

Inhibitory Effects of Baicalein Derived from Japanese Traditional Herbal Medicine on SN-38 Glucuronidation

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ABSTRACT - Purpose: The chemotherapeutic agent irinotecan is hydrolyzed to its active form SN-38 by human carboxyesterases, but SN-38 is converted into the inactive form SN-38G by hepatic UDP-glucuronosyltransferases (UGTs). The aim of the present study was to evaluate the inhibitory effects of two β -glucuronidase-treated Japanese traditional herbal medicines (*kampo*), Hange-Shashin-To (TJ-14) and Sairei-To (TJ-114) on SN-38 glucuronidation, and the deglycosylation of baicalin (BG) and glycyrrhizic acid (GL) derived from TJ-14 and TJ-114 to form their respective aglycones, baicalein (BA) and glycyrrhetic acid (GA). **Methods:** The inhibitory effects of β -glucuronidase-treated TJ-14 and TJ-114 on SN-38 glucuronidation by human liver microsomes were examined. BA and GA, which were enzymatically converted from BG and GL present in TJ-14 and TJ-114, were examined in the same manner. Furthermore, the enzymatic activities were measured by using recombinant UGT1A1 and UGT1A9 isoforms instead of human liver microsomes. BA, GA, SN-38, and their glycosides/glucuronides were analyzed with an LC-MS system. **Results:** As regards the linear initial reaction rate, SN-38 glucuronidation by human liver microsomes was significantly inhibited by the addition of β -glucuronidase-untreated TJ-14 and TJ-114, but was more strongly inhibited by the addition of β -glucuronidase-treated TJ-14 and TJ-114. The results of LC-MS analysis and pharmacokinetic studies suggested that BA is the main inhibitor of SN-38 glucuronidation. In the Dixon plot, BA showed competitive inhibition of SN-38 glucuronidation, and the inhibition constant was $8.70 \pm 3.24 \mu\text{M}$. Previous reports, studies of recombinant UGT isoforms indicated that SN-38 glucuronidation was mainly catalyzed by UGT1A1. **Conclusions:** These findings strongly suggested that SN-38 glucuronidation is inhibited by BA. BA could act as a pharmacokinetic regulating factor associated with SN-38 glucuronidation.

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INTRODUCTION

Irinotecan [7 - ethyl - 10 - [4 - (1 - piperidino) - 1 - piperidino] carbonyloxy camptothecin (CPT-11) is a chemotherapeutic agent that exerts antitumor activity against several solid tumors. CPT-11 is predominantly hydrolyzed to a potent topoisomerase I inhibitor, 7 - ethyl - 10 - hydroxycamptothecin (SN-38), by human carboxyesterases (1). The second metabolic pathway of CPT-11 is through cytochrome P450. CPT-11 is oxidized to 7 - ethyl - 10 [4 - N - (5 - aminopentanoic acid) - 1 - piperidino] carbonyloxy camptothecin (APC) and 7 - ethyl - 10 - (4 - amino - 1 - piperidino) carbonyloxy camptothecin (NPC)

by CYP3A4. Both APC and NPC are much less cytotoxic than CPT-11. However, carboxyesterase can hydrolyze NPC into SN-38 but not APC (2-4). The active form, SN-38, is subsequently converted into the inactive form, 7 - ethyl - 10 - hydroxycamptothecin glucuronide (SN-38G), by hepatic UDP - glucuronosyltransferases (UGTs) (5, 6).

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UGTs represent one of the major classes of enzymes and are involved in phase II conjugative metabolism. UGTs catalyze the transfer of a

glucuronic acid moiety from uridine 5'-diphosphoglucuronic acid (UDPGA) to a wide range of structurally diverse endogenous compounds (e.g., bile acid, bilirubin, and steroids) and xenobiotics (e.g., such drugs as SN-38 and environmental toxicants). The resulting glucuronide conjugates are more polar than their parent compounds and are subsequently eliminated in bile or urine (5). Human UGTs are classified into four families: UGT1, UGT2, UGT3, and UGT8 (7). The most important drug-conjugating UGTs belong to UGT1 and UGT2 families. Among the UGT1A isoforms, UGT1A1, 1A4, 1A6, and 1A9 show moderate expression in the liver (7, 8). UGT1A1 is mainly responsible for the formation of SN-38G (6, 9, 10).

The inactive form, SN-38G, is excreted mainly into bile via such transporters as breast cancer resistance protein (BCRP), multidrug resistance-associated protein (MRP), and P-glycoprotein (11, 12). SN-38G is deconjugated by β -glucuronidase present in intestinal microflora and regenerated to SN-38. As a result, SN-38 causes gastrointestinal toxicity by inducing mucosal damage. The major dose-limiting toxicity after administration of CPT-11 is severe myelosuppression (13) and late-onset diarrhea (14). In many cases, neutropenia caused by myelosuppression converges in a relatively short time and is treated with granulocyte colony-stimulating factor (G-CSF) (15, 16). On the other hand, late-onset diarrhea continues to be a serious, debilitating, and life-threatening condition that leads to dehydration (17).

The Japanese traditional herbal medicine (*kampo*) Hange-Shashin-To (TJ-14) (17) and antibiotics, such as cefixime (18), are orally administered to ameliorate gastrointestinal toxicity because they inactivate bacterial β -glucuronidase, thereby suppressing the formation of toxic SN-38 from SN-38G (19). *Kampo* is prepared by combining multiple crude drugs and thus it contains many substances. As *kampo* is a combination of many substances, its effect is a combination of various interactions of the constituent substances (20). TJ-14 is commonly used to treat gastrointestinal disorders, such as acute and chronic gastrointestinal catarrh, fermentative diarrhea, and acute gastroenteritis. TJ-14 is composed of seven medicinal herbs, one of which is *Scutellariae radix* (skullcap), the active ingredient of which is baicalin (BG). BG is the glucuronide of baicalein. A glucuronide is also a glycoside and the most specific term in the field. BG has one glucuronic acid molecule and is a natural glucuronide conjugate. The mechanism of action of

TJ-14 toward SN-38-induced late-onset diarrhea is considered to be bacterial β -glucuronidase inhibition by BG. As a result, BG alleviates SN-38-induced mucosal damage (21) and enzymatically hydrolyzed BA is absorbed by small intestinal epithelial cells through passive diffusion. TJ-14 also contains *Glycyrrhizae radix* (licorice). Glycyrrhizic acid (GL) is one of the active ingredients of licorice; it has two glucuronic acid molecules and is the glucuronide of glycyrrhetic acid (GA). Orally administered GL is enzymatically hydrolyzed via 3-monoglucuronyl-glycyrrhetic acid (3MGA) to more bioactive GA by bacterial β -glucuronidase. Then, the deglycosylated forms, GA and BA, are absorbed into bloodstream and converted via 3MGA into GL and BG by hepatic UGTs (22, 23). On the other hand, SN-38 deconjugated by bacterial β -glucuronidase in intestine is taken up by the organic anion transporting polypeptide (OATP) 2B1 located at the apical membrane of enterocytes (24, 25) and absorbed into bloodstream. Based on the above findings, we hypothesized that BA and GA might influence hepatic UGT-mediated SN-38 glucuronidation. In this study, we examined whether the inhibitory effects of BA and GA originating from TJ-14 on UGT activity could be a pharmacokinetic regulating factor associated with SN-38-induced late-onset diarrhea *in vitro*. In addition to TJ-14, another Japanese traditional herbal medicine, Sairei-To (TJ-114), which is composed of *Scutellariae radix* and *Glycyrrhizae radix* and used to treat severe diarrhea, was also examined.

MATERIALS AND METHODS

Materials

7-Ethyl-10-hydroxycamptothecin (SN-38) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Glycyrrhetic acid (GA) and 7-ethyl-10-hydroxycamptothecin glucuronide (SN-38G) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Alamethicin, uridine 5'-diphosphoglucuronic acid trisodium salt (UDPGA), and β -glucuronidase type HP-2 from *Helix pomatia* were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Pooled human liver microsomes BD Ultra Pool™ HLM 150 (lot number 38291), recombinant UDP-glucuronosyltransferase (rUGT) 1A1 and rUGT1A9 (Human UGT1A1 Supersomes™ (lot number 4265007) and Human UGT1A9 Supersomes™ (lot number 6307002)) were purchased from Corning Gentest (Woburn, MA, U.S.A.). Baicalin (BG) and glycyrrhizic acid (GL)

were purchased from Wako Pure Chemical Industries (Osaka, Japan). Baicalein (BA) was purchased from Tocris Bioscience (Minneapolis, MN, U.S.A.). 3-Monoglucuronyl-glycyrrhetic acid (3MGA) was purchased from Nacalai Tesque (Kyoto, Japan). Hange-Shashin-To (TJ-14) and Sairei-To (TJ-114) were obtained from Tsumura & Co. (Tokyo, Japan). All other chemicals used in the experiments were of the highest purity commercially available.

Composition of Japanese Traditional Herbal Medicine

Japanese traditional herbal medicines TJ-14 and TJ-114 were spray-dried hot-water extracts of a mixture of herbal crude drugs. According to the manufacturer's information, the total daily dose of 7.5 g of TJ-14 (three times a day) consists of 4.5 g of water-extracted herbal powder and 3.0 g of additives. The water-extracted powder (4.5 g) is prepared from a decoction of seven medicinal herbs: *Pinelliae tuber* 5.0 g; *Scutellariae radix* 2.5 g; *Glycyrrhizae radix* 2.5 g; *Zizyphi fructus* 2.5 g; *Ginseng radix* 2.5 g; *Zingiberis siccatum rhizoma* 2.5 g; and *Coptidis rhizome* 1.0 g. On the other hand, the total daily dose of 9.0 g TJ-114 (three times a day) consists of 6.0 g of water-extracted herbal powder and 3.0 g of additives. The water-extracted powder (6.0 g) is prepared from a decoction of twelve medicinal herbs: *Bupleuri radix*, 7.0 g; *Pinelliae tuber*, 5.0 g; *Alismatis rhizome*, 5.0 g; *Scutellariae radix*, 3.0 g; *Ginseng radix*, 3.0 g; *Zizyphi fructus*, 3.0 g; *Poria*, 3.0 g; *Polyporus*, 3.0 g; *Atractylodis lanceae rhizome*, 3.0 g; *Cinnamomi cortex*, 2.0 g; *Glycyrrhizae radix*, 2.0 g; and *Zingiberis rhizome*, 1.0 g. Generally, *kampo* including TJ-14 and TJ-114 is taken with half a glass (50–100 mL) of water or warm water.

β -Glucuronidase Treatment of Japanese Traditional Herbal Medicine

The deglycosylation of BG to BA and GL via 3MGA to GA in TJ-14 and TJ-114 was conducted according to a previous report (26) with minor modifications. Ground TJ-14 and TJ-114 were added to 50 mM sodium acetate buffer (pH 5.0). The 5.0 w/v% suspensions were incubated without or with 200 units/mL β -glucuronidase for 8 hr at 37 °C. A portion of the reaction solutions were passed through a solid-phase extraction cartridge (Sep-Pak C18 Plus Short Cartridge, Waters, Milford, MA, U.S.A.) and elution was carried out with methanol. The eluates were diluted to 0.5 w/v% solutions using 50 vol%

methanol and filtered through 0.45 μ m membrane filter. The filtrate diluted to one-tenth its original concentration was injected into a liquid chromatograph-mass spectrometer (LC-MS) system.

Glucuronidation of SN-38

The inhibitory effects of β -glucuronidase-treated TJ-14 and TJ-114 on SN-38 glucuronidation in human liver microsomes were examined. Ten μ L of a 0.5 w/v% solution of β -glucuronidase-treated or untreated TJ-14 or TJ-114 was added into a microtube and the solution was evaporated under reduced pressure to give a residue. The residue was added to an incubation mixture composed of 50 mM Tris-HCl buffer (pH 7.4) containing 10 mM MgCl₂, 0.2 mg protein/mL human liver microsomes, and 25 μ g/mL alamethicin, and the mixture was kept on ice for 10 min. Pre-incubation was carried out after the addition of SN-38 methanolic solution for 5 min at 37 °C. The final concentration of methanol was adjusted to 1.0 vol%. The reaction was initiated by adding 5 mM UDPGA in a final volume of 100 μ L, followed by incubation for 0–60 min at 37 °C. The reaction was terminated by adding 100 μ L of ice-cold acetonitrile to the reaction mixture. Precipitated proteins were removed by centrifugation for 5 min at 5,000 rpm and the supernatant was filtered through 0.45 μ m membrane filter. The filtrate was injected into a LC-MS system.

The inhibitory effects of BA and GA on SN-38 glucuronidation were examined in the same manner as described above except for the addition of β -glucuronidase-treated or untreated TJ-14 or TJ-114. Furthermore, except for the addition of rUGT1A1 or rUGT1A9 instead of human liver microsomes, the incubation was performed in the same manner as that described above.

LC-MS System

The mass chromatographs of BA, GA, SN-38, and their glycosides/glucuronides were measured with an LC-MS system (LCMS-2020 equipped with a model LC-20AD dual pump and an SPD-M20A UV detector at 254 nm) with a reversed-phase column (2.2 μ m particle size, 50 \times 2.0 mm, i.d.; SHIM-PACK XR-ODS (Shimadzu, Kyoto, Japan)) at the constant temperature of 40 °C. Chromatography was performed as follows.

The mobile phase consisted of solvents (A) methanol/water/formic acid (2:98:0.1, v/v/v) and (B) methanol/water/formic acid (98:2:0.1, v/v/v). The gradient program for GL, 3MGA, and GA analysis was 0–3.5 min, 80–100% B; 3.5–5.5 min, 100% B;

and 5.5–8.5 min, 80% B at the flow rate of 0.20 mL/min, and that for SN-38G, BG, SN-38, and BA analysis was 0–5.0 min, 40–62.5% B; 5.0–7.5 min, 62.5–100% B; 7.5–9.5 min, 100% B; and 9.5–12.5 min, 40% B at the flow rate of 0.17 mL/min. The retention times of GL, 3MGA, GA, SN-38G, BG, SN-38, and BA were 1.4 min, 2.7 min, 4.2 min, 3.2 min, 5.2 min, 6.7 min, and 7.5 min, respectively.

MS analysis was performed at the electrospray ionization interface with probe voltage set at 4.5 kV and -3.5 kV. Desolvation line and block heater temperatures were set at 250 °C and 400 °C, respectively. Nitrogen was used as the nebulizing and drying gas at the flow rates of 1.5 L/min and 15 L/min, respectively. Target ions (polarity) of GA, 3MGA, GL, BA, BG, SN-38, and SN-38G were set at m/z 471.3 (+), 647.4 (+), 821.6 (-), 268.9 (-), 445.0 (-), 393.6 (+), and 569.0 (+), respectively. Chromatographic analysis was carried out on Shimadzu model 2020 data analysis software Lab Solutions Version 5.55.

DATA ANALYSIS

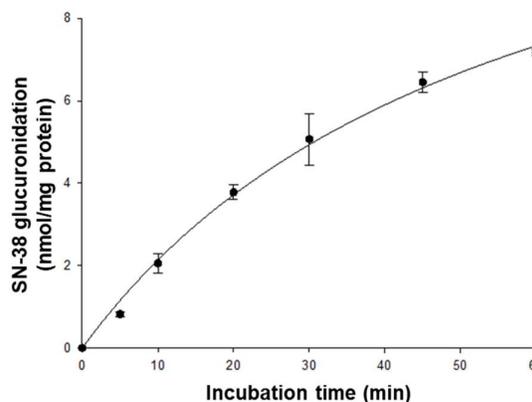
Kinetic parameters (V_{max} , K_m , and K_{si} (substrate inhibition constant)) were estimated by fitting the Michaelis-Menten and substrate inhibition equations to the substrate concentrations and the initial reaction rates. Data analysis was performed with Enzyme Kinetics Module of SigmaPlot 13 (Systat Software, San Jose, CA, U.S.A.). The goodness of fit was evaluated on the basis of R^2 values, AICc (Akaike's Information Criterion with adjustment for small sample size), and Sy.x (standard error of the regression). For the test of the difference between two independent groups, the Student's *t* test was conducted after the dispersion test by the *F* test. The test of the difference between three independent groups was processed using the Dunnett method and the Tukey method after the dispersion test.

RESULTS

Time Course and Kinetic Profiles of SN-38 Glucuronidation in Human Liver Microsomes

In order to clarify the kinetic characterization of SN-38 glucuronidation, the concentration and the incubation time for SN-38 glucuronidation were set to 1.5 μ M and 20 min in the following experiments unless otherwise noted.

SN-38 glucuronidation, the effect of incubation time on SN-38 glucuronidation were examined. Human liver microsomes were incubated with 10 μ M SN-38 for 60 min (Figure 1). SN-38 glucuronidation activity increased in a time-dependent manner and the increase was linear up to 20 min. Then, the effect of substrate concentration on SN-38 glucuronidation in human liver microsomes was examined. Human liver microsomes were incubated with 0–100 μ M SN-38 for 20 min (Figure 2). SN-38 glucuronidation increased in a concentration-dependent manner up to 10 μ M and the increase was linear up to 2.0 μ M (inset in Figure 2). However, at concentrations higher than 20 μ M, the kinetic profile of SN-38 glucuronidation indicated substrate inhibition. The enzyme kinetic parameters of K_m , V_{max} , CL_{int} , and K_{si} were 9.0 ± 0.79 μ M, 521 ± 45.5 pmol/mg protein/min, 58.3 ± 3.3 μ L/mg protein/min, and 48.5 ± 3.1 μ M, respectively. The influence of TJ-14 and TJ-114 and their ingredients on SN-38 glucuronidation had to be examined under



conditions that were not affected by substrate inhibition. From the kinetic parameters, K_{si}/K_m ratio showed a high value of 5.4, and thus we used a concentration of SN-38 that was lower than the K_m value and range of the initial reaction rate period.

Figure 1. Effect of incubation time on SN-38 glucuronidation in human liver microsomes. SN-38 concentration was set to 10 μ M. Each value represents the mean \pm S.D. of triplicate determinations.

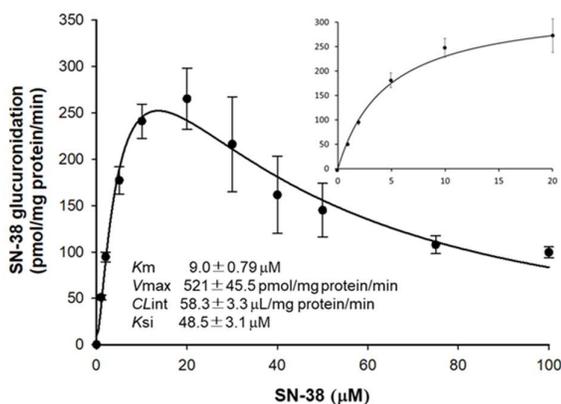


Figure 2. Kinetic profile and parameters of SN-38 glucuronidation in human liver microsomes. The incubation was performed for 20 min. Inset shows low concentration of SN-38. Each value represents the mean \pm S.D. of triplicate determinations.

Inhibitory Effects of TJ-14 and TJ-114 on SN-38

Glucuronidation in Human Liver Microsomes

The inhibitory effects of β -glucuronidase-treated or untreated TJ-14 or TJ-114 on SN-38 glucuronidation were investigated. In Figure 3, the control activity of SN-38 glucuronidation in human liver microsomes was $71.0 \pm 3.92 \text{ pmol/mg protein/min}$. Co-incubation with untreated TJ-14 significantly decreased SN-38 glucuronidation by 54% and co-incubation with β -glucuronidase-treated TJ-14 significantly reduced SN-38 glucuronidation by 87%. Similarly, co-incubation with untreated TJ-114 significantly decreased SN-38 glucuronidation by 48% and co-incubation with β -glucuronidase-treated TJ-114 significantly reduced SN-38 glucuronidation by 72%.

Table 1 shows the contents of five active ingredients BG, BA, GL, 3MGA, and GA in β -glucuronidase-treated or untreated TJ-14 and TJ-114. The results indicated that deglycosylation by β -glucuronidase markedly increased BA obtained from BG but slightly increased GA from GL. Accordingly, it was considered that SN-38 glucuronidation is strongly inhibited by aglycone BA rather than glycosides BG and GL, as shown in Figure 3.

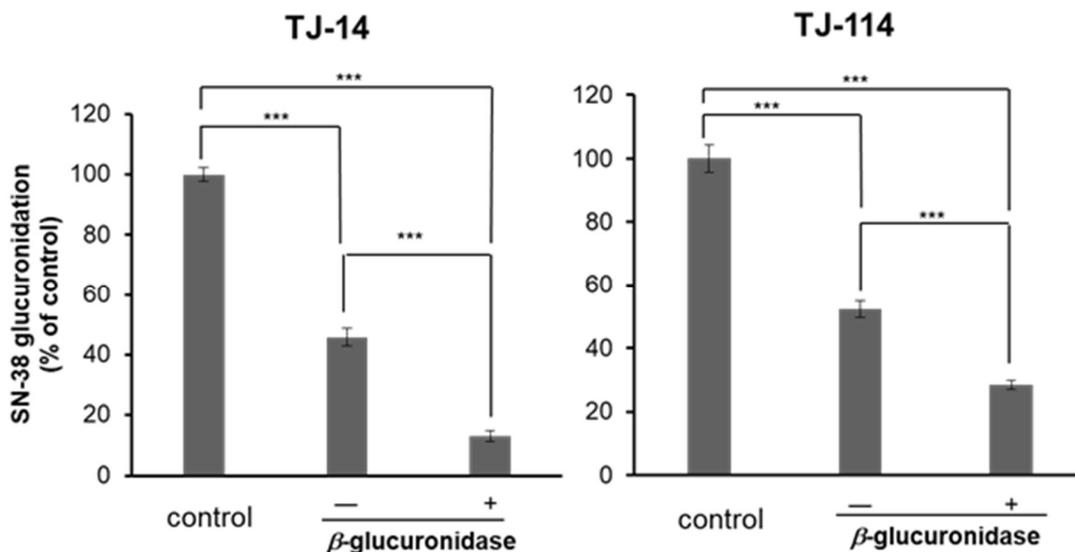


Figure 3. Inhibitory effects of β -glucuronidase-treated or untreated TJ-14 or TJ-114 on SN-38 glucuronidation in human liver microsomes. SN-38 concentration was set to $1.5 \mu\text{M}$ and incubation was performed for 20 min. Control activity was $71.0 \pm 3.92 \text{ pmol/mg protein/min}$. The final concentrations of TJ-14 and TJ-114 in the incubation mixture were set to 0.05 w/v%. Each value represents the mean \pm S.D. of triplicate determinations. Significantly different from control, $p < 0.001$.

Table 1. Contents of active ingredients derived from β -glucuronidase-treated or untreated TJ-14 and TJ-114

Ingredient	TJ-14		TJ-114	
	untreated	treated	untreated	treated
BG	1,832 \pm 63.2	N.D.	1,365 \pm 23.4	N.D.
BA	135 \pm 5.23	2,128 \pm 21.2 **	39.7 \pm 4.60	1,696 \pm 137 **
GL	297 \pm 9.27	178 \pm 1.43 ***	166 \pm 9.82	39.0 \pm 1.20 **
3MGA	N.D.	5.16 \pm 0.26	N.D.	1.36 \pm 0.01
GA	N.D.	18.4 \pm 0.13	N.D.	36.6 \pm 0.27

The 5.0 w/v% suspensions were incubated without or with 200 units/mL β -glucuronidase for 8 hr at 37 °C. Each value represents the mean \pm S.D. of triplicate determinations. Significantly different from control, ** $p < 0.01$, *** $p < 0.001$. N.D.: less than 20 fmol, 40 fmol, 10 fmol, 2.5 fmol, and 1.3 fmol for BG, BA, GL, 3MGA, and GA, respectively.

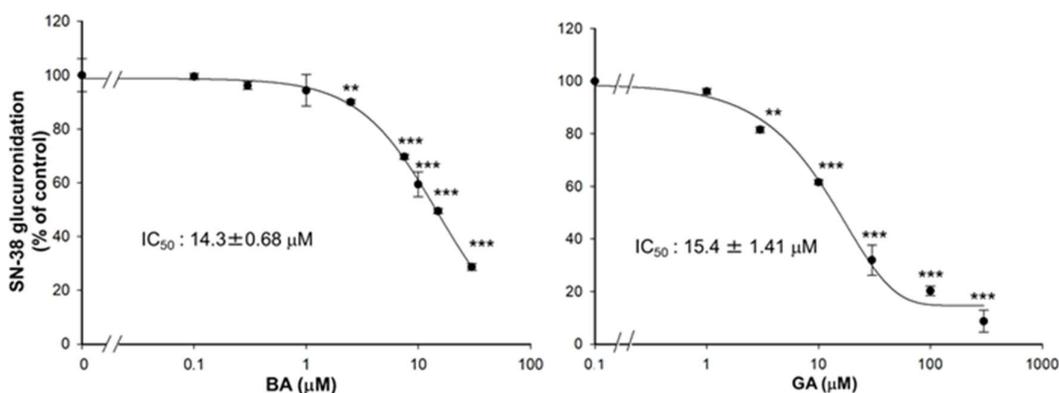


Figure 4. Concentration-dependent inhibition curves of BA and GA on SN-38 glucuronidation in human liver microsomes. SN-38 was set to 1.5 μ M and incubation was performed for 20 min. Each value represents the mean \pm S.D. of triplicate determinations. Significantly different from control, $p < 0.01$, $p < 0.001$.

Inhibitory Effects of BA and GA on SN-38 Glucuronidation in Human Liver Microsomes

The inhibitory effects of BA and GA on SN-38 glucuronidation in human liver microsomes were investigated. The concentration-dependent inhibition curves are shown in Figure 4. Co-incubation with more than 3.0 μ M BA or GA significantly decreased SN-38 glucuronidation. Fifty percent inhibitory concentration (IC_{50}) values of BA and GA were 14.3 \pm 0.68 μ M and 15.4 \pm 1.41 μ M, respectively.

Then, the Dixon plots were analyzed to clarify the types of inhibition and the inhibition constant (K_i) of BA in human liver microsomes. In Figure 5, the concentrations of SN-38 were set at 0.5, 0.75 (lower than K_m value), and 1.5 μ M (K_m value). BA showed competitive inhibition of SN-38

glucuronidation and the corresponding K_i value was 8.70 \pm 3.24 μ M.

SN-38 Glucuronidation by rUGT1A1 and rUGT1A9

SN-38 is mainly converted into inactive SN38G by UGT1A1 (6) and UGT1A9 (27) in the liver. Therefore, we investigated the kinetic profiles of SN-38 glucuronidation by rUGT1A1 and rUGT1A9. Recombinant UGT1A1 or rUGT1A9 was incubated with 0–75 μ M SN-38 for 20 min (Figure 6). SN-38 glucuronidation increased in a concentration-dependent manner up to 20 μ M and the increase was linear up to 5.0 μ M. In the same way as human liver microsomes at concentrations higher than 20 μ M, the kinetic profiles of SN-38 glucuronidation indicated substrate inhibition (10). The enzyme kinetic parameters of K_m , V_{max} , CL_{int} , and K_{si} by

rUGT1A1 were $11.4 \pm 1.2 \mu\text{M}$, $194 \pm 13.5 \text{ pmol/mg protein/min}$, $17.1 \pm 0.6 \mu\text{L/mg protein/min}$, and $26.9 \pm 2.2 \mu\text{M}$, respectively, and those by rUGT1A9 were $12.8 \pm 2.7 \mu\text{M}$, $8.5 \pm 2.7 \text{ pmol/mg protein/min}$, $0.68 \pm 0.09 \mu\text{L/mg protein/min}$, and $23.7 \pm 16.1 \mu\text{M}$, respectively.

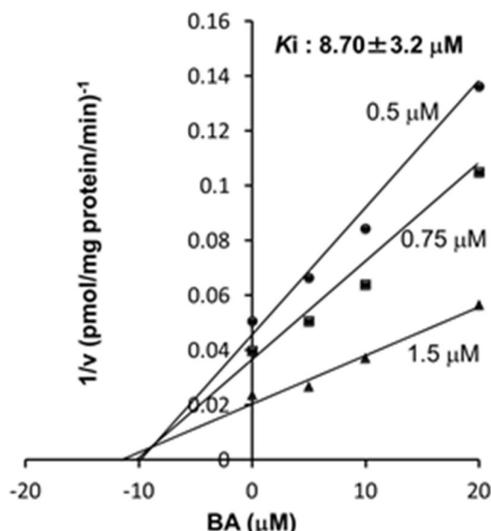


Figure 5. Dixon plots of BA on SN-38 glucuronidation in human liver microsomes. Incubation was performed for 20 min. Each value represents the mean of triplicate determinations.

Inhibitory Effect of BA on SN-38 Glucuronidation by rUGT1A1

The inhibitory effect of BA on SN-38 glucuronidation by rUGT1A1 was investigated (Figure 7). Co-incubation with BA concentration-dependently decreased SN-38 glucuronidation significantly; the addition of $5 \mu\text{M}$ and $10 \mu\text{M}$ BA reduced SN-38 glucuronidation by 42% and 68%, respectively. On the other hand, SN-38 glucuronidation by rUGT1A9 showed very low activity; hence, SN-38G could not be detected when BA was added to the incubation mixture.

DISCUSSION

The kinetic profiles of SN-38 glucuronidation in human liver microsomes were clarified. SN-38 glucuronidation increased in a time-dependent (Figure 1) and concentration-dependent manner and showed the highest activity ($250 \text{ pmol/mg protein/min}$) at approximately $20 \mu\text{M}$ (Figure 2). However, at concentrations higher than $20 \mu\text{M}$, the activity gradually decreased, indicating substrate

inhibition. Some studies of SN-38 glucuronidation in human liver microsomes reported the Michaelis-Menten type profile (6, 27-29), whereas Wu *et al.* (10) reported substrate inhibition profiles in human liver microsomes and rUGT1A1, which were similar to our results. Luukkanen *et al.* (30) pointed out that the bisubstrate (drug and UDPGA) kinetics of seven recombinant human UGTs 1A1, 1A3, 1A6, 1A7, 1A8, 1A9, and 1A10 indicated substrate inhibition. However, they concluded that these substrate inhibitions did not occur under physiological conditions. We speculate that the difference in kinetic profiles among research groups is probably due to differences in incubation conditions, including the lot number of human liver microsomes, the incubation time, and the ratio of UDPGA to SN-38. In particular, human liver microsomes may be affected by not only UGT contents in donor liver, but also the difference in abundance ratio of such genotypes as UGT1A1*1 and UGT1A1*28 (31). From our results, the K_{si}/K_m ratio of SN-38 glucuronidation in human liver microsomes was 5.4 (see Figure 2). Consequently, if the concentration of SN-38 in the reaction were set to a value lower than K_m , a reaction system that does not undergo substrate inhibition would be realized. From the above, the concentration of SN-38 in the reaction was set to $1.5 \mu\text{M}$, which was within the linear initial reaction rate and six times lower than the K_m value.

Such glycosides as BG and GL, which contain a glucuronic acid moiety, are considered natural prodrugs in herbal medicine. Orally administered glycosides are hydrolyzed by β -glucuronidase in the intestinal microflora to form more bioactive aglycones that are absorbed and exhibit various effects. Hence, the inhibitory effects of β -glucuronidase-treated or untreated TJ-14 and TJ-114 on SN-38 glucuronidation in human liver microsomes were examined. As shown in Figure 3, the addition of 0.05 w/v% (final concentration) each of β -glucuronidase untreated TJ-14 and TJ-114 to the incubation mixture significantly inhibited SN-38 glucuronidation by 46% and 52% as residual activity, respectively. Katoh *et al.* (32) reported that when 0.1 w/v% each of TJ-14 and TJ-114, the amount of which was twice ours, was added to the incubation mixture, the residual activities were approximately 20% and 30%, respectively. Their results coincided with ours.

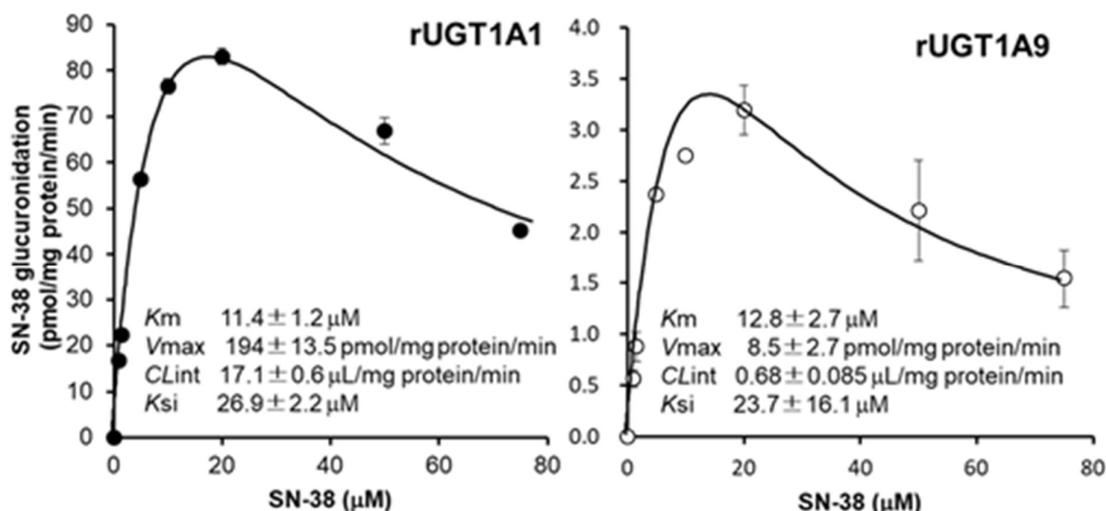


Figure 6. Kinetic profiles and parameters of SN-38 glucuronidation by rUGT1A1 and rUGT1A9. Incubation was performed for 20 min. Each value represents the mean \pm S.D. of triplicate determinations.

We therefore suggest that TJ-14 and TJ-114 contain active components that inhibit SN-38 glucuronidation. However, because *kampo* is characterized by its complex composition, it may be difficult to demonstrate its mechanism or mode of action (20). On the assumption that SN-38G was hydrolyzed by β -glucuronidase from intestinal microflora and converted into the aglycone, β -glucuronidase-treated TJ-14 and TJ-114 were added to the incubation mixture. As a result, the residual activities of TJ-14 and TJ-114 were 13% and 28%, respectively. Our results indicate that the inhibitory effect on SN-38 glucuronidation is enhanced in deglycosylated Japanese traditional herbal medicine. Katoh *et al.* (32) reported that such aglycones as BA and GA exert a greater inhibitory effect on SN-38 glucuronidation than their respective glycosides BG and GL.

The contents of the active ingredients in 5.0 w/v% suspensions of β -glucuronidase-treated or untreated TJ-14 and TJ-114 are indicated in Table 1. These 5.0 w/v% suspensions corresponded to orally administered 2.5 g/dose (TJ-14) or 3.0 g/dose (TJ-114) with approximately 50 mL of water. The contents of BA in β -glucuronidase-untreated TJ-14 and TJ-114 were approximately 135 μM and 40 μM , respectively, whereas the contents of BG in β -glucuronidase-treated TJ-14 and TJ-114 were completely converted into BA, resulting in approximately 2,130 μM and 1,700 μM , respectively. On the other hand, 3MGA and GA

were not detected in β -glucuronidase-untreated TJ-14 and TJ-114, whereas 3MGA and GA in β -glucuronidase-treated TJ-14 and TJ-114 were slightly increased to 1–5 μM and 18–37 μM from GL, respectively. BG could have inhibited GL hydrolysis, and this could be the reason why GL was not completely hydrolyzed to GA by β -glucuronidase in the same way as BG hydrolysis to BA (26).

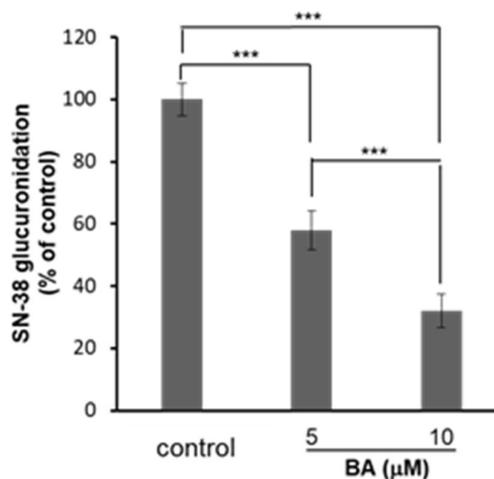


Figure 7. Inhibitory effects of BA on SN-38 glucuronidation by recombinant UGT1A1. SN-38 was set to 1.5 μM and incubation was performed for 20 min. Each value represents the mean \pm S.D. of triplicate determinations. Significantly different from control, $p < 0.001$.

The 5.0 w/v% suspensions of TJ-14 and TJ-114 shown in Table 1 were diluted to 100-fold their amounts by treatment with solid-phase extract and added to the reaction mixture. Therefore, the final concentrations of TJ-14 and TJ-114 in Figure 3 were 0.05 w/v%. When intestinal volume was estimated to be 500 mL (33), the concentrations of TJ-14 and TJ-114 were approximately 0.5 w/v%, and this value was further multiplied by 10 as the dilution coefficient. Thus, BA derived from TJ-14 and TJ-114 corresponded to the addition of approximately 21 μM and 17 μM , respectively, to the incubation mixture. Both concentrations almost agreed with 14 μM , which is the IC_{50} of BA, as shown in Figure 4. However, conflicting results were obtained between GA contents treated with β -glucuronidase corresponding to less than 0.4 μM in Table 1 and the IC_{50} of 15 μM in Figure 4.

SN-38 glucuronidation is mainly inhibited by BA in human liver microsomes. In order to clarify the type of inhibition and the K_i value of BA, a Dixon plot was drawn (Figure 5). BA showed competitive inhibition and the corresponding K_i value was 8.7 μM . Previous reports of human (32) and rat (34) liver microsomes yielded three times (26.9 μM , mixed type) and one hundred times (970 μM , mixed type) higher K_i values than ours. This may indicate species difference in the SN-38 glucuronidation inhibition process.

According to Hanioka *et al.* (35), SN-38 glucuronidation was catalyzed by UGT1A1, UGT1A3, UGT1A6, and UGT1A9 as well as by human liver microsomes. Among these UGT isoforms, UGT1A1 showed the highest activity for SN-38 glucuronidation at both low (1 μM) and high (200 μM) substrate concentrations. The ranking in order of activity at low and high substrate concentrations was $\text{UGT1A1} > \text{UGT1A9} > \text{UGT1A6} > \text{UGT1A3}$ and $\text{UGT1A1} > \text{UGT1A3} > \text{UGT1A6} \geq \text{UGT1A9}$. Based on this report, the kinetic profiles of SN-38 glucuronidation catalyzed by rUGT1A1 and rUGT1A9 are shown in Figure 6. Both isoforms showed a substrate inhibition profile similar to human liver microsomes, but the CL_{int} value of rUGT1A1 was approximately 25 times higher than that of rUGT1A9. These results suggest that SN-38 glucuronidation in human liver is catalyzed primarily by UGT1A1. However, even if standardization is carried out by the negative control microsomes, each UGT expression level in the recombinant system is different from that in human liver microsomes. Therefore, care is necessary to compare with glucuronidation activities of each

rUGT isoform. This UGT1A1-catalyzed SN-38 glucuronidation was inhibited in a concentration-dependent manner by the addition of BA (Figure 7), suggesting that BA is strongly involved in the blockade of this pathway. The maximum plasma concentrations of GA and BA in humans are 0.10–0.43 μM (36–38) and 0.49 μM (39), respectively. In the present study, the K_i value of BA in human liver microsomes was 8.7 μM (Figure 5), which was higher than the plasma concentration. Although BA concentration in liver may be higher than that in plasma, it is very difficult to compare liver concentration due to lack of information.

Hirose *et al.* (40) reported the relationship between SN-38G/SN-38 concentration ratio and neutropenia in 70 Japanese colorectal cancer patients. The SN-38G/SN-38 concentration ratio was lower in patients who were homozygous for UGT1A1*6, heterozygous for UGT1A1*6 or UGT1A1*28, or double heterozygotes than in patients with wild-type genes. It has been proposed that BG from TJ-14 and TJ-114 inhibits bacterial β -glucuronidase (17), and the uptake of SN-38 mediated by OATP2B1 in the apical side of intestinal epithelial cells (24, 25), thereby reducing the risk of SN-38-induced late-onset diarrhea. On the other hand, the deglycosylated form, BA, may raise plasma SN-38 levels by inhibiting hepatic UGT1A1 activity as well as UGT1A1*6 or UGT1A1*28, and consequently decrease neutrophil count. Further research is required to prove this speculation.

We summarize our findings as follows: when TJ-14 and TJ-114 are orally administered, BG derived from *Scutellariae radix* is hydrolyzed to aglycone BA by bacterial β -glucuronidase in the intestinal tract. As the deglycosylation of GL derived from *Glycyrrhizae radix* is inhibited by BG, a sufficient amount of GA could not be produced. These results led us to suggest that GA does not contribute to SN-38 glucuronidation. BA is transported to liver via bloodstream. CPT-11 is hydrolyzed by carboxyesterase and converted into SN-38, and SN-38 glucuronidation is mainly catalyzed by UGT1A1 in the liver. Our results strongly suggest that SN-38 glucuronidation is inhibited by BA.

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