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ORIGINAL ARTICLE

Streptomycin alleviates irinotecan-induced delayed-onset diarrhea in rats by a mechanism other than inhibition of β-glucuronidase activity in intestinal lumen

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Abstract Irinotecan hydrochloride (CPT-11) is a useful drug for cancer chemotherapy but sometimes induces severe diarrhea clinically. CPT-11 is mainly activated to SN-38 by carboxylesterase (CES) and then detoxified to SN-38 glucuronide (SN-38G) by UDP-glucuronosyltransferase (UGT) in the liver. SN-38G is excreted via bile and de-conjugated to SN-38 by β-glucuronidase (β-GLU) in the intestinal content. In order to clarify the alleviative effect of antibiotics on CPT-11-induced diarrhea, we examined whether penicillin G and streptomycin (SM) alleviate CPT-11-induced delayed-onset diarrhea using three diarrheal models, i.e., Wistar rats with repeated dosing of CPT-11 (60 mg/kg/day i.v. for 4 consecutive days) and Wistar and Gunn rats with a single dosing of CPT-11 (200 and 20 mg/kg i.v., respectively). Gunn rats have an inherited deficiency of UGT1A and cannot conjugate SN-38 to SN-38G. Therefore, onset of CPT-11-induced diarrhea in Gunn rats is not affected by β-GLU activity. SM alleviated diarrhea in all three diarrheal models. The alleviation of diarrhea by SM in Gunn rats indicated that the effect of SM occurred by a mechanism other than the inhibition of β-GLU activity. SM decreased CPT-11 and/or SN-38 concentrations in intestinal tissues and alleviated epithelial damage from the ileum to colon. SM did not inhibit β-GLU activity in the cecal content. SM also inhibited the intestinal absorption of CPT-11 and decreased CES activity and increased UGT activity in the intestinal epithelium. These findings indicated that SM decreased the exposure of CPT-11 and SN-38 to the intestinal epithelium by inhibiting the absorption of CPT-11 from the intestinal lumen and the change of CES and UGT activities in the intestinal epithelium and alleviated delayed-onset diarrhea.

Keywords Irinotecan hydrochloride · CPT-11 · Diarrhea · Streptomycin · Intestinal absorption

Abbreviations

CPT-11	Irinotecan hydrochloride
SN-38	7-ethyl-10-hydroxycamptothecin
SN-38G	SN-38 glucuronide
CES	Carboxylesterase
UGT	UDP-glucuronosyltransferase
β-GLU	β-glucuronidase
C_{max}	Maximum concentration
AUC	Area under the concentration–time curve
MRT	Mean resident time
CL_{tot}	Total clearance
Vd_{ss}	Volume of distribution at steady state
$t_{1/2}$	Half-life
IS	Internal standard
i.v.	Intravenous(ly)
p.o.	Oral(ly)
SM	Streptomycin sulfate
PG	Penicillin G potassium
SI	Small intestine

Introduction

Irinotecan hydrochloride (CPT-11), a water-soluble derivative of camptothecin [6, 18], is used clinically to treat colorectal, gastric, lung, uterine cervical, and ovarian cancers, malignant lymphoma, and other malignancies [5,

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23, 29, 30, 34]. However, CPT-11 sometimes causes severe adverse effects, such as diarrhea and myelosuppression. These dose-limiting adverse effects prevent the continuation of CPT-11-based chemotherapy [1, 5, 21, 22, 25, 29].

Diarrhea usually appears in acute and/or delayed-onset settings [26]. The mechanism of acute diarrhea is assumed to be that the cholinergic activity of CPT-11 stimulates intestinal contractility and disturbs normal intestinal absorptive and secretory functions [7, 13, 23]. Acute diarrhea is transient and can be suppressed with atropine [7, 26]. In contrast, delayed-onset diarrhea is so severe that it can be sometimes life-threatening. A possible explanation for delayed-onset diarrhea is that 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of CPT-11, damages the intestinal epithelium both structurally and functionally [8, 9, 14, 17, 23, 31, 32].

CPT-11 is mainly activated to SN-38 by carboxylesterase (CES) in the human liver and in the liver and plasma of rats and mice. SN-38 is detoxified to SN-38 glucuronide (SN-38G) by UDP-glucuronosyltransferase (UGT) in the liver, and CPT-11 and its metabolites are excreted via bile and urine [3, 12]. SN-38G excreted via bile is de-conjugated to SN-38 in the cecum and large intestine by β-glucuronidase (β-GLU) produced by the intestinal microflora [32, 33]. It has been reported that pre-treatment with a combination of streptomycin and penicillin G alleviated CPT-11-induced diarrhea and potently suppressed β-GLU activity of the intestinal luminal content [32, 33]. These findings suggest that SN-38 de-conjugated from SN-38G by β-GLU in the cecal and colonal content directly damages the intestinal epithelium and induces delayed-onset diarrhea.

We previously reported that a much lower dose of CPT-11 than in normal rats induced severe diarrhea in Gunn rats [24], which have an inherited deficiency of UGT1A and cannot conjugate SN-38 to SN-38G. Therefore, the onset of CPT-11-induced diarrhea in Gunn rats is not affected by β-GLU activity. If the inhibition mechanism of the antibiotics on diarrhea induction described previously is correct, antibiotics will not affect diarrhea induced by CPT-11.

In the present study, we found the pre-treatment of streptomycin alleviated CPT-11-induced delayed-onset diarrhea in Wistar and Gunn rats and proposed a new mechanism of SM alleviation other than the inhibition of β-GLU in the intestine.

Materials and methods

Materials and reagents

CPT-11 (Lot 115126, 115234, 115A100, 115A197), SN-38 (Lot 300917R), and SN-38G (Lot 970326, MS0366) were provided by Yakult Honsha Co. (Tokyo, Japan). Camptothecin was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium 1-decanesulfonate was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Penicillin G potassium (PG) and streptomycin sulfate (SM) were purchased from Wako Pure Chemicals (Tokyo, Japan). The water was of Milli-Q grade (Millipore Co., Bedford, MA), and all other chemicals were of analytical or HPLC grade obtained from commercial sources.

Animals

Male Wistar and Gunn rats were purchased at 6 and 7 weeks old from Japan SLC (Hamamatsu, Japan), respectively, and were used for experiments after at least 1 week of acclimatization with free access to tap water and commercial animal chow (F-2; Funabashi Farm, Funabashi, Japan). Body weights of Wistar and Gunn rats were 156–189 g and 186–230 g at the start of antibiotics treatment and 185–232 g and 206–254 g at the start of CPT-11 administration, respectively.

Animal operation

In studies using Wistar rats in the single dosing model except biliary excretion and in the study of intestinal absorption of CPT-11, the rats were cannulated into the right cervical vein (Intramedic PE-50; Clay Adams, Parsippany, NI) under anesthesia with pentobarbital on Day −2 and were kept in free-movement cannulation (FMC) cages (Tsumura, Tokyo, Japan) with free access to an ordinary diet and tap water with or without antibiotics.

In the biliary excretion study, rats were cannulated into the right femoral vein and bile duct (PE-50 and PE-10, respectively) under light ether anesthesia before CPT-11 administration on Day 0 and placed in Bollman cages with free access to an ordinary diet and water.

Antibiotics and CPT-11 treatment

The dosing schedule of CPT-11 and antibiotics in three rat models is shown in Fig. 1.

PG at 1 mg/mL, SM at 2 mg/mL, or a combination of both antibiotics at 1 and 2 mg/mL, respectively (PG/SM), was given in drinking water to rats from Day −5 to 6 or from Day −5 to −1.

In Wistar rats in the repeated dosing model, CPT-11 was i.v. administered at a dose of 60 mg/kg once daily as a bolus injection via the tail vein from Day 0 to 3 (CPT). Vehicle (physiological saline) instead of CPT-11 was administered at 3 mL/kg to the rats with no treatment (Control group) and treatment of only PG/SM (PG/SM group).

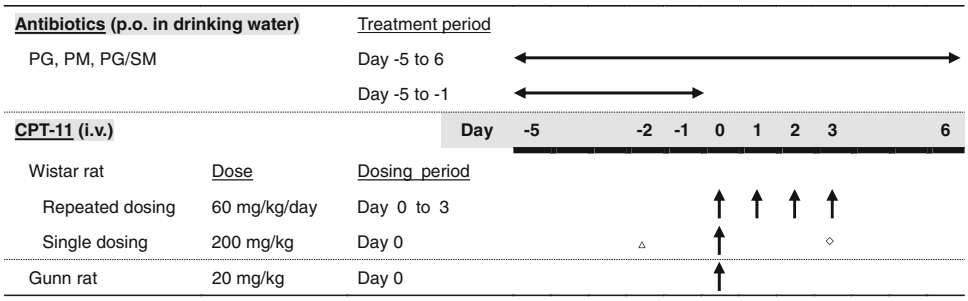

Fig. 1 Dosing schedule of CPT-11 and antibiotics in the monitoring study of delayed-onset diarrhea. *PG*: penicillin G, *SM*: streptomycin, *open triangle*: cannulation into the right cervical vein, *open diamond*: dissection for histological analysis of intestine

In Wistar rats in the single dosing model, CPT-11 was i.v. administered at a dose of 200 mg/kg as 0.5-h infusion via the cervical vein cannula by a syringe pump (Harvard Apparatus, Holliston, MA) on Day 0 (CPT).

In the Gunn rat model, CPT-11 was i.v. administered at a dose of 20 mg/kg as a bolus injection via the tail vein on Day 0 (CPT).

Monitoring of diarrhea

All rats after administration of CPT-11 or vehicle were monitored for weight and feces condition throughout the experimental period. In Wistar rats in the single dosing model, the rats were transferred from FMC to normal cages 6 h after the start of CPT-11 infusion. Diarrhea within 6 h after the start of CPT-11 administration was defined as acute diarrhea and later as delayed-onset diarrhea. The severity of diarrhea was scored as follows: 0, normal; 1, soft feces or small black feces; 2, muddy feces; 3, watery feces or mucous feces.

Histological and hematological analyses in Wistar rats in the single dosing model

Animals were dissected on Day 3. The upper small intestine (SI) (duodenum and jejunum), lower SI (ileum), cecum, and colon were removed immediately after exsanguination and fixed in 10% neutral-buffered formalin. After fixation, the tissues were embedded in paraffin, sectioned about 5 μm thick, and stained with hematoxylin-eosin for histopathological examination.

Pharmacokinetics after i.v. administration of CPT-11 in Wistar rats in the single dosing model

To examine plasma concentration profiles, 0.2 mL of blood was sampled via the cervical vein cannula at 1, 2, 4, 8, 12, 24, and 48 h after the start of CPT-11 infusion. The plasma was separated immediately after blood sampling. The plasma was rapidly diluted fivefold with 0.15 M H_3PO_4 and then added to an equal volume of internal standard (IS) solution (0.15 M H_3PO_4 containing 1 μg/mL camptothecin as IS).

To measure the concentrations in intestinal tissues and contents, animals were killed by exsanguination at 3, 6, 12, 24, and 48 h after the start of CPT-11 infusion. Their intestines were removed immediately and cooled in cold physiological saline. The intestine was then divided into five parts (upper, middle, and lower SI, cecum, colon), and the intestinal content of each part was collected separately. Proximal sections of the upper, middle, and lower SI (corresponding to the duodenum, jejunum, and ileum, respectively) and center sections of the cecum and colon (approximately 0.1 g for each section) were collected. Each intestinal tissue section and intestinal content was homogenized with a fivefold volume of 0.15 M H_3PO_4 on ice with a Teflon homogenizer and sonicator (frequency; 20 kHz, amplitude; 250 μm, output; 10 W, time; 30 s, Model 450 Sonifier; Branson Ultrasonics Co., Danbury, CT), respectively. The homogenate was mixed with a fourfold volume of cold methanol and centrifuged at 15,000 rpm for 5 min. The supernatant was diluted fivefold with IS solution.

To examine biliary excretion, CPT-11 was infused via the femoral vein cannula after waking completely from anesthesia. Bile was collected on ice for 24 h after the start of CPT-11 infusion. The bile was diluted 500-fold with water and then added to an equal volume of IS solution.

Intestinal absorption of CPT-11

CPT-11 was dissolved with water at 20 mg/mL and mixed with a fourfold volume of the bile collected from untreated rats. The mixture (5 mL/kg) was administered orally on Day 0 to rats with no treatment or SM treatment from Day −5 to −1 (SM1), or Day −7 to −3 (SM2), and 0.2 mL of blood was sampled via the cervical vein cannula at 0.5, 1, 2, 4, 8, and 12 h after CPT-11 administration.

Metabolic enzyme assay

Microsomes of the liver and SI epithelium were prepared from animals with or without SM treatment for 5 days. SI epithelium was scraped from the intestine with slide glass.

Tissues were homogenized in 1.15% KCl on ice by a Teflon homogenizer, followed by centrifugation at 9,000×*g* for 10 min at 4°C. The supernatant was re-centrifuged at 105,000×*g* for 60 min at 4°C, resulting in sedimentation of the microsome. The microsomal pellet was re-suspended in 1.15% KCl. To measure CES activity, microsomes (protein content: 1 mg/mL) were incubated with CPT-11 (100 μg/mL) in phosphate-buffered saline (PBS) at 37°C, and 50 μL aliquots were sampled up to 20 min. To measure UGT activity, microsomes (protein content: 1 mg/mL) were incubated with SN-38 (5 μM) and UDP-glucuronic acid (5 mM) in a 0.1 M Tris–HCl buffer medium (pH 7.4) containing $MgCl_2$ (10 mM) at 37°C, and 50-μL aliquots were sampled up to 60 min [11].

To measure *β*-GLU activity in intestinal content, cecal contents were obtained from animals with no treatment, SM, or PG/SM treatment for 5 days. Cecal contents were suspended in PBS, followed by centrifugation at 3,000 rpm for 15 min at 4°C, and the supernatants were obtained. To measure *β*-GLU activity, the supernatant (protein content: 1 mg/mL) was incubated with SN-38G (10 μg/mL) at 37°C, and 50 μL aliquots were sampled up to 30 min.

The sampled aliquots were rapidly diluted fivefold with 0.15 M H_3PO_4 and then added to an equal volume of IS solution.

Determination of CPT-11, SN-38, and SN-38G concentrations

A previously reported high-performance liquid chromatographic method with a fully automated on-line solid phase extraction system (PROSPEKT; Spark Holland, Emmen, The Netherlands) [19] was used. Briefly, 50 μL of each prepared sample was used for solid phase extraction with a Cartridge-C18 Analytichem (Spark Holland). A C_{18} reverse-phase column (Symmetry Column C18, 150 mm × 4.6 mm I.D., 5 μm; Waters, Milford, MA) was used at 50°C for chromatography. The fluorescence detector (470 scanning fluorescence detector; Waters) was set at 373 and 428 nm (excitation and emission, respectively) for 0–2.7 min and 3.8–8.5 min and 380 and 540 nm for 2.7–3.8 min. The mobile phase consisted of 0.05 M KH_2PO_4: acetonitrile (70:30, v/v) containing 4 mM sodium 1-decanesulfonate (pH 3.5 with H_3PO_4) and the flow rate was 1.5 mL/min. The retention times of SN-38G, SN-38, camptothecin (IS), and CPT-11 were approximately 1.5, 3.3, 4.2, and 7.2 min, respectively. The lower limits of quantification of CPT-11, SN-38, and SN-38G were 2.5, 2.5, and 5 ng/mL for the plasma and sample in the 'Metabolic enzyme assay', 100, 20, and 100 ng/mL for bile, and 200, 20, and 40 ng/g for intestinal content, respectively. Those of CPT-11 and SN-38 in intestinal tissue were 100 and 20 ng/g, respectively.

Pharmacokinetic analysis

The area under the concentration–time curve (AUC) of plasma and intestinal tissues was calculated by the trapezoidal rule with estimation of AUC from the last sampling time to infinity using Equation A.

$$\int_{\text{last}}^{\infty} C dt = C_{\text{last}}/\text{last log} - \text{linear phase slope (A)}$$

where C_{last} is the concentration at the last sampling time.

The following plasma pharmacokinetic parameters of CPT-11 were calculated by the non-compartmental model. Total clearance (CL_{tot}), mean resident time (MRT), and volume of distribution at steady state (Vd_{ss}) were calculated as follows.

$$CL_{tot} = \text{Dose}/\text{AUC}$$

$$\text{MRT} = \text{AUMC}/\text{AUC}$$

(AUMC : area under the moment curve)

$$Vd_{ss} = CL_{tot} \times \text{MRT}.$$

Statistical analysis

Differences were considered significant based on Student's *t* test or Dunnett's multiple comparison test except for diarrheal scores, which were analyzed using Wilcoxon's rank sum test.

Results

The objective of the present study was detailed research into the mechanism by which antibiotics (PG and SM) alleviated CPT-11-induced delayed-onset diarrhea. For that purpose, we used a new established diarrheal model, a Wistar rat with a single dosing of CPT-11, in addition to a conventional diarrheal model, a Wistar rat with repeated dosing of CPT-11 [31–33]. We also used a Gunn rat diarrheal model [24]. Gunn rats cannot conjugate SN-38 to SN-38G because of an inherited deficiency of UGT1A. Therefore, *β*-GLU activity in intestinal content does not contribute to the induction of diarrhea in Gunn rats.

As a result, SM alleviated CPT-11-induced diarrhea in all three diarrheal models. We examined the effect of SM on pharmacokinetics in Wistar rats with a single dosing model. The results of each study are described in the following sections.

Acute symptoms after administration of CPT-11

Acute diarrhea was observed immediately after a single dosing of CPT-11 in Wistar and Gunn rats and after the

final administration of repeated dosing of CPT-11 in Wistar rats. The diarrheal symptoms were severe (muddy or watery feces) in rats with antibiotics treatment in both strains. Other acute symptoms, such as tremors, prone/lateral body position, and lacrimation, were observed in all three models irrespective of antibiotics treatment, and their severity in Wistar rats depended on the CPT-11 dose. All of these acute symptoms almost disappeared within 3 h after the end of CPT-11 administration (data not shown).

Delayed-onset diarrhea after administration of CPT-11

Body weight changes and diarrheal symptoms after administration of CPT-11 in three diarrheal models are shown in Fig. 2 and Tables 1, 2, 3, respectively.

In Wistar rats with repeated dosing of CPT-11, there was no apparent difference in both the body weight profile and fecal condition between the Control and PG/SM groups. In the CPT group, body weight decreased gradually until Day 5 and severe diarrhea (muddy and watery feces) was observed in most rats on Day 4. The decrease in body weight in groups treated with both CPT-11 and antibiotics was smaller than in the CPT group, and the alleviating effect on body weight decrease depended on the antibiotics treatment period (Day −5 to 6 > Day −5 to −1). Diarrheal symptoms were significantly alleviated by all antibiotics treatment from Day −5 to 6 and by SM treatment from Day −5 to −1.

In Wistar and Gunn rats with a single dosing of CPT-11, body weight in the CPT groups decreased markedly until Day 4 or 5 after administration of CPT-11, and severe diarrhea (watery and mucous feces) was observed on Day 2 to 4. These symptoms recovered gradually, but two Gunn rats in the CPT group died without recovery on Day 5 or 6. In the CPT+SM and CPT+PG/SM groups of both strains, the decrease in body weight and diarrheal symptoms was alleviated compared to the CPT groups. SM exerted a significant alleviating effect irrespective of the treatment period, but PG/SM treatment from Day −5 to −1 degraded the effect compared with that from Day −5 to 6. The effect of PG was apparently weaker than each SM and PG/SM, and one Wistar rat in the CPT+PG group from Day −5 to −1 died on Day 5.

Histological and hematological findings

The effects of SM on intestinal epithelial damage 3 days after i.v. single administration of CPT-11 at a dose of 200 mg/kg are shown in Fig. 3.

In the CPT group, the degeneration of crypts was severe in the lower SI, cecum, and colon but mild in the upper SI. The villi of lower SI almost disappeared, but short villi were observed in the upper SI. In the CPT + SM group, the degeneration of crypts in the lower SI, cecum, and colon and the shortening of villi in the lower SI were observed, but this histological damage was milder than in the CPT group.

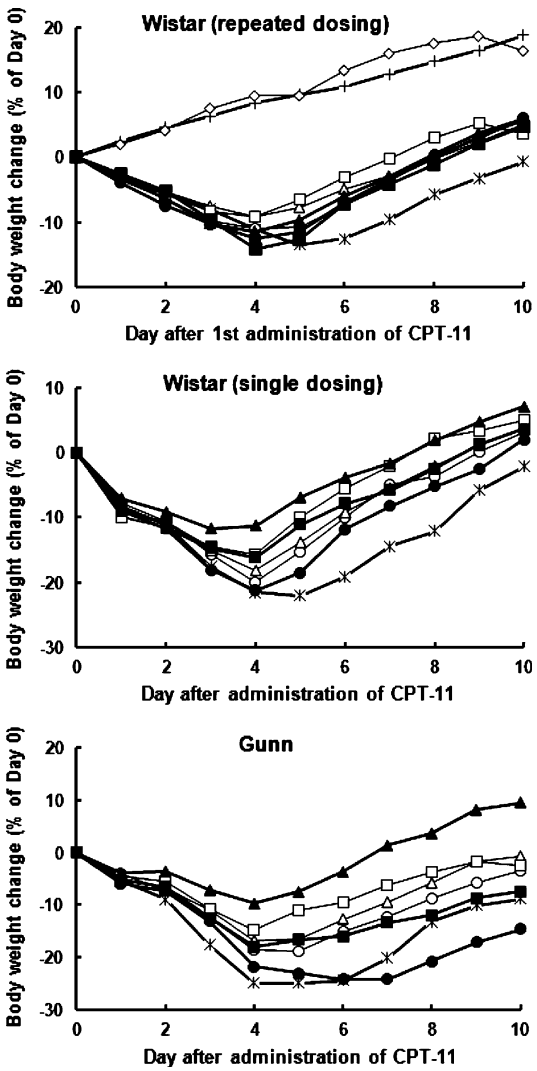

Fig. 2 Effects of antibiotics on body weight after i.v. administration of CPT-11 to Wistar and Gunn rats. CPT-11 was i.v. administered at a dose of 60 mg/kg once daily from Day 0 to 3 to repeated dosing groups or 200 mg/kg on Day 0 to single dosing groups of Wistar rats, or 20 mg/kg on Day 0 to Gunn rats (CPT). Penicillin G at 1 mg/mL (PG), streptomycin at 2 mg/mL (SM), or both antibiotics (PG/SM) were given in drinking water from Day −5 to 6 (*open symbol*) or from Day −5 to −1 (*closed symbol*). +: Control, *open diamond*: PG/SM, *Asterisks*: CPT, *open and closed circle*: CPT+PG, *open and closed triangle*: CPT+SM, *open and closed square*: CPT+PG/SM

Pharmacokinetics

Concentration profiles in plasma after a single infusion of CPT-11 are shown in Fig. 4. In the CPT group, CPT-11 concentration in plasma decreased exponentially and

Table 1 Effects of antibiotics on delayed-onset diarrhea in Wistar rats with repeated dosing of CPT-11

Antibiotics treatment period	Group	*n*	Diarrheal score																	
			Day 3				Day 4					Day 5					Day 6			
			0	1	2	3	0	1	2	3		0	1	2	3		0	1	2	3
	Control	3	3				3					3					3			
	CPT	9	3	1		**5**	1		**2**	**6**		1	4	**3**	**1**		4	5		
Day −5 to 6	PG/SM	3	3				3					3					3			
	CPT + PG	10	5	3	**1**	**1**	8	1	**1**		*	5	4	**1**			4	6		
	CPT + SM	10	6	4			10				*	9	1			*	9	1		
	CPT + PG/SM	9	5	4			9				*	7	2			*	5	4		
Day −5 to −1	CPT + PG	10	3	2	**2**	**3**	3	2	**2**	**3**		2	6	**2**			7	3		
	CPT + SM	10	5	4	**1**		9	1			*	6	4			*	10			*
	CPT + PG/SM	10	5	1	**3**	**1**	5	1		**4**		3	2	**5**			7	3		

CPT-11 was i.v. administered at a dose of 60 mg/kg once daily from Days 0 to 3 (CPT)

Penicillin G at 1 mg/mL (PG), streptomycin at 2 mg/mL (SM), or both antibiotics (PG/SM) were given in drinking water

Diarrheal score: 0, normal feces; 1, soft feces or small black feces; 2, muddy feces; 3, watery or mucous feces

Bold values indicate the number of rats which showed heavy diarrheal symptoms

* The mean was significantly different from the CPT group ($P < 0.05$ by Wilcoxon's rank sum test)

Table 2 Effects of antibiotics on delayed-onset diarrhea in Wistar rats with a single dosing of CPT-11

Antibiotics treatment period	Group	*n*	Diarrheal score																					
			Day 2					Day 3					Day 4					Day 5							
			0	1	2	3		0	1	2	3		0	1	2	3		0	1	2	3				
	CPT	10		1		**9**					**10**			1	**5**	**4**			7	**2**	**1**				
Day −5 to 6	CPT + PG	6		3		**3**					**6**				**4**	**2**			5	**1**				
	CPT + SM	6		6			*			**4**	**2**			1	**5**				6					
	CPT + PG/SM	5		5			*		5			*		5			*		5					
Day −5 to −1	CPT + PG	12		1	**2**	**9**					**12**			7	**3**	**1**	#	1	9	**1**				
	CPT + SM	11	3	8			*		9	**2**		*	3	7	**1**		*	4	7			*		
	CPT + PG/SM	12	1	11			*	1	11			*		12			*	2	10					

CPT-11 was i.v. administered at a dose of 200 mg/kg on Day 0 (CPT)

Penicillin G at 1 mg/mL (PG), streptomycin at 2 mg/mL (SM), or both antibiotics (PG/SM) were given in drinking water

Diarrheal score: 0, normal feces; 1, soft feces or small black feces; 2, muddy feces; 3, watery or mucous feces

Bold values indicate the number of rats which showed heavy diarrheal symptoms

* The mean was significantly different from the CPT group ($P < 0.05$ by Wilcoxon's rank sum test)

One rat in the CPT + PG group from Day −5 to −1 did not defecate on Day 4 and died on Day 5

SN-38 concentration decreased rapidly until 4 h and gradually thereafter. In the CPT+SM group, CPT-11 and SN-38 concentration profiles were similar to those in the CPT group, but CPT-11 concentrations at 24 and 48 h were lower than in the CPT group (421 vs. 920 ng/mL at 24 h, 18.0 vs. 45.8 ng/mL at 48 h), and MRT, $t_{1/2}$, and Vd_{ss} values were significantly smaller (Table 4). SN-38 concentration was also lower at some time points (e.g., 7.9 vs 10.2 ng/mL at 24 h), and the AUC value decreased significantly (Table 4).

Concentrations of CPT-11 and SN-38 in the ileum of the CPT group peaked at 6 and 12 h, respectively, and decreased after 12 h, but no increase in these concentrations was observed in the CPT+SM group (Fig. 5).

Table 3 Effects of antibiotics on delayed-onset diarrhea in Gunn rats with a single dosing of CPT-11

Antibiotics treatment period	Group	*n*	Diarrheal score																	
			Day 2					Day 3					Day 4					Day 5			
			0	1	2	3		0	1	2	3		0	1	2	3		0	1	2	3
	CPT	9				**9**					**9**					**8**	[a]		4	**2**	**1**[b]
Day −5 to 6	CPT + PG	5	1	3		**1**	*		2		**3**			1	**2**	**2**		2	1	**2**	
	CPT + SM	5	4	1			*	3	2			*			**5**			2	1	**2**	
	CPT + PG/SM	5		5			*	4	1			*		4	**1**		*		5		
Day −5 to −1	CPT + PG	5	3	2			*				**5**					**5**			1	**2**	**2**
	CPT + SM	4	4				*	2	1	**1**		*	2	1	**1**		*	4			
	CPT + PG/SM	4	2	2			*		1	**1**	**2**			1	**1**	**2**		2		**1**	**1**

CPT-11 was i.v. administered at a dose of 20 mg/kg on Day 0 (CPT)

Penicillin G at 1 mg/mL (PG), streptomycin at 2 mg/mL (SM), or both antibiotics (PG/SM) were given in drinking water

Diarrheal score: 0, normal feces; 1, soft feces or small black feces; 2, muddy feces; 3, watery or mucous feces

Bold values indicate the number of rats which showed heavy diarrheal symptoms

[a] One rat in the CPT group did not defecate on Day 4 and died on Day 5

[b] One rat in the CPT group did not defecate on Day 5

* The mean was significantly different from the CPT group ($P < 0.05$ by Wilcoxon's rank sum test)

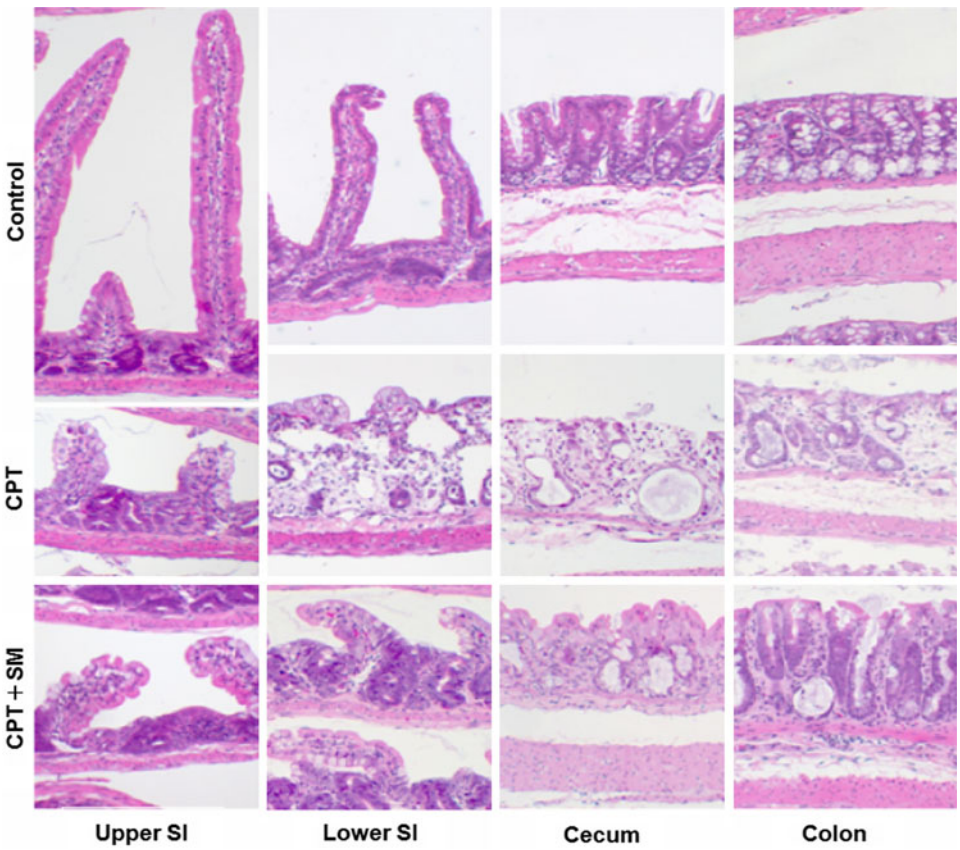

Fig. 3 Effect of streptomycin on epithelial damage to the intestinal tract by CPT-11. CPT-11 was i.v. administered at 200 mg/kg on Day 0 to Wistar rats (*CPT*). Streptomycin was given from Day −5 to −1 (SM). Damage to the upper and lower small intestine (*SI*), cecum, and colon was observed on Day 3. Histological slides were stained with hematoxylin-eosin

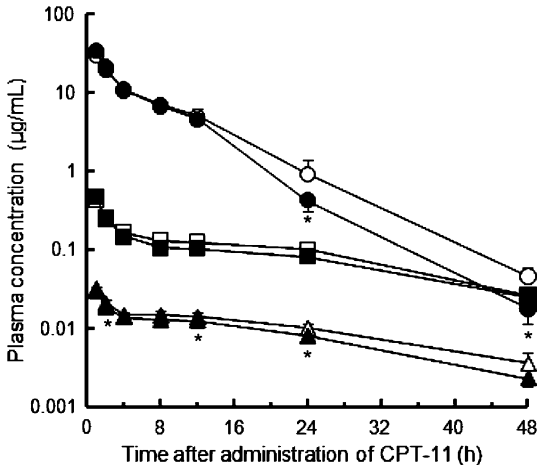

Fig. 4 Plasma concentration profiles of CPT-11, SN-38, and SN-38G after i.v. administration of CPT-11. CPT-11 was i.v. administered at 200 mg/kg on Day 0 to Wistar rats (CPT). Streptomycin was given from Day −5 to −1 (SM). *Open symbol*: CPT, *closed symbol*: CPT+SM. *open and closed circle*: CPT-11, *open and closed triangle*: SN-38, *open and closed square*: SN-38G. Each point represents the mean ± standard deviation from five or six animals. *Asterisks*: The mean was significantly different from that of the CPT group ($P < 0.05$ by Student's t test)

The order of AUC values of intestinal tissues in the CPT group was as follows: CPT-11; ileum > cecum, colon > duodenum, jejunum: SN-38; cecum > colon > ileum > duodenum, jejunum. In the CPT+SM group, the AUC value of CPT-11 in the ileum was half of that in the CPT group. The AUC values of SN-38 in cecum and colon were about 0.4-fold, and those of other intestinal tissues were also lower than in the CPT group (Table 5).

There was no apparent difference between CPT and CPT + SM groups in cumulative biliary excretion rates for 24 h (CPT-11: 32.1 ± 3.2 versus 35.1 ± 2.6%, SN-38: 1.13 ± 0.18 versus 1.16 ± 0.28%, SN-38G: 2.47 ± 0.43 versus 2.45 ± 0.53%, respectively).

SN-38 and SN-38G concentrations and β-GLU activities in intestinal contents

7-ethyl-10-hydroxycamptothecin (SN-38) and SN-38G concentrations in the ileal and cecal contents 6 h after CPT-11 administration are shown in Fig. 6. SN-38 and SN-38G concentrations in ileal contents were almost identical among treatments. However, SN-38G in cecal contents was hardly detected in CPT and CPT+SM groups, whereas it was detected at threefold the SN-38 level in the CPT+PG/SM group. SN-38 concentration in the cecal content of the CPT+SM group was about one-third of that of the CPT group.

β-GLU activities in the cecal content of the Control, SM, and PG/SM groups without CPT-11 administration were 55.8 ± 18.8, 17.0 ± 9.27, and 1.84 ± 1.44 nmol/min/g protein, respectively.

Table 4 Pharmacokinetic parameters after i.v. administration of CPT-11 in Wistar rats

	Parameter	CPT ($n = 6$)	CPT + SM ($n = 5$)
CPT-11	$AUC_{0\text{-inf}}$ (μg h/mL)	163 ± 19	153 ± 6
	$MRT_{0\text{-inf}}$ (h)	7.75 ± 0.93	**6.41 ± 0.21***
	$t_{1/2}$ (h)	5.38 ± 0.34	**4.60 ± 0.30***
	CL_{tot} (L/h/kg)	1.24 ± 0.13	1.31 ± 0.05
	Vd_{ss} (L/kg)	9.56 ± 0.97	**8.38 ± 0.50***
SN-38	$AUC_{0\text{-inf}}$ (μg h/mL)	0.599 ± 0.110	**0.452 ± 0.028***
	$t_{1/2}$ (h)	18.7 ± 5.9	14.6 ± 1.5
SN-38G	$AUC_{0\text{-inf}}$ (μg h/mL)	5.36 ± 0.71	4.78 ± 0.47
	$t_{1/2}$ (h)	16.0 ± 3.5	17.1 ± 2.3

CPT-11 was i.v. administered at a dose of 200 mg/kg (CPT)

Streptomycin at 2 mg/mL was given in drinking water for 5 days before administration of CPT-11 (SM)

Bold values indicate the parameters with significant difference between CPT and CPT+SM groups

* The mean was significantly different from the CPT group ($P < 0.05$, Student's t test)

Intestinal absorption of CPT-11

To examine the effect of SM on the intestinal absorption of CPT-11 excreted via bile, CPT-11 concentration profiles in plasma after p.o. administration of the mixture of CPT-11 and bile were determined (Fig. 7). In the CPT group, CPT-11 concentration peaked 2 h after CPT-11 administration and gradually decreased after 4 h. In the CPT+SM1 group, CPT-11 concentration increased gradually until 2 h and decreased thereafter, and the AUC value was about half of the CPT group (0.679 ± 0.245 vs 1.41 ± 0.33 μg h/mL, respectively). Two-day cessation (Day −2 to 0) in the CPT+SM2 group did not affect the SM effect, as the AUC value of CPT-11 in the CPT+SM2 group, 0.495 ± 0.055 μg h/mL, was comparable to that of the CPT+SM1 group.

Enzyme activity in liver and epithelium of SI

The effect of SM on CES and UGT activities in microsomes of the liver and SI epithelium is shown in Table 6. In the SI epithelium, CES activities fell about half and UGT activities increased about eightfold by SM treatment, whereas these enzyme activities in liver microsomes remained unchanged.

Discussion

In a few studies using normal rats, it has been reported that CPT-11-induced delayed-onset diarrhea is inhibited by treatment with a combination of PG and SM (PG/SM),

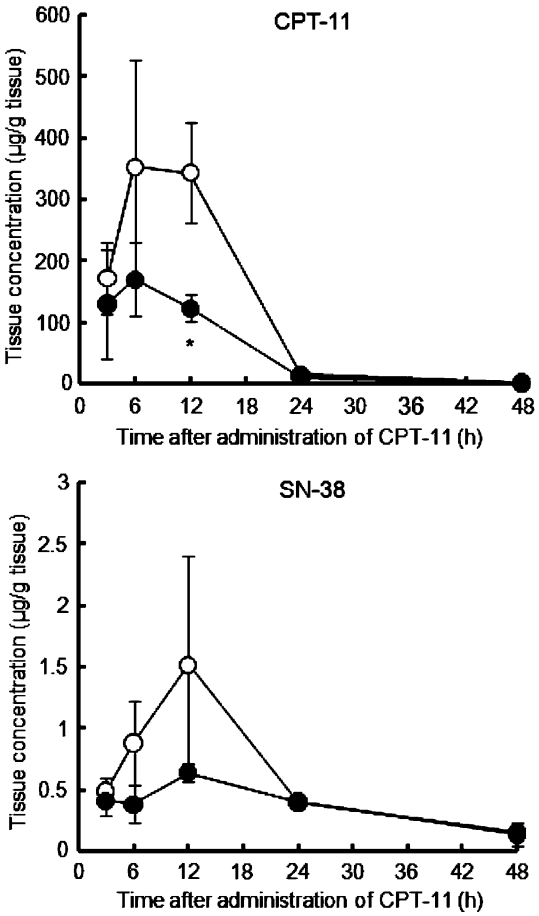

Fig. 5 Tissue concentration profiles of CPT-11 and SN-38 in ileum after i.v. administration of CPT-11. CPT-11 was i.v. administered at 200 mg/kg on Day 0 to Wistar rats (CPT). Streptomycin was given from Days −5 to −1 (SM). *Open circle*: CPT, *closed circle*: CPT+SM. Each point represents the mean ± standard deviation from three animals. *Asterisk*: The mean was significantly different from that of the CPT group ($P < 0.05$ by Student's *t* test)

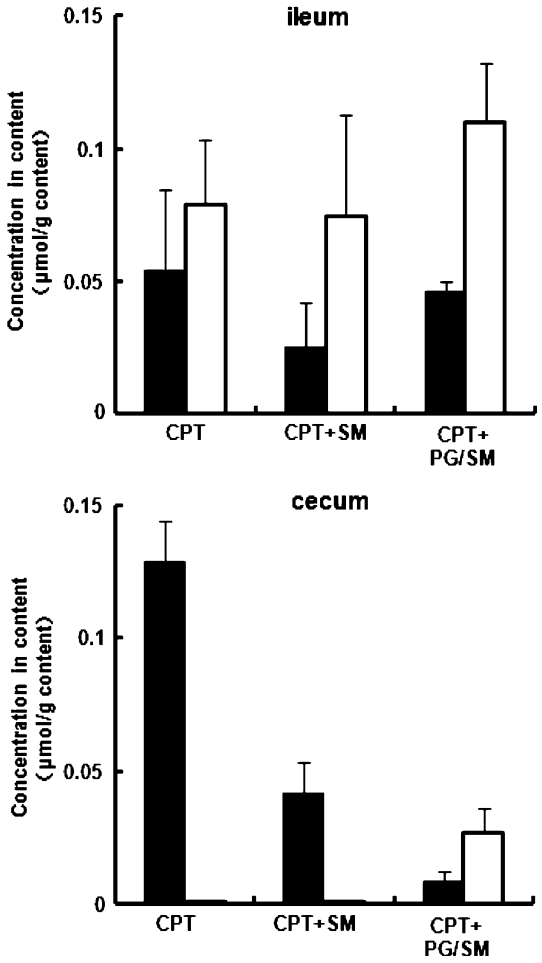

Fig. 6 SN-38 and SN-38G concentrations in intestinal contents 6 h after i.v. administration of CPT-11. CPT-11 was i.v. administered at 200 mg/kg on Day 0 to Wistar rats (CPT). Streptomycin (SM), or both penicillin G and streptomycin (PG/SM), was given from Day −5 to −1. *Closed column*: SN-38, *Open column*: SN-38G. Each *column* and *bar* represents the mean and standard deviation from three animals

Table 5 AUC values of CPT-11 and SN-38 in intestinal tissues in Wistar rats after i.v. administration of CPT-11

	Group	AUC_{0-48} (μg h/g tissue)				
		Duodenum	Jejunum	Ileum	Cecum	Colon
CPT-11	CPT	1,950	1,691	4,497	3,104	3,222
	CPT + SM	1,801	1,371	2,197	2,496	2,562
	Ratio	0.92	0.81	0.49	0.80	0.80
SN-38	CPT	10.8	14.1	25.7	69.3	46.9
	CPT + SM	6.29	10.9	17.1	27.5	17.2
	Ratio	0.58	0.77	0.67	0.40	0.37

CPT-11 was i.v. administered at a dose of 200 mg/kg (CPT)

Streptomycin at 2 mg/mL was given in drinking water for 5 days before administration of CPT-11 (SM)

suggesting that SN-38G de-conjugation by β-GLU in the intestinal content plays a key role in the development of diarrhea [32, 33]. However, in our preliminary study, oral administration of SN-38 or SN-38G did not induce delayed-onset diarrhea at an equimolar dose of CPT-11 that induced severe diarrhea by i.v. administration. Therefore, we considered that direct injury of the intestinal epithelium by SN-38 de-conjugated from SN-38G was not the main mechanism of delayed-onset diarrhea and studied a new mechanism.

In the present study, we used not only normal rats but also Gunn rats with an inherited deficiency of UGT1A. UGT1A detoxifies SN-38, and it has been reported that some polymorphisms of the UGT1A gene (e.g., UGT1A1*28 and −3156G>A genotypes) are associated with severe toxicity (neutropenia and/or diarrhea) of

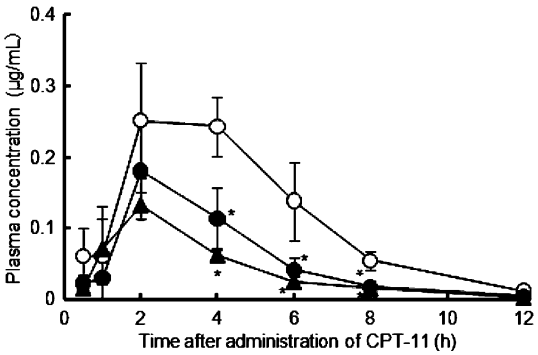

Fig. 7 CPT-11 concentration profiles in plasma after p.o. administration of CPT-11. CPT-11 mixture was p.o. administered at 20 mg/kg on Day 0 to Wistar rats (CPT). Streptomycin was given from Day −5 to −1 (SM1), or form Day −7 to −3 (SM2). *Open circle*: CPT, *closed circle*: CPT+SM1, *closed triangle*: CPT+SM2. Each point represents the mean ± standard deviation from three to six animals. *Asterisks*: The mean was significantly different from that of the CPT group ($P < 0.05$ by Dunnett's multiple comparison test)

Table 6 Effect of streptomycin on carboxylesterase (CES) and UDP-glucuronosyltransferase (UGT) activities in microsomes of liver and epithelium of small intestine in Wistar rats

	Enzyme	*n*	Enzyme activity (nmol/min/g protein)	
			Control	SM
Liver	CES	4	8.94 ± 0.87	9.00 ± 0.36
	UGT	4	281 ± 111	306 ± 48
Epithelium of Small intestine	CES	4	5.06 ± 1.31	**2.29 ± 1.16***
	UGT	3	17.5 ± 2.4	**137 ± 58***

Streptomycin at 2 mg/mL was given in drinking water for 5 days before the collection of liver and small intestine (SM)

Bold values indicate the parameters with significant difference between CPT and CPT+SM groups

* The mean was significantly different from that of the Control group ($P < 0.05$ by Student's *t* test)

CPT-11 clinically [2, 10]. In Gunn rats, β-GLU activity in the intestinal content does not contribute to the induction of diarrhea by CPT-11 because SN-38G does not exist in bile [24]. The Gunn rat model would be suitable to study the inhibition mechanism of antibiotics in CPT-11-induced diarrhea other than the inhibition of β-GLU activity in intestinal content.

We first assessed the alleviation effect of each PG and SM on delayed-onset diarrhea using three diarrheal models, that is, Wistar rats with repeated and single dosing models and the Gunn rat model (Tables 1, 2, 3, respectively). SM or PG/SM treatment exerted an alleviation effect on delayed-onset diarrhea in Gunn rats as well as in the two Wistar rat models. The inhibition of SM to β-GLU in the cecal content was weaker than PG/SM and did not block the de-conjugation of SN-38G in Wistar rats (Fig. 6). Moreover, severe epithelial damage was observed in SI as well as the cecum and colon in Wistar rats with a single dosing model (Fig. 3), although SN-38G was not de-conjugated in SI content (Fig. 6). These findings indicated that the de-conjugation of SN-38G excreted via bile contributed little to the induction of both intestinal damage in SI and delayed-onset diarrhea and that SM alleviated diarrhea by a mechanism other than the inhibition of β-GLU in the intestinal lumen.

Next, we examined the effects of SM on the pharmacokinetics after CPT-11 administration using Wistar rats with a single dosing model. SM treatment decreased the plasma concentration of CPT-11 and SN-38 in the terminal phase (Fig. 4). SM did not affect the biliary excretion of CPT-11 and its metabolites but inhibited the intestinal absorption of CPT-11 (Fig. 7). These findings indicated that acceleration of the disappearance of CPT-11 and SN-38 was caused not by the induction of biliary excretion of CPT-11 and its metabolites, but by the inhibition of intestinal absorption of CPT-11.

Exposure of CPT-11 and SN-38 in the ileum, cecum, and colon was markedly higher than in the duodenum and jejunum (Table 5; Fig. 5), and the amount of exposure seemed to well correlate with the severity of intestinal epithelial damage (Fig. 3). On the other hand, SM treatment significantly decreased the tissue exposure of CPT-11 in the ileum and of SN-38 in the cecum and colon and alleviated epithelial damage in these intestinal regions. As the ileum was suggested to be the main area for CPT-11 absorption because the AUC value of CPT-11 in the ileum was much higher than in other intestinal regions, SM could alleviate damage to the epithelium, especially in the ileum, due to decreased CPT-11 exposure caused by the inhibition of CPT-11 absorption.

SM treatment from Day −7 to −3 also alleviated CPT-11-induced diarrhea in Wistar rats with a single dosing of CPT-11 in a preliminary study (data not shown), and the inhibitory effect on intestinal absorption of CPT-11 by SM was confirmed after two-day cessation (Fig. 7). From these findings, we speculated that SM did not inhibit the intestinal absorption of CPT-11 by any direct means, such as competitive inhibition or formation of the complex with CPT-11. As the pH value of the cecal content of Wistar rats with and without SM treatment was 5.98 ± 0.29 and 5.81 ± 0.32, respectively, the equilibrium between CPT-11 lactone and carboxylate forms would not shift to the lactone form, which is transported into intestinal cells much greater than the carboxylate form [16]. SM might induce P-glycoprotein, the efflux transporter of CPT-11 [20], in the intestinal epithelium. The inhibition mechanism of the intestinal absorption of CPT-11 by SM is being studied at present.

SM affected the activities of metabolic enzymes for CPT-11 in the intestinal epithelium (Table 6), namely, decreased CES activity and increased UGT activity. As the AUC values of SN-38 in intestinal tissues (10.8–69.3 μg h/g tissue) were much higher than in plasma (0.599 μg h/mL), SN-38 converted from CPT-11 in intestinal tissues would contribute greatly to the exposure to SN-38 rather than SN-38 via the bloodstream. Changes of metabolic enzyme activities by SM would decrease exposure to SN-38 in the intestinal tissues.

The mechanism of changes of CES and UGT activities by SM is not unexplained. It is reported that the activities of some enzymes in the distal small intestine in rats decrease by oral treatment of high-dose neomycin, an aminoglycoside antibiotic similar to SM [35], and that hepatic and intestinal UGTs are induced by some exogenous effects, such as dietary compounds, drugs, and so on [28, 36]. SM would induce changes to these enzyme activities in the intestinal epithelium directly and/or indirectly by changes to intestinal flora by antibiotic effect of SM, and SM would not change these enzyme activities in the liver, presumably because of poor intestinal absorption. A study using germ-free rats as well as conventional rats will give some important data for elucidating the mechanism.

SM would also decrease exposure to SN-38 from the intestinal lumen in the cecum because it decreased SN-38 concentration in the cecal content (Fig. 6). As the cecum in animals with antibiotics treatment was apparently larger than without treatment, SN-38 would be diluted by the greater cecal content.

Based on the results of the present study, we speculated the pathway of CPT-11 and SN-38 to the intestinal epithelium and the effects of SM (Fig. 8). CPT-11 and SN-38 are distributed to the intestinal tissues via the bloodstream and/or by intestinal absorption. Moreover, CPT-11 converts to SN-38 by CES, followed by conjugation to SN-38G in the intestinal tissues. When CPT-11 exists in the intestinal tissues at a much higher level, the conversion of CPT-11 to SN-38 in intestinal tissues probably contributes to the increase in SN-38 concentration in intestinal tissues much more than either distribution via the bloodstream or intestinal absorption of SN-38. The distribution of massive CPT-11 to the intestinal tissues would produce more SN-38 than the conjugation capacity by UGT. Consequently, SN-38 would be maintained at a high level for a long time and induce severe epithelial damage by subsequent delayed-onset diarrhea.

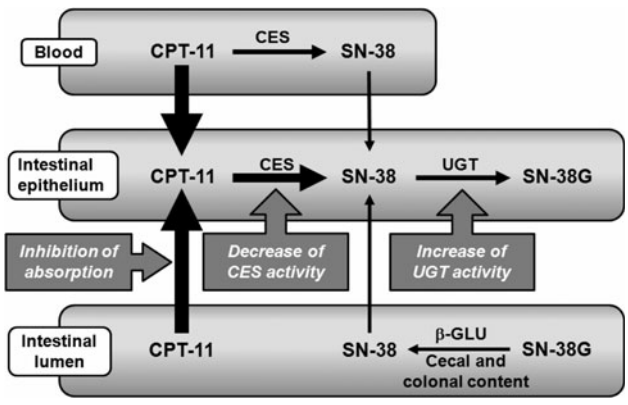

Fig. 8 Effect of streptomycin (*SM*) on the pharmacokinetic pathway of CPT-11, SN-38, and SN-38G around the intestinal epithelium. *Italics* indicate the effect of SM on the pathway. *CES*: carboxylesterase, *UGT*: UDP-glucuronosyltransferase, *β- GLU*: *β*-glucuronidase

SM would decrease the exposure of CPT-11 to the intestinal epithelium by inhibiting the intestinal absorption of CPT-11, and the decrease in CPT-11 concentration in the intestinal epithelium by the inhibition of CPT-11 absorption would reduce SN-38 production by CES in the tissue. Moreover, SM would also decrease SN-38 production by both the decrease in CES activity and the increase in GUT activity in the intestinal epithelium. Therefore, SM would alleviate delayed-onset diarrhea by reducing SN-38 exposure in the intestinal epithelium.

It is reported that neomycin was clinically examined for the prevention of CPT-11-induced delayed-onset diarrhea, and it alleviated diarrhea in patients with experience of CPT-11-induced diarrhea graded ≥2 [4, 15, 27]. As SN-38G was detected in the feces of patients without neomycin treatment, *β*-GLU activity in the intestinal content of these patients would be weak and the inhibition of *β*-GLU would not be so effective to decrease exposure to SN-38 in the intestinal epithelium compared to in rats. Neomycin might alleviate CPT-11-induced diarrhea not only by inhibiting *β*-GLU, but also by other mechanisms.

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