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ORIGINAL ARTICLE

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Activation and antitumor activity of CPT-11 in plasma esterase-deficient mice

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Abstract *Purpose*: To examine the antitumor activity and the pharmacokinetics of CPT-11 (irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin) in a plasma esterase-deficient scid mouse model, bearing human tumor xenografts. *Experimental design*: Plasma carboxylesterase (CE)-deficient mice were bred with scid animals to develop a strain that would allow growth of human tumor xenografts. Following xenotransplantation, the effect of the plasma esterase on antitumor activity following CPT-11 administration was assessed. In addition, detailed pharmacokinetic studies examining plasma and biliary disposition of CPT-11 and its metabolites were performed. *Results*: In mice lacking plasma carboxylesterase, the mean SN-38 systemic exposures were approximately fourfold less than that observed in control animals. Consistent with the pharmacokinetic data, four to fivefold more CPT-11 was required to induce regressions in human Rh30 xenografts grown in esterase-deficient scid mice, as opposed to those grown in scid animals. Additionally, the route of elimination of CPT-11, SN-38, and SN-38 glucuronide (SN-38G) was principally in the bile. *Conclusions*: The pharmacokinetic profile for CPT-11 and its metabolites in the esterase-deficient mice more closely reflects that seen in humans. Hence, these mice may represent a more accurate model for antitumor studies with this drug and other agents metabolized by CEs.

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Abbreviations $AUC_{0-\infty}$: Area under the concentration-time curve from time 0 to infinity · CE: Carboxylesterase · CL: Clearance · CPT-11: Irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycamptothecin · HPLC: High-performance liquid chromatography · *o*-NPA: *o*-nitrophenyl acetate · SN-38: 7-ethyl-10-hydroxycamptothecin · SN-38G: SN-38 glucuronide · V_c: Volume of the central compartment

Introduction

Irinotecan, 7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin (CPT-11) is a prodrug that is activated by esterases to yield the potent topoisomerase I inhibitor, SN-38 (7-ethyl-10-hydroxycamptothecin [20]). CPT-11 has been approved for use in both front line and drug refractory colon cancer, and has demonstrated remarkable antitumor activity in a wide variety of pediatric solid malignancies [5]. As a consequence, this agent is being used in a variety of clinical trials to assess its efficacy against many different adult and pediatric cancers.

Biochemical evidence and subsequent animal studies indicate that the primary enzymes involved in CPT-11 activation are carboxylesterases (CEs) [10, 11, 13, 14, 17]. Cell lines engineered to overexpress these enzymes are sensitized to the drug, and in xenograft studies, complete regressions of human tumors can be achieved with CPT-11 after exogenous CE expression [3, 14, 15, 22]. However, in vivo, the levels of CPT-11 activation differ markedly between humans and rodents. For example, after a bolus administration of CPT-11 to humans (e.g., 350 mg/m^2), less than 5% of the drug is converted to SN-38 [16]; however, if lower CPT-11 dosages are used in humans (e.g., 20 mg/m^2), then a higher percentage of the drug is converted to SN-38

(e.g., ~40%). In contrast, greater than 50% of CPT-11 is activated after intravenous injection in mice [13, 12]. The differences observed are probably due to several factors including the affinity of the enzymes for the drug, the levels of CPT-11-activating enzymes expressed, and the presence of CE in rodent blood.

Human blood lacks CE activity, and consequently the level of CPT-11 activation seen by human plasma in vitro is very low [7]. However, plasma derived from mice and rats is very proficient at CPT-11 activation, with greater than 50% of the drug converted to SN-38 within 1 hour of incubation [12]. Hence, animal models designed to predict tumor responses in humans may overestimate the efficacy of the drug due to the increased plasma activation of CPT-11.

Recently, we identified a mouse strain (*Es1*^{e}) with reduced plasma esterase activity and demonstrated that plasma obtained from these animals was very inefficient at CPT-11 metabolism. [12, 13, 18]. Preliminary pharmacokinetic studies demonstrated that less SN-38 was measured in the plasma of these mice, as compared to control mice, after CPT-11 administration. We now detail the pharmacokinetics of CPT-11, SN-38, and SN-38 glucuronide (SN-38G) in both plasma and bile, and assess the response of a human rhabdomyosarcoma xenograft (Rh30) to CPT-11 when grown in immune-deprived *Es1*^{e} mice. These studies demonstrate the contribution of blood CE toward the antitumor activity of CPT-11, and indicate that biliary excretion of the drug may account for the delayed diarrhea frequently observed after CPT-11 administration.

Materials and methods

Drugs

CPT-11 and SN-38 were kindly provided by Dr. J.P. McGovren. SN-38G was obtained from Yakult Honsha Co. Ltd., Tokyo, Japan.

Animals

B6D2 and *Es1*^{e} mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and the latter were bred in an isolator, housed at Charles River Laboratories (Worcester, MA, USA). Crosses with C.B-17/IcrCrl-scid-BR mice were performed at Charles River and the colony was maintained at that site. Upon arrival at St. Jude Children's Research Hospital, animals were housed in an AALAC accredited vivarium and were handled using an approved IACUC protocol following the Guide to the Care and Use of Laboratory Animals.

Pharmacokinetic studies

Mice were anesthetized with isoflurane and CPT-11 (10 mg/kg), dissolved in 0.9% sodium chloride, was administered by retro-orbital injection. Plasma was harvested at time intervals ranging from 5 min to 24 h after CPT-11 administration, and immediately mixed with an equal volume of cold acid methanol. At the time of plasma isolation, bile was collected from the gall bladder using a 29-gauge needle attached to a 0.3-cc insulin syringe and was diluted with an equal volume of cold acid methanol as indicated above. All samples were centrifuged at 14,000 g for 10 min at 4°C, and total (lactone and carboxylate) CPT-11 and metabolite concentrations were determined by HPLC.

A minimum of three animals was used for each time point and data from *Es1*^{e} and B6D2 mice were fitted separately to a 5-compartment model using maximum likelihood estimation (ADAPT II; [2]). Parameters estimated included inter-compartment rate constants (k_{13}, k_{35}), elimination rate constants (k_{30}, k_{50}), and V_c (volume of the central compartment). Clearance of CPT-11 (CL) and area under the concentration–time curve from time 0 to infinity ($AUC_{0-\infty}$) were calculated using standard equations.

Carboxylesterase activity

CE activity was determined spectrophotometrically using *o*-nitrophenyl acetate (*o*-NPA) as a substrate [1, 14]. Data were expressed as nanomoles of *o*-nitrophenol produced per milligram of protein for cell sonicates or per milliliter for plasma.

In vitro CPT-11 conversion assays

The ability of samples to metabolize CPT-11 in vitro was assessed by incubation with 5 µM drug in 50 mM Hepes pH7.4 in a total volume of 200 µl. After incubation at 37°C for 1 h, reactions were terminated by the addition of an equal volume of cold acid methanol and stored at −70°C for a minimum of 1 h. Particulate matter was removed by centrifugation at 20,000 g for 30 min at 4°C prior to HPLC analysis.

Preparation of gut extracts

The entire alimentary canal was removed from *Es1*^{e} or control mice and divided into 1-cm sections. Following removal of the gut contents by rinsing with cold PBS, the sections were finely minced on ice and transferred to ice cold 50 mM Hepes pH7.4. Samples were sonicated twice using a Cole Parmer 4710 ultrasonic homogenizer for 10 s on ice. Sonicates were not centrifuged since CEs are present in the membrane fraction of cell extracts.

Detection of CPT-11, SN-38, and SN-38G

CPT-11 and its metabolites were separated by reverse phase HPLC and detected using a Jasco 920FP

fluorimeter as previously described [8]. Briefly, an equal volume of acidified methanol was added to each sample, and after centrifugation to remove particulate material, the supernatant was applied to a 300×3.9 mm NovaPak 4 μm C18 column using 25% acetonitrile/75 mM ammonium acetate pH 4.0 as a mobile phase. Under these conditions, SN-38G, CPT-11, and SN-38 eluted at 2.5, 5.0 and 7.5 min, respectively. The excitation wavelength was set at 375 nm and the emission wavelength varied to allow optimal quantitation of drug metabolites. To facilitate detection of SN-38G, the emission wavelength of the fluorimeter was set to 420 nm for the initial 3.5 min of the run, and subsequently increased to 550 nm to ensure maximal sensitivity for SN-38 determination. The sensitivity of this system was 20, 1.5 and 10 pg/μl for CPT-11, SN-38, and SN-38G, respectively.

Toxicity studies

CPT-11 toxicity was assessed by weight loss of animals. Groups of seven animals were weighed and the total weight recorded on days Monday through Friday. Mice were treated with 25, 50 or 75 mg/kg of CPT-11 on a daily ×5 schedule repeated twice in a 3-week period ([(d×5)2]3) and three complete courses of drug were given. Results were calculated as the percentage of body weight compared to day 1. Groups of animals that lost more than 15% of their original weight were euthanized.

Xenograft studies

Methods for tumor transplantation and statistical analysis of xenograft studies were performed as previously described [9]. Briefly, mice bearing advanced s.c. Rh30 human rhabdomyosarcoma xenografts (typically 1 cm^3) were treated with CPT-11 given i.v., on a daily ×5 schedule repeated for 2 weeks in a 3-week cycle ([(d×5)2]3), at dosages ranging from 0.28 mg/kg/day to 2.5 mg/kg/day. These doses are within the range that are tolerated in humans and yield good antitumor responses with this xenograft model. Tumor volumes were measured at weekly intervals using digital vernier calipers and data transferred directly to a Microsoft Excel spreadsheet. Tumor volumes were calculated using the formula $[(\pi/6)\times d^3]$ where d is the diameter.

Results

Pharmacokinetics of CPT-11 in *Es1*^{e} and control mice

We have previously described the plasma pharmacokinetics of CPT-11 and SN-38 in *Es1*^{e} mice [12], and demonstrated that the SN-38 plasma concentrations were considerably lower in these animals as compared to control mice. We have furthered these studies by examining the concentrations of CPT-11, SN-38, and SN-38G in both plasma and bile after a single 10 mg/kg retro-orbital injection of CPT-11.

Plasma pharmacokinetics

To confirm that reduced CPT-11 metabolism would occur in *Es1*^{e} mice, we monitored the formation of SN-38 and SN-38G after retro-orbital injection of 10 mg/kg CPT-11. With the inclusion of the SN-38G concentrations, the data were fitted to a five-compartment model as indicated in Fig. 1. Plasma SN-38 concentrations were about fourfold lower in the *Es1*^{e} mice compared to the control B6D2 mice as depicted in Fig. 2. The plasma SN-38 $AUC_{0\rightarrow\infty}$ for *Es1*^{e} mice was also approximately fourfold lower than that observed in control mice (Table 1). Interestingly, the elimination of SN-38 (k_{30}) was ninefold higher in control mice but the conversion to SN-38G (k_{35}) was approximately sixfold lower. In contrast, the SN-38G $AUC_{0\rightarrow\infty}$ values were similar between the two strains of animals (597 versus 651 ng/ml*h), due to the higher conversion rate for SN-38 to the glucuronide (k_{35}) in *Es1*^{e} mice.

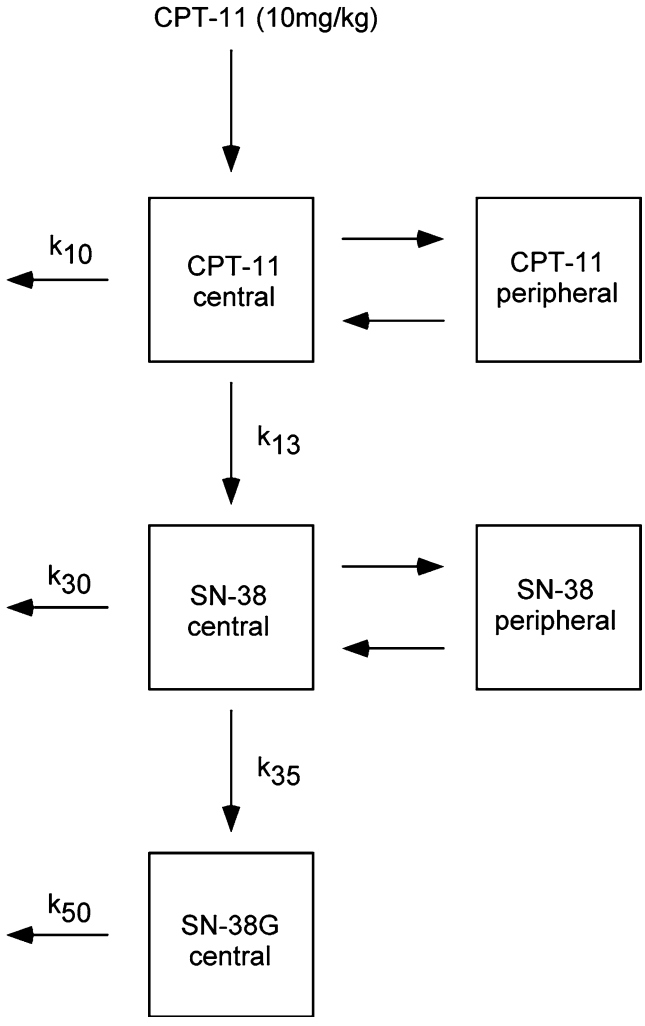

Fig. 1 The five-compartment model to which the pharmacokinetic data were fitted

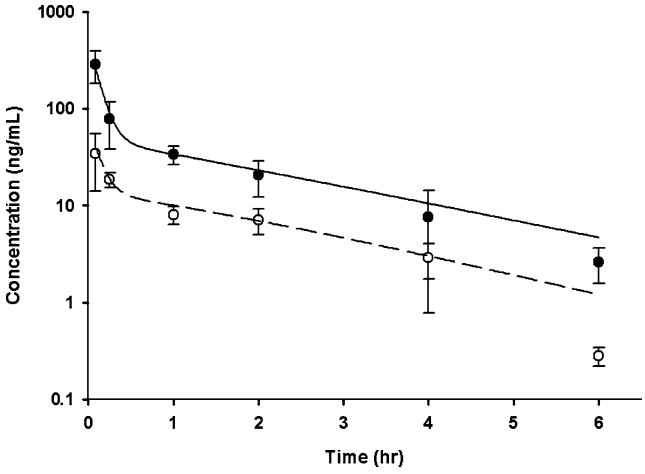

Fig. 2 SN-38 plasma concentrations versus time in control B6D2 (*solid line with filled circle*) and *Es1*^{e} (*broken line with open circle*) mice following retro-orbital injection of 10 mg/kg CPT-11

Table 1 Plasma pharmacokinetic parameters obtained from B6D2 and *Es1*^{e} mice following a single injection of 10 mg/kg of CPT-11

Pharmacokinetic parameter	B6D2	*Es1*^{e}
CPT-11		
$AUC_{0-\infty}$(ng/ml × h)	731	1,044
$k_{13}(h^{-1})$	5.5	3.4
CL (l/h/m^2)	46.4	31.2
SN-38		
$AUC_{0-\infty}$(ng/ml × h)	163	41
k_{30} (h^{-1})	9.64	1.05
k_{35} (h^{-1})	14.8	85.3
SN-38G		
$AUC_{0-\infty}$(ng/ml × h)	597	651
$k_{50}(h^{-1})$	4.0	5.4

Bile pharmacokinetics

Mean maximum biliary concentrations of CPT-11, SN-38, and SN-38G in B6D2 mice were ~600, 150, and 73 μM, respectively, compared to ~260, 116, and 34 μM in *Es1*^{e} mice. We estimated cumulative biliary excretion of CPT-11 and its metabolites in the two strains of animals. Table 2 indicates the amounts of CPT-11, SN-38, and SN-38G that were recovered in the bile. Accordingly, the cumulative biliary secretion of all three compounds was very high. As indicated in Table 2, B6D2 mice had a 1.7-, 1.3-, and 2.2-fold higher CPT-11, SN-38, and SN-38 cumulative biliary excretion, respectively, as compared to *Es1*^{e} mice. The average cumulative biliary excretion of CPT-11, SN-38, and SN-38G from two experiments in B6D2 and *Es1*^{e} mice is presented in Fig. 3.

Esterase metabolism of CPT-11 by gut extracts

Since the bile contained very high CPT-11 concentrations, and we have previously reported that the human small intestine can efficiently activate CPT-11 to SN-38 [11, 12], we determined the ability of the entire length of the mouse gastrointestinal tract to metabolize CPT-11. CE activity was also determined in identical samples using *o*-NPA as a substrate. Figure 4 demonstrates that peak CE and CPT-11 conversion occurred immediately distal to the stomach in the upper portion of the small intestine. This region of the gut is coincident with the entrance of the bile duct into the intestine. Therefore, after CPT-11 administration, very high CPT-11 and SN-38 concentrations would be deposited into the duodenum, the organ which contains peak levels of both CE and CPT-11-converting activities (Fig. 4). Hence, our data support the hypothesis that the delayed diarrhea associated with CPT-11 treatment may be due to direct conversion of the drug to SN-38 by enzymes located within the epithelial lining of the small intestine [6, 19]. This would result in local damage to proliferating tissues within the gut, i.e., the epithelial stem cells within the crypts, and consequently, diarrhea.

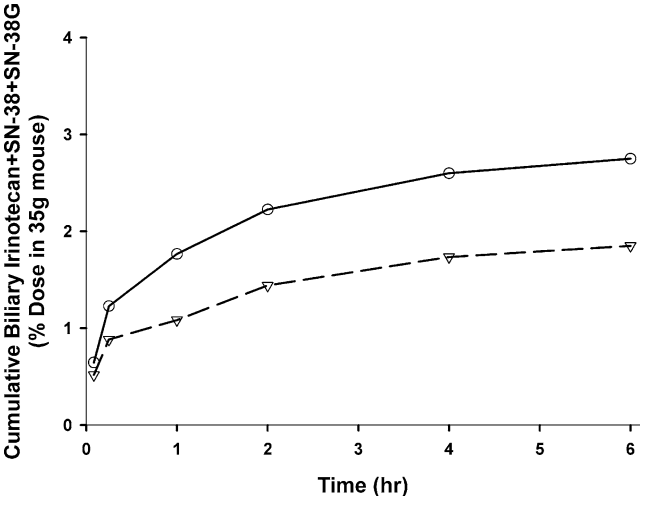

Fig. 3 Average cumulative biliary excretion of CPT-11, SN-38, and SN-38G in B6D2 (*broken line with inverted triangle*) and *Es1*^{e} (*solid line with open circle*) mice following injection with 10 mg/kg CPT-11. Data represents the mean values obtained from two independent experiments

Table 2 Observed concentrations of CPT-11, SN-38, and SN-38G in bile following injection of 10 mg/kg of CPT-11

Mouse	Average bile concentration (μM)			Cumulative amount of drug in bile (nmol)		
	CPT-11	SN-38	SN-38G	CPT-11	SN-38	SN-38G
Es1^e	163 ± 32	71.7 ± 4.9	19.8 ± 3.9	6.2 ± 5.4	8.4 ± 4.3	1.8 ± 0.6
B6D2	275 ± 71	95.9 ± 35	44.5 ± 15	7.3 ± 1.9	2.6 ± 0.8	1.2 ± 0.4

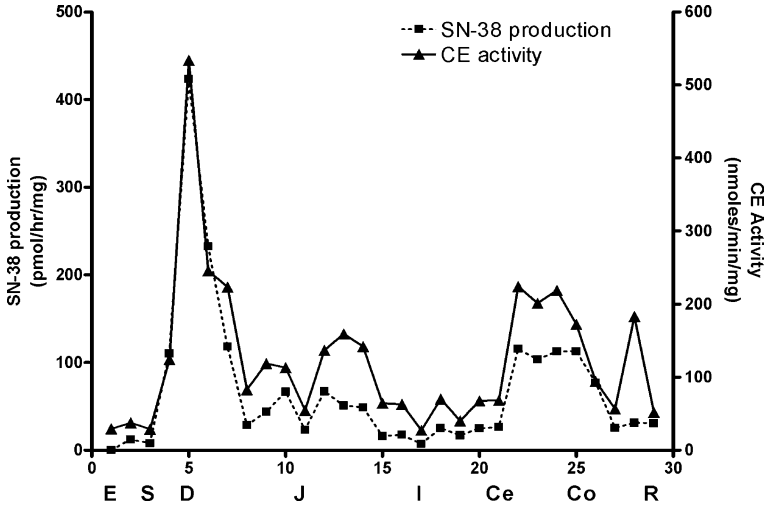

Fig. 4 Esterase activity in section of mouse gut. Metabolism of *o*-NPA (*solid line with filled triangle*) and CPT-11 (*broken line with filled square*) were assessed as described in the Material and methods. Fraction 1, esophagus (*E*); fractions 2–3, stomach (*S*); fractions 4–21, small intestine [duodenum (*D*), jejunum (*J*), ileum (*I*)]; Fractions 22–29, large intestine [cecum (*Ce*), colon (*Co*) and rectum (*R*)]. The bile duct enters the gut at the duodenum (fraction 5)

Toxicity of CPT-11 to *Es1*^{e} and control mice

Since the plasma SN-38 concentrations were lower in *Es1*^{e} mice, we would predict that these animals would be less sensitive to the toxic effects of CPT-11. To determine if this was the case, mice were treated with increasing dosages of CPT-11 using the [(*d* ×5)2]3 administration schedule. At the 75 and 50 mg/kg dosages, the *Es1*^{e} animals lost less weight than control B6D2 mice, with the latter being terminated after 10 days treatment at the higher dose (Fig. 5a). By comparison, *Es1*^{e} animals treated with 75 mg/kg CPT-11 were not terminated until day 33 (Fig. 5b). A similar difference was observed with animals treated with 50 mg/kg CPT-11; however, essentially no difference was observed at the lowest CPT-11 dose. This is probably due to the fact that at this dose level, both B6D2 mice and *Es1*^{e} animals tolerate the toxic effects of SN-38.

Generation of *Es1*^{e}/scid mice

To perform human tumor xenograft studies with the *Es1*^{e} mice, we generated scid variants that would support xenotransplantation. *Es1*^{e} mice were bred with C.B-17/IcrCrl-scid-BR animals and the offspring were screened for plasma esterase activity and immunoglobulin levels. Table 3 indicates the results of these crosses. As noted, of 121 F1 animals, 36 had plasma esterase activity below 900 nmoles/min/ml, of which 13 were also scid. Crossing of these mice to yield an F2 generation produced 16 animals, all of which demonstrated the *Es1*^{e} and scid phenotypes. These animals were then used for

Table 3 Plasma CE activities in mice following breeding of *Es1*^{e} and scid animals

Mouse	Plasma CE activity (nmoles/min/ml; ean ± S.D.)	No. of mice	No. of scids
C.B-17/IcrCrl-scid-BR	3043	NA[a]	NA
Es1^e	610	NA	NA
F1 cross	< 900 (717 ± 70)	36	13
	> 900 (2869 ± 90)	85	ND[b]
F2 cross	< 900 (550 ± 66)	16	16
	> 900	0	NA

Enzyme activity was determined spectrophotometrically using *o*-NPA as a substrate (see Materials and methods for details)
[a]NA not applicable
[b]ND not determined

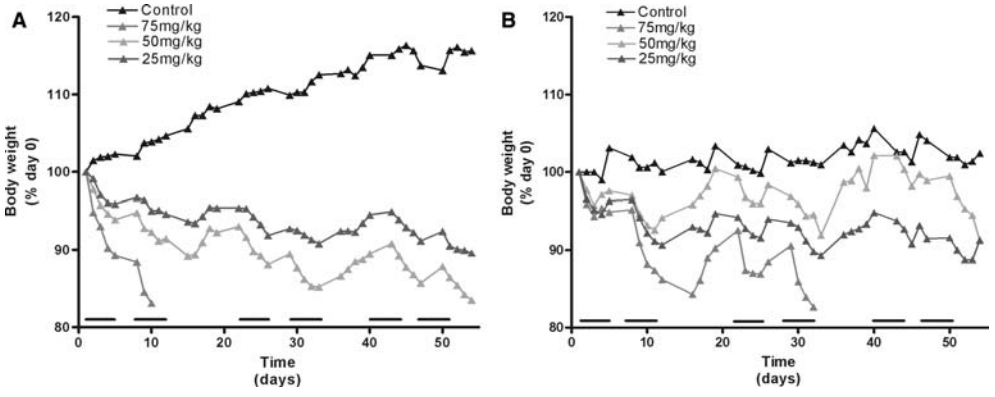

Fig. 5 Weights of B6D2 (**a**) and *Es1*^{e} (**b**) mice following treatment with 25 (*blue line*), 50 (*red line*) or 75 mg/kg (*green line*) of CPT-11. Drug was given i.v. on a daily ×5 schedule, repeated for 2 weeks in a 3-week cycle ([(*d* ×5)2]3), as indicated by the *black bars* adjacent to the abscissa

human tumor xenograft drug studies using the rhabdomyosarcoma Rh30 [4].

Xenograft studies in scid and *Es1*^{e}/scid mice

The sensitivity of Rh30 xenografts in scid and *Es1*^{e}/scid mice was assessed by monitoring growth of advanced s.c. tumors after treatment with CPT-11 using the [(d×5)2]3 schedule for three complete courses. Figure 6 demonstrates that a dose of 0.28 mg/kg results in partial regression of tumors in scid animals, whereas 1.25 mg/kg CPT-11 is required for an equivalent response in *Es1*^{e}/scid mice. Hence, approximately four to five times more CPT-11 is necessary to achieve similar antitumor responses in the *Es1*^{e}/scid animals. These data are consistent with our pharmacokinetic analyses, which indicated that the plasma SN-38 $AUC_{0 \rightarrow \infty}$ in the *Es1*^{e} mice was approximately four times less than that observed in the B6D2 animals (Table 1).

Discussion

The use of human tumor xenografts implanted into animal models to monitor the extent of drug activation and antitumor responses has been extensively used in preclinical studies. However, implicit in these experiments is the belief that drug disposition is similar between both species, and that the toxicities observed might predict results in clinical trials. Since CPT-11 is a prodrug that must be converted to SN-38 prior to demonstrating antitumor activity, any difference in the specificity and/or activity of the human or animal enzymes involved in this conversion, should be apparent in in vitro studies. In such studies, we observed that plasma from scid mice was greater than 4,000-fold more efficient at CPT-11 activation than human plasma (data not shown), indicating that either the specificity, or level of esterases in this fluid differed enormously. In contrast, plasma derived from *Es1*^{e} mice demonstrated similar levels of drug conversion to those of human plasma and we have proposed therefore that this strain of animals is a more suitable model for preclinical evaluation of CPT-11 activation and antitumor activity [12].

Based upon these hypotheses, we performed detailed plasma pharmacokinetic studies that indicated that less SN-38 was produced in *Es1*^{e} mice and that the rate of conversion from CPT-11 to SN-38 was about half of that in the control B6D2 mice. Also, the level of SN-38 systemic exposure was about fourfold less in *Es1*^{e} mice as compared to control animals (Table 1). This decrease in SN-38 production was evident in xenograft studies, which indicated that the *Es1*^{e}/scid mice required a fourfold higher dose of CPT-11 to achieve an equivalent antitumor response (Fig. 5). Therefore, it is likely that the *Es1*^{e}/scid mouse represent a more appropriate model to predict antitumor activity of CPT-11 in humans. Studies to assess the influence of plasma CE activity on the antitumor activity of this drug are currently underway.

Interestingly, very high levels of CPT-11 and its metabolites were detected in the bile of both B6D2 and *Es1*^{e} mice, consistent with this being a major route of elimination of the drug from these animals. However, analysis of the CE and CPT-11 converting activities of the gut suggest that the duodenum into which the bile is secreted contains the highest level of drug activating

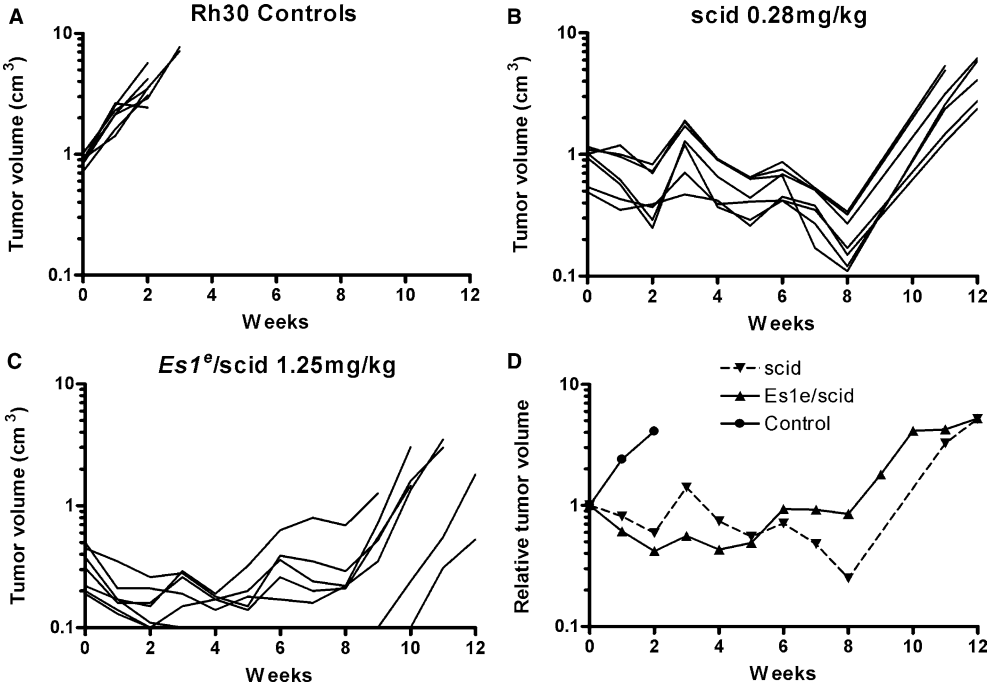

Fig. 6 Response of Rh30 xenografts following treatment with CPT-11 when grown in scid or *Es1*^{e}/scid mice. CPT-11 was given by i.v. on a daily ×5 schedule repeated for 2 weeks in a 3-week cycle ([(*d* ×5)2]3). **a** Tumor volumes for nondrug-treated mice. **b** Volumes of tumors grown in scid mice following treatment with 0.28 mg/kg of CPT-11. **c** rowth of xenografts in *Es1*^{e}/scid mice following administration of 1.25 mg/kg of CPT-11. **d** Relative tumor volumes of all datasets shown in panels **a**–**c**

enzymes (Fig. 3). Hence, any free CPT-11 in the bile could be converted to SN-38 upon entering the gastrointestinal tract and would result in gut toxicity. Since it would be very difficult to obtain consecutive biopsies of human gut, it is unknown whether human duodenum has similar abilities to activate CPT-11. Previously, we have demonstrated that the human small intestine is capable of drug activation [11], although the extent to which this occurs in vivo is unknown. However, the data presented here indicate that if the metabolic fate of CPT-11 is similar in humans to that observed in *Es1*^{e} mice, very high levels of CPT-11 would be deposited into the gut where it would be converted to SN-38. This would likely result in local tissue damage and diarrhea. Since human cells and tissues are considerably more sensitive to SN-38 than mouse cells, diarrhea from CPT-11 administration occurs at much lower doses in patients than in mice [5]. Nonetheless, the diarrhea would occur by a similar mechanism in the two species.

Analysis of the cumulative amounts of CPT-11, SN-38, and SN-38G that were present in the bile indicated that exceptional high amounts of all three metabolites could be detected in this fluid (Table 2). Indeed the concentrations of drug and metabolites that we observed were potentially the highest that have ever been recorded for any biological sample. This is exemplified by SN-38, where average concentrations as high as 70–95 μM were observed in the bile. This concentration is ~1,000-fold greater than that require to kill human tumor cell lines in vitro [8, 21]. However, it is apparent from our direct measurements that very large amounts of all three compounds are excreted via this mechanism.

Overall, the studies presented in this paper demonstrate the contribution of plasma esterase in CPT-11 activation in mice. Pharmacokinetic experiments confirmed the differences in SN-38 $AUC_{0 \rightarrow \infty}$ levels between B6D2 and *Es1*^{e} mice, and this translated into a requirement for more CPT-11 to achieve the same antitumor activity in *Es1*^{e}/scid animals. We are currently using the *Es1*^{e}/scid mouse model to assess the effectiveness of CPT-11 in a variety of human tumor xenografts and to monitor drug metabolism. Ultimately, these studies may allow us to use CPT-11 more effectively in the clinic.

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