Reduced Gastrointestinal Toxicity following Inhibition of the Biliary Excretion of Irinotecan and its Metabolites by Probenecid in Rats

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Purpose. To ameliorate the late-onset of severe gastrointestinal toxicity provoked by irinotecan (CPT-11), which may be related to the biliary excretion of CPT-11 and/or its metabolites.

Methods. Effects of probenecid, an inhibitor of MRP2/ABCC2, on the biliary excretion and mucosal intestinal tissue concentration of CPT-11 and its metabolites were examined in rats. CPT-11-induced late-onset gastrointestinal toxicity was also evaluated.

Results. Coadministration of probenecid reduced the biliary excretion of CPT-11, an active metabolite (SN-38) and its glucuronide by half with a concomitant increase in their plasma concentration. When the dose of CPT-11, in the presence of probenecid, was set at half that in its absence, the plasma SN-38 concentration was maintained at the same level as the control, whereas the mucosal intestinal tissue concentration of SN-38 was reduced. Under this condition, CPT-11-induced watery diarrhea, changes in intestinal marker enzymes and body weight reduction were much less in the probenecid-treated group, although the degree of bone marrow suppression was almost the same as that in the control.

Conclusions. Coadministration of probenecid with a reduced dose of CPT-11 potently reduces both SN-38 exposure and CPT-11-induced late-onset toxicity in gastrointestinal tissues, possibly by inhibiting the biliary excretion of CPT-11 and/or its metabolites.

KEY WORDS: CPT-11; SN-38; probenecid; biliary excretion; MRP2.

INTRODUCTION

Irinotecan hydrochloride, 7-ethyl-10-[10-4-(1-piperidino)-1-piperidino] -carbonyloxy- camptothecin, CPT-11, is an anticancer agent that exhibits its activity by interfering with mammalian DNA topoisomerase I (1). CPT-11 is a prodrug that is split into the primary active metabolite, SN-38 (7-ethyl-10-hydroxycamptothecin), by the enzyme carboxylesterase (2). SN-38 subsequently undergoes glucuronic acid conjugation to form the corresponding glucuronide, SN-38 glucuronide (SN38-Glu) (3). The major dose-limiting toxicity of CPT-11 is myelosuppression and late-onset severe diarrhea (4). Such severe diarrhea, however, exhibits a large degree of interpatient variability (5–6) and does not always respond adequately to conventional antidiarrheal agents (7). Because biliary excretion is a major elimination pathway for CPT-11 and its metabolites, several hypotheses for the mechanism of this diarrhea involve the biliary excretion of CPT-11 and/or its metabolites.

Gupta *et al.* reported that the excessive biliary excretion of SN-38, which follows low-glucuronidation activity in the liver, might cause this diarrhea (8). They also demonstrated that the biliary index (the product of the relative area ratio of SN-38 to SN38-Glu multiplied by that of CPT-11 under the plasma concentration-time curve) correlates with the degree of diarrhea in humans (8).

Another theory for the mechanism of this intestinal toxicity involves SN-38 production via deconjugation of SN38-Glu by β-glucuronidase of the intestinal microflora (9–10). Takasuna *et al.* examined the possible inhibitory effect of a Chinese herbal medicine (Hange-Shashin-To, Tsumura Co. Ltd.; Tokyo, Japan) on CPT-11-induced chronic diarrheal symptoms and verified the efficacy of the method, which may be based on a competitive inhibition of β-glucuronidase by baicalin, a major component of the Chinese herbal medicine (11).

Furthermore, the parent compound itself is mainly excreted into bile both in rats (>32%) and humans (>26%) (12–13), and it has also been demonstrated that CPT-11 is converted to SN-38 by human intestinal carboxylesterase (CEs) (14). Therefore, the intestinal toxicity may be due, at least in part, to direct drug conversion by CEs present within the small intestine.

In the light of these hypotheses, it is likely that inhibition of the biliary excretion of CPT-11, and its metabolites could reduce the severity of this adverse effect. We have previously shown that CPT-11 is excreted mainly via P-glycoprotein and, to a much lesser degree, via canalicular multispecific organic anion transporter/multidrug resistance associated protein-2 (MRP2/ ABCC2), whereas a large part of the transport of SN-38 and SN38-Glu is mediated by MRP2 (15–17). Therefore, MRP2 is a possible target. Among several MRP2 inhibitors, probenecid inhibits the transport of methotrexate by MRP2 on canalicular membranes with an inhibition constant (K_i) of 50 μM (18), which is within the range of the observed free plasma concentration of probenecid in clinical situations (19). Therefore, in the present study, we examined the inhibitory effect of this compound on the biliary excretion of CPT-11 and its metabolites, with the aim of reducing the late-onset diarrhea observed following CPT-11 administration.

MATERIALS AND METHODS

Materials

CPT-11, SN-38 and SN38-Glu were obtained from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan) and Yakult Honsha Co. Ltd. (Tokyo, Japan). Probenecid, ATP, AMP, creatine phosphate, and creatine phosphokinase were purchased from Sigma (St. Louis, MO, USA). Benemid (containing 250 mg probenecid/tablet) was purchased from Banyu Pharmaceutical Co. Ltd. (Tokyo, Japan). [^{3}H] taurocholate

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ABBREVIATIONS: CPT-11, irinotecan; SN-38, 7-ethyl-10-hydroxycamptothecin; SN38-Glu, SN-38 glucuronide; CMV, canalicular membrane vesicles; AUC, area under the plasma concentration time curve.

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(3.47 μCi/nmol) was purchased from New England Nuclear (Boston, MA, USA). [^{3}H] S-(2,4-dinitrophenyl) glutathione (50.0 μCi/nmol) was synthesized by the method of Saxena and Henderson (20). [^{14}C] thymidine (50.0 μCi/μmol) was purchased from NEN Life Science Products, Inc. (Bevererly, MA, USA). All other chemicals were commercial products of analytical grade.

Isolation of canalicular membrane vesicles (CMVs)

Male Sprague-Dawley rats (250–320 g body weight) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). CMVs from rats were prepared as described previously (16).

The purity of the prepared CMVs was checked by determining the activity of alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (γ-GTP) using, respectively, the method of Yachi *et al.* (21) and an assay kit for γ-GTP (Wako Pure Chemical Industries, Ltd.; Osaka, Japan). The ALP and γ-GTP enrichment factors in CMVs compared with the corresponding activity in liver homogenate were 60.3 ± 6.4 and 48.5 ± 11.2 (mean ± SE, n = 5), respectively. The activity of CMVs used in the present study was also checked by measuring the ATP-dependent uptake of standard substrates, [^{3}H] taurocholic acid (1 μM) and [^{3}H] S-(2,4-dinitrophenyl) glutathione (1 μM), in a 2-min incubation performed at 37°C. These values were 85.6 ± 6.8 and 60.1 ± 7.4 μl/min/mg protein, respectively (n = 3).

Uptake Study by CMVs

The uptake study using the carboxylate forms of CPT-11, and its metabolites were performed as reported previously (15–17).

In Vivo Study

Inhibition of the Biliary Excretion of CPT-11 and Its Metabolites

The surgical operation was performed according to Chu *et al.* (16). Probenecid dissolved in 100 mM phosphate buffered saline (PBS) was infused into a femoral vein through a catheter at a rate of 37.7 mg/4 mL/hr/kg until the end of the experiments after a 12.8 mg/kg loading dose. As a control experiment, 100 mM PBS was administered in the same manner. One hour after starting the probenecid infusion, CPT-11 (5 mg/2 mL saline/kg) was injected as a bolus dose. Aliquots of approximately 0.3 mL blood were collected at 0.0833, 0.5, 2, 4, and 6 h. Bile samples were collected at 0 to 1, 1 to 2, 2 to 3, 3 to 4, 4 to 5, and 5 to 6 h. The concentrations of CPT-11 and its metabolites were determined by HPLC as described below.

Amelioration of Gastrointestinal Toxicity of CPT-11 *in Vivo*

Benemid tablets were ground down in a triturator and suspended in 0.5%methylcellulose (0.5% MC). Probenecid (300 mg/kg) was administered orally twice daily (9:00 AM and 5:00 PM) from 1 day before the start of CPT-11 injection at an IV dose of 10 mg/2 mL saline/kg via a penile vein five times daily (10:00 AM, 1:00, 4:00, 7:00, and 10:00 PM) for 4 successive days (days 1–4) and one-time-only on day 5 (10:00 AM). In our preliminary study, this dose of probenecid resulted in a plasma unbound concentration at 8 h after administration of 38 μM, which was comparable to that found in clinical situations (~50 μM), (18, 19). For comparison, four other groups received the following treatments: vehicle (0.5% MC) + saline, probenecid + saline, vehicle + CPT-11 (10 mg/kg), and vehicle + CPT-11 (20 mg/kg). The incidence and severity of diarrhea was monitored throughout the experimental period (days 0–7). Diarrhea starting after day 3 was defined as delayed diarrhea. The severity of this delayed diarrhea was scored as follows: 0) normal stools, 1) wet and unformed stools, 2) watery stools. To monitor the plasma concentration of CPT-11 and its metabolites, plasma samples were collected 2 h after the second CPT-11 administration for 4 days (days 1–4) and 0.0833, 2, 4, 8, and 24 h after the final CPT-11 administration (day 5). In addition, to estimate the change in hematological parameters, other blood samples were collected at day 4 or day 6, 2 h after the third administration of CPT-11. The numbers of blood cells in the samples were counted in an automatic hemocytometer (Toa-iyou Co. Ltd.; Kakogawa, Japan). The aspartate amino transferase (AST) and blood urea nitrogen (BUN) values were determined using assay kits for AST and BUN (Wako Pure Chemical Industries, Ltd.), respectively. At the end of the experiments (day 7), rats were sacrificed, and their intestines were excised. The excised intestinal tract was opened longitudinally and gently rinsed with saline to remove the luminal contents. The mucosa was scraped off and homogenized with 2 mL 100 mM Tris-HCl buffer (pH 8.0) on ice. Samples were then centrifuged at 100,000 × g for 60 min at 4°C. The activity of alkaline phosphatase (ALP), a marker enzyme for the villus area, and the activity of thymidine kinase (TK), a marker enzyme for the crypt area, were estimated by the procedure of Takasuna *et al.* (7) and Klemperer *et al.* (22), respectively.

In the first experiment, we measured the body weight, diarrheal score, activities of ALP and TK, numbers of the different types of blood cells (full blood count), and plasma concentration of each compound in the vehicle (0.5% MC) + saline, probenecid + saline, probenecid + CPT-11 (10 mg/kg), and vehicle + CPT-11 (20 mg/kg) treatment groups. In a similar fashion, a second experiment was performed in which we measured the body weight, diarrheal score, plasma concentration of AST and BUN, and plasma concentration of each compound in the vehicle + saline, probenecid + saline, probenecid + CPT-11 (10 mg/kg), vehicle + CPT-11 (10 mg/kg), and vehicle + CPT-11 (20 mg/kg) treatment groups. Data obtained from these two experiments were integrated and are presented below.

Determination of the Concentration of CPT-11 and Its Metabolites in the Gastrointestinal Tract

The surgical operation was also performed according to Chu *et al.* (16). Probenecid was infused into a femoral vein as described above. An hour after reaching steady state, CPT-11 was infused at a rate of 150 μg/min/kg for 4 h. As a control experiment, two other groups received a PBS infusion and a CPT-11 infusion (150 and 300 μg/min/kg). Blood and urine samples were collected at 0.0833, 1, 2, 3, 4 h and 0–1, 1–2, 2–3, and 3–4 h, respectively. At the end of the experiments, rats were sacrificed and the duodenum, jejunum, ileum, and colon were excised collecting lengths of 10, 15, 15, and 10 cm, respectively. The excised tissue from each part of the intestinal

tract was rinsed with 10, 15, 15, and 10 mL of 100 mM phosphate buffer (pH 7.4), respectively. The washed intestinal tracts were opened longitudinally and mucosa was scraped off with a glass slide and homogenized with 2, 3, 3, and 2 mL cold 100 mM phosphate buffer (pH 7.4), respectively, on ice. The concentration of tissue homogenate from each intestinal segment was determined, and the obtained value was corrected by the weight of mucosa scrapped off from the original tissues. This concentration of mucosa was expressed as the mucosal concentration of each segment.

HPLC Analysis

Determination of CPT-11 and its metabolites was carried out by HPLC as described previously (16) with a minor modification of the HPLC system. Briefly, the column was a J'sphere ODS-H80 column (250 × 4.6 mm inner diameter, YMC Co. Ltd.; Kyoto, Japan) with a C-KGC-H80-3 guard column (YMC). The mobile phase consisted of acetonitrile/50 mM phosphate buffer (pH 6.0) (solvent A; 10/90, solvent B; 30/70). The total elution time was 25 min (0–7 min; solvent A 100%, 7.1–22 min; solvent B 100%, 22.1–25 min; solvent A). The flow rate was 1.0 mL/min.

Data Analysis

The AUC values for CPT-11 and its metabolites (SN-38 and SN38-Glu) were calculated by the trapezoidal rule. The inhibition constant (K_i) of probenecid for the SN-38 transport in CMVs was obtained by fitting the following equation:

$$V_{(+I)}/V_{(-I)} = 1/(1 + I_u/K_i) \quad (1)$$

where $V_{(+I)}$ and $V_{(-I)}$ represent the transport velocity in the presence and absence of inhibitor, respectively: I_u is the inhibitor concentration in the medium (μM). This equation was derived based on the assumption of competitive or noncompetitive inhibition and the fact that the SN-38 concentration (5 μM) was much lower than its K_m value (69 μM) (15). The K_i for SN38-Glu transport was obtained by fitting the following equation:

$$V_{(+I)}/V_{(-I)} = 1/[1 + I_u \cdot K_m/K_i \cdot (K_m + S)] \quad (2)$$

where S is the substrate (SN38-Glu) concentration (5 μM) and K_m was set at 2.77 μM (15). The above fitting was performed by an iterative nonlinear least-squares method using a MULTI program (23) to obtain estimates of the K_i values.

Statistical Analysis

The results are shown as mean ± SE values for the number of determinations. For most comparisons, results were analyzed by student's *t* test or ANOVA using Dunnett's multiple comparisons for significance. Wilcoxon's rank sum test was also used to determine the significance of differences in diarrheal score between day 0 and the other days. P values of less than .05 or .01 were considered statistically significant.

RESULTS

Inhibition of the Biliary Excretion of CPT-11 and Its Metabolites by Probenecid

The biliary excretion of CPT-11, SN-38, and SN38-Glu was reduced by the coadministration of probenecid, whereas the plasma concentrations of these compounds were increased (Fig. 1). The $AUC_{(0-6hr)}$ of SN-38 was 0.288 ± 0.014 and 0.198 ± 0.028 μg · h/mL in the presence and absence of probenecid, respectively. Probenecid reduced the ATP-dependent uptake of SN-38 and SN38-Glu in a concentration-dependent manner (Fig. 2B, 2C) with a K_i of 35.7 and 22.5 μM, respectively. The effect of probenecid on the transport of CPT-11 was minimal (Fig. 2A).

Effect of Probenecid on the Exposure of Intestinal tissues to CPT-11 and Its Metabolites

The mucosal tissue concentrations of CPT-11 and its metabolites were determined during the IV infusion of CPT-11 (Table I). At 150 μg/min/kg of CPT-11, a significant reduction in concentration by probenecid was found only for SN-38 in the ileum and colon. Almost similar plasma SN-38 concentrations were found when comparing CPT-11 (150 μg/min/kg) + probenecid and CPT-11 (300 μg/min/kg) alone (Table I). Comparing these two conditions, the mucosal tissue concentration and mucosa-to-plasma concentration ratio of CPT-11 and its metabolites were significantly lower under the former condition for the majority of intestinal tissues (Table I).

Effects of Probenecid on Hepatic and Renal Concentration and Renal Excretion of CPT-11 and Its Metabolites

At 150 μg/min/kg of CPT-11, probenecid reduced the renal excretion of SN-38, whereas its effect on that of CPT-11 and SN38-Glu was minimal (Table II). The effects of probenecid on the hepatic and renal concentrations of CPT-11 were insignificant, whereas probenecid produced a one and a half- to two-fold increase in the hepatic concentration of SN-38 and the hepatic and renal concentrations of SN38-Glu (Table I). In both the liver and kidney, the tissue-to-plasma concentration ratio of SN-38 at 150 μg/min/kg of CPT-11 in the presence of probenecid was not very different (at most a 40% difference) from that in its absence and that following administration of CPT-11 (300 μg/min/kg) alone (Table I).

Effects of Probenecid on the Gastrointestinal Toxicity and Pharmacokinetics of CPT-11 and Its Metabolites

Injection of CPT-11 (20 mg/kg) alone resulted in severe (grade 2) late-onset diarrhea during day 4 through to day 7 (Table III). Injection of CPT-11 (10 mg/kg) alone induced diarrhea in some rats, the diarrheal score being at most 1 (Table III). When probenecid was coadministered, a diarrheal score of 1, but not 2, was also observed (Table III). The diarrheal score for CPT-11 (10 mg/kg) + probenecid was not obviously different from that for CPT-11 (10 mg/kg) alone, but significantly different from that for CPT-11 (20 mg/kg) alone (Table III). In this toxicology study, the plasma SN-38 concentration profile was almost the same for CPT-11 (10 mg/kg) + probenecid and CPT-11 (20 mg/kg) alone (Fig. 4).

In control rats, the body weight increased continuously, whereas the body weight of rats treated with CPT-11 (10 mg/kg) alone started to fall at day 2–3, and this reduction was more marked for CPT-11 (20 mg/kg) alone (Fig. 3). Coadministration of probenecid ameliorated this body weight loss (Fig. 3), although probenecid alone suppressed the increase of body weight to some extent (Fig. 3), possibly due to an adverse-effect of probenecid itself.

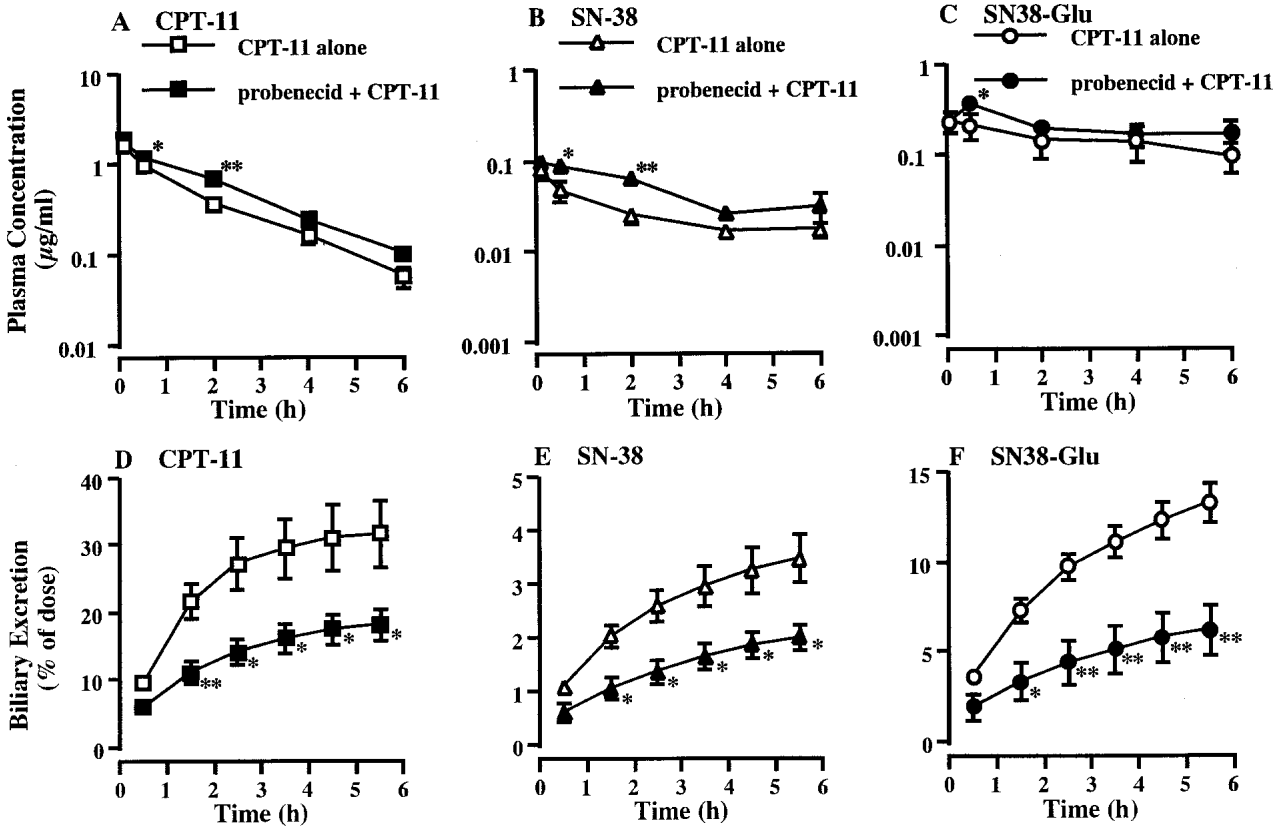

Fig. 1. Effects of probenecid on the plasma concentration (A, B, C) and biliary excretion (D, E, F) of CPT-11 (A, D), SN-38 (B, E) and SN38-Glu (C,F). Probenecid was administered intravenously at a rate of 0 (open symbol) or 37.7 (closed symbol) mg/hr/kg and CPT-11 was administered intravenously as a 5 mg/kg bolus. Each data point represents the mean ± SE of 5 different rats. *$p < 0.05$, **$p < 0.01$, significantly different from the control (student's *t*-test).

At the end of this experiment, the activity of the marker enzymes ALP and TK in intestinal tissues was determined (Table IV). Injection of CPT-11 (20 mg/kg) alone reduced ALP activity in both the ileum and colon and increased TK activity in the duodenum, jejunum and ileum compared with the injection of vehicle plus saline (Table IV). On the other hand, injection of CPT-11 (10 mg/kg) with probenecid resulted in only a minimal change in these enzyme activities (Table IV). The white blood cell (WBC) count was reduced by CPT-11 (20 mg/kg) alone and CPT-11 (10 mg/kg) + pro-

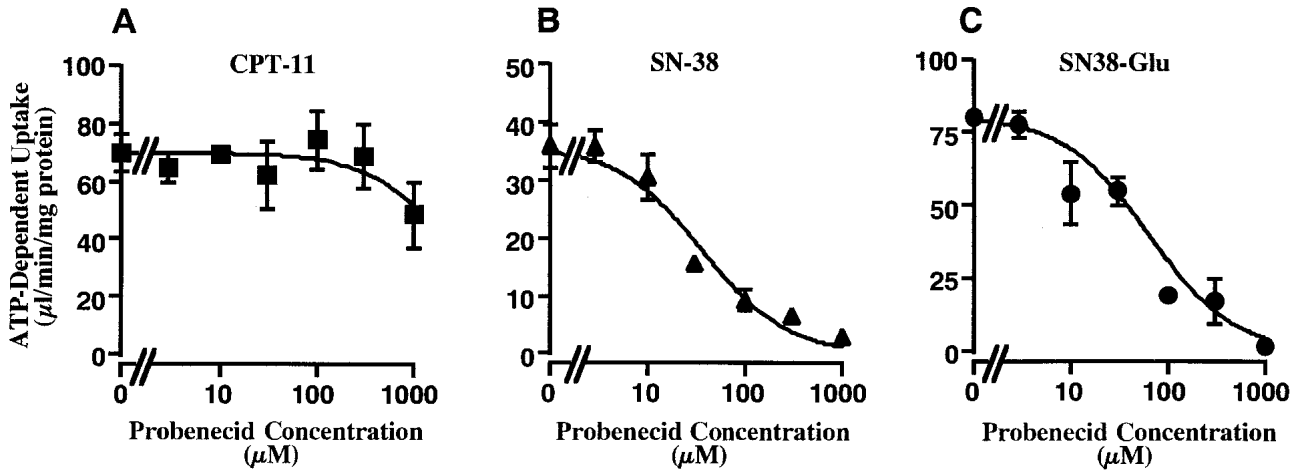

Fig. 2. Inhibition of the primary active transport of CPT-11 and its metabolites by probenecid in CMVs. CMVs were incubated for 2 min with 5 μM of the carboxylate forms of CPT-11 (■), SN-38 (▲) and SN38-Glu (●) in the presence of the indicated concentration of probenecid. The ATP-dependent uptake was calculated by subtracting the uptake in the absence of ATP from that in its presence. The solid lines represent the fitted curves based on Eqs. (1) for SN-38 and (2) for SN38-Glu. Each data point represents the mean ± SE of 3 experiments from 6 preparations.

Table I. Concentration of CPT-11 and Its Metabolites in Mucosal Intestinal Tissue, Liver, Kidney, and Plasma during the Intravenous Infusion of CPT-11[a]

i) CPT-11

Treatment	Infusion rate[b] (μg/min/kg)	n	Duodenum	Jejunum	Ileum	Colon	Liver	Kidney	Plasma (4 h)[d]
			Concentration (μg/g tissue or μg/ml plasma)						
Vehicle	150	5	43.8 ± 12.2	44.6 ± 19.3	33.9 ± 12.6	24.0 ± 12.8	46.2 ± 7.7	48.9 ± 15.3	2.74 ± 0.41
Probenecid	150	5	15.8[f] ± 4.7	14.0[f] ± 6.9	20.3 ± 12.8	12.5[f] ± 7.4	55.2[f] ± 8.6	54.1[f] ± 8.2	4.29[f] ± 0.20
Vehicle	300	5	77.1 ± 23.3	63.0 ± 19.0	75.1 ± 22.8	73.3 ± 19.3	125 ± 14	169 ± 39	6.34 ± 1.01
			The Mucosa-to-Plasma Concentration Ratio[c]						
Vehicle	150	5	19.3 ± 6.6	21.8 ± 11.4	16.0 ± 7.9	9.81 ± 4.68	16.6 ± 0.5	15.4 ± 3.3	
Probenecid	150	5	3.77[e,f] ± 1.13	3.44 ± 1.67	4.81 ± 2.99	2.98[f] ± 1.74	13.7 ± 3.0	12.9[f] ± 2.2	
Vehicle	300	5	11.5 ± 3.1	10.7 ± 3.5	11.8 ± 3.2	11.4 ± 2.6	20.5 ± 1.4	27.5 ± 6.0	

ii) SN-38

Treatment	Infusion Rate (μg/min/kg)	n	Duodenum	Jejunum	Ileum	Colon	Liver	Kidney	Plasma (4 h)
			Concentration (μg/g tissue or μg/ml plasma)						
Vehicle	150	5	0.251 ± 0.074	0.308 ± 0.077	0.791 ± 0.139	0.393 ± 0.084	0.243 ± 0.020	0.119 ± 0.012	0.480 ± 0.0070
Probenecid	150	5	0.0993[f] ± 0.0199	0.112 ± 0.037	0.155[e,f] ± 0.060	0.0863[e,f] ± 0.0298	0.409[e] ± 0.010	0.281 ± 0.080	0.0944[e] ± 0.0094
Vehicle	300	5	0.475 ± 0.117	0.608 ± 0.271	1.60 ± 0.27	0.837 ± 0.191	0.472 ± 0.076	0.212 ± 0.026	0.104 ± 0.016
			The Mucosa-to-Plasma Concentration Ratio						
Vehicle	150	5	5.26 ± 1.83	6.73 ± 1.86	16.8 ± 1.7	8.08 ± 0.94	5.74 ± 1.23	2.70 ± 0.19	
Probenecid	150	5	1.05[f] ± 0.20	1.14[e] ± 0.36	1.69[e,f] ± 0.62	0.926[e,f] ± 0.306	4.43 ± 0.35	3.35 ± 1.26	
Vehicle	300	5	4.73 ± 1.32	5.88 ± 2.85	15.8 ± 2.8	8.02 ± 1.50	4.68 ± 0.91	2.09 ± 0.26	

iii) SN38-Glu

Treatment	Infusion Rate (μg/min/kg)	n	Duodenum	Jejunum	Ileum	Colon	Liver	Kidney	Plasma (4 h)
			Concentration (μg/g tissue or μg/ml plasma)						
Vehicle	150	5	0.695 ± 0.330	1.11 ± 0.54	1.97 ± 0.77	0.812 ± 0.462	0.947 ± 0.042	0.828 ± 0.171	0.292 ± 0.029
Probenecid	150	5	0.288[f] ± 0.095	0.437[f] ± 0.225	0.580 ± 0.367	0.221[f] ± 0.098	1.50[e] ± 0.09	1.67[e,f] ± 0.10	0.508 ± 0.090
Vehicle	300	5	1.14 ± 0.17	3.98 ± 0.89	2.69 ± 1.43	1.07 ± 0.26	1.61 ± 0.22	2.09 ± 0.12	0.545 ± 0.015
			The Mucosa-to-Plasma Concentration Ratio						
Vehicle	150	5	2.39 ± 1.05	3.73 ± 1.71	6.80 ± 2.63	2.69 ± 1.44	3.39 ± 0.40	3.03 ± 0.81	
Probenecid	150	5	0.779[f] ± 0.323	1.27[f] ± 0.77	1.77 ± 1.25	0.629[f] ± 0.328	3.28 ± 0.52	3.69 ± 0.62	
Vehicle	300	5	2.09 ± 0.32	7.44 ± 1.77	4.82 ± 2.51	1.96 ± 0.44	2.94 ± 0.31	3.85 ± 0.27	

[a] After a 4 h infusion of CPT-11, each tissue was washed and the mucosal tissue was resected, the amount of each compound being determined. Data are represented as mean ± SE.
[b] Infusion rate of CPT-11.
[c] The ratio of the concentration in tissue to that in circulating plasma.
[d] Plasma concentration of CPT-11 and its metabolites 4 hours after the start of CPT-11 infusion.
[e] $p < .05$; significantly different from vehicle + CPT-11 (150 μg/min/kg) treatment (student's *t*-test).
[f] $p < .05$; significantly different from vehicle + CPT-11 (300 μg/min/kg) treatment (student's *t* test).

Table II. Renal Excretion of CPT-11 and Its Metabolites during Intravenous Infusion of CPT-11[a]

Treatment	Infusion rate[b] (μg/min/kg)	n	CPT-11	SN-38	SN38-Glu
			Urinary Excretion Rate (μg/min/kg)		
Vehicle	150	5	16.1 ± 3.3	0.497 ± 0.077	3.10 ± 0.39
Probenecid	150	5	21.9 ± 5.2	0.234[d,e] ± 0.038	3.71[e] ± 1.01
Vehicle	300	5	32.9 ± 2.8	0.711 ± 0.134	6.29 ± 1.07
			Urinary Excreted Amount/AUC (mL/min/kg)[c]		
Vehicle	150	5	7.51 ± 0.93	11.4 ± 2.5	12.8 ± 1.9
Probenecid	150	5	8.72 ± 1.72	2.86[d,e] ± 0.35	9.60 ± 2.77
Vehicle	300	5	8.85 ± 1.31	8.98 ± 2.16	13.7 ± 2.0

[a] Data are represented as mean ± SE.
[b] Infusion rate of CPT-11.
[c] Cumulative excreted amount into the urine divided by AUC from time 0 to 4 h.
[d] $p < .05$; significantly different from vehicle + CPT-11 (150 μg/min/kg) treatment (student's *t*-test).
[e] $p < .05$; significantly different from vehicle + CPT-11 (300 μg/min/kg) treatment) student's *t*-test).

benecid, the fall being fairly similar for each treatment (Table V). Injection of CPT-11 (20 mg/kg) alone slightly increased the AST, a marker for hepatic function, whereas the effect of CPT-11 (10 mg/kg) + probenecid on this enzyme was minimal (Table V). The effect of each treatment on red blood cell (RBC) and platelet (PLT) counts and BUN was minimal under the experimental conditions used (Table V).

DISCUSSION

Due to the unpredictable severe diarrhea observed in patients treated with CPT-11, the clinical use of this anticancer agent has remained limited. Because it has been proposed that the severe gastrointestinal toxicity results from exposure of intestinal tissues to SN-38, due to its biliary excretion and/or deconjugation of SN38-Glu (15,16), we investigated a potential strategy to inhibit their biliary excretion to reduce exposure of the intestines to SN-38. Probenecid has been used to treat gout for a long time, and it inhibits various types of organic anion transporting systems (24–26). With this in mind, we examined the use of probenecid as an inhibitor of the biliary excretion of SN-38 and SN38-Glu to demonstrate the importance of biliary excretion in late-onset diarrhea induced following injection of CPT-11 in rats.

Probenecid inhibits the biliary excretion of both SN-38 and SN38-Glu (Fig. 1) and the primary active transport of these compounds, which is mediated by MRP2 (15, 16), in CMVs (Fig. 2). Considering that the biliary excretion rate of SN-38 and SN38-Glu was reduced (Fig. 1) whereas their hepatic concentrations were increased (Table I), the biliary clearance defined with respect to the hepatic concentration should also be reduced by probenecid, suggesting that the excretion of these compounds across the bile canalicular membrane is inhibited by probenecid. In addition, in our previous analysis, we determined the unbound probenecid concentration in both plasma and liver and found this to be 59 and 42 μM, respectively, at a probenecid infusion rate of 37.7 mg/hr/kg (18). Because these concentration values were com-

Table III. Incidence of Chronic Diarrheal Symptoms Caused by CPT-11, with or without Probenecid, in Rats[a]

Treatment	Dose[b] (mg/kg)	n	(Delayed diarrheal score)[c,d] Day 0			Day 1			Day 2			Day 3			Day 4			Day 5			Day 6			Day 7		
			0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2	0	1	2
Vehicle + Saline (mean)[e]	0	12	12	0	0	12	0	0	12	0	0	12	0	0	12	0	0	12	0	0	12	0	0	12	0	0
			(0.00)			(0.00)			(0.00)			(0.00)			(0.00)			(0.00)			(0.00)			(0.00)		
Probenecid + Saline (mean)	0	12	12	0	0	12	0	0	12	0	0	12	0	0	12	0	0	12	0	0	12	0	0	12	0	0
			(0.00)			(0.00)			(0.00)			(0.00)			(0.00)			(0.00)			(0.00)			(0.00)		
Vehicle + CPT-11 (mean)	10	6	6	0	0	6	0	0	6	0	0	6	0	0	4	2	0	3	3	0	3	3	0	3	3	0
			(0.00)			(0.00)			(0.00)			(0.00)			(0.333)			(0.500)			(0.500)			(0.500)		
Probenecid + CPT-11 (mean)	10	12	12	0	0	12	0	0	12	0	0	12	0	0	10	2	0	9	3	0	9	3	0	10	2	0
			(0.00)			(0.00)			(0.00)			(0.00)			(0.167)			(0.250)			(0.250)			(0.167)		
Vehicle + CPT-11 (mean)	20	12	12	0	0	12	0	0	12	0	0	10	2	0	1	3	8	0	0	12	0	0	12	0	0	12
			(0.00)			(0.00)			(0.00)			(0.167)			(1.58[f,g])			(2.00[f,g])			(2.00[f,g])			(2.00[f,g])		

[a] Probenecid was given orally at dose of 300 mg/kg twice daily for 5 consecutive days (day 0–5).
[b] Dose of CPT-11. CPT-11 was given intravenously 5 times daily for 4 consecutive days (day 1–4).
[c] Diarrheal score was defined as follows: 0, no diarrhea; 1, diarrhea; 2, watery diarrhea.
[d] Number of rats for each score.
[e] The average of diarrheal score.
[f] $p < .05$; significantly different from vehicle + saline treatment (non-parametric Dunnett's post hoc test).
[g] $p < .05$; significantly different from day 0 (Wilcoxons rank sum test).

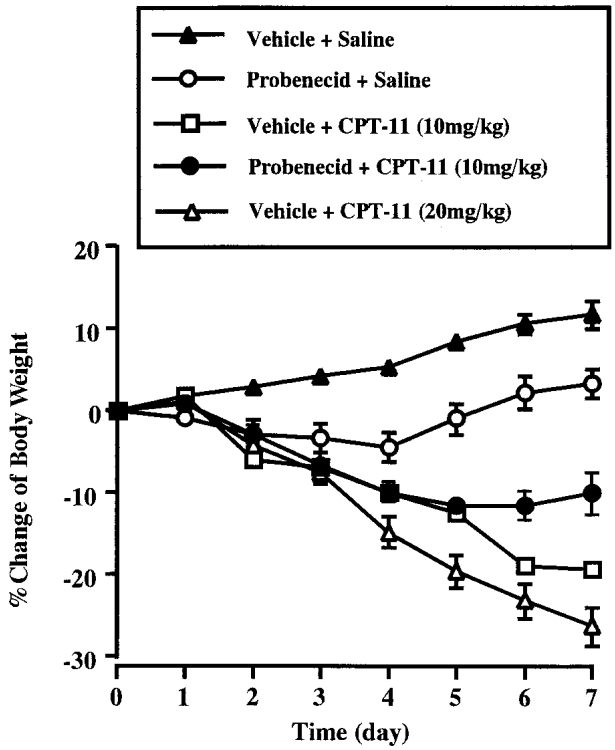

Fig. 3. Effect of probenecid on body weight loss in rats during and after repeated administration of CPT-11.Probenecid was given orally at 0 (▲, □, △) or 300 (○, ●) mg/kg twice daily before and throughout CPT-11 administration at 0 (▲, ○), 10 (□, ●), or 20 (△) mg/kg 5 times daily. Each data point represents the mean ± SE of 6–12 different rats.

parable to the K_i for the transport of both SN-38 and SN38-Glu found in Fig. 2, it is reasonable to assume that the inhibition of the biliary excretion of both SN-38 and SN38-Glu is due, at least partially, to inhibition at the bile canalicular membranes.

Next, we attempted to examine whether or not this strategy ameliorates the gastrointestinal toxicity of CPT-11. Because probenecid also increases the systemic exposure to SN-38 (Fig. 1), the use of this compound in clinical situations may result in an increase in other dose-limiting toxicities including myelosuppression. Here, we attempted to compare the gastrointestinal toxicity by setting the dose of CPT-11 in the presence of probenecid (10 mg/kg) at half that in its absence (20 mg/kg) (Tables III, IV, Fig. 3). Because the plasma concentration profile of SN-38 in this toxicity study was almost the same for CPT-11 (10 mg/kg) + probenecid and CPT-11 (20 mg/kg) alone (Fig. 4), the systemic exposure to SN-38 under these two sets of conditions is almost identical. Nevertheless, the observed gastrointestinal toxicity, assessed as the diarrheal score, body weight change and marker enzyme activity, was much less with the former dose regimen than with the latter (Tables III, IV, Fig. 3). Thus, coadministration of probenecid with a reduced dose of CPT-11 results in the observed amelioration of gastrointestinal toxicity with a minimal change in the systemic SN-38 concentration profile. The degree of myelosuppression was almost identical under these two sets of conditions (Table V). This is reasonable when we consider the similar plasma concentration profiles of SN-38 in these groups (Fig. 4). Concerning the possible increase in hepatic and/or renal exposure to SN-38 due to the inhibition of biliary and renal excretion (Tables I, II, Fig. 1), the tissue-to-plasma concentration ratio of SN-38 was not affected as much by probenecid (Table I), indicating a parallel change in both the plasma and tissue concentrations. Treatment with

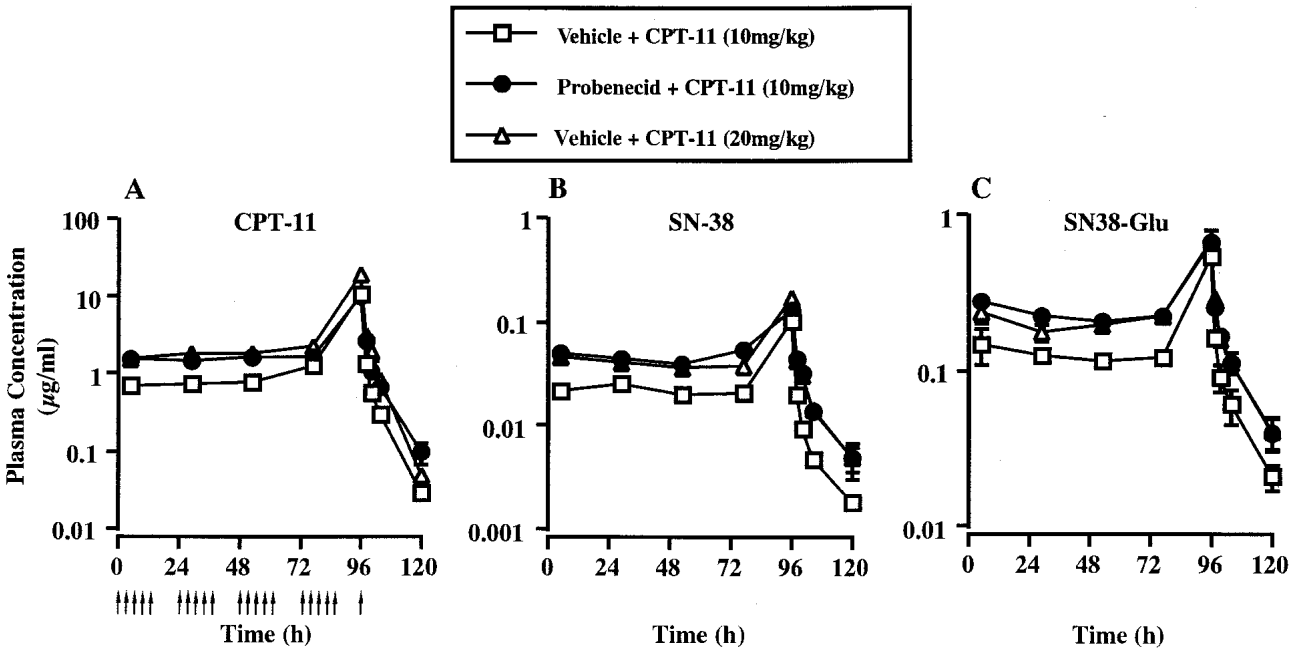

Fig. 4. Plasma concentrations of CPT-11 (A), SN-38 (B) and SN38-Glu (C) during and after repeated administration of CPT-11. Probenecid was given orally at 0 (□, △) or 300 (●) mg/kg twice daily before and throughout CPT-11 administration at 10 (□, ●) or 20 (△) mg/kg 5 times daily. Each data point represents the mean ± SE of 6–12 different rats. Each arrow below the figure indicates the administration time of CPT-11 (1st; 10:00 AM, 2nd; 1:00, 3rd; 4:00, 4th; 7:00, 5th; 10:00 PM).

Table IV. Change in Intestinal Marker Enzymes Caused by CPT-11, with or without Probenecid, in Rats[a]

Treatment	Dose[b] (mg/kg)	n	ALP[c] Activity (nmol/min/mg protein)			
			Duodenum	Jejunum	Ileum	Colon
Vehicle + Saline	0	6	919 ± 78	592 ± 22	94.7 ± 16.4	135 ± 12
Probenecid + Saline	0	6	732 ± 75	599 ± 73	94.3 ± 11.6	132 ± 8
Vehicle + CPT-11	10	6	ND[d]	ND	ND	ND
Probenecid + CPT-11	10	6	687 ± 62	543 ± 85	135 ± 17	112 ± 10
Vehicle + CPT-11	20	6	640 ± 115	417 ± 91	33.1[f] ± 5.1	49.7[f] ± 4.9
Experimental Treatment	Dose (mg/kg)	n	TK[e] Activity (pmol/min/mg protein)			
			Duodenum	Jejunum	Ileum	Colon
Vehicle + Saline	0	6	6.25 ± 0.57	5.43 ± 1.03	4.37 ± 0.31	4.53 ± 0.25
Probenecid + Saline	0	6	4.71 ± 0.49	5.10 ± 0.94	3.37 ± 0.16	5.94 ± 0.99
Vehicle + CPT-11	10	6	ND	ND	ND	ND
Probenecid + CPT-11	10	6	5.64 ± 0.88	3.65 ± 0.56	4.10 ± 0.34	6.23 ± 1.27
Vehicle + CPT-11	20	6	14.0[f] ± 2.1	11.8[f] ± 1.5	11.5[f] ± 0.8	7.55 ± 1.29

[a] Data are represented as mean ± SE.
[b] Dose of CPT-11.
[c] Alkaline phosphatase.
[d] Not determined.
[e] Thymidine kinase.
[f] $p < .05$; significantly different from vehicle + saline treatment (Dunnett's post hoc test).

CPT-11 (10 mg/kg) + probenecid did not show an increase in AST, whereas a slight increase was found in the case of CPT-11 (20 mg/kg) alone (Table V), suggesting that the hepatotoxicity may not be due to the use of probenecid with a lower dose of CPT-11.

The inhibition of processes other than biliary excretion by probenecid might also be involved. In the present study, the amount of CPT-11 excreted into the bile in unchanged form accounts for ~30% of the dose up to 6 h (Fig. 1D) whereas the biliary clearance (cumulative excretion divided by the AUC from time 0 to 6 h) of CPT-11 was reduced to 37.7% of the control by probenecid with a minimal effect of probenecid on the renal clearance (Table II). Therefore, approximately 20%of the increase in CPT-11 concentration in the circulating plasma produced by probenecid can be explained by the inhibition of its biliary excretion. The actual increase in the $AUC_{(0-6h)}$ (Fig. 1) and plasma concentration (Table I) of CPT-11 produced by probenecid was 34–57% of the control. Therefore, inhibition of the metabolic pathway for CPT-11 may also partially account for such an increase. Slatter *et al.* reported that probenecid inhibits esterase activity in human hepatic microsomes as far as CPT-11 is concerned, with 25 μM probenecid reducing it to 87% of the control (27). Considering the higher plasma unbound concentration of probenecid found in the present study, it may be that the esterase activity is partially inhibited by probenecid. On the other hand, the reason for the increase in the plasma concentration of SN-38 produced by probenecid (Table I, Fig. 1) is likely to be complex since this concentration can be affected by many factors, including esterase and glucuronide formation, and biliary and urinary excretion of SN-38. Considering that the plasma concentration of CPT-11 is also increased by probenecid (Table I, Fig. 1), one possible reason may be simply that the esterase-mediated formation of SN-38

Table V. Change in Hematologic Parameters Caused by CPT-11, with or without Probenecid, in Rats[a]

Treatment	Dose[b] (mg/kg)	n	WBC[c] ($\times 10^2$ cells/μl)	RBC[d] ($\times 10^4$ cells/μl)	PLT[e] ($\times 10^4$ cells/μl)	AST[f] (IU/l)	BUN[g] (mg/dl)
Vehicle + Saline	0	6	84.0 ± 6.7	437 ± 35	54.2 ± 4.7	51.3 ± 5.3	8.29 ± 0.23
Probenecid + Saline	0	6	84.0 ± 3.8	534 ± 27	71.7 ± 8.6	57.9 ± 4.6	10.9 ± 1.4
Vehicle + CPT-11	10	6	ND[h]	ND	ND	50.5 ± 2.3	9.35 ± 0.87
Probenecid + CPT-11	10	6	36.4[i] ± 6.6	431 ± 72	63.8 ± 3.6	61.2 ± 10.6	8.64 ± 1.00
Vehicle + CPT-11	20	6	34.3[i] ± 2.9	551 ± 82	59.4 ± 9.6	80.9[i] ± 2.9	8.80 ± 1.02

[a] Data are represented as mean ± SE.
[b] Dose of CPT-11.
[c] Numbers of white blood cells determined at day 6.
[d] Numbers of red blood cells determined at day 6.
[e] Numbers of blood platelet cells determined at day 6.
[f] Aspartate amino transferase level in plasma determined at day 4.
[g] Blood urea nitrogen level in plasma determined at day 4.
[h] Not determined.
[i] $p < .05$; significantly different from vehicle + saline treatment (Dunnett's post hoc test).

is increased due to the increase in CPT-11 concentration. However, if we assume inhibition of esterase activity by probenecid as discussed above, we should also consider a possible reduction in the systemic clearance of SN-38. SN-38 formed in the body is mainly recovered as the glucuronide, because both the urinary and biliary excretion of SN38-Glu is much higher than that of SN-38 (Table II, Fig. 1). Therefore, the systemic elimination of SN-38 can mainly be accounted for by glucuronide formation. Thus, inhibition of biliary and urinary SN-38 excretion (Fig. 1, Table II) cannot fully account for the increase in plasma SN-38 concentration, and glucuronide formation may be inhibited by probenecid. Considering these complex factors, we need to consider the possible inhibition of both esterase activity and glucuronide formation by probenecid to explain all these experimental results.

The reduction in the renal excretion of SN-38 by probenecid suggests the existence of a transport mechanism for SN-38. This hypothesis is in agreement with our previous report that the renal clearance of the carboxylate form of SN-38 exhibited saturation when the dose of CPT-11 was increased and such clearance at a lower dose was reduced in Eisai hyperbilirubinemic rats, which have a hereditary deficiency in MRP2. Considering that MRP2 is also expressed in the kidney (28), further studies are needed to examine its possible function as an excretion mechanism for SN-38.

In conclusion, the present study demonstrates that the coadministration of probenecid with a reduced dose of CPT-11 lowers the late onset gastrointestinal toxicity found during treatment with CPT-11 by inhibiting the biliary excretion and subsequent exposure of intestinal tissues to SN-38 without any significant effect on both systemic SN-38 concentration and bone marrow suppression.

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