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Inhibition of UDP-glucuronosyltransferase by Aglycons of Natural Glucuronides in *Kampo* Medicines Using SN-38 as a Substrate

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7-Ethyl-10-[4-(piperidino)-1-piperidino]carbonyloxycamptothecin (CPT-11), a potent anticancer agent for lung and gynecological cancers, is metabolized *in vivo* to the active compound, 7-ethyl-10-hydroxycamptothecin (SN-38), which is subsequently conjugated to SN-38-glucuronide by UDP-glucuronosyltransferase (UDP-GT). Three purified aglycons of natural glucuronides, baicalein, luteolin and glycyrrhetic acid, inhibited UDP-GT activity towards SN-38 as a substrate. The inhibitory potencies of these aglycons toward UDP-GT were similar to that of 1-naphthol. Based on these results, together with our previous finding that the corresponding glucuronides used in the present study strongly inhibited β-glucuronidase in gut flora, we propose that materials in *Kampo* (Japanese herbal) medicines containing these aglycons of natural glucuronides could be used *in vivo* to decrease the enterohepatic circulation of SN-38 and other drugs.

Key words: SN-38-glucuronide — Baicalein — Glycyrrhetic acid — Luteolin — 1-Naphthol

CPT-11,[1)] a water-soluble semisynthetic derivative of CPT[6] in use as a new anticancer agent, is metabolized to SN-38, which shows a potent anticancer activity against a variety of experimental tumors[2–4)] through the inhibition of DNA topoisomerase I.[5)] Upon administration of SN-38 to rats, SN-38-glucuronide is excreted into the bile.[1,6,7)] The metabolite SN-38-glucuronide is deconjugated by β-glucuronidase in intestinal microflora to form SN-38,[1)] which is assumed to be the cause of diarrhea, one of the side effects of CPT-11.[8,9)]

Glucuronidation catalyzed by UDP-GT is one of the most important detoxication processes in the liver. No potent inhibitors of UDP-GT that could be used *in vivo*, especially for clinical studies, have been developed. Thus, it is of considerable importance to develop a UDP-GT inhibitor which could be used *in vivo* to decrease the enterohepatic circulation of drugs. It is known that natural glucuronides and their aglycons exist in *Kampo* (Japanese herbal) medicines. In this study, purified natural aglycons of glucuronides, namely, baicalein, luteolin, glycyrrhetic acid and glycyrrhetic acid methyl ester, and an aglycon of the glycoside genipin (Fig. 1) were isolated from plant materials of *Kampo* medicines, and were studied to determine if they were capable of inhibiting UDP-GT. To evaluate the ability of these aglycons of natural glucuronides to inhibit UDP-GT, SN-38 was used as a substrate.

MATERIALS AND METHODS

Chemicals SN-38 and SN-38-glucuronide were synthesized and provided by the Research Laboratory of Yakult Co., Tokyo.[10)] The purity of SN-38 used in these experiments was >99.5% as judged by high-performance liquid chromatography.[7)] Aglycons of natural glucuronides, such as baicalein, luteolin, glycyrrhetic acid and glycyrrhetic acid methyl ester were provided by the Research Institute for Biology and Chemistry of Tsumura & Co., Ibaraki.[11)] An aglycon of a natural glycoside, genipin, was also provided by Tsumura & Co. UDP-GA was obtained from Sigma, St. Louis, MO. All other chemicals were commercial products of analytical grade. The aglycons were dissolved in DMSO and then diluted with 0.2 *M* Tris-HCl (pH 7.4). The final concentration of DMSO was 0.5% in the reaction mixture. DMSO did not inhibit UDP-GT at this concentration. CPT was dissolved in 0.1 *M* NaOH to prepare a stock solution and then diluted with 0.01 *M* HCl before use.

Preparation of microsomal fraction as an enzyme source Rat liver microsomes were prepared and used as an

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[6] Abbreviations: CPT, camptothecin; CPT-11, 7-ethyl-10-[4-(piperidino)-1-piperidino]carbonyloxycamptothecin; DMSO, dimethyl sulfoxide; SN-38, 7-ethyl-10-hydroxycamptothecin; SN-38-glucuronide, 7-ethyl-10-hydroxycamptothecin glucuronide; UDP-GA, uridine 5′-diphosphoglucuronic acid; UDP-GT, UDP-glucuronosyltransferase.

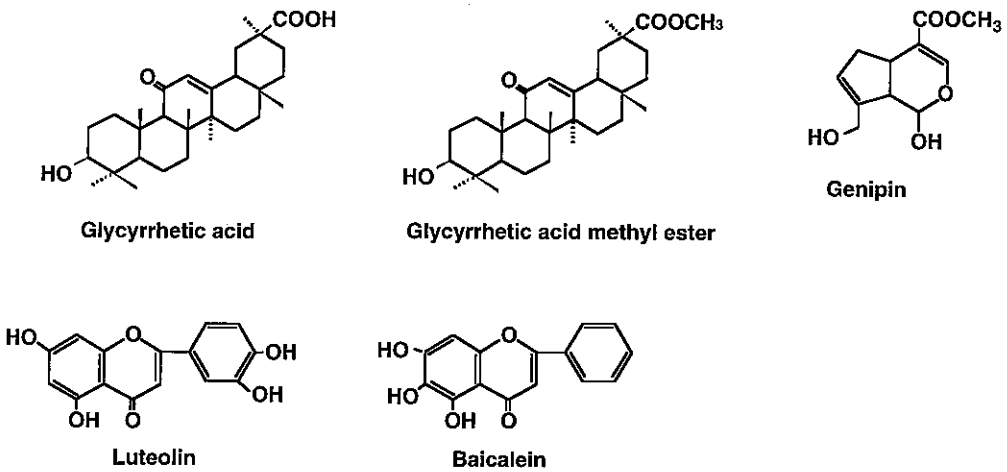

Fig. 1. Chemical structures of aglycons of natural glucuronides and genipin.

enzyme source of UDP-GT as described by Bock *et al.*[12] Briefly, male rats (8-week-old, Wistar strain) were decapitated and exsanguinated, and their livers were immediately removed. Liver microsomes were prepared as described previously.[13] Microsomes (5 mg/ml) were pretreated with 0.25% sodium cholate at 4°C for 30 min. The protein content was determined by the method of Lowry *et al.*[14] using bovine serum albumin as the standard.

Determination of glucuronides of SN-38 and *p*-nitrophenol *in vitro* An aglycon of a natural glucuronide in DMSO (0–2.5 m*M*) was added to an incubation mixture (200 μl) containing 100 μM SN-38, 0.2 *M* Tris-HCl buffer (pH 7.4), 5 m*M* magnesium chloride, 5 m*M* UDP-GA and the desired amounts of liver microsomes. Incubations were carried out at 37°C for desired periods. The reaction was terminated by the addition of trichloroacetic acid at a final concentration of 2.5%. After centrifugation at 5,000*g* for 10 min, the supernatant was collected and the specific fluorescence due to SN-38-glucuronide was measured with a fluorescence detector (model 650-10LC, Hitachi, Tokyo) with excitation at 375 nm and emission at 424 nm, according to the method of Atumi *et al.*[15] Authentic SN-38-glucuronide was used as the standard. *p*-Nitrophenol was used as another substrate for UDP-GT at a final concentration of 1 m*M*. The measurement was carried out according to the method of Mahu *et al.*[16] Reaction conditions were the same as for the SN-38 assay as mentioned above. Solubilized microsomes were added and the mixture was incubated at 37°C for 10 min. The reaction was terminated by the addition of five volumes of water-saturated ethyl acetate, mixed well and centrifuged at 3,000 rpm for 10 min. The resulting water phase was extracted again with the same volume of water-saturated ethyl acetate. β-Glucuronidase (60 FU/ml) in 0.5 *M* acetate buffer (pH 4.5) was added to the water phase and incubated at 37°C for 30 min. A solution of NaOH (0.1 *N*, 2.7 ml) was added, and then the absorbance at 400 nm was measured. *p*-Nitrophenol glucuronide was used as the standard. One Fishman unit (FU) was defined as the amount of enzyme catalyzing 1.0 μg of substrate per hour at 37°C at the appropriate pH.

RESULTS

Formation of SN-38-glucuronide from SN-38 as a function of UDP-GT concentration, incubation time or SN-38 concentration As SN-38-glucuronide is excreted in bile,[7] the possibility of SN-38 being a good substrate for UDP-GT was examined *in vitro* (Fig. 2). The SN-38 incubated with various amounts of solubilized microsomes as the source of UDP-GT in the presence of UDP-GA produced SN-38-glucuronide linearly with the amounts of microsomal protein (Fig. 2A). The formation of SN-38-glucuronide reached a maximal level when amounts of microsomes greater than 0.3 mg/ml were added to the incubation mixture. The amount of SN-38-glucuronide increased linearly with incubation time up to about 2 h (Fig. 2B). The rate of formation of SN-38-glucuronide was linear at concentrations of SN-38 below 40 μM in the incubation mixture (Fig. 2C). Thus, incubation mixtures for the assay of UDP-GT contained 100 μM SN-38, 0.3 mg of microsomal protein and other assay components and were incubated at 37°C for 60 min in further experiments.

Inhibition of UDP-GT by natural glucuronides and their aglycons measured as SN-38-glucuronide production Inhibition of UDP-GT by four aglycons of natural glucuronides was examined using SN-38 as a substrate. All

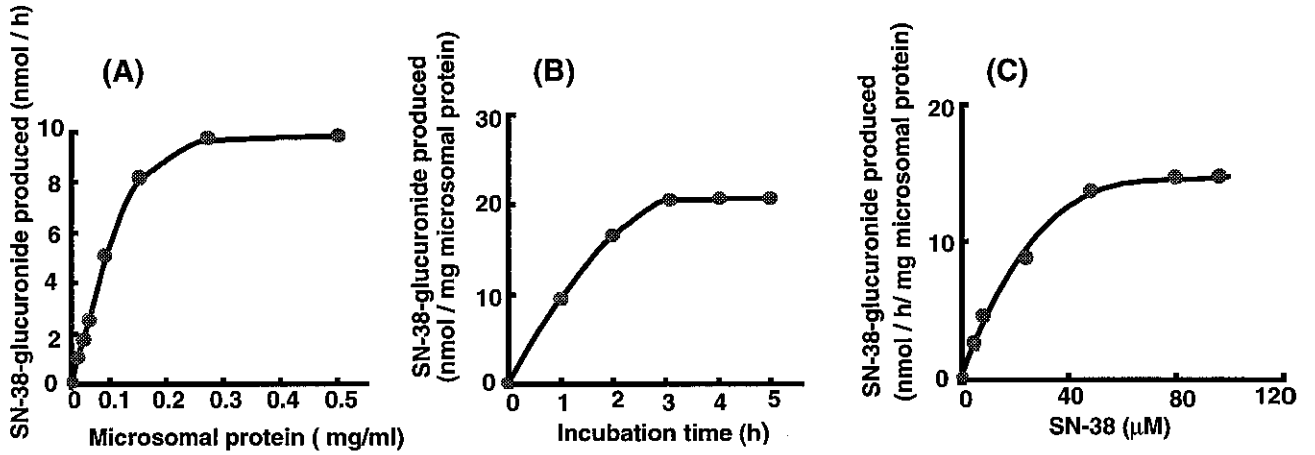

Fig. 2. Formation of SN-38-glucuronide from SN-38 as a function of (A) the amount of microsomes, (B) incubation time, or (C) SN-38 concentration. Unless specified, a standard incubation mixture contained 0.2 *M* Tris-HCl buffer (pH 7.4), 5 m*M* magnesium chloride, 5 m*M* UDP-GA, 0.3 mg/ml microsomal fraction and 100 μM SN-38 as a substrate, and was incubated at 37°C for 60 min.

compounds except glycyrrhetic acid methyl ester were found to inhibit UDP-GT as potently as 1-naphthol (Fig. 3). Genipin, which is an aglycon of a glycoside (Fig. 1), showed no inhibitory activity. The mechanism involved in the inhibition of UDP-GT by these aglycons of natural glucuronides was examined (Fig. 4). Baicalein, glycyrrhetic acid and luteolin inhibited UDP-GT in a mixed type competitive manner. Inhibitory potencies of the aglycons were calculated, and the results are summarized in Table I. Glycyrrhetic acid and luteolin showed IC_{50} and K_i values similar to those of 1-naphthol.

Inhibition of UDP-GT by aglycons using *p*-nitrophenol as a substrate To determine whether the aglycons act as inhibitors of UDP-GT in general, the inhibition was examined using *p*-nitrophenol as a substrate. Glycyrrhetic acid and luteolin inhibited the activity (Fig. 5). Luteolin showed almost the same IC_{50} and K_i values as 1-naphthol (data not shown). SN-38 also inhibited the activity strongly.

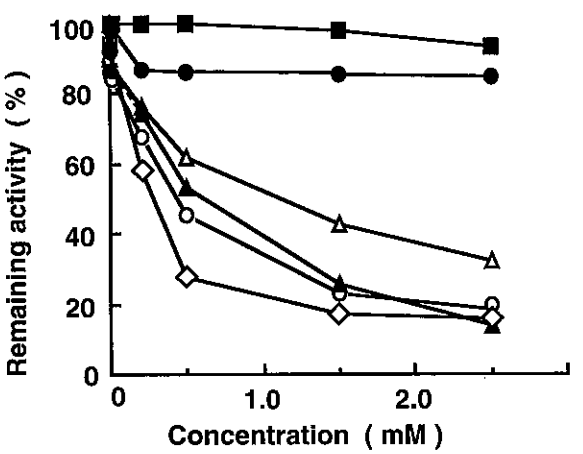

Fig. 3. Inhibition by aglycons of natural glucuronides, genipin and 1-naphthol of UDP-GT with SN-38 as a substrate. Various concentrations of genipin (■), glycyrrhetic acid methyl ester (●), baicalein (△), glycyrrhetic acid (○), luteolin (◇) and 1-naphthol (▲) were added to the incubation mixture containing SN-38. The activity of UDP-GT using SN-38 as a substrate in the absence of an inhibitor was 41.6 nmol of SN-38-glucuronide formed/h per mg microsomal protein, and this was taken as 100%.

DISCUSSION

CPT-11, one of the most potent anticancer agents, has been found to exert significant activity against a variety of tumors such as colon cancer, gastric cancer, melanoma, lung cancer and malignant lymphoma[8] through the inhibition of DNA topoisomerase I.[5] However, this drug also shows certain side-effects, including a decrease in blood cells, especially neutrophils,[8] as well as alopoecia, vomiting, leukopenia, nausea and gastro-intestinal toxicities including diarrhea.[17,18] Among these side-effects, diarrhea is the most serious problem forcing cessation of therapy.

CPT-11 is metabolized to SN-38, which subsequently undergoes glucuronidation in the liver.[1] The glucuronide thus formed is excreted in urine and bile.[6] The SN-38-glucuronide excreted in bile is hydrolyzed by β-glucuronidase present in intestinal microflora to regenerate SN-38. Most of the biological activities of CPT-11, the side-effects as well as the anticancer activity, could be ac-

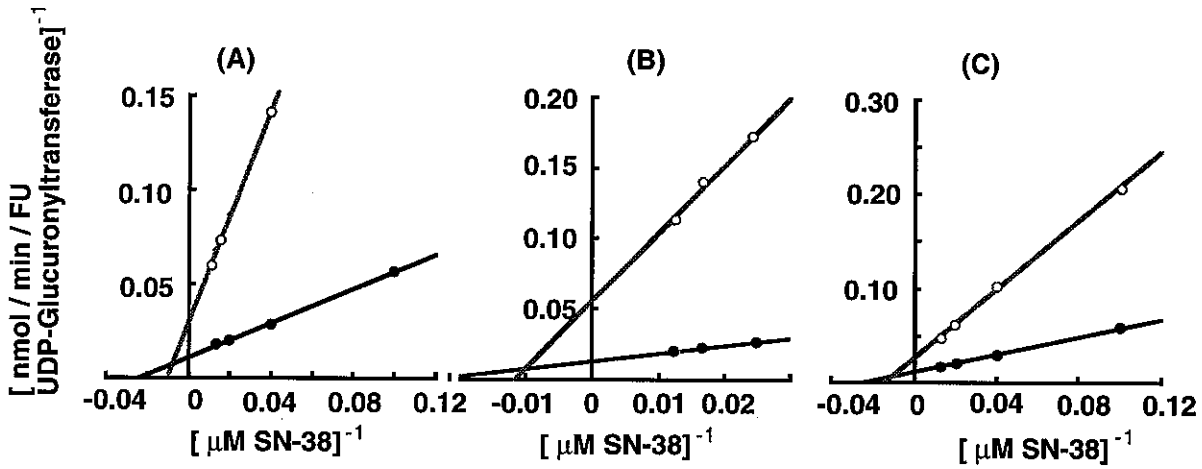

Fig. 4. Lineweaver-Burk plots for inhibition of UDP-GT by aglycons of natural glucuronides. The inhibition pattern was drawn with three or four concentrations of SN-38 (10.0, 25.0, 50.0 and 66.7 μM) as a substrate and (A) glycyrrhetic acid, (B) luteolin and (C) baicalein as inhibitors (○) and without inhibitors (●).

Table I. Kinetic Constants for Inhibition of UDP-GT by Aglycons of Natural Glucuronides Measured as SN-38-glucuronide Production

Inhibitor	IC_{50} (μM)	K_i (μM)
Baicalein	1117	970
Glycyrrhetic acid	440	241
Luteolin	277	446
1-Naphthol	620	690

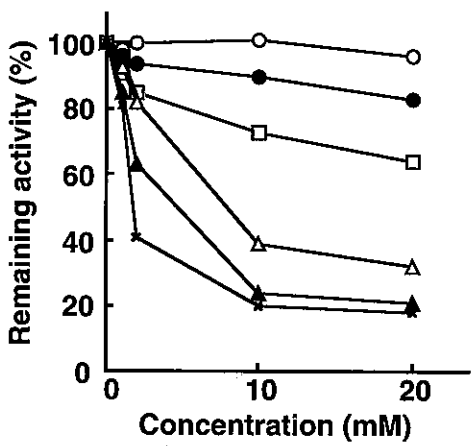

Fig. 5. Inhibition by aglycons of natural glucuronides, genipin, 1-naphthol and SN-38 of UDP-GT activity with *p*-nitrophenol as a substrate. Various concentrations of genipin (○), baicalein (●), glycyrrhetic acid (□), luteolin (△), 1-naphthol (▲) and SN-38 (×) were added to the incubation mixture containing *p*-nitrophenol. The activity of UDP-GT using *p*-nitrophenol as a substrate in the absence of an inhibitor was 11.2 nmol *p*-nitrophenol glucuronide formed/min per mg microsomal protein, and this was taken as 100%.

counted for by the metabolite, SN-38, which has at least 1,000-fold greater antitumor activity than the parental CPT-11.[7,19] The direct cause of diarrhea associated with CPT-11 administration is considered to be enterocolitis caused by high levels of SN-38 retained for a long period in the intestine.[9] In our previous report, we demonstrated that naturally occurring glucuronides inhibit the β-glucuronidase acting on SN-38-glucuronide as a substrate.[20] The inhibitory potency of these glucuronides was similar to that of saccharic acid 1,4-lactone, a known inhibitor of β-glucuronidase. Here we have demonstrated that the corresponding naturally occurring aglycons inhibit UDP-GT, which catalyzes glucuronidation of SN-38 in the liver. Based on these results, we consider that natural aglycons and corresponding glucuronides present in *Kampo* medicines act to inhibit the inactivation of SN-38 in the liver, and to inhibit the regeneration of SN-38, a possible causal factor of diarrhea, in the intestine. Therefore, we propose that a therapeutic concentration of SN-38, concomitantly with reduced side effects, should be obtainable *in vivo* with less amount of CPT-11 is coadministered with some *Kampo* medicines.

Supporting our hypothesis, our preliminary studies have shown that treatment of rats with baicalin or some antibiotics efficiently prevented the diarrhea caused by CPT-11.

Advantages in using the natural glucuronides are that they can be used *in vivo*, since some *Kampo* medicines contain these compounds as ingredients, and that *Kampo* medicines are given to patients orally. The aglycons

examined in the present study are given to patients at doses much greater than the estimated amounts of SN-38 in the liver. Therefore, it seems reasonable to assume that aglycons of natural glucuronides inhibit UDP-GT to prevent the formation of SN-38-glucuronide in the liver.

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