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ORIGINAL ARTICLE

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Biliary transport of irinotecan and metabolites in normal and P-glycoprotein-deficient mice

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Abstract *Purpose:* The extensive and unpredictable biliary excretion of CPT-11 and its metabolites, SN-38 and SN-38 glucuronide (SN-38G) may contribute to the wide interpatient variability reported in the disposition and gastrointestinal toxicity of CPT-11. We studied the role of P-glycoprotein (P-gp) in in vivo biliary excretion of CPT-11, SN-38 and SN-38G in mice lacking mdr1-type P-gp [*mdr1a/1b*(–/–)] in the presence of the multidrug resistance (MDR) reversal agent, PSC833. *Methods:* Wild-type (Wt) and *mdr1a/1b*(–/–) mice ($n = 3$ or 4) were treated intragastrically with PSC833 (50 mg/kg) or vehicle 2 h prior to i.v. CPT-11 dosing (10 mg/kg), and bile samples were collected. *Results and conclusions:* P-gp was found to play an important role in CPT-11 biliary excretion, as there was a significant (40%, $P < 0.05$) decrease in its biliary recovery in 90 min in *mdr1a/1b*(–/–) mice ($6.6 \pm 0.6\%$ dose) compared with Wt mice ($11 \pm 1.2\%$). This also implied a major role of other undetermined non-P-gp-mediated mechanism(s) for hepatic transport of CPT-11, which was inhibited by PSC833 ($1.8 \pm 0.8\%$ with PSC833, $6.6 \pm 0.6\%$ without PSC833) in *mdr1a/1b*(–/–) mice. SN-38 and SN-38G biliary transport was unchanged in mice lacking P-gp after vehicle treatment, indicating a lack of P-gp mediation in their transport. PSC833 significantly reduced (56–89%) SN-38 and SN-38G biliary transport in Wt and *mdr1a/1b*(–/–) mice, suggesting that PSC833 may be a candidate to modulate biliary excretion of SN-38 with potential use in reducing CPT-11 toxicity.

Keywords Irinotecan · Transporters · CPT-11 · PSC833 · P-glycoprotein

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Introduction

Irinotecan (7-ethyl-10-[4-(1-piperidino)-1-piperidino] carbonyloxycamptothecin, CPT-11) is a topoisomerase I enzyme inhibitor approved for the treatment of metastatic colorectal cancer. It is biotransformed via a complex metabolic cascade involving an initial activation by carboxylesterase enzyme (hCE-2 in humans) to SN-38 (7-ethyl-10-hydroxycamptothecin) [15, 23], which is then conjugated with glucuronic acid by hepatic uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) enzyme to SN-38 glucuronide (SN-38G) [16]. CPT-11 can also undergo oxidation to APC (7-ethyl-10-[4-*N*-(5-aminopentanoic acid)-1-piperidino]carbonyloxycamptothecin) [24] and NPC (7-ethyl-10-(4-amino-1-piperidino) carbonyloxycamptothecin) [13] via CYP3A4 (cytochrome P450 3A4) enzyme activity. Therapy with CPT-11 is characterized by the severe dose-limiting toxicities of diarrhea and myelosuppression, thought to be caused by the active metabolite, SN-38 [1].

Significant interpatient variability in pharmacokinetics and toxicity has been observed after CPT-11 administration in cancer patients [10, 12]. This may, in part, be due to the extensive and unpredictable biliary excretion reported for CPT-11 and its metabolites, SN-38 and SN-38G [2, 9, 19]. Different primary active transport mechanisms have been proposed for CPT-11 and its metabolites [5, 6, 7, 8, 29]. The anionic forms of SN-38 and SN-38G have been shown to be transported

into the bile via canalicular multispecific organic anion transporter/multidrug resistance-associated protein 2 (cMOAT/MRP2, symbol ABCC2) [6, 7]. Using an in vitro transport system across isolated rat canalicular membrane vesicles (CMVs), Chu et al. [8] have recently shown the possible involvement of P-glycoprotein (P-gp, symbol ABCB1) in the biliary excretion of CPT-11.

The current study was performed to investigate the role of P-gp in in vivo biliary transport of CPT-11 and its metabolites (SN-38, SN-38G and APC) in a mouse model of mdr1-type P-gp deficiency [(*mdr1a/1b*(–/–)] [26]. The effect of pretreatment with the multidrug resistance (MDR) reversal agent, PSC833 [(3′-oxo-4-butenyl-4-methyl-threonine[1])-(Val[2])-cyclosporin] on biliary excretion of CPT-11 and metabolites was also investigated.

Materials and methods

Chemicals

CPT-11 was kindly provided by Dr. Kiyoshi Terada (Yakult Honsha, Tokyo, Japan). SN38 and APC were obtained from Pharmacia and Upjohn (Kalamazoo, Mich.). PSC833 was a kind gift from Novartis Pharma (Basel, Switzerland). Acetonitrile, methanol, potassium dihydrogen phosphate, sodium dihydrogen phosphate, disodium hydrogen phosphate, hydrochloric acid and phosphoric acid were obtained from Fisher Scientific (Itasca, Ill.). Camptothecin (CPT), sodium heptane sulfonic acid and β-glucuronidase from *E. coli* were obtained from Sigma Chemical Company (St. Louis, Mo.). Ketamine (Ketamin KH) and xylazine HCl (Rompun 2%) were obtained from aniMedica West (Senden-Bösensell, Germany) and Bayer (Leverkusen, Germany), respectively.

Bile collection

Normal (wild-type, Wt) and *mdr1a/1b*(–/–) mice (female, 10–14 weeks of age) were obtained from the Netherlands Cancer Institute. The genetic background was 99% FVB. All animal experiments were carried out according to institutional guidelines and in compliance with German national law. The Wt and m*dr1a/1b*(–/–) mice were each divided into groups of four to receive PSC833 or corresponding vehicle (see below) pretreatments (except three *mdr1a/1b*(–/–) mice in the vehicle pretreatment group). Bile cannulation experiments were carried out as described previously [21]. Briefly, the abdominal cavity was opened after induction of anesthesia with ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). The common bile duct was ligated and a catheter was inserted into the gallbladder. After i.v. injection of CPT-11, bile was collected for 90 min, after which the mice were sacrificed.

CPT-11 solution in 0.9% saline was administered at a dose of 10 mg/kg (i.v. bolus). A solution of PSC833 in ethanol (33% v/v) and Cremophor EL (65% v/v) after further dilution in 5% (w/v) glucose, was administered orally (50 mg/kg) by intragastric injection 2 h prior to CPT-11 administration. Vehicle-treated groups were included, as Cremophor EL has itself been shown to reverse MDR [30]. In addition, control treatments were included with CPT-11 alone, i.e. without PSC833 or its vehicle. No acute side effects of PSC833 or CPT-11 were observed in Wt or *mdr1a/1b*(–/–) mice.

Assay of CPT-11 and metabolites (SN-38, SN-38G and APC) in bile

The concentrations of CPT-11 and metabolites were determined using a high-performance liquid chromatography (HPLC) system (Hitachi Instruments, San Jose, Calif.) with fluorescence detection at 355 nm (λ_{ex}) and 515 nm (λ_{em}). Bile samples (20 µl) were combined with 20 µl internal standard (CPT, 55 µg/ml), 200 µl 0.1 *M* sodium phosphate buffer (pH 6.4) and 1 ml methanol. Samples were acidified by the addition of 300 µl 0.5 *M* HCl, and 75-µl aliquots were injected into the HPLC system. The compounds were separated using a reversed-phase µBondapak C18 column (10 µm, 3.9×300 mm; Waters Corporation, Milford, Mass.) preceded by a µBondapak C18 guardpak (Waters). The mobile phase consisted of 28% acetonitrile and 72% 0.1 *M* KH_2PO_4 containing 3 m*M* sodium heptane sulfonate (pH adjusted to 4 with 8.5% H_3PO_4). At a flow of 0.8 ml/min the retention times of CPT-11, SN-38, APC and CPT were 10.7, 14.5, 7.5 and 16.3 min, respectively. Standard curves of CPT11, SN38 and APC were linear ($r^2 = 0.99$) within the ranges 10.1–149.2, 6.0–81.5 and 1.0–12.2 µg/ml, respectively.

SN-38G concentrations were determined as equivalents of SN-38 levels after incubation with β-glucuronidase enzyme. A 20-µl aliquot of bile was combined with 20 µl internal standard (CPT, 55 µg/ml), and 1000 U β-glucuronidase enzyme dissolved in 200 µl 0.1 *M* sodium phosphate buffer, pH 6.4. After a 2-h incubation at room temperature, 1 ml methanol and 300 µl 0.5 *M* HCl were added to the samples. Aliquots of 75 µl were analyzed by HPLC.

Statistical analysis

The amounts of CPT-11 and its metabolites excreted in bile over 90 min were expressed as percentages of the total CPT-11 dose administered in each mouse. Student's *t*-test (unpaired) was used to test for the significance of differences between two treatments using GraphPad Prism (GraphPad Software, San Diego, Calif.).

Results

The biliary excretions of CPT-11, SN-38 and SN-38G over 90 min after CPT-11 administration, expressed as percentages of CPT-11 dose, are shown in Fig. 1. About 11% of the CPT-11 dose was recovered unchanged in the bile during this period in Wt mice in the absence of PSC833 treatment (Fig. 1A). Recovery of SN-38 and SN-38G in bile was relatively minor (about 2% each, Fig. 1B, C) in these mice. Biliary excretion of APC was negligible (0.4%, data not shown) during this period. Vehicle (Cremophor EL) treatment did not significantly alter transport of CPT-11, SN-38 or SN-38G in either Wt or *mdr1a1b*(–/–) mice (Fig. 1A, B, C, respectively). Other studies have shown higher levels (9–55%) of biliary elimination of CPT-11, SN-38 and SN-38G [2, 19, 20], but these data were generated in rats and humans over longer periods of 24 to 48 h, in contrast to our study in mice over 1.5 h. The duration of bile collection was limited to 1.5 h in our study because of the difficulty of keeping the mice alive for longer periods as a result of the stress of laparotomy. It is quite possible that more CPT-11 may be recovered if the bile collections were performed for longer periods.

There was a 40% decrease ($P < 0.05$) in biliary recovery of CPT-11 over 90 min in the *mdr1a/1b*(–/–) mice (6.6 ± 0.6% of CPT-11 dose) in comparison with that observed in Wt mice (11 ± 1.2%), in the vehicle-treated groups (Fig. 1A). This indicates a significant role of P-gp in biliary transport of CPT-11. In this regard, our results are in agreement with the report that P-gp is a possible candidate for the high-affinity

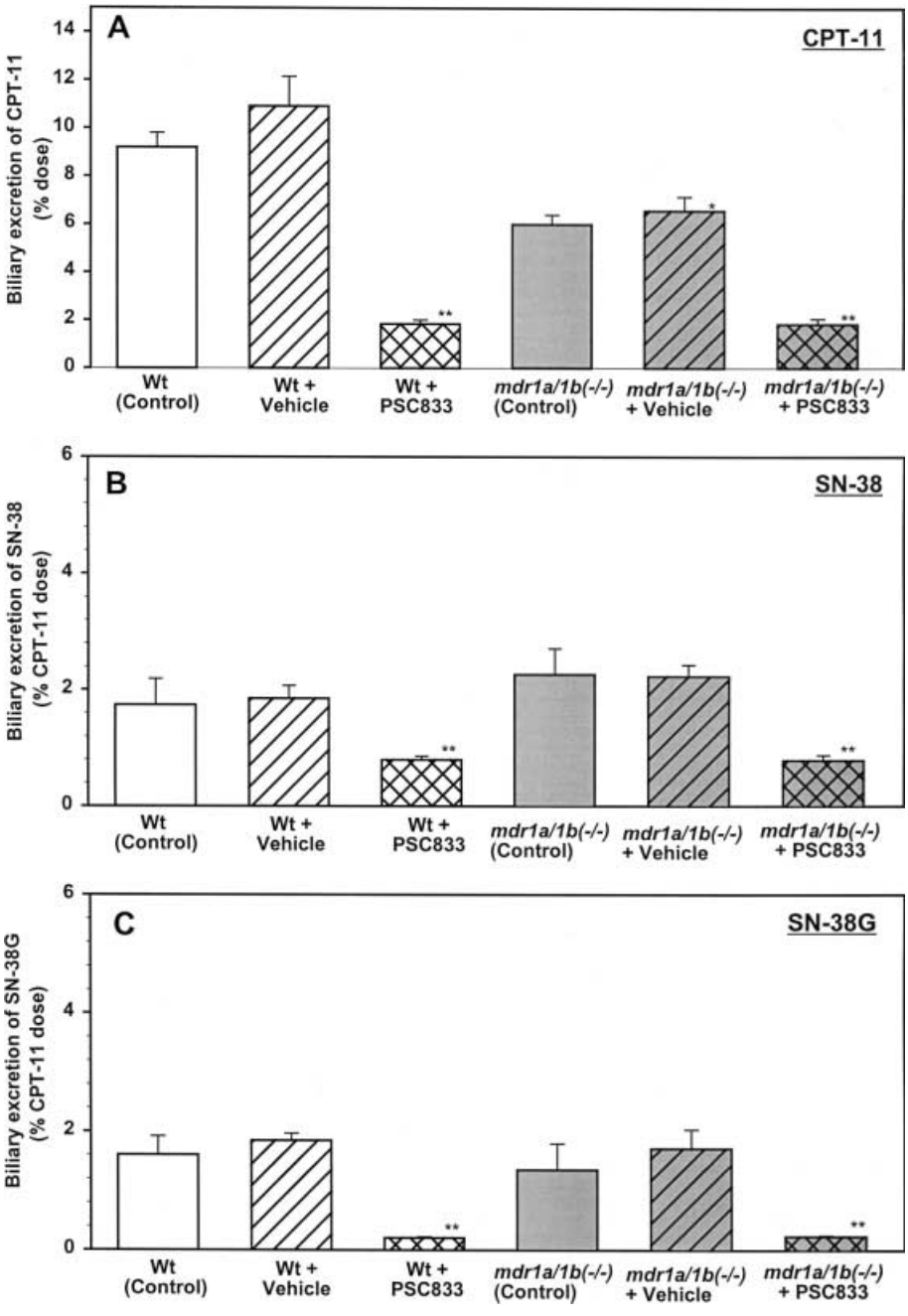

Fig. 1A–C Biliary excretion of CPT-11 (**A**), SN-38 (**B**) and SN-38G (**C**) over 90 min after CPT-11 administration (10 mg/kg, i.v.) in wild-type (Wt) and *mdr1a1b*(–/–) mice with and without PSC833 pretreatment (50 mg/kg, intragastric). Control rats were treated with CPT-11 alone, without Cremophor or PSC833. Each bar represents the mean ± SEM from three or four mice. *$P < 0.05$ *mdr1a/1b*(–/–) mice vs Wt mice after vehicle pretreatment; **$P < 0.05$ *mdr1a/1b*(–/–) and Wt mice after PSC833 treatment versus vehicle treatment (unpaired Student's *t*-test)

component of CPT-11 transport across isolated rat bile CMVs [8]. However, there was significant residual biliary excretion of CPT-11 in this mouse model of P-gp deficiency, suggesting that there are additional biliary transport mechanisms for CPT-11. In contrast to the biliary excretion CPT-11, those of SN-38 and SN-38G were not significantly different between the Wt and *mdr1a/1b*(–/–) vehicle-treated mice (Fig. 1B, C), ruling out the possibility of P-gp mediation in their transport. This supports the previous finding that carboxylate forms of SN-38 and SN-38G and the lactone form of SN-38G undergo biliary elimination mainly via cMOAT/MRP2 [12, 14].

Treatment with PSC833 resulted in a major reduction (84%, $P < 0.05$) in CPT-11 biliary excretion in Wt mice (1.8 ± 0.2% of CPT-11 dose) compared with the corresponding controls (11 ± 1.2%; Fig. 1A). In addition, PSC833 significantly reduced (73%, $P < 0.05$) CPT-11 transport in *mdr1a/1b*(–/–) mice (1.8 ± 0.8%) when compared with vehicle treatment (6.6 ± 0.6%; Fig. 1A). This provides evidence that CPT-11 is also eliminated into the bile via transport mechanisms other than P-gp, which are inhibited by PSC833. A possible mechanism would be via the breast cancer resistance protein (BCRP, symbol ABCG2), which is known to be a transporter of CPT-11 (and SN-38) [25]. PSC833 has been shown to

inhibit non-P-gp-mediated hepatic transport of other compounds, such as [^{3}H]digoxin [21]. However, its role on BCRP-mediated transport is not clear.

It is interesting that PSC833 significantly ($P < 0.05$) decreased biliary transport of SN-38 and SN-38G in Wt and *mdr1a/1b*(–/–) mice (Fig. 1B, C). There was a 56% and 89% decrease in SN-38 and SN-38G biliary elimination in Wt mice, respectively, after PSC833 treatment. Corresponding reductions in the P-gp knockout mice were 64% and 88%, respectively. This effect may have been due to inhibition of cMOAT/MRP2, which is known to mediate transport of SN-38 and SN-38G [5, 7], or due to inhibition of other unknown transporters by PSC833.

There was a residual level of biliary excretion of all three compounds (0.2–2%) after PSC833 treatment in both Wt and *mdr1a/1b*(–/–) mice (not significantly different; Fig. 1A, B, C). This may have been a result of incomplete inhibition of their transport by PSC833 or due to the presence of PSC833-resistant transporters present in normal and P-gp-deficient mice [21].

Discussion

The major route of elimination of CPT-11 and its metabolites (SN-38 and SN-38G) in humans is via biliary excretion, which may be an important contributing factor in the wide interpatient variability in the disposition and toxicity of CPT-11. Cumulative biliary excretion of CPT-11, SN-38 and SN-38G in two patients ranged from 25% to 50% of the CPT-11 dose [19]. Large bile to plasma concentration ratios have been reported for CPT-11 (70 and 135) and SN-38 (29 and 57) in the 1st and 3rd weeks during a weekly administration of CPT-11 in patients, respectively [9], suggesting possible intestinal accumulation after repeated dosing. It has been suggested that coadministration of cyclosporine (CSA) with CPT-11 may reduce SN-38 biliary excretion and diarrhea [22]. Studies of the modulation of the pharmacokinetics of CPT-11 with CSA have shown an increased systemic availability of CPT-11, SN-38 and SN-38G in rats [11] and in patients with cancer [22], possibly due to lowered biliary excretion. However, the specific mechanism for this CSA-mediated decrease in biliary excretion of these compounds is not clear.

The current study was performed to elucidate the in vivo role of P-gp in biliary transport of CPT-11 and its metabolites, using mice that lack P-gp, i.e. the *mdr 1a/1b*(–/–) mice. These mice have a genetic deficiency in *mdr1a* and *mdr1b* genes that encode P-gps with essentially the same drug-transporting functions and tissue distribution as the human MDR1 P-gp [21]. The effect of the MDR modulator, PSC833, in altering biliary transport of CPT-11 and its metabolites was also investigated. PSC833 is a non-immunosuppressive and non-nephrotoxic analog of CSA and has exhibited promising results in clinical trials in reversing MDR [28].

The following conclusions can be drawn from our experiments. First, P-gp plays an important role in the biliary transport of CPT-11. About 40% of the biliary recovery of CPT-11 over 90 min in mice may be attributed to transport via P-gp. Second, there is a significant non-P-gp-mediated component (60%) to CPT-11 transport, a major fraction (73%) of which is inhibited by PSC833. Third, the biliary excretion of SN-38 and SN-38G is not mediated by P-gp and is substantially reduced by PSC833.

This is the first study in which the effect of PSC833 on in vivo biliary transport of CPT-11 and its metabolites has been investigated. Our finding that PSC833 reduced biliary excretion of CPT-11, SN-38 and its glucuronide may be of significance, to prevent and/or reduce the extent of diarrhea after CPT-11 treatment. This reduction may have been due to inhibitory effects of PSC833 on P-gp (CPT-11), cMOAT/MRP2 (SN-38 and SN-38G) and possibly other unknown transporters. Previous studies have utilized CSA to modulate the pharmacokinetics of CPT-11 by reducing biliary transport of SN-38 [11, 22]. PSC833 and CSA have been shown to exhibit differences in their potency to inhibit transporters. CSA is more effective in reducing cMOAT-mediated leukotriene C_4 (LTC_4) transport (K_i 4.7 μM) than PSC833 (K_i 29 μM) [4] and therefore might be better than PSC833 in reducing biliary transport of SN-38 and SN-38G. PSC833 inhibits P-gp-mediated daunorubicin transport (K_i 0.3 μM) more specifically than CSA (K_i 1.5 μM) [3].

The attenuation of SN-38 transport by PSC833 may be expected to be greater with intravenous dosing rather than by gastric lavage, as was used in this study. The dose of PSC833 (50 mg/kg) used in this study was higher than that used in other studies, which could have resulted in a loss of specificity for P-gp inhibition. Further studies are required to compare the efficacies of CSA and PSC833 used in different routes and schedules, on the modulation of the disposition of CPT-11, SN-38 and SN-38G. In addition, it is quite possible that the *mdr1a/1b* knockout mice may have altered patterns of expression of CYP3A4 levels, as shown in other studies [27]. More detailed studies are required to investigate the complete pharmacokinetic and metabolic profile of CPT-11 in environments lacking P-gp activity.

Genetic differences in both hepatic metabolism and transport of CPT-11 may be responsible for the considerable interpatient variability in disposition and toxicity of CPT-11. CPT-11-induced diarrhea has been attributed to the enterocolitis caused by high levels of SN-38 and/or CPT-11 in the intestine [1, 5]. Biliary excretion of SN-38G may also be of importance as SN-38G can undergo deconjugation by intestinal β-glucuronidase to regenerate SN-38 [18]. The contribution of genetic differences between subjects in SN-38 glucuronidation to CPT-11-induced diarrhea is under active investigation [16, 17]. The TATA polymorphism [$(TA)_7TAA$ versus $(TA)_6TAA$)] in *UGT1A1* has been

shown to correlate with in vivo glucuronidation rates of SN-38 and CPT-11 toxicity in cancer patients [17].

It is unclear whether the modest role (less than 50%) of P-gp in biliary transport of CPT-11 will be clinically significant. Further studies are being planned to address the effect of alterations in P-gp transport on the complete pharmacokinetic profile of CPT-11. If P-gp is found to significantly influence the disposition of CPT-11, then our findings may add a new variable in understanding the pharmacogenetics of this drug. Functional polymorphisms have recently been described in the human *MDR-1* gene [14]. Patients homozygous to a polymorphism in exon 26 (C3435T) of the *MDR-1* gene have significantly lower duodenal expression levels of MDR-1 and high circulating digoxin levels. Hence, chemotherapy with CPT-11 may be influenced by multiple genetic factors that control its hepatic metabolism and transport.

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