 4. Effect of AGE on Five Human CYP Isoform-Catalyzed Reactions in HLMs

Probe substrates for five isoforms (CYP1A2, 2C9, 2C19, 2D6, 3A4) of human CYP were incubated with HLMs in the presence and absence of AGE at 0.01–0.3 mg material/mL. The substrate metabolites formed were analyzed with LC-MS/MS. Data points represent the mean $\pm$ S.D. ($n=3$). *a*) Midazolam 1'-hydroxylation. *b*) Testosterone 6 β -hydroxylation.

Fig. 5. Metabolic Stability of NAc-SAC, NAc-SMC, and NAc-SIPC against HLMs

After the incubation of HLMs with NAc-SAC (A), NAc-SMC (B), and NAc-SIPC (C) at 0.01 mM for 15–90 min, the amount of their unchanged and deacetylated forms was quantified with LC-MS/MS. Data points represent the mean $\pm$ S.D. ($n=3$).

SAC, SMC, and SIPC, respectively, to a limited extent (Fig. 5). After the 90-min incubation, the remaining amount (% of the initial concentration) of the three compounds was 94% (NAc-SAC), 85% (NAc-SMC), and 87% (NAc-SIPC). After the incubation for 15–90 min, total amount (% of the initial concentration, on a molar basis) of the unchanged and deacetylated forms was almost 100% for NAc-SAC and NAc-SMC, whereas that of NAc-SIPC was 91–100%.

DISCUSSION

A significant number of adverse events occur from DDI due to the inhibition of CYP-mediated drug metabolism. With the increased concomitant use of garlic preparations and prescription medications, a number of clinical and preclinical studies have examined the potential of garlic products to inhibit human CYP.

The effect of chemicals on the activities of CYP enzymes is typically evaluated using two *in vitro* assay systems, each employing recombinant CYP isoforms with synthetic fluorometric substrates²²⁾ and pooled HLMs with probe substrates for CYP isoforms.²³⁾ In particular, the system of HLMs has been successfully employed to predict the interactions of chemicals with CYP *in vivo*.^{24,25)} In this study, three orally absorbable organosulfur compounds in AGE, namely SAC, SMC, and SIPC, were examined for their effects on the activities of five major isoforms of human CYP (CYP1A2, 2C9, 2C19, 2D6, 3A4), by evaluating CYP isoform-specific metabolism of probe substrates in HLMs.

To start, we examined the effect of allicin, which is a major component of some garlic preparations, but is not detected in AGE by its HPLC analysis. Zou *et al.* reported that allicin is a potent inhibitor of human CYP isoforms.²¹⁾ We confirmed that allicin exhibited potent inhibition of CYP1A2 and CYP3A4, with the IC_{50} values of 67 and 50–165 μ M, respectively (Fig. 2). In contrast, allicin increased the metabolite formation of probe substrates catalyzed by CYP2C9 and CYP2C19 at 3–300 μ M, which was not previously reported.²¹⁾ To date, the increase of CYP2C19 activity by certain chemicals has been little reported. On the other hand, several studies have demonstrated the enhancement of CYP2C9-mediated metabolism by some drugs, suggesting a two-site binding model as the underlying mechanism of the activation.^{26–28)} The exact reason for the different results between the studies by us and Zou *et al.* is un-

clear, but the assay systems used may be the cause. We used the system of HLMs and probe substrates, whereas Zou *et al.* employed the system of recombinant isoforms and synthetic substrates. We need to examine and compare the effects of allicin on CYP2C9 and CYP2C19 activities using the two assay systems in the future.

In the present study, SAC, SMC, and SIPC did not show any effect on five isoform-specific metabolisms, even at the highest concentration tested (1 mM), except that SIPC inhibited CYP3A4-catalyzed midazolam 1'-hydroxylation by 31% (Figs. 3A–C). CYP3A4 is the most abundant isoform of human CYP and is responsible for the metabolism of more than 50% of clinically available drugs.²⁹⁾ The involvement of this isoform in the majority of drug metabolism is largely attributable to its broad substrate specificity, and therefore the evaluation with the use of two or more probe substrates from distinct groups has been recommended to identify CYP3A4 inhibitors.³⁰⁾ Different from our results, Greenblatt *et al.* reported that SAC and SMC showed moderate inhibition, by over 50% at a concentration of 0.1 mM, of CYP3A4-mediated metabolism.¹⁷⁾ Our study employed the two most popular probe substrates for CYP3A4, midazolam and testosterone, whereas Greenblatt *et al.* used only one substrate, triazolam. At present, we do not know the exact reason for the difference in the inhibitory potential of SAC and SMC between the two studies because similar inhibition sensitivity was reported for midazolam and triazolam.³⁰⁾ In any case, SAC and SMC are very unlikely to inhibit CYP3A4 activity *in vivo*; a preliminary pharmacokinetic study of AGE demonstrated that the maximum plasma concentration of SAC was 140 nM,³¹⁾ which is lower by three orders of magnitude than the concentration (0.1 mM) used by Greenblatt *et al.*¹⁷⁾

Recently, we examined the metabolism and pharmacokinetics of SAC, SMC, and SIPC in rats and dogs and found that these compounds undergo *N*-acetylation and *S*-oxidation metabolism.^{18,19)} In both species, the *N*-acetylation is the major metabolic pathway to eliminate SAC and SIPC. In contrast, SMC is metabolized to NAc-SMC only to a small extent. We examined the effects of NAc-SAC, NAc-SMC, and NAc-SIPC on the activities of the five isoforms (Figs. 3D–F). At 0.01 mM, the three *N*-acetylated compounds did not show any effect on all the isoforms studied. At 1 mM, NAc-SMC exhibited a weak inhibition of CYP1A2 activity by 26%, whereas NAc-SIPC had weak to modest inhibition (19–49%) of the activities of

CYP1A2, 2C19, 2D6, and 3A4 (midazolam 1'-hydroxylation). Our previous *in vitro* metabolic study also demonstrated that there was a species difference in the *N*-acetylation metabolism of SAC, SMC, and SIPC between animals and humans, suggesting that in humans, *N*-acetylated metabolites are formed to a lesser extent.^{18,19)} Taken together, it is unlikely that the *N*-acetylated metabolites of SAC, SMC, and SIPC cause DDI due to CYP inhibition in humans. We also evaluated the effects of the minor metabolites (NAc-SACS, NAc-SMCS, NAc-SIPCS) on the activities of CYP isoforms and found that all the compounds had little influence on the five isoform-mediated metabolisms (Figs. 3G–I).

We examined the stability of SAC, SMC, SIPC, and their *N*-acetylated and *N*-acetylated/*S*-oxidized metabolites in the incubation with HLMs, and found that NAc-SAC, NAc-SMC, and NAc-SIPC underwent deacetylation to a limited extent (Fig. 5). Our previous work showed that the enzymatic activity of human liver to deacetylate NAc-SAC, NAc-SMC, and NAc-SIPC was primarily localized to the cytosol fraction (data not shown), which allowed the evaluation of the effects of these three compounds on CYP activities using the liver microsomal fraction.

For the evaluation of herbal products *in vitro*, the appropriate selection of test concentration is critical. Foster *et al.* reported the potent inhibitory activities of 10 garlic preparations against human CYP isoforms³²⁾; however, the study doses of garlic preparations used were likely too high, making it difficult to translate the *in vitro* results into *in vivo* situations. In order to address this issue, Strandell *et al.* proposed that CYP inhibitory potential is expressed as the volume (L/dose unit) with which recommended human doses of natural remedies are diluted to yield the solutions at concentrations of IC₅₀ values obtained in a CYP inhibition study,³³⁾ suggesting that further evaluation should be undertaken when the calculated volume approaches 4 L/dose unit, corresponding to human blood volume. Our present study examined the inhibitory potency of AGE (recommended dose of 0.3–0.6 g) for the first time. The maximal test concentration of AGE was set at 0.3 mg material/mL, with the content of SAC, SMC, and SIPC in the range of 0.24–7.8 μ M. AGE did not have any influence on the activities of the five isoforms at 0.01–0.3 mg material/mL (Fig. 4), suggesting that the calculated volume of AGE is, at the most, 1 L/dose unit and therefore AGE has less potential to inhibit CYP enzymes in its human dose.

In conclusion, the present study showed that SAC, SMC, SIPC, and their *N*-acetylated and *N*-acetylated/*S*-oxidized metabolites had little effect on the metabolism of probe substrates catalyzed by five major isoforms of human CYP (CYP1A2, 2C9, 2C19, 2D6, 3A4) with their IC₅₀ values of more than 1 mM. Our results suggested that it is extremely unlikely that these orally absorbable organosulfur components cause adverse events due to CYP inhibition or activation after the human intake of AGE.

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Conflict of Interest The authors are employees of Wakunaga Pharmaceutical Co., Ltd.

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