OATP1A/1B transporters affect irinotecan and SN-38 pharmacokinetics and carboxylesterase expression in knockout and humanized transgenic mice

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Abstract (247/250)

Organic anion-transporting polypeptides (OATPs) mediate the hepatic uptake of many drugs, thus co-determining their clearance. Impaired hepatic clearance due to low-activity polymorphisms in human OATP1B1 may increase systemic exposure to SN-38, the active and toxic metabolite of the anticancer pro-drug irinotecan. We investigated the pharmacokinetics and toxicity of irinotecan and SN-38 in Oatp1a/1b-null mice: plasma exposure of irinotecan and SN-38 was increased 2-3 fold after irinotecan dosing (10 mg/kg, i.v.) compared to wild-type mice. Also, liver-to-plasma ratios were significantly reduced, suggesting impaired hepatic uptake of both compounds. After 6 daily doses of irinotecan, Oatp1a/1b-null mice suffered from increased toxicity. However, Oatp1a/1b-null mice had increased levels of carboxylesterase (Ces) enzymes, which caused higher conversion of irinotecan to SN-38 in plasma, potentially complicating pharmacokinetic analyses. Ces inhibitors blocked this increased conversion. Interestingly, liver-specific humanized OATP1B1 and OATP1B3 transgenic mice had normalized hepatic expression of Ces1 genes. Whereas irinotecan liver-to-plasma ratios in these humanized mice were similar to those in Oatp1a/1b-null mice, SN-38 liver-to-plasma ratios returned to wild-type levels, suggesting that human OATP1B proteins mediate SN-38, but not irinotecan uptake in vivo. Upon direct administration of SN-38 (1 mg/kg, i.v.), Oatp1a/1b-null mice had increased SN-38 plasma levels, lower liver concentrations and decreased cumulative biliary excretion of SN-38. Conclusion: Mouse Oatp1a/1b transporters have a role in the plasma clearance of irinotecan and SN-38, while human OATP1B transporters may only affect SN-38 disposition. Oatp1a/1b-null mice have increased expression and activity of Ces1 enzymes, while humanized mice provide a rescue of this phenotype.
Introduction

Organic anion-transporting polypeptides (human: OATPs; rodents: Oatps; gene names: \textit{SLCO}, \textit{Slco}) are uptake transporters which mediate the cellular uptake of various endogenous and exogenous compounds (1). Members of the OATP1A/1B subfamilies are of interest for their impact on the pharmacokinetics and hence the therapeutic efficacy of many drugs due to their tissue localization (liver, small intestine, and kidney) and wide substrate specificity. In the liver, human OATP1B1 and OATP1B3 and mouse Oatp1a1, -1a4 and -1b2 are expressed on the basolateral membrane of hepatocytes, where they mediate the hepatic uptake, and therefore the clearance of many (anticancer) drugs (2;3). The substrate specificity of both mouse and human OATP1A/1B proteins is very broad, from charged anionic drugs, to polar zwitterionic compounds, and even highly lipophylic drugs (3;4). Accordingly, a reliable common predictive structural feature has not been identified yet for OATP1A/1B substrates.

Irinotecan (CPT-11) is an anticancer drug, widely used in the treatment of colorectal, ovarian and lung cancer (5). Irinotecan’s therapeutic index is quite low, mainly due to its complex pharmacokinetics which involves many metabolic enzymes and drug transporters (6). The pro-drug irinotecan is hydrolyzed to its primary pharmacologically active (cytotoxic) metabolite, SN-38, mostly by carboxylesterase enzymes (CES in humans; Ces in mice), mainly in liver, but also in plasma and small intestine (5). Both irinotecan and SN-38 exist in two forms, lactone and free acid (Supplemental Figure 1), which adds to the complexity of their pharmacokinetics. Irinotecan is administered to patients in an acidic solution (pH 3.5) which contains mostly the lactone form. Upon intravenous administration, blood pH-dependent hydrolysis occurs and irinotecan is mostly found in its carboxylate form. Uptake into the liver might be mediated by OATP1B transporters, because SN-38 has been described as an OATP1B1 and OATP1B3 substrate \textit{in vitro}, whereas the pro-drug irinotecan did not appear to be a substrate of these transporters \textit{in vitro} (7-9). In the liver, SN-38 is further metabolized by UGT1A1 to an inactive form, SN-38 glucuronide. Both SN-38 and its glucuronide can be excreted into the bile, mainly by ABCC2 and ABCB1, and to a lesser extent by ABCG2 (6;10-12).

Severe unpredictable toxicities, mainly diarrhea and neutropenia, limit irinotecan’s therapeutic use. The incidence of toxic effects correlates well with higher systemic exposure to SN-38(11). Alterations in the activity of metabolizing enzymes and transporters regulating the pharmacokinetics of SN-38 can lead to increased toxicity. Indeed, low-activity polymorphic variants of OATP1B1, which lead to impaired hepatic
clearance of irinotecan and/or SN-38, are associated with higher exposure to SN-38 and life-threatening toxicity (13-16). These polymorphic variants have quite high allele frequencies in the American, European and Middle-Eastern populations, namely ~20% for 521T>C and ~50% for 388A>G (17). The risk of toxicity may be even greater for individuals with a complete deficiency of OATP1B1 or OATP1B3, which probably occur at a significant frequency, likely in the order of 1 in 2,000-20,000, dependent on the population. Also the very rare Rotor syndrome patients (~1 in 10^6), which are deficient in both OATP1B1 and OATP1B3 (18;19), are potentially at risk when treated with irinotecan.

Several single (Oatp1b2, Oatp1a1, Oatp1a4) and combined Oatp1a/1b knockout mouse strains are available and these have provided valuable insights into the physiological and pharmacological functions of OATP1A/1B transporters (3). In addition, liver-specific humanized OATP1A/1B transgenic mice have been recently characterized and they are excellent tools to study the functions of human OATP1A/1B transporters in vivo (4). Here, we aimed to investigate the impact of mouse and human OATP1A/1B transporters on pharmacokinetics and toxicity of irinotecan and SN-38 in vivo using Oatp1a/1b knockout mice and liver-specific humanized OATP1B transgenic mice.
Materials and methods

Animals

Mice were housed in small groups in a temperature-controlled environment with a 12-hour light/12-hour dark cycle. They received a standard diet (AM-II; Hope Farms) and acidified water *ad libitum*. All mouse experiments complied with Dutch legislation. Female wild-type, *Slco1a/1b(-/-)* (Oatp1a/1b knockout), *Slco1a/1b(-/-);1B1(tg)* and *Slco1a/1b(-/-);1B3(tg)* (liver-specific OATP1B1 and OATP1B3 humanized transgenic) mice of comparable genetic background (>99% FVB) between 8 and 14 weeks of age were used (4).

Chemicals and reagents

Irinotecan (Irinotecan HCl-trihydrate) was from Hospira Benelux BVBA (Brussels, Belgium). SN-38 was purchased from Sequoia Research Products (Pangbourne, UK). Bis(4-nitrophenyl) phosphate (BNPP) and simvastatin lactone were from Sigma-Aldrich (Steinheim, Germany), isoflurane (Forane) from Abbott Laboratories (Queenborough, Kent, UK) and heparin (5,000 IE/ml) was from Leo Pharma BV (Breda, The Netherlands). Bovine serum albumin (BSA), Fraction V was from Roche (Mannheim, Germany) and drug-free human plasma was obtained from healthy volunteers. Tetra-n-butylammonium bromide (TBABr) was from Merck Schuchardt (Hohenbrunn, Germany). All other reagents were obtained from Sigma-Aldrich (Steinheim, Germany).

Plasma and tissue pharmacokinetic experiments

Patients receive a treatment of 350 mg/m² every three weeks for six cycles, which would amount to a physiologically equivalent bolus dose in a mouse of ~120 mg/kg. However, we chose a lower dosage (10 mg/kg), based on previous pharmacokinetic studies and the sensitivity of the HPLC analytical method (20;21). Irinotecan (20 mg/mL in water-based solution containing NaOH, lactic acid and sorbitol) was diluted with saline (to 2 mg/mL) for administration of 10 mg/kg; 5 µl/g bodyweight were administered intravenously to mice. SN-38 was dissolved in DMSO (1 mg/mL) and 1 µl/g body weight was administered intravenously to mice to achieve a dosage of 1 mg/kg. The experiments were terminated by isoflurane anaesthesia, heparin-blood sampling by cardiac puncture followed by cervical dislocation and tissue collection.
Blood samples were centrifuged at 5,200g for 5 min at 4°C and plasma was collected and stored at -30°C until analysis.

**Drug analysis**

Concentrations of irinotecan and its metabolite SN-38 (as total amount of irinotecan and SN-38 after converting the carboxylate form into lactone by acidification of the samples prior to analysis) in plasma, organs (homogenized in appropriate volumes of ice-cold 4% (w/v) BSA) and bile (diluted 10-100-fold with human blank plasma) were determined by HPLC analysis as previously described (21). Because irinotecan and SN-38 have different molecular weights, we expressed the plasma concentrations in μM.

**Toxicity studies**

For this experiment, individually housed mice received a daily dose of 30 mg/kg irinotecan intravenously for 6 days. Mice were checked daily for changes in body weight, general appearance and incidence of diarrhea. One day prior to, and 24 hours after the last administration, blood for hematological and drug analysis was isolated from the tail vein. Under isoflurane anaesthesia, the mice were sacrificed by cervical dislocation and several organs (small intestine, stomach, spleen, sternum and extremities) were collected for histological analysis.

**Hematological analysis**

Hemoglobin level, hematocrit, mean corpuscular volume, red and white blood cells, and platelet counts were analyzed in peripheral blood on a Beckman Coulter analyzer (Florida, US).

**Histological analysis**

Isolated tissues and organs were handled as described previously and hematoxylin and eosin (H& E) stained sections were analyzed for pathological changes as previously described (22).

**Ex vivo carboxylesterase activity measurement and inhibition of carboxylesterase activity**

Esterase activity in mouse plasma was measured by monitoring in vitro conversion of irinotecan to SN-38 using previously described methods with slight modifications (23). In short, 50 μl 1 mM irinotecan was mixed with 950 μl 20 mM Tris-
HCl buffer (pH 7.5) in a 1.5 ml microcentrifuge tube and incubated at 37°C for 30 min to reach equilibrium between the lactone and carboxylate forms of irinotecan. 540 μl fresh plasma collected from wild-type and Slco1a/1b(-/-) mice (n = 3) was mixed with 60 μl of the irinotecan solution (50 μM) (final irinotecan concentration 5 μM) and the mixtures were kept at 37 °C with shaking. At 1, 3, 5, 10, 15, 30, 60, 120 and 240 minutes, 60 μl samples were collected for the determination of irinotecan and SN-38 concentrations. For the inhibition experiment, the non-specific CES inhibitor BNPP (24) (1 mM), the human CES1 and CES2 inhibitor simvastatin (100 μM) (25), BNPP vehicle (0.5% DMSO in water), and simvastatin vehicle (0.5% polysorbate 80 and 0.5% ethanol in water) were preincubated in mouse plasma for 15 min at 37 °C. Reactions were started by adding irinotecan (5 μM) and stopped after 60 min by putting the samples on dry ice. Sample pretreatment and drug analysis were as described above.

**RNA isolation, cDNA synthesis and RT-PCR**

RNA isolation from mouse liver, kidney, and small intestine and subsequent cDNA synthesis and RT-PCR were performed as described (26). Specific primers (QIAGEN, Hilden, Germany) were used to detect expression levels of the following mouse esterase genes: Ces1b, Ces1c, Ces1d, Ces1e, Ces1f, Ces1g, Ces2a, Ces2e, Ces3a, Ces3b, Aadac, Pon1, Pon2, Pon3 and Bche (26).

**Pharmacokinetic and statistical analysis**

Averaged plasma concentrations for each time point were used to calculate the area under the blood concentration versus time curve (AUC) from t = 0 to the last sampling time point by the linear trapezoidal rule; S.E. was calculated by the law of propagation of errors (27).

The two-sided unpaired Student’s t-test was used throughout the study to assess the statistical significance of differences between two sets of data. Statistical significance of differences between wild-type and Slco1a/1b<sup>+</sup>, Slco1a/1b<sup>-/-;1B1(tg)</sup> or Slco1a/1b<sup>-/-;1B3(tg)</sup> or between Slco1a/1b<sup>-/-</sup> mice and Slco1a/1b<sup>-/-;1B1(tg)</sup> or Slco1a/1b<sup>-/-;1B3(tg)</sup> mice was assessed by one-way ANOVA followed by Dunett’s multiple comparison test. Results are presented as the means ± S.D. Differences were considered to be statistically significant when P < 0.05.
Results

Altered disposition of irinotecan and SN-38 in Oatp1a/1b-null mice

We assessed the role of Oatp1a/1b transporters in the disposition of irinotecan and SN-38 after intravenous administration of 10 mg/kg irinotecan to wild-type and Slco1a/1b(-/-) mice. Despite the fact that this mouse dosage is lower than the physiological equivalent of the human dosage (350 mg/m²), mouse plasma concentrations of both irinotecan and SN-38 early after i.v. administration were similar to those observed in patients after a 90 minutes infusion (10).

Absence of Oatp1a/1b transporters resulted in significantly increased plasma concentrations of irinotecan and SN-38 at almost all the time points after administration (Figure 1A, B), suggesting a role for Oatp1a/1b transporters in the plasma clearance of irinotecan and SN-38. At 30 min after administration, irinotecan plasma concentrations in Slco1a/1b(-/-) mice were 1.9-fold higher than in the wild-type mice (1.89 μM versus 1.01 μM, respectively, Figure 1A), while SN-38 plasma concentrations of Slco1a/1b(-/-) mice were 8-fold higher compared with wild-type mice (0.4 μg/ml versus 0.05 μg/ml, respectively, Figure 1B). Overall plasma exposure (AUC(5-240)) of irinotecan was 1.7-fold higher in Oatp1a/1b knockout mice versus wild-type mice (209.8 ± 6.7 versus 120.9 ± 4.4 μM·min, P < 0.01), and 2.9-fold higher for SN-38 (50 ± 2.9 versus 12 ± 2 μM·min, P < 0.001). The fraction of SN-38 recovered in the plasma represented ~10% of the irinotecan fraction in plasma of wild-type mice, and ~23% in plasma of Oatp1a/1b knockout mice.

Oatp1a/1b uptake transporters control the plasma clearance of many compounds mainly by mediating their liver uptake. There was a slight trend of lower irinotecan and SN-38 liver concentrations in Oatp1a/1b knockout than in wild-type mice, though the differences were mostly not significant (Figure 1C, D). However, liver-to-plasma ratios of both irinotecan and SN-38 were markedly and significantly lower in Slco1a/1b(-/-) than in wild-type mice at most time points (Figure 1E, F), suggesting an impaired liver uptake of both compounds in the absence of Oatp1a/1b transporters. We also measured the small intestinal tissue and content concentrations of irinotecan and SN-38 and as the profile of the data was very similar between the two matrices, we present them together (Supplemental Figure 2). Here, we observed a similar pattern as for the liver data, with little differences in absolute drug concentrations and intestinal-to-plasma ratios of both irinotecan and SN-38 lower in Oatp1a/1b knockout mice than in the wild-type. In the
kidney, irinotecan concentrations were very similar between the two strains of mice and the kidney-to-plasma ratios of irinotecan significantly decreased in the Oatp1a/1b knockout mice from 30 min onwards, reflecting the higher plasma concentrations in the Oatp1a/1b-null mice (Supplemental Figure 3A, C). Interestingly, SN-38 kidney concentrations were significantly higher in Oatp1a/1b knockout mice than wild-type mice from the first time point on (5 min) (Supplemental Figure 3B). This might be a reflection of the consistently higher SN-38 plasma concentrations in Slco1a/1b(-/-) than in wild-type mice (Figure 1B). Indeed, the kidney-to-plasma ratios of SN-38 showed significantly lower values in Oatp1a/1b-null than in wild-type mice, suggesting that relative SN-38 uptake into the kidney was in fact reduced in the knockout mice (Supplemental Figure 3D).

*Increased systemic toxicity in Oatp1a/1b knockout mice after repeated irinotecan administration*

In patients, low-activity polymorphisms in genes encoding OATP1B1 are associated with increased toxicity due to increased plasma concentrations of SN-38 (6;28). We therefore investigated if Oatp1a/1b knockout mice suffer from more toxicity after 6 daily administrations of 30 mg/kg intravenous irinotecan. Up to day 3-4, the loss in body weight was similar between the two strains (~5 %), after which wild-type mice did not lose additional body weight, whereas Oatp1a/1b knockout mice continued to lose significantly more body weight (a cumulative ~10% by the last day) (Figure 2A). Although both strains suffered from neutropenia (lower white blood cells counts) on day 7, this was more pronounced for the Oatp1a/1b-null mice. Wild-type mice had 42% reduced white blood cell counts, but Oatp1a/1b-null mice had 72% reduced white blood cell counts (Figure 2B). These effects were probably a consequence of the increased SN-38 systemic exposure in Oatp1a/1b-null mice, which had 75-fold higher SN-38 trough blood concentrations at 24 hours after the last administration (0.015 μM in wild-type mice versus 1.10 μM in Oatp1a/1b-null mice, \( P < 0.001 \)) (Figure 2C), whereas differences for irinotecan were not significant.

Accordingly, pathological examination performed 24 hours after the last administration revealed more toxicity in the bone marrow, thymus and small intestine of Oatp1a/1b knockout mice (Figure 3). We compared untreated wild-type mice (Figure 3A, D, G), with treated wild-type (Figure 3B, E, H) and treated Oatp1a/1b knockout mice (Figure 3C, F, I). Tissues of untreated Oatp1a/1b knockout mice were similar to
untreated wild-type tissues. When comparing bone marrow (dark blue cells) in sternum sections (Figure 3B versus 3C), it was clear that Oatp1a/1b knockout mice had severe depletion of the bone marrow and dilatation of blood vessels, which is in line with the more severe neutropenia observed (Figure 2B). In normal healthy thymus, the cortex (dark blue cells on the rim) is densely populated with rapidly proliferating cells (Figure 3D). When treated, both wild-type and Oatp1a/1b knockout mice suffered from a depletion of the cortex, but this was more severe in the knockout mice (Figure 3E versus 3F). We found the most striking difference in the small intestine. In the untreated mice the architecture of the small intestine was normal (Figure 3G). In the treated wild-type mice, there is a partial depletion of cells in the villi, which lead to an altered architecture of the small intestine (Figure 3H). In the knockout mice, we observed severe toxicity with many apoptotic cells and drastically shortened or absent villi in the Oatp1a/1b-null mice, together with an increased number of leukocytes (Figure 3I versus H).

**Increased plasma conversion of irinotecan to SN-38 in Oatp1a/1b knockout mice**

After 6 consecutive administrations of irinotecan, only the SN-38 trough concentrations were increased in the knockout mice, but not the irinotecan concentrations (Figure 2C). We wondered what could explain this higher accumulation of SN-38 in knockout mice. We therefore evaluated the *in vitro* conversion of irinotecan to SN-38 in freshly isolated plasma from wild-type and Oatp1a/1b knockout mice. Surprisingly, we found that this conversion was very slow in wild-type plasma, but highly increased in the Oatp1a/1b knockout plasma (Figure 4A). In plasma from Oatp1a/1b knockout mice, irinotecan was rapidly converted to SN-38, with as early as 10 minutes ~48% of irinotecan converted to SN-38, while in the wild-type mice, this fraction was negligible, only ~0.2%. By 60 minutes nearly all irinotecan was transformed to SN-38 in the knockout mice (88%). In contrast, in wild-type plasma the amount of SN-38 was 125-fold lower (0.7%) (Figure 4A). The conversion of irinotecan to SN-38 can be mediated by plasma (carboxyl)esterases, which are generally much more abundant in mouse plasma than in human plasma (29-31).
Several Ces1 genes are upregulated in Oatp1a/1b knockout mice

Conversion of irinotecan to SN-38 can be mediated by several esterases (32), which are usually synthesized in the liver, but some also in the small intestine and kidney (29;33). Therefore, we tested expression levels by RT-PCR of all obvious candidate carboxylesterase, paraoxonase and butyrylcholinesterase genes (34) in the liver, small intestine and kidney of wild-type and Oatp1a/1b knockout, but also of liver-specific humanized OATP1B1 and OATP1B3 transgenic mice (Figure 4B, C, D and Supplemental Table 1-3). The latter can display a rescue of phenotypic changes caused by deletion of the Oatp1a/1b genes (4).

Several members of the Ces1 family were highly upregulated in the livers of Oatp1a/1b knockout relative to wild-type mice: Ces1b (10-fold), Ces1c (80-fold), Ces1d (90-fold) and Ces1e (10-fold) (Figure 4B). Expression levels of other esterases, including Ces2a and butyrylcholinesterase (Bche) (35), an enzyme present in both human and mouse plasma which is thought to play a role in the conversion of irinotecan to SN-38, were unchanged in Oatp1a/1b knockout or transgenic mice (Supplemental Table 1-3).

The high upregulation of Ces1 genes was surprising because a previous analysis of changes in gene expression of several metabolizing enzymes and influx and efflux drug transporters in Oatp1a/1b knockout mice had revealed very few changes in comparison with wild-type mice, although Ces genes were not directly tested (4). Also a genome wide RNA microarray screen had not identified significant changes in Ces genes. Interestingly, OATP1B1- and OATP1B3-humanized transgenic mice had strongly reduced mRNA levels of the enzymes that were upregulated in the Oatp1a/1b knockout mice, back to, or even below, the wild-type levels (Figure 4B, Supplemental Table 1). In the small intestine, Ces1d was highly upregulated (~2700-fold, albeit from an extremely low baseline expression level) and Ces1e ~6-fold, but Ces1b and Ces1c were unchanged (Figure 4C, Supplemental Table 2). In the humanized mice, the expression of these genes was again roughly back to wild-type levels (Figure 4C). Note that even the highly upregulated Ces1d RNA levels in small intestine were still quite low in terms of absolute expression and relative to liver expression, making it unlikely that this would have much functional impact (Supplemental Table 2). In the kidney there was a similar pattern, with primarily Ces1d, but not Ces1b or Ces1c, highly upregulated in the Oatp1a/1b knockout mice and much reduced in the humanized mice (Figure 4D, Supplemental Table 3).
Collectively, these data suggest that one or more of the upregulated Ces1 enzymes is responsible for the conversion of irinotecan to SN-38 in Oatp1a/1b knockout mice.

*Increased conversion of irinotecan to SN-38 in plasma can be completely inhibited by BNPP and partially by simvastatin*

To further corroborate the involvement of Ces1 enzymes in the increased conversion of irinotecan to SN-38 in plasma of Oatp1a/1b knockout mice, we investigated if this conversion could be inhibited by BNPP, a general (carboxyl-)esterase inhibitor (24), and simvastatin, a human CES1 and CES2 inhibitor (25). We incubated irinotecan (5 µM) in Oatp1a/1b knockout plasma for 30 minutes with BNPP (1 mM) or simvastatin (100 µM) and measured the disappearance of irinotecan and formation of SN-38. In wild-type plasma there was hardly any conversion of irinotecan to SN-38 (Figure 4A), and preincubation with inhibitors did not have an additional effect on the formation of SN-38 (data not shown). In contrast, in Oatp1a/1b knockout plasma, after 30 minutes, ~75% of irinotecan was converted to SN-38, and this conversion was almost completely inhibited by BNPP, while simvastatin had a modest inhibitory capacity (1.5-fold) (Figure 4E). Note that the vehicle of simvastatin (water containing 0.5% polysorbate 80 and 0.5% ethanol) also had a minor inhibitory effect (Figure 4E). These inhibition experiments support that one or more of the upregulated carboxylesterase (Ces1) enzymes are responsible for the conversion of irinotecan to SN-38 in Oatp1a/1b knockout mouse plasma.

*Role of human OATP1B1 and OATP1B3 in disposition of irinotecan and SN-38 after irinotecan administration*

Increased Ces1 activity in plasma of Oatp1a/1b knockout mice obviously complicates pharmacokinetic studies with irinotecan. Humanized transgenic mice, with roughly normal levels of the main Ces enzymes, might be more suitable for studying the impact of OATP1B transporters on the liver uptake of irinotecan and SN-38. Therefore, in an independent experiment, we administered irinotecan (10 mg/kg i.v.) to these mice (and also to wild-type and knockout mice) and evaluated the plasma and liver levels at 15 and 60 minutes (Figure 5).

Plasma levels of irinotecan were significantly higher in the liver-specific OATP1B1 or OATP1B3 humanized mice than in wild-type mice, both at 15 and 60
minutes (Figure 5A). Also liver-to-plasma ratios were significantly decreased in the humanized mice in comparison with wild-type mice, suggesting an impaired liver uptake (Figure 5E). These results show that in the humanized mice, irinotecan could not be cleared as efficiently as in the wild-type mice. This suggests that single human OATP1B1 or OATP1B3 cannot mediate efficient liver uptake of irinotecan in vivo. The data also suggest that one or more of the deleted mouse hepatic Oatp1a/1b transporters (Oatp1a1, -1a4 and -1b2) can mediate efficient irinotecan uptake, since replacement of mouse Oatp1a/1b with human OATP1B1 or OATP1B3 resulted in significantly increased plasma levels and decreased liver-to-plasma ratios in comparison with wild-type mice, whereas the Ces1 expression between these strains was similar.

In contrast, plasma levels of SN-38 were similar between humanized and wild-type mice, and significantly decreased in comparison with the Oatp1a/1b knockout mice (Figure 5B). In view of the normalized Ces1 expression in the humanized mice, this suggests that human OATP1B1 and OATP1B3 can efficiently clear SN-38 from the plasma, to a similar extent as the Oatp1a/1b transporters in the wild-type mouse. Also the liver-to-plasma ratios of SN-38 support this, indicating an equal relative liver uptake in the wild-type and the humanized mice, and an impaired liver uptake in the Oatp1a/1b knockout mice (Figure 5F).

**Impact of mouse OATPs on SN-38 disposition after direct administration of SN-38**

We next studied the impact of mouse Oatp1a/1b transporters on the disposition of SN-38 after its direct administration in order to bypass the increased formation of SN-38 by upregulated Ces1 enzymes in these mice. Shortly after administration (3 min) of SN-38 (1 mg/kg i.v.), Oatp1a/1b knockout mice had modestly, but significantly increased plasma levels of SN-38 (Figure 6A). In the same mice, liver levels were significantly reduced at 5 min after SN-38 dosing (Figure 6B, C). Liver-to-plasma ratios at various time points were also lower in the absence of Oatp1a/1b transporters, suggesting an impaired liver uptake of SN-38 shortly after intravenous dosing (Figure 6D). An impaired early liver uptake of SN-38 was also obvious in an independent experiment, from the reduced cumulative biliary excretion of SN-38 in gall-bladder cannulated mice, which were also dosed with SN-38 (1 mg/kg) (Figure 6E, F). These results further support that mouse hepatic Oatp1a/1b transporters have a role in the disposition of SN-38. However, it should be noted that these experiments were technically challenging due to the extremely poor solubility of SN-38, which necessitated direct i.v. administration in a small
volume of DMSO. This may have contributed to the high experimental variation evident in Figure 6 B-F.
Discussion

In this study we show that mouse Oatp1a/1b transporters contribute to the plasma clearance of both irinotecan and SN-38. In contrast, human OATPB1 and/or OATP1B3 in humanized transgenic mice do not mediate marked clearance of irinotecan, whereas they do appear to contribute to the hepatic uptake and clearance of SN-38. Unexpectedly, Oatp1a/1b knockout mice displayed highly increased expression and activity of plasma carboxylesterases, with the Ces1c enzyme as the main plasma carboxylesterase (see below) presumably responsible for the increased formation of SN-38 from irinotecan. In addition, we show that Oatp1a/1b knockout mice suffer from increased toxicity after repeated irinotecan administration, correlating with elevated systemic exposure to SN-38.

Low-activity polymorphic variants of \textit{SLCO1B1} (e.g. haplotype *15) have been associated with increased plasma exposure of irinotecan and SN-38 in Asian patients (15). In another study, carriers of the same polymorphic variant correlated with increased exposure to SN-38, but not irinotecan (13). Patients with other \textit{SLCO1B1} genotypes associated with low activity (521 T>C and 388 GG) suffered from increased neutropenia and diarrhea, respectively (13). In addition, there were two isolated cases reported of life-threatening toxicities after irinotecan administration to patients who were carriers of low-activity polymorphic variants of \textit{SLCO1B1} (14;16). These data correlate with our own findings in the Oatp1a/1b knockout mice which suffer from increased neutropenia and intestinal toxicity associated with increased plasma exposure to SN-38.

Irinotecan was found not to be transported by human OATP1B1 or OATP1B3 \textit{in vitro} (7-9), which is consistent with the absence of detectable \textit{in vivo} transport that we observed in the OATP1B1- and OATP1B3-humanized mice. On the other hand, our data suggest that mouse Oatp1a/1b transporters do contribute to the plasma clearance and liver uptake of irinotecan. Despite the increased conversion of irinotecan to SN-38 in plasma of the Oatp1a/1b knockout mice, these mice still displayed a clearly increased plasma exposure of irinotecan after intravenous administration, most likely due to impaired liver uptake. This is also evident from the significantly decreased liver-to-plasma ratios of irinotecan (Figure 1). An explanation for this human-mouse difference might be that in mouse liver there are three uptake transporters expressed (Oatp1a1, Oatp1a4 and Oatp1b2), and due to species differences one or more of these could be
good irinotecan transporters, in contrast to human OATP1B1 and OATP1B3. Possibly also expression levels of the responsible mouse transporters are relatively high.

Interpretation of data regarding the SN-38 disposition after irinotecan administration was complicated by the increased conversion of irinotecan to SN-38 in plasma of the Oatp1a/1b knockout mice, which might itself contribute to higher plasma levels of SN-38. However, the clearly decreased liver-to-plasma ratios of SN-38 in the knockout mice (Figure 1) strongly supported decreased hepatic uptake. Also after direct administration of SN-38, we could observe an impact of mouse Oatp1a/1b transporters on the plasma clearance and hepatic uptake of SN-38, especially early after intravenous administration. This was also reflected in the decreased biliary output of SN-38 in the absence of Oatp1a/1b transporters. The role of each of the human OATP1B transporters in plasma clearance and liver uptake of SN-38 appeared to be similar to that of the mouse Oatp1a/1b transporters (Figure 5). This would be consistent with previously reported in vitro data showing transport of SN-38 by both human transporters (7-9), and it is also in line with the increased irinotecan/SN-38 toxicity observed in patients with low-activity OATP1B1 variants.

The impact of mouse and/or human OATP1A/1B transporters on the irinotecan or SN-38 distribution are mostly evident through altered plasma levels, and liver-to-plasma ratios, without much affecting the liver absolute concentrations (Figures 1 and 5). This is well in line with our previous studies with rosvastatin and pravastatin in Oatp1a/1b-knockout mice and the findings of a physiologically-based pharmacokinetic model by Watanabe et al (36-38). In short, this model predicts that a reduction in the hepatic uptake will have strong effects on the plasma exposure, and little effect on the liver exposure for drugs that are mostly hepatically cleared and for which there is no substantial alternative clearance route.

Perhaps the most intriguing finding of this study is that Oatp1a/1b knockout mice have highly increased expression of Ces1 enzymes in liver, small intestine and kidney. This correlates with an increased plasma esterase activity leading to far higher formation of SN-38 from irinotecan in plasma. Several Ces1 enzymes were upregulated, of which Ces1c and Ces1d had the highest fold increase (80-90-fold) in expression in the livers of the Oatp1a/1b knockout mice. This increase in expression correlates well with a ~120-fold increase in the conversion rate of irinotecan to SN-38 (Figure 4A). A recent study reported that, in mouse liver, the absolute mRNA expression levels of Ces1c are 1000-fold higher than those of Ces1d (34). Moreover, Ces1d (NCBI Reference Sequence:
NP_444430.2) contains an ER retention signal at its C-terminus (EHVEL), which prevents it from being released into the plasma (39). In contrast, Ces1c lacks this retention signal (NCBI Reference Sequence: NP_031980.2), meaning that it can be secreted into the plasma (39). In addition, mutant Ces1c-deficient mice display a much lower exposure to SN-38 after irinotecan administration, and the in vitro conversion rate of irinotecan to SN-38 in plasma isolated from these mice is markedly reduced (20;40). Collectively, these results strongly implicate Ces1c as the main plasma carboxylesterase responsible for the increased conversion of irinotecan to SN-38 in the Oatp1a/1b knockout mice. We also demonstrated that BNPP (24), a general CES inhibitor, can almost completely block the conversion of irinotecan to SN-38 in Oatp1a/1b-null plasma. Simvastatin, an inhibitor of human CES1 and CES2 (25), inhibited less than 50% of the conversion activity of mouse Ces1 enzymes. This lower inhibitory capacity might be explained by a difference in affinity for simvastatin between mouse and human Ces enzymes.

It is as yet unclear how Oatp1a/1b deficiency causes Ces1 upregulation. Regulation of Ces1 enzymes is complex and not yet fully elucidated, with many xenobiotics acting as inducers of Ces enzymes, presumably through nuclear receptors (39;41). A recent study tested the impact of a few microsomal enzyme inducers on the mouse Ces enzymes, but it did not find a substantial effect on the mRNA expression levels of the Ces1 subfamily (34). Interestingly, we recently also observed Ces1 upregulation in P-gp- and Cyp3a-deficient mice similar to that in Oatp1a/1b knockout mice (42). This suggests that when normal detoxification processes of endogenous and/or dietary-derived components are reduced (by knockout of transporters or metabolizing enzymes), Ces1 upregulation can occur. Whatever the underlying mechanism of the upregulation, hepatic expression of human OATP1B1 or OATP1B3 in the humanized Oatp1a/1b-null mice largely normalized this situation. Apparently the detoxification of relevant compounds is sufficiently recovered to normalize expression of the Ces1 enzymes levels as well. More in depth studies will be required to establish the exact mechanism of Ces1 upregulation in Oatp1a/1b knockout mice.

Although human CES activity in plasma is very low, CES1 and CES2 are abundant in human liver and small intestine, respectively, where they can mediate the formation of SN-38, but CES2 has a 12.5-fold higher affinity and a 5-fold higher conversion rate (29;33). This is due to the preference of CES2 for hydrolyzing esters with a bulky alcohol side group (e.g. irinotecan), while CES1 generally hydrolyzes esters.
with a small alcohol side group, like methyl or ethyl groups. Clinically, this preference of irinotecan for CES2 would be offset by much higher exposure to liver CES1 of intravenously administered irinotecan. However, the ester side group size preference of CES1 vs CES2 is not absolute, and mouse Ces1c may well further differ from human CES1 in preference for certain drug substrates. Indeed, our own preliminary data with other drugs (data not shown) suggest that there are species differences between substrate specificity of mouse Ces1c and human CES1.

There are several polymorphic variants described for both CES1 and CES2 enzymes (reviewed in (43)). However, their impact on irinotecan pharmacokinetics and toxicity has not yet been proven (44-48), with the exception of one study which indicated a possible dose-relationship between several functional variants of CES1 and an elevated exposure to SN-38 and/or SN-38 glucuronide (49). CES activity might be of importance in tumor cells, where CES expression can increase sensitivity to irinotecan treatment (50), although further studies are necessary to establish their exact contribution. In addition, expression of CES enzymes can be induced by drugs, e.g. dexamethasone, and this might also lead to undesired drug-drug interactions.

Obviously, the Ces1 upregulation in the Oatp1a/1b knockout mice, and its subsequent normalization in the humanized OATP1B1 and OATP1B3 mice, should be taken into account when performing pharmacokinetic and toxicological studies in these mouse strains with (pro-)drugs that might be affected by Ces activity. For instance, it may be that the highly increased toxicity of irinotecan treatment that we observed in the Oatp1a/1b knockout mice (Figures 2 and 3) was not only caused by delayed SN-38 clearance, but also in part by increased SN-38 formation. Therefore considerable caution should be exercised when interpreting results obtained with such drugs in these mouse models. Nevertheless, keeping in mind the possible complications brought about by the upregulated Ces1 enzymes for pharmacokinetic studies with irinotecan, we can still conclude that mouse Oatp1a/1b transporters have a role in the plasma clearance of irinotecan and SN-38, while human OATP1B transporters have only an impact on SN-38 disposition.

Acknowledgements

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Reference List


mice.


Legends for figures:

Figure 1. Role of Oatp1a/1b uptake transporters in the plasma and liver exposure of irinotecan and SN-38 after intravenous administration of irinotecan (10 mg/kg) to female wild-type and Oatp1a/1b knockout mice. Plasma concentrations of (A) irinotecan and (B) SN-38. Liver concentrations (as % of dose) of (C) irinotecan and (D) SN-38. Liver-to-plasma ratios of (E) irinotecan and (F) SN-38. Data are presented as mean ± S.D. (n = 5-6, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type).

Figure 2. Role of Oatp1a/1b uptake transporters in irinotecan-induced toxicity after administration of irinotecan (30 mg/kg i.v. daily) for 6 days to female wild-type and Oatp1a/1b knockout mice. (A) Changes in body weight (as % of difference in comparison with the weight on day 0) measured daily. Arrows below the X-axis indicate irinotecan administrations. (B) White blood cell count on day 0 and day 7 of the experiment. (C) Blood concentrations of irinotecan and SN-38 on day 6 of the experiment (6 hours after the last administration). Data are presented as mean ± S.D. (n = 5-6, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type).

Figure 3. Role of Oatp1a/1b uptake transporters in irinotecan/SN-38-induced toxicity after administration of irinotecan (30 mg/kg i.v. daily) for 6 days to female wild-type and Oatp1a/1b knockout mice assessed with microphotographs of H&E sections of tissues. Untreated wild-type mice: (A) the bone marrow with high density of cells (dark blue), (D) thymus with high number of proliferating cells in the cortex (outer rim filled with dark blue cells) and (G) the small intestine with intact villi. Irinotecan-treated wild-type mice: (B) minor depletion of the bone marrow, (E) moderate depletion of the cortex in the thymus and (H) moderate toxicity in the small intestine, with shortened villi. Irinotecan-treated Oatp1a/1b knockout mice: (C) severe depletion of bone marrow, (F) severe depletion of the cortex in the thymus and (I) severe toxicity in the small intestine with very short or absent villi and apoptotic cells. Tissues for the treated mice were isolated at 24 hours after the last administration.

Figure 4. Oatp1a/1b knockout mice have higher in vitro carboxylesterase activity (A, E) and expression of Ces enzymes (B, C, D). (A) Concentrations of irinotecan (dashed lines) and SN-38 (black lines) over time after addition of irinotecan (5 μM) to freshly isolated wild-type plasma (black symbols) and Oatp1a/1b knockout plasma (white symbols) Data are presented as mean ± S.D (n = 3, *, P < 0.05; ***, P < 0.001 for irinotecan. (B, C, D), Ces1 family gene mRNA expression measured by RT-PCR. Results are expressed as the fold change in expression of murine Ces1 genes in female Oatp1a/1b knockout mice and transgenic mice with liver-specific expression of OATP1B1 and OATP1B3 in liver, small intestine and kidney relative to wild-type mice. Data are presented as mean ± S.D. (n=3). n.d.: not detectable. (E) Cumulative concentrations of irinotecan (black bars) and SN-38 (white bars) in plasma isolated from female Oatp1a/1b knockout mice after addition of irinotecan (5 μM) with or without inhibitors at 0 and 30 minutes. Total bar height indicates the sum of irinotecan and SN-38 concentrations. Data are presented as mean ± S.D (n = 3, *, P < 0.05; ***, P < 0.001 for irinotecan and #, P < 0.05; ##, P < 0.01; ###, P < 0.001 for SN-38 when compared with no inhibition condition).
Figure 5. Role of human OATP1B1/1B3 uptake transporters in the pharmacokinetics of irinotecan and SN-38 after intravenous administration of irinotecan (10 mg/kg) to female wild-type, Oatp1a/1b knockout and OATP1B1 and OATP1B3 humanized transgenic mice. Plasma concentrations of (A) irinotecan and (B) SN-38. Liver concentrations (as % of dose) of (C) irinotecan and (D) SN-38. Liver-to-plasma ratio of (E) irinotecan and (F) SN-38 at various time points. Data are presented as mean ± SD (n = 4-5, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type; #, P < 0.05; ##, P < 0.01; ###, P < 0.001 when compared with Slco1a/1b(-/-)).

Figure 6. Role of Oatp1a/1b uptake transporters in the pharmacokinetics of SN-38 after intravenous administration of SN-38 (1 mg/kg) to female wild-type and Oatp1a/1b knockout mice. (A) Plasma (in μM) and (B) liver concentrations (in μg/g) and (C) (as % of dose) of SN-38 versus time curve. (D) Liver-to-plasma ratio of SN-38 versus time curve. (E) Cumulative biliary excretion of SN-38 (% of dose) and (F) fractional bile output of SN-38 (% of dose) over time. Data are presented as mean ± SD (n = 4-5, *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type).
Figure 1

A. Plasma irinotecan (μM)

B. Plasma SN-38 (μM)

C. Liver irinotecan (% of dose)

D. Liver SN-38 (% of dose)

E. Liver/plasma ratio irinotecan

F. Liver/plasma ratio SN-38

Time (min)
Figure 5
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OATP1A/1B transporters affect irinotecan and SN-38 pharmacokinetics and carboxylesterase expression in knockout and humanized transgenic mice

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