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Inhibition of intestinal microflora β-glucuronidase modifies the distribution of the active metabolite of the antitumor agent, irinotecan hydrochloride (CPT-11) in rats

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Abstract *Purpose*: SN-38, a metabolite of irinotecan hydrochloride (CPT-11), is considered to play a key role in the development of diarrhea as well as in the antitumor activity of CPT-11. We have previously found that the inhibition of β-glucuronidase, which hydrolyzes detoxified SN-38 (SN-38 glucuronide) to reform SN-38, in the lumen by eliminating the intestinal microflora with antibiotics, markedly ameliorates the intestinal toxicity of CPT-11 in rats. In this study we compared the disposition of CPT-11 and its metabolites in rats treated with and without antibiotics. *Methods*: Rats were given drinking water containing 1 mg/ml penicillin and 2 mg/ml streptomycin from 5 days before the administration of CPT-11 (60 mg/kg i.v.) and throughout the experiment. CPT-11, SN-38 glucuronide and SN-38 concentrations in the blood, intestinal tissues and intestinal luminal contents were determined by HPLC. *Results*: Antibiotics had little or no effect on the pharmacokinetics of CPT-11, SN-38 glucuronide or SN-38 in the blood, or in the tissues or contents of the small intestine, which has less β-glucuronidase activity in its luminal contents. In contrast, antibiotics markedly reduced the $AUC_{1-24\ h}$ of SN-38 (by about 85%) in the large intestine tissue without changing that of CPT-11, and this was accompanied by a complete inhibition of the deconjugation of SN-38 glucuronide in the luminal contents. *Conclusions*: These results suggest that SN-38, which results from the hydrolysis of SN-38 glucuronide by β-glucuronidase in the intestinal microflora, contributes considerably to the distribution of SN-38 in the large intestine tissue, and that inhibition of the β-glucuronidase activity by antibiotics results in decreased accumulation of SN-38 in the large intestine.

Key words CPT-11 · SN-38 · SN-38 glucuronide · Diarrhea · β-Glucuronidase

Abbreviations *CPT-11* [(+)-(4S)-4,11-diethyl-4-hydroxy-9-[(4-piperidinopiperidino) carbonyloxy]-1H-pyrano [3′,4′:6,7]indolizino[1,2-b]quinoline-3,14 (4H, 12H)-dione hydrochloride trihydrate] · *SN-38* 7-ethyl-10-hydroxycamptothecin · *SN-38 glucuronide* glucuronic conjugate of 7-ethyl-10-hydroxycamptothecin

Introduction

In clinical trials of the new antitumor camptothecin derivative, irinotecan hydrochloride (CPT-11), the occurrence of severe, uncontrollable diarrhea has limited the further evaluation of more aggressive antitumor regimens using CPT-11 [6, 13, 14, 17]. Despite great interest in this diarrhea and a large amount of effort put into its investigation, only modest progress has been made in understanding the precise mechanism of induction of diarrhea by CPT-11 [3, 7, 21–24].

CPT-11 is a real prodrug, because its metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) has much stronger antitumor activity [9, 10]. SN-38 is mainly detoxified to SN-38 glucuronide in the liver and excreted into the bile with the other major components, CPT-11 and SN-38. Most of the SN-38 glucuronide, however, is hydrolysed to reform SN-38 in the presence of β-glucuronidase by the intestinal microflora [4, 8]. Recently, we have found that the inhibition of β-glucuronidase in

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the intestinal microflora by antibiotics markedly reduces the intestinal toxicity of CPT-11 in rats, suggesting that SN-38, which is generated from SN-38 glucuronide by β-glucuronidase in the intestinal microflora, plays a key role in the exacerbation of the intestinal toxicity of CPT-11 [25]. However, there is uncertainty as to whether or not the protective effects of antibiotics depend on the reduction in the distribution of SN-38 to the intestinal tissue via inhibition of the deconjugation of SN-38 glucuronide. Also, the question remains as to whether the fact that the small intestine suffers less damage than the large intestine is attributable to the lack of β-glucuronidase activity in its luminal contents leading to decreased accumulation of SN-38 in the small intestine tissue.

To address these questions, we investigated the pharmacokinetics of CPT-11 and its metabolites to evaluate the hypothesis that the inhibition of SN-38 glucuronide deconjugation in the intestinal lumen by antibiotics reduces the accumulation of SN-38 in the intestinal tissue, thereby improving CPT-11-induced diarrhea.

Materials and methods

Animals

The experiments were conducted using male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing 190–220 g ($n = 4$ at each point). The animal room was maintained at a temperature of 23 ± 2 °C and a relative humidity of 55 ± 15% with a 12-h light–dark cycle. A commercial animal chow (F-2, Funabashi Farm, Funabashi, Japan) and tap-water were freely available throughout the acclimatization and experimental periods.

Experimental schedule

Animals were given drinking water containing 1 mg/ml penicillin and 2 mg/ml streptomycin from 5 days before the start of the administration of CPT-11 and throughout the experiment. This regimen has been shown to inhibit completely fecal β-glucuronidase activity and to ameliorate the intestinal toxicity induced by CPT-11 [25]. A blood sample of about 2 ml was taken from the carotid artery under ether anesthesia 1, 3, 5, 7, 9 and 24 h after a single i.v. administration of CPT-11 (60 mg/kg) into the tail vein. Animals were then sacrificed by exsanguination, and all the intestinal tissue was excised and opened longitudinally. The luminal contents were removed and weighed. The intestinal tissue was rinsed until clear with cold physiological saline, blotted and weighed. Immediately after the procedures, each blood, luminal content and intestinal tissue sample were directly suspended (or homogenized) in an appropriate volume (five fold for blood and ten-fold for luminal content and intestinal tissue) of cold 10% 0.1 *N* HCl/methanol. The suspension or homogenate was centrifuged at 1600 *g* for 30 min and the supernatant was stored at 4 °C until assayed for the concentrations of CPT-11, SN-38 and SN-38 glucuronide.

Determination of CPT-11 and its metabolites in the samples

The total CPT-11, SN-38 and SN-38 glucuronide concentrations in the supernatant were determined by high-performance liquid chromatography (HPLC). A TSK gel ODS-80Ts column (4.6 mm × 150 mm; Tosoh Co., Tokyo, Japan) was used for separation. The mobile phases consisted of 12% tetrahydrofuran (THF)/50 m*M* phosphate buffer (pH 2.3) and 30% THF/50 m*M* phosphate buffer (pH 2.3) for CPT-11 and SN-38, respectively. The flow rate was 1.0 ml/min at 30 °C. Fluorescence detection was performed using a fluorescence spectrophotometer (F-1050, Hitachi, Tokyo, Japan) with a λ_{ex} of 370 nm and a λ_{em} of 430 nm for CPT-11, and a λ_{ex} of 380 nm and a λ_{em} of 550 nm for SN-38. SN-38 glucuronide was determined by the same HPLC method as was used for CPT-11. The detection limits of CPT-11, SN-38 and SN-38 glucuronide were 0.5 ng/ml, 1 ng/ml and 0.5 ng/ml, respectively. The assay was reproducible with < 5% inter- and intraday coefficients of variation.

Drugs

CPT-11 was supplied by Yakult Honsha (Tokyo, Japan). Penicillin G and streptomycin were purchased from Sigma (St. Louis, Mo.).

Statistics

The results except for AUC are expressed as mean ± standard error, and differences between the control and antibiotics groups were considered statistically significant when Student's *t*-test showed $P < 0.05$. A single AUC value was calculated for the period 1–24 h ($AUC_{1-24\ h}$) from the mean values at each time point for each curve using the trapezoid rule.

Results

Time course profiles in whole blood

Blood concentration-time profiles of CPT-11 and its metabolites are shown in Fig. 1. The blood concentrations of CPT-11, SN-38 glucuronide and SN-38 1 h after the administration of CPT-11 were about 4 μg/ml, 150 ng/ml and 7 ng/ml, respectively. The concentrations of CPT-11 and SN-38 glucuronide decreased rapidly, but that of SN-38 was sustained even at lower levels, which resulted in a decrease in the ratios of the concentrations of CPT-11 and SN-38 glucuronide to

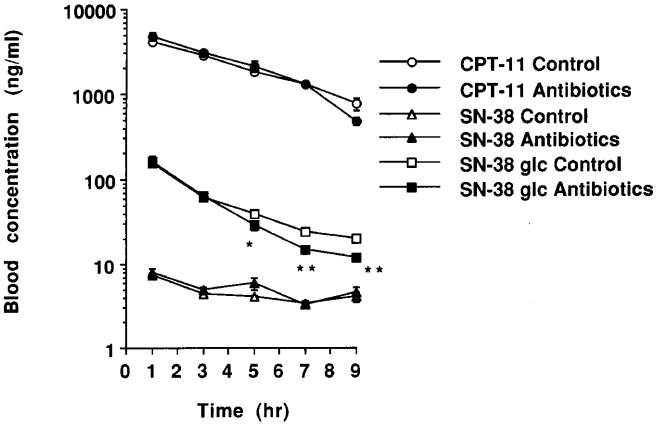


Fig. 1 Effects of antibiotics on the blood concentration-time curves for CPT-11 and its metabolites after a single dose of CPT-11 (60 mg/kg, i.v.) in rats. Antibiotics (penicillin 1 mg and streptomycin 2 mg per ml drinking water) were administered from 5 days before the start of CPT-11 administration. Each point represents the mean ± standard error from four animals. *$P < 0.05$, **$P < 0.01$; vs the group treated with CPT-11 alone (*t*-test)

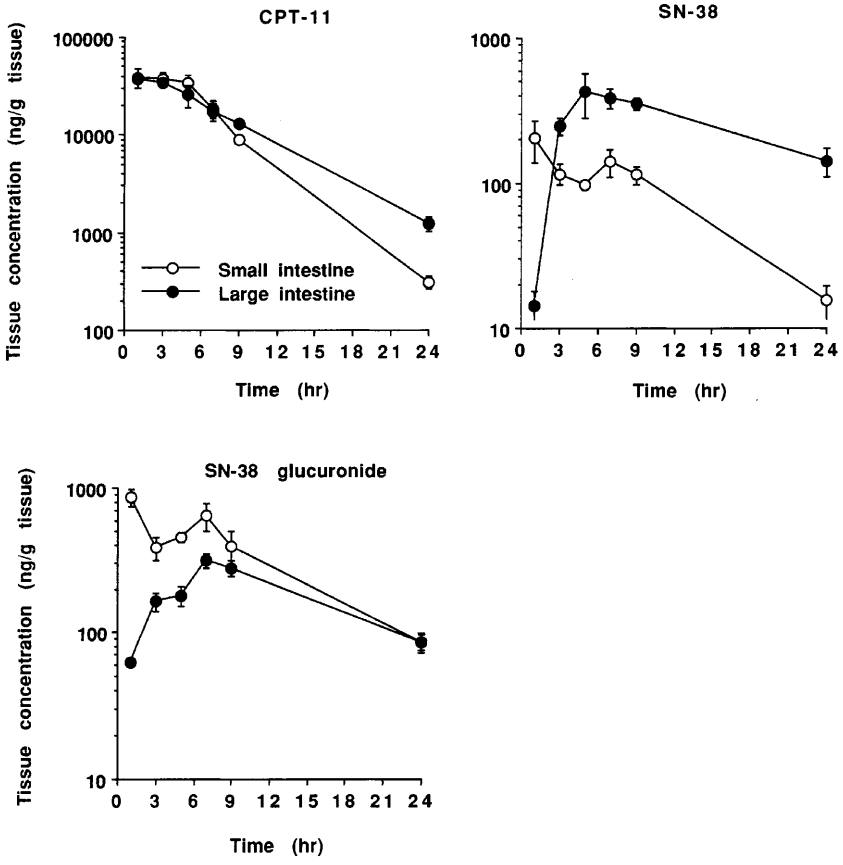


Fig. 2 Comparison of tissue concentration-time curves for CPT-11 and its metabolites between the small and the large intestines after a single dose of CPT-11 (60 mg/kg, i.v.) in rats. Each point represents the mean ± standard error from four animals

SN-38 from 1 to 9 h. All the concentrations fell to below the detection limit 24 h after dosing.

Antibiotics had little or no effect on the blood concentration-time profiles of CPT-11 or SN-38. The concentration of SN-38 glucuronide decreased faster in antibiotic-treated animals than in control animals.

Time course profiles in small and large intestines

Following i.v. injection of CPT-11, there were much higher concentrations of CPT-11 in both the small and large intestine, when compared with its metabolites. The predominant metabolite was SN-38 glucuronide during 1–24 h in the small intestine as was seen in the blood, but the ratio of the concentration of SN-38 glucuronide to that of SN-38, unlike that in the blood, was almost constant (at about 5). In the large intestine, the main metabolite, unlike those in the blood and the small intestine, was SN-38 except at 1 h (Fig. 2).

There was little or no difference in the pharmacokinetics of CPT-11 between the small and large intestine during 1 to 9 h. The higher concentrations of SN-38 glucuronide and SN-38, however, were detected in the small intestine 1 h after dosing at levels about 15 times those in the large intestine. Thereafter, the concentrations of both compounds in the small intestine decreased biphasically, with a second peak at about 7 h after dosing. The concentration of SN-38 glucuronide in the large intestine rose from 1 h onward, to peak at 7 h after dosing, but it remained at lower levels than that in the small intestine and had about one-half the $AUC_{1-24\ h}$ of the small intestine (Table 1). On the other hand, the concentration of SN-38 in the large intestine rose sharply from 1 h after dosing, to reach a peak at about 5 h and was still high even 24 h later. SN-38 levels in the large intestine from hours 3 to 24 exceeded those in the small intestine, and the $AUC_{1-24\ h}$ of SN-38 was about three times as high (Fig. 2, Table 1).

Antibiotics had no significant effect on the tissue concentration-time profiles of CPT-11 or its metabolites in the small intestine (Fig. 3). In the large intestine, antibiotics had little or no effect on the pharmacokinetics of CPT-11, but markedly decreased the concentrations of SN-38 between hours 3 and 24, with a decrease in $AUC_{1-24\ h}$. The concentrations of SN-38 glucuronide slightly increased from hours 5 to 24, with an accompanying increase in $AUC_{1-24\ h}$ in antibiotic-treated animals (Fig. 4, Table 1).

Time course profiles in intestinal luminal contents

Following i.v. injection of CPT-11, there were higher concentrations of CPT-11 and its metabolites in the luminal contents of both the small and large intestines as compared with those of the respective tissues. The concentrations of CPT-11 and its metabolites in the

Table 1 Comparison of the disposition of CPT-11, SN-38 and SN-38 glucuronide in the control and antibiotic-cotreated rats after the intravenous administration of CPT-11 60 mg/kg. A single AUC value was calculated for the period 1–24 h from the mean values ($n = 4$) at each time point for each curve using trapezoid rule

	$AUC_{1-24\ h}$(ng · h/ml or g)		
	CPT-11	SN-38	SN-38 glucuronide
Blood			
Control	22438	66	574
Antibiotics	21753	75	483
Small intestine			
Control	293599	1989	7834
Antibiotics	264601	1963	8845
Large intestine			
Control	308826	6187	4376
Antibiotics	243229	1020	6585

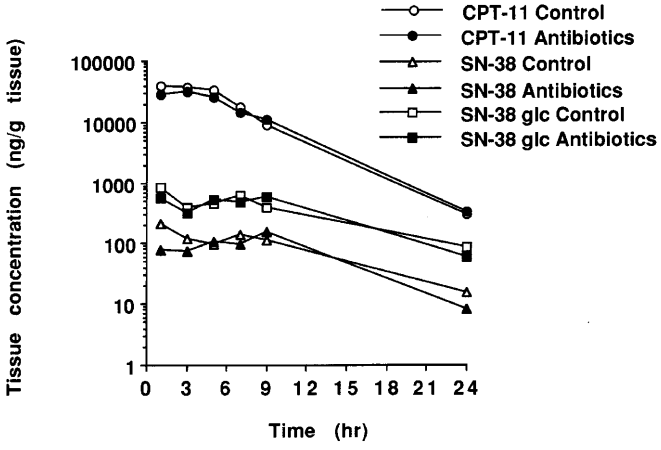


Fig. 3 Effects of antibiotics on the small intestine tissue concentration-time curves for CPT-11 and its metabolites after a single dose of CPT-11 (60 mg/kg, i.v.) in rats. Antibiotics (penicillin 1 mg and streptomycin 2 mg per ml drinking water) were administered from 5 days before the start of CPT-11 dosing. Each point represents the mean ± standard error from four animals

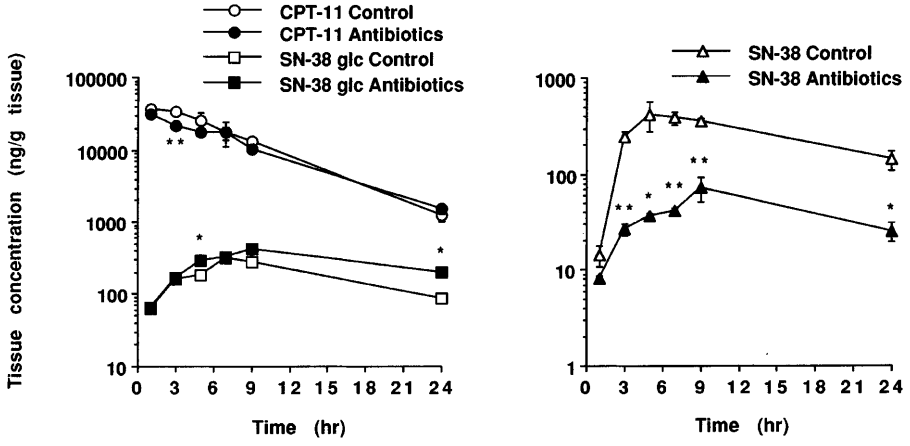
small intestine contents were almost constant from hours 1 to 9. The major metabolite was SN-38 glucuronide with levels approximately five times higher than those of SN-38, as was seen in the small intestine tissue (Fig. 5). The concentrations of CPT-11 and its metabolites in the large intestine contents were lower than those in the small intestine contents at 1 h, but increased sharply from hours 1 to 3, and the levels of CPT-11 and SN-38 exceeded those in the small intestine contents. The predominant metabolite was SN-38 in the luminal content of the large intestine from hours 1 to 24, with levels about 2 to 30 times those of SN-38 glucuronide (Fig. 6).

Antibiotics had little or no effect on the concentration-time profiles of CPT-11 or its metabolites in the luminal contents of the small intestine (Fig. 5). There was an increase of about three times in the mass of the large intestine contents, which is typical when the intestinal microflora is eliminated by antibiotics. Such an alteration clearly decreased the concentrations of CPT-11. The concentration of SN-38, however, decreased beyond that estimated from the increased mass of the contents, and that of SN-38 glucuronide markedly increased (Fig. 6). These changes resulted in a ratio of the concentration of SN-38 glucuronide to that of SN-38 similar to that of the small intestine contents.

Discussion

Although many clinical pharmacokinetic analyses of CPT-11 have been conducted [7, 11, 15, 22], the wide interpatient variations in the pharmacokinetics and the occurrence of diarrhea make the prediction of

Fig. 4 Effects of antibiotics on the large intestine tissue concentration-time curves for CPT-11 and its metabolites after a single dose of CPT-11 (60 mg/kg, i.v.) in rats. Antibiotics (penicillin 1 mg and streptomycin 2 mg per ml drinking water) were administered from 5 days before the start of dosing with CPT-11. Each point represents the mean ± standard error from four animals. $*P < 0.05$, $**P < 0.01$; vs the group treated with CPT-11 alone (*t*-test)



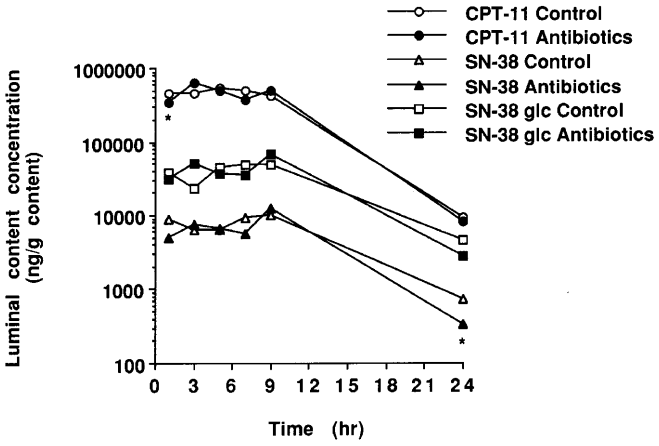


Fig. 5 Effects of antibiotics on the luminal content concentration-time curves for CPT-11 and its metabolites after a single dose of CPT-11 (60 mg/kg, i.v.) in the rat small intestine. Antibiotics (penicillin 1 mg and streptomycin 2 mg per ml drinking water) were administered from 5 days before the start of CPT-11 dosing. Each point represents the mean ± standard error from four animals. $^{*}P < 0.05$; vs the group treated with CPT-11 alone (*t*-test)

CPT-11-induced diarrhea difficult. The precise mechanisms of and reliable treatments for CPT-11-induced diarrhea are also still unknown [6, 7, 12, 20, 23]. We have recently found that the inhibition of β-glucuronidase in the intestinal microflora by antibiotics inhibits the conversion of SN-38 glucuronide to SN-38, thereby ameliorating the intestinal toxicity of CPT-11 [25]. The present study provides a pharmacokinetic basis for the hypothesis that the amount of SN-38, which is deconjugated from SN-38 glucuronide by β-glucuronidase in the intestinal microflora, remains higher for a longer period in the large intestine tissue and is involved in the development of intestinal toxicity (particularly the delayed onset of diarrhea accompanied by intestinal epithelial damage) induced by CPT-11.

After the administration of CPT-11 (60 mg/kg, i.v.), its two metabolites, SN-38 and SN-38 glucuronide, were detected in all the samples tested. The predominant metabolite in the blood, small intestine tissue, and small intestine contents was SN-38 glucuronide. This is consistent with the results of Gupta et al. [7], Lokiec et al. [12] and Rivory and Robert [16], who presented pharmacokinetic data on CPT-11 in human plasma and bile. The principal metabolite in the large intestine tissue and contents, on the other hand, was SN-38, except in the tissue at 1 h when SN-38 glucuronide was predominant. Although there was little or no difference in the distribution of the parent compound, CPT-11, between the small and large intestines, a higher amount of SN-38 was detected for a longer period in the large intestine tissue: the $AUC_{1-24\ h}$ of SN-38 in the large intestine was about three times higher than that in the small intestine.

It is suggested that the lower exposure of the small intestine to SN-38 is not due to segmental differences in the disposition of the parent compound or SN-38 (or both) carried via the bloodstream. The higher amount of SN-38 seen in the small intestine than in the large intestine at 1 h may reflect the higher activity of carboxylesterase [25], an enzyme which converts CPT-11 to SN-38. Since there was a good correlation between the β-glucuronidase activity in the intestinal lumen and the degree of intestinal damage [25], we have previously suggested that SN-38, which mainly results from the hydrolysis of SN-38 glucuronide by β-glucuronidase, may play a key role in the exacerbation of intestinal toxicity induced by CPT-11. The present pharmacokinetic study of the intestinal distribution of SN-38 suggests that the lesser degree of damage found in the small intestine than in the large intestine could be explained by the lower exposure of the tissue to SN-38, which may have been due to the smaller amount of β-glucuronidase activity in the luminal contents of the small intestine.

The inhibition of β-glucuronidase activity in the intestinal lumen by antibiotics had little or no effect on the concentration-time profiles of CPT-11, SN-38, or SN-38 glucuronide in the blood, or in the tissue or contents of the small intestine, except for the faster decline of SN-38 glucuronide in the blood. In contrast, antibiotics markedly reduced the $AUC_{1-24\ h}$ of SN-38 (by about

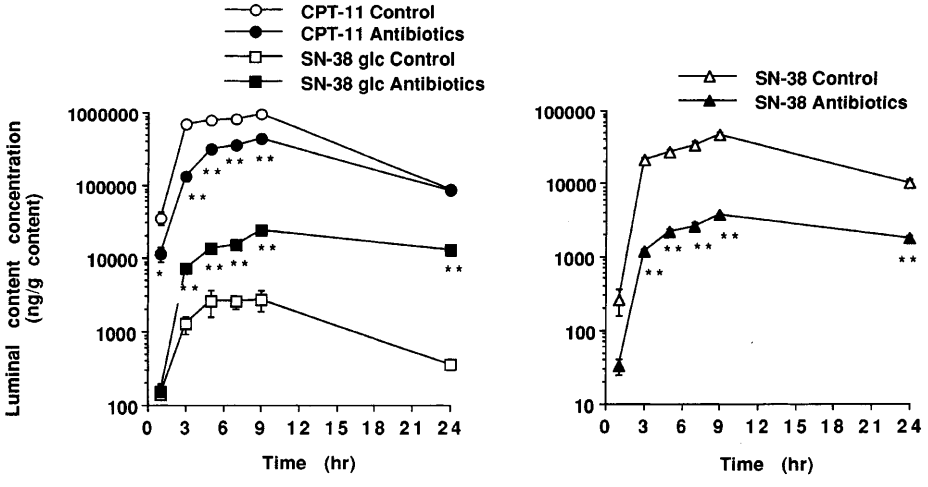


Fig. 6 Effects of antibiotics on the luminal content concentration-time curves for CPT-11 and its metabolites after a single dose of CPT-11 (60 mg/kg, i.v.) in the rat large intestine. Antibiotics (penicillin 1 mg and streptomycin 2 mg per ml drinking water) were administered from 5 days before the start of dosing with CPT-11. Each point represents the mean ± standard error from four animals. $^{*}P < 0.05$, $^{**}P < 0.01$; vs the group treated with CPT-11 alone (*t*-test)

85%) in the large intestine tissue, with a slight increase in the $AUC_{1-24\ h}$ of SN-38 glucuronide. Since the concentration-time trends of SN-38 in the large intestine were very similar to those of SN-38 in the luminal contents, it is supposed that SN-38 carried in the large intestine lumen may contribute considerably to the distribution of SN-38 in the large intestine tissue. The lower distribution of SN-38 in the large intestine in the antibiotic-cotreated group could have been due to inhibition of the conversion of SN-38 glucuronide to SN-38 by β-glucuronidase in the lumen, which led to a decreased SN-38 concentration in the luminal contents. No change in the concentration-time trends of CPT-11, SN-38 glucuronide and SN-38 in the luminal contents of the small intestine by antibiotics further supports the above results.

We have shown that CPT-11 impairs normal intestinal mucosal absorptive and secretory functions, resulting in watery diarrhea of acute onset [23]. Recently, Sakai et al. [21] have reported that not only CPT-11 but also SN-38 induces active anion (Cl^-) secretion in the rat colon in vitro. Thus, inhibition of the conversion of SN-38 glucuronide to SN-38 in the large intestine lumen should improve not only structural injuries but also functional injuries, both of which may be induced by SN-38.

Prior pharmacokinetic studies aimed at improving our understanding of the mechanisms of CPT-11-induced diarrhea, or at predicting the incidence of diarrhea have shown inconsistent relationships between the pharmacokinetics and gastrointestinal toxicity of CPT-11 [11, 13, 15, 18, 19, 22]. Although it is presently unknown whether CPT-11 causes diarrhea by the same mechanisms in humans as in our rat model, the pharmacokinetics of CPT-11 and its metabolites in rats were similar to those in humans in terms of higher plasma and bile concentrations of SN-38 glucuronide as compared with SN-38, and of higher elimination by biliary excretion than urinary excretion [4, 7, 12, 16]. The present results suggest the significance of intestinal bacterial enzymes, particularly β-glucuronidase, in the gastrointestinal toxicity of CPT-11. Moreover, no change in the disposition of CPT-11 and its metabolites in the plasma between the control and the antibiotic-cotreated rats which suffered less gastrointestinal toxicity may indicate that it is difficult to predict the occurrence of diarrhea from the pharmacokinetic analyses of plasma CPT-11 and its metabolites in clinical trials. Recently, early use of intensive loperamide therapy after the first diarrheal episode has been shown to be successful in controlling delayed onset diarrhea of CPT-11 in the United State and France [1, 2, 5, 19], but not in Japan because of a limitation on the maximum daily dosage of loperamide. Thus, an approach for predicting and ameliorating CPT-11-induced diarrhea in patients such as determining the fecal β-glucuronidase activity and/or inhibiting the enzyme activity may be valuable.

In conclusion, the present results suggest that SN-38, which results from the hydrolysis of SN-38 glucuronide by β-glucuronidase in the large intestinal microflora, contributes considerably to the distribution of SN-38 in the large intestine. The protective effects of antibiotics against CPT-11-induced intestinal toxicity in rats could be, at least in part, due to the decrease of exposure of the large intestine to SN-38, which, in turn, results from the inhibition of bacterial β-glucuronidase activity. However, since bacteria or bacterial products in the intestinal lumen could play a part in the progression of inflammatory lesions to chronicity [26], further studies will concentrate on elucidating other protective mechanisms of antibiotics.

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