Involvement of Up-Regulation of Hepatic Breast Cancer Resistance Protein in Decreased Plasma Concentration of 7-Ethyl-10-hydroxycamptothecin (SN-38) by Coadministration of S-1 in Rats

Koji Yokoo, Akinobu Hamada, Hiroshi Watanabe, Takanobu Matsuzaki, Tomoyuki Imai, Hiromi Fujimoto, Kengo Masa, Teruko Imai, and Hideyuki Saito

Department of Pharmacy, Kumamoto University Hospital, Kumamoto, Japan (K.Y., A.H., T.M., To.I., H.F., K.M., H.S.); and Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan (H.W., Te.I.)

Received February 13, 2007; accepted May 24, 2007

ABSTRACT:
The safety and efficacy of combination therapy with 7-ethyl-10-[4-\{1-piperidino\}-1-piperidino]carbonyloxycamptothecin (CPT-11, irinotecan) and S-1 composed of tegafur, a prodrug of 5-fluorouracil, gimeracil, and potassium oxonate, have been confirmed in patients with colorectal cancer. Previously, we showed that p.o. coadministration of S-1 decreased the plasma concentration of both CPT-11 and its metabolites in a patient with advanced colorectal cancer. The aim of this study was to clarify the mechanism of drug interaction using both in vivo and in vitro methods. Rats were administered S-1 p.o. (10 mg/kg) once a day for 7 consecutive days. On day 7, CPT-11 (10 mg/kg) was administered by i.v. injection. Coadministration of S-1 affected the pharmacokinetic behavior of CPT-11 and its metabolites, with a decrease in the C_{max} and area under the plasma concentration curve (AUC) of the active metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) lactone form. Furthermore, the rate of biliary excretion of the SN-38 carboxylate form increased on coadministration of S-1. In the liver, the level of breast cancer resistance protein (BCRP), but not P-glycoprotein and multidrug resistance-associated protein 2, was elevated after administration of S-1. Enzymatic conversion of CPT-11 to SN-38 by carboxylesterase (CES) was unaffected by the liver microsomes of rats treated with S-1. In addition, components of S-1 did not inhibit the hydrolysis of p-nitrophenylacetate, a substrate of CES, in the S9 fraction of HepG2 cells. Therefore, the mechanism of drug interaction between CPT-11 and S-1 appears to involve up-regulation of BCRP in the liver, resulting in an increased rate of biliary excretion of SN-38 accompanied by a decrease in the C_{max} and AUC of SN-38.

Irinotecan (CPT-11), an inhibitor of DNA topoisomerase I, is widely used in the treatment of several types of solid tumors, including colorectal cancer (Wiseman and Markham, 1996). CPT-11 exerts its antitumor activity after enzymatic transformation to its more active metabolite, SN-38, by hepatic carboxylesterases (CES) (Fig. 1) (Hammerichkouse et al., 2000). In vitro study suggested that antitumor effects of SN-38 are 100 to 1000 times more potent than those of CPT-11 (Kawato et al., 1991). SN-38 is subsequently conjugated in the liver by UDP glucuronosyltransferase (UGT) 1A, thereby forming the inactive metabolite SN-38 glucuronide (SN-38G) (Fig. 1) (Rivory and Robert, 1995). The biliary-excreted SN-38G is deconjugated by bacterial β-glucuronidases in the intestinal lumen (Takasuna et al., 1996). Furthermore, a portion of CPT-11, SN-38, and SN-38G in bile is reabsorbed from the intestinal lumen to enter an enterohepatic recirculation loop. Biliary excretion is the major elimination route for CPT-11 and its metabolites, with urinary excretion being a less important pathway. P-glycoprotein (P-gp/ABCB1), multidrug resistance-associated protein 2 (MRP2/ABCC2), and breast cancer resistance protein (BCRP/ABCG2), expressed at the bile canalicular membrane, appear to be responsible for the biliary excretion of CPT-11 and its metabolites (Sugiyama et al., 1998; Nakatomi et al., 2001). On the other hand, CPT-11, SN-38, and SN-38G have a labile lactone ring, which can undergo reversible pH-dependent hydrolysis in aqueous solution (Fig. 1) (Rivory et al., 1994). Under acidic conditions the lactone form is the predominant species, whereas under physiological conditions hydrolysis of the lactone yields the carboxylate form. The carboxylate form of SN-38 has much weaker antitumor activity than the lactone form of SN-38 (Rivory et al., 1994). The complex metabolic profile of CPT-11 explains the observed interin...
individual variability in the pharmacokinetics and toxicity of this compound.

S-1 is an oral anticancer agent composed of tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP), and potassium oxonate (Oxo) at a molar ratio of 1:0:4:1 (Shirasaka et al., 1996). FT, a prodrug of 5-fluorouracil (5-FU), is converted to 5-FU mainly in the liver and tumor cells (Ikeda et al., 2000). CDHP enhances the concentration of 5-FU in the plasma and tumor cells by competitive inhibition of dihydroxy-pyrimidine dehydrogenase, which is responsible for the catabolism of 5-FU (Takechi et al., 2002). A side effect of 5-FU is diarrhea, which is caused by its phosphorylation in the intestine by orotate phosphoribosyltransferase. Oxo, a specific inhibitor of orotate phosphoribosyltransferase, is a protective agent against 5-FU–induced diarrhea (Shirasaka et al., 1996). In phase II studies, it was shown that a single treatment with S-1 had a high response rate, ranging from 19 to 39%, and good compliance in patients with advanced colorectal cancer (Ohtsuka et al., 2000; Van den Brande et al., 2003; Shirao et al., 2004).

The safety and efficacy of combination therapy with CPT-11 and S-1 were investigated in a phase II study of patients with colorectal cancer (Yokoo et al., 2006). In particular, coadministration of S-1 resulted in a significant decrease in the plasma concentration of SN-38 compared with a single treatment with CPT-11. However, the mechanisms of this drug interaction have not been elucidated. Therefore, we explored the effect of coadministration of S-1 on the pharmacokinetics of CPT-11 and its metabolites in rats.

**Materials and Methods**

**Materials.** CPT-11, SN-38, and SN-38G were provided by Daiichi Sankyo Co. Ltd. (Tokyo, Japan). S-1 was provided by Taiho Pharmaceutical Co. Ltd. (Tokyo, Japan). p-Nitrophenylacetate (p-NPA) and p-nitrophenol were purchased from Sigma-Aldrich (St. Louis, MO). All the other chemicals were commercially available products and of analytical grade.

**Experimental Animals.** Male Sprague-Dawley rats (7 weeks old) were purchased from Kyudo Co. Ltd. (Kumamoto, Japan). The rats were housed in a standard animal maintenance facility at constant temperature (21–23°C), humidity (50–70%), and a 12-h light/dark cycle for at least 1 week before the day of the experiment. All the animal experiments were conducted according to the guidelines of Kumamoto University for the care and use of laboratory animals.

**Pharmacokinetic and Biliary Excretion Analyses.** Rats were administered p.o. the carboxyl methyl cellulose (CMC) (0.5 w/v%) (without S-1) or S-1 (10 mg/kg) (with S-1) once a day for 7 consecutive days. On day 6, the rats were fasted overnight with free access to drinking water before commencement of the experiments. On day 7, CPT-11 was i.v. administered to rats at 10 mg/kg via the left jugular vein about 1 min after p.o. administration of CMC or S-1. Blood samples were collected from the right jugular vein at 5, 15, 30, and 45 min after injection of CPT-11. Bile was collected from a cannula implanted in the bile duct. Samples were collected at various intervals from the injection of CPT-11 to 5 min, 5 to 15 min, 15 to 30 min, 30 to 45 min, 45 min to 1 h, or more than 1 h. Thereafter, bile was collected over 30-min intervals. The blood samples were centrifuged at 3000g for 5 min, and the plasma and bile samples were stored at −80°C until analysis. The concentration of the carboxylate and lactone forms of CPT-11, SN-38, and SN-38G was measured by high-performance liquid chromatography as described previously (Hamada et al., 2005). Pharmacokinetic parameters for CPT-11, SN-38, and SN-38G were estimated by noncompartmental model methods with the use of WinNonlin version 3.1 software (Pharsight, Cary, NC).

**Inhibition Studies on the Hydrolysis of p-NPA by 9000g Supernatant (SN) of HepG2 Cells.** HepG2 cells cultured for 7 days in 75-cm² culture flasks were washed with ice-cold phosphate-buffered saline (PBS) (without CaCl₂ and MgCl₂) and then removed with a cell scraper. The cells were suspended in 50 mM HEPES buffer (pH 7.4). The sonicated cells were then homogenized under ice-cold conditions. After centrifugation of the cell homogenate at 9000g for 20 min at 4°C, the supernatant (SN) was obtained. Protein content was determined by the method described by Bradford with bovine serum albumin as the standard.

The hydrolysis reaction was initiated by the addition of p-NPA after preincubation (37°C) of SN fraction for 5 min, and the spontaneous rate of hydrolysis was recorded. The enzymatic hydrolysis of p-NPA was followed spectrophotometrically at 400 nm as described previously (Yoshigae et al., 1998). In experiments involving inhibition by CPT-11, FT, 5-FU, CDHP, and Oxo, the enzyme solution of SN fraction was preincubated (37°C) for 5 min with various concentrations of them dissolved in HEPES buffer or without them in controls.

**Hydrolysis of CPT-11 by Rat Liver Microsomes.** Livers were harvested from male Sprague-Dawley rats that had been administrated CMC or S-1 (10 mg/kg). The livers were stored at 80°C until use. Hepatic microsomes were prepared by differential centrifugation. The rat liver homogenates were centrifuged at 9000g for 20 min at 4°C. The supernatant was then centrifuged at 105,000g for 1 h at 4°C. The microsomes were stored at −80°C in PBS (10 mg/ml) until use. Incubations of microsomes with CPT-11 were carried out in PBS at 37°C in 0.5-ml Eppendorf tubes. A stock solution of CPT-11 was prepared directly in PBS, and the pH was corrected to pH 7.4. An aliquot of the enzyme stock (25 µl) was added to 125 µl of PBS. After 5 min of equilibration at 37°C, the reaction was initiated by the addition of the required dilution of the CPT-11 stock (100 µl) to yield concentrations of CPT-11 ranging from 1 to 1000 µM in a total of 250 µl. The tubes were agitated in a shaking water bath maintained at 37°C. Aliquots (50 µl) were withdrawn at regular intervals and immediately mixed with 100 µl of ice-cold methanol to quench the reaction. Samples were then assayed for the total forms (carboxylate form plus lactone form) of SN-38 as detailed earlier.

**Western Blot Analysis.** Livers were homogenized in a homogenization buffer composed of 230 mM sucrose, 5 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml peptatin A. The homogenate was centrifuged twice at 3000g for 15 min, and the supernatant was further centrifuged at 105,000g for 30 min. The resultant pellet is referred to as the crude membrane fraction. After measurement of the protein content using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL), each sample was mixed in a loading buffer (2% SDS, 125 mM...
Tris-HCl, pH 6.4, 20% glycerol, and 5% 2-mercaptoethanol) and heated at 100°C for 2 min. The samples were separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) by semidy electrophoretic transfer. The blots were blocked overnight at 4°C with 2% enhanced chemiluminescence advanced blocking agents (GE Healthcare, Little Chalfont, Buckinghamshire, UK) in Tris-buffered saline containing 0.3% Tween 20 and incubated for 1 h at room temperature with primary antibody specific for P-gp (C219 monoclonal antibody, Signet Laboratories, Inc., Dedham, MA), MRP2 (MRP2 monoclonal antibody, CHEMICON International, Inc., Temecula, CA), and BCRP (ABCG2 polyclonal antibody, Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The blots were washed with Tris-buffered saline containing 0.3% Tween 20 and incubated with the secondary antibody [horseradish peroxidase-linked anti-rabbit immunoglobulin F(ab)'2 or horseradish peroxidase-linked anti-mouse immunoglobulin F(ab)'2; both from GE Healthcare] for 1 h at room temperature. Immunoblots were visualized with an enhanced chemiluminescence system (ECL Advance Western Blotting Detection Kit, GE Healthcare). The relative amount of each band was determined densitometrically using Densitograph Imaging Software (ATTO Corporation, Tokyo, Japan). Densitometric ratios were normalized to the value obtained without S-1.

**Statistical Analysis.** The differences were analyzed statistically using Student's unpaired t test. A value of \( P < 0.05 \) was considered significant.

**Results**

Effect of S-1 on the Pharmacokinetics of CPT-11 and Its Metabolites. Figure 2 shows the plasma concentration-time profiles of the carboxylate form, lactone form, and total form (i.e., carboxylate form plus lactone form) of CPT-11, active metabolite SN-38, and inactive metabolite SN-38G after i.v. administration of CPT-11 at a dose of 10 mg/kg i.v. administration in the absence or presence of S-1 (10 mg/kg/day) in rats. Coadministration p.o. of S-1 appeared to affect the pharmacokinetic parameters of CPT-11, SN-38, and SN-38G (Table 1). In particular, the maximum plasma concentration \( (C_{\text{max}}) \) of the SN-38 lactone form decreased by 30.1% from 144.1 ± 15.8 ng/ml (without S-1) to 100.8 ± 8.9 ng/ml (with S-1). Furthermore, the AUC of the SN-38 lactone form decreased by 33.8% from 235.8 ± 43.6 ng · h/ml (without S-1) to 156.2 ± 15.0 ng · h/ml (with S-1). However, \( C_{\text{max}} \) and AUC of the SN-38 carboxylate form were unchanged by coadministration of S-1, whereas \( C_{\text{max}} \) and AUC of both CPT-11 and SN-38G showed a slight decrease in concentration on coadministration with S-1.

Effect of S-1 on the Biliary Excretion of CPT-11 and Its Metabolites. Figure 3 shows the cumulative biliary excretion curves of the carboxylate form, lactone form, and total forms of CPT-11, active metabolite SN-38, and inactive metabolite SN-38G after i.v. administration of CPT-11 at a dose of 10 mg/kg with or without S-1 (10 mg/kg/day) in rats. Excretion of CPT-11 carboxylate and lactone forms into bile was unaltered by coadministration of S-1. SN-38 was excreted into bile mostly as the lactone form in the absence of S-1, whereas with S-1 treatment, a greater proportion of SN-38 was excreted into bile as the carboxylate form.

**Fig. 2.** Plasma concentration-time profiles of CPT-11 and its metabolites in the absence or presence of S-1. Plasma concentration-time profiles of the carboxylate form (A, D, G), lactone form (B, E, H), and total forms (i.e., carboxylate form plus lactone form) (C, F, I) of CPT-11 (○, ●), SN-38 (△, ▲), and SN-38G (■, □) in rats after receiving CPT-11 at 10 mg/kg i.v. administration in the absence (○, △, □) or presence of S-1 (●, ▲, ■) at a dosage of 10 mg/kg/day. All the data are expressed as mean ± S.D., \( n = 5 \).
SN-38 carbohydrate form, whereas SN-38G was excreted into bile mostly as the SN-38G lactone form. The biliary excretion of both forms of CPT-11 and SN-38G or the SN-38 lactone form was unaltered by administration of S-1. However, the biliary excretion of the SN-38 carbohydrate form was significantly increased by coadministration of S-1. The amount of the SN-38 carbohydrate form excreted via bile significantly increased from 9.7 ± 3.3% (without S-1) to 14.9 ± 3.7% (with S-1) 4 h after coadministration of S-1. In addition, the total amount of the total forms of SN-38 also significantly increased after coadministration of S-1 from 12.4 ± 2.8% (without S-1) to 17.6 ± 1.0% (with S-1).

**Effect of Each Component of S-1 and 5-FU on the Relative Inhibition of p-NPA Hydrolysis.** Kinetic analysis of p-NPA hydrolysis at a range of concentrations (0.1–5.0 mM) was performed (Fig. 4A). The hydrolysis of p-NPA was best fitted by a single Michaelis-Menten equation with a Michaelis-Menten constant (K_m) of 599 μM and maximum hydrolysis rate (V_max) of 8.38 μM/min. The effects of CPT-11, FT, 5-FU, CDHP, and Oxo on p-NPA (500 μM) hydrolysis were examined by incubating with the S9 fraction of HepG2 cells (Fig. 4B). CPT-11 was found to be a potent inhibitor of p-NPA hydrolysis, whereas FT, 5-FU, CDHP, and Oxo did not inhibit p-NPA hydrolysis.

**Effect of S-1 on the Hydrolysis of CPT-11 by Rat Liver Microsomes.** Production of SN-38 from 10 μM CPT-11 over a 2-h incubation period with rat liver microsomes in the absence of S-1 is shown in Fig. 5A. A linear regression of the concentration versus time plot from 15 min onward yielded a steady-state velocity (r² = 0.99). The kinetics of production of the total forms of SN-38 from CPT-11 at a range of concentrations (0.1–300 μM) was investigated at 1 h (Fig. 5B). The K_m and V_max of hydrolysis of CPT-11 with rat liver microsomes were unchanged by administration of S-1 for 7 consecutive days. The K_m and V_max values for rat liver microsomes in the absence or presence of S-1 were 4.08 μM and 466 nM/h or 5.23 μM and 472 nM/h, respectively.

**Effect of S-1 on P-gp, MR2, and BCRP Protein Expression in the Liver.** Potential changes to the expression of P-gp, MR2, and BCRP protein in the crude plasma membrane from rat liver in the absence or presence of S-1 were explored by Western blot analyses (Fig. 6A). The presence of S-1 significantly increased the level of BCRP protein in the crude plasma membrane preparation of the rat liver. The relative amount of BCRP with S-1 was about 1.6-fold higher than that without S-1. By contrast, administration of S-1 had no significant effect on the P-gp and MR2 protein levels.

**Discussion**

The efficacy of combination therapy with various anticancer agents has now been generally recognized. However, there is little information on the pharmacokinetic interaction of combination chemotherapy. Recently, we reported the pharmacokinetic changes of CPT-11 and its metabolites in a patient with colorectal cancer given S-1 p.o. (Yokoo et al., 2006). Here, we investigated the effect of S-1 on the pharmacokinetics of CPT-11 and its metabolites in rats to explore the possible mechanism of drug interaction.

Our results show that p.o. coadministration of S-1 causes a significant decrease in the plasma concentration of an active metabolite, SN-38. A previous pharmacokinetic study of patients has now been generally recognized. However, there is little information on the pharmacokinetic interaction of combination chemotherapy. Recently, we reported the pharmacokinetic changes of CPT-11 and its metabolites in a patient with colorectal cancer given S-1 p.o. (Yokoo et al., 2006). Here, we investigated the effect of S-1 on the pharmacokinetics of CPT-11 and its metabolites in rats to explore the possible mechanism of drug interaction.
was reported that the biliary excretion and the intestinal exsorption of CPT-11 or SN-38 were significantly inhibited by coadministration of cyclosporin in rats (Arimori et al., 2003).

Figure 3 shows that the rate of biliary excretion of the SN-38 carboxylate form increased on coadministration of S-1. The total amount of SN-38 carboxylate form excreted in the absence or presence of S-1 was 9.7 and 14.9% of the dose 4 h after administration, respectively ($P < 0.01$). Figure 6 shows that the BCRP protein level in crude plasma membrane from the liver with S-1 was 1.6-fold higher than that without S-1 ($P < 0.05$). On the other hand, the mRNA level of BCRP was measured in both the intestine and kidney with or without S-1, and we found that the mRNA level was not affected by administration of S-1 in both tissues (data not shown). Therefore, an increase in the level of BCRP protein may result in increased biliary excretion of the SN-38 carboxylate form. In previous studies, sulfasalazine, various aryl hydrocarbon receptor agonists, and progesterone were found to significantly increase the level of BCRP protein in vitro (van der Heijden et al., 2004; Ebert et al., 2005; Wang et al., 2006). By contrast, 17$\beta$-estradiol significantly decreases the level of BCRP protein in vitro (Wang et al., 2006). However, there are few previous reports of drug-related regulation of BCRP protein levels in vivo (Han and Sugiyama, 2006). Here, we report the S-1–mediated induction of BCRP protein in vivo. Our study shows that the rate of biliary excretion of the SN-38 carboxylate form is significantly increased by coadministration of S-1, whereas that of the SN-38 lactone form is unaltered. These results suggest BCRP may display a higher affinity for the SN-38 carboxylate form relative to the SN-38 lactone form. Further studies are required to confirm this hypothesis.

Although the rate of biliary excretion of the SN-38 carboxylate form increased on coadministration of S-1, $C_{\text{max}}$ and AUC of the
SN-38 lactone form decreased. The predominant CPT-11 metabolite in rat liver is the SN-38 carboxylate form rather than the SN-38 lactone form. Four hours after i.v. administration of CPT-11 in the absence of S-1, the concentration of the SN-38 carboxylate and lactone forms in the liver was 0.7 ± 0.2 and 0.2 ± 0.1 ng/g of tissue, respectively. Thus, the ratio of the carboxylate or lactone forms of SN-38 to the total amount of SN-38 was 77.8 and 22.2%, respectively. Similar results were obtained after coadministration of CPT-11 with S-1 (data not shown). These results suggest that the induction of the BCRP protein level by coadministration of S-1 increased the rate of biliary excretion of the SN-38 carboxylate form, followed by hydrolytic cleavage of the lactone, yielding the carboxylate form of SN-38 in the liver. The lower rate of secretion of the SN-38 lactone form from liver to blood compared with that of the SN-38 carboxylate form could explain the lower $C_{\text{max}}$ and AUC of the SN-38 lactone form.

Previous studies of drug interaction between CPT-11 and 5-FU suggested that 5-FU inhibits the enzymatic hydrolysis of CPT-11 to SN-38 in humans and rats (Sasaki et al., 1994; Umezawa et al., 2000). However, the hydrolysis of p-NPA was not inhibited by components of S-1 and 5-FU (Fig. 4). Furthermore, administration of S-1 did not affect the hydrolysis activity of rat liver microsomes (Fig. 5). Therefore, inhibition of the hydrolysis activity by coadministration of S-1 cannot account for the pharmacokinetic parameters of SN-38.

The piperidine ring of CPT-11 is oxidized to 7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]carbonyloxy camptothecin and 7-ethyl-10-(4-amino-1-piperidino)carbonyloxy camptothecin by CYP3A4 and CYP3A5 in the liver (Santos et al., 2000). A decrease in the $C_{\text{max}}$ and AUC of SN-38 lactone form could be caused by enhanced activity of CYP3A on coadministration of S-1. Indeed, a previous study showed that chronic p.o. administration of FT (100 mg/kg daily for 20 days) resulted in the induction of CYP3A in rat liver (Yamazaki et al., 2001). However, no increase in the concentration of CYP3A-derived metabolites of CPT-11 could be detected (data not shown). Therefore, we conclude that the effect of coadministration of S-1 on CYP3A activity can be ignored.

SN-38 is conjugated with glucuronic acid by hepatic UGT1A to form the inactive metabolite SN-38G (Rivory and Robert, 1995). Induction or activation of UGT1A by treatment with S-1 is a potential mechanism for the observed drug interaction. If this is the case, we may anticipate an increase in the plasma concentration of SN-38G. However, we found that the plasma concentration of SN-38G was
lower on coadministration of S-1, suggesting that S-1 has no effect on UGT1A activity.

In summary, we have shown significant drug interaction between CPT-11 and S-1 in rats. In particular, Cmax and AUC of the SN-38 lactone form were markedly reduced by coadministration of S-1. Induction of BCRP protein was observed in the rat liver with S-1, resulting in an increased rate of biliary excretion of the SN-38 carboxylate form. We suggest our data reveal the underlying mechanism of drug interaction between CPT-11 and S-1 that has been observed in previous clinical case reports. Further studies are needed to establish the most effective regimen for combination therapy based on the pharmacokinetic and pharmacodynamic data.

References


Address correspondence to: Hideyuki Saito, Department of Pharmacy, Kumamoto University Hospital, 1-1-1 Honjo, Kumamoto 860-8556, Japan. E-mail: saitohide@fc.kuh.kumamoto-u.ac.jp