Cancer Therapy: Preclinical

P-glycoprotein (P-gp/Abcb1), Abcc2, and Abcc3 Determine the Pharmacokinetics of Etoposide

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Abstract

Purpose: Despite the extensive use of etoposide for the treatment of different malignant neoplasms, its main pharmacokinetic determinants are not completely defined. We aimed to study the impact of P-glycoprotein (P-gp/ABCB1) and the multidrug resistance proteins ABCC2 (MRP2) and ABCC3 (MRP3) on the pharmacokinetics of etoposide.

Experimental Design: Abcb1a/1b−/−, Abcc2−/−, Abcc3−/−, Abcb1a/1b;Abcc2−/−, and Abcc2;Abcc3−/− mice were used to investigate the separate and combined impact of P-gp, Abcc2, and Abcc3 on the in vivo behavior of etoposide.

Results: P-gp restricted the oral (re)uptake of unchanged etoposide, and mediated its excretion across the gut wall. In contrast, hepatobiliary excretion was almost entirely dependent on Abcc2. Yet, complete loss of Abcc2 did not result in elevated liver or plasma concentrations of etoposide. Instead, Abcc2−/− mice displayed an increased hepatic formation of etoposide glucuronide, which was secreted via Abcc3 from the liver to the blood circulation and eliminated with the urine. Combination Abcc2;Abcc3−/− mice had highly increased accumulation of etoposide glucuronide in their livers, whereas both single knockouts did not, indicating that Abcc2 and Abcc3 provide alternative pathways for the hepatic elimination of etoposide glucuronide.

Conclusions: P-gp, Abcc2, and Abcc3 significantly affect the pharmacokinetics of etoposide and/or etoposide glucuronide. Variation in transporter expression or activity may explain the high variation in oral availability of etoposide (25-80%) among cancer patients. However, despite the fact that substantial variations in transporter activity can occur, we believe that cancer patients are often relatively protected from etoposide toxicity due to overlapping functions of these transporters in the elimination of etoposide.

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These transporters can have a profound effect on the toxicity and therapeutic efficacy of anticancer drugs. The aim of the present study was to investigate the separate and combined impact of the multidrug transporters P-gp, Abcc2, and Abcc3 on the in vivo pharmacokinetic behavior of etoposide. For this purpose, we used single Abcb1a/b-/-, Abcc2-/-, and Abcc3-/- mice. We also used the currently available combination knockout strains, i.e., Abcb1a/b-/-; Abcc2-/- and Abcc2;Abcc3-/- mice, to study the overlapping functions of these transporters in the pharmacokinetics of etoposide.

Materials and Methods

Chemicals. Etoposide, formulated as solution for i.v. injection (Toposin; 20 mg/mL), originated from Pharmacia. Teniposide, formulated as solution for i.v. injection (Vumon; 10 mg/mL) was from Bristol-Myers Squibb. [3H]etoposide (specific activity 5 Ci/mmol) was obtained from Campro Scientific. Ketamine (Ketanest-S) was from Pfizer. Xylazine was from Sigma Chemical Co. Methoxyflurane (Metofane) was from Medical Developments Australia. Heparin (5,000 IE/mL) was from Leo Pharma BV. β-Glucuronidase from Helix pomatia [aqueous solution, ≥ 85,000 units/mL] originated from Sigma-Aldrich. Bovine serum albumin, fraction V, was from Roche. The organic solvents methanol, acetonitril (both high performance liquid chromatography grade), and 1,2-dichloroethane were from Merck. Blank human plasma was from healthy volunteers. All other chemicals and reagents were obtained from Sigma-Aldrich.

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. The animals used in this study were male Abcb1a/b-/- (8), Abcc2-/- (9), Abcc3-/- (10), Abcb1a/b;Abcc2-/- (11), Abcc2;Abcc3-/- (12), and WT mice, all of a >99% FVB genetic background, between 9 and 15 wk of age. The body weights of the mice ranged from 24 to 30 g, and there were no significant differences in body weight among the genotypes. The animals were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle and received a standard diet (AM-IL, Hope Farms) and acidified water ad libitum.

Plasma pharmacokinetics. For oral administration, etoposide (Toposin; 20 mg/mL) was 6.7-fold diluted with a 5% glucose solution in water, and a total volume of 10 mL/kg (30 mg/kg) body weight was administered by gavage into the stomach, using a blunt-ended needle. To minimize variation in absorption, the mice were fasted 3 h before drug administration. For i.v. administration, etoposide (Toposin; 20 mg/mL) was 3.3-fold diluted with a saline solution (0.9% NaCl), and a total volume of 5 mL/kg (30 mg/kg) body weight was injected into a tail vein. Blood samples were collected by cardiac puncture under methoxyflurane anesthesia. Animals were sacrificed at 7.5 (i.v.), 15 (oral), and 30 min, and 1, 2, and 4 h (both series) after etoposide administration. Blood samples were centrifuged at 2,100 g for 6 min at 4°C, and the plasma fraction was collected and stored at -20°C until analysis.

Translational Relevance

In this study we show that the ATP-binding cassette (ABC) transporters P-gp, ABC2, and ABC3 can markedly affect the pharmacokinetics of etoposide and/or etoposide glucuronide, the main metabolite of etoposide. These findings may be important for the treatment of cancer patients with etoposide, because for each of these drug transporters polymorphisms and mutations have been identified that affect their expression and/or activity. These polymorphisms and mutations may partly explain the high variability in the oral availability of etoposide (25-80%) observed among cancer patients. On the other hand, we also show that these ABC transporters have redundant functions in the elimination of etoposide. This overlap in functionality is favorable during patient treatment, as it reduces the risk of dramatically increased etoposide exposure and toxicity due to variable expression and/or function of just one of these multidrug transporters. These insights may be used to further optimize pharmacotherapy with etoposide and possibly with related anticancer agents.
Fecal and urinary excretion. Excretion of etoposide in urine and feces was studied as previously described (11). Briefly, mice were individually housed in Rucro Type M/1 stainless-steel metabolic cages. They were allowed 2 d to adapt, before 30 mg/kg etoposide, supplemented with $[^3H]$etoposide ($\sim$0.5 $\mu$Ci per animal), was injected into a tail vein. Feces and urine were collected over a 24-h period and feces were homogenized in 4% bovine serum albumin (1 mL/100 mg feces). Part of the samples was used to determine levels of radioactivity by liquid scintillation counting and the rest was stored at -20°C until analysis.

Biliary excretion. In gall bladder cannulation experiments the mice were anesthetized by i.p. injection of a combination of ketamine (100 mg/kg) and xylazine (6.7 mg/kg), in a volume of 4.33 μL/g body weight. After opening the abdominal cavity and distal ligation of the common bile duct, a polythene catheter (Portex Limited) with an inner diameter of 0.28 mm was inserted into the incised gallbladder and fixed with an additional ligation. Bile was collected for 60 min after i.v. injection of 30 mg/kg etoposide, supplemented with $[^3H]$etoposide ($\sim$0.5 $\mu$Ci per animal). At the end of the experiment, blood was collected by cardiac puncture and the mice were sacrificed by cervical dislocation. Several tissues were removed and homogenized in 4% bovine serum albumin. Intestinal contents were separated from intestinal tissues prior to homogenization. Part of the tissue homogenates, bile and plasma samples was used to determine levels of radioactivity by liquid scintillation counting. The rest was stored at -20°C until analysis.

Drug analysis. Etoposide concentrations were measured with a previously described high performance liquid chromatography method with minor modifications (13). A volume of 20 μL bile or 50 μL plasma, urine or tissue homogenate was completed to 500 μL with drug-free human plasma. Teniposide (Vumon; 10 mg/mL) was 500-fold diluted with methanol to 20 μg/mL, and 100 μL of this solution were added as internal standard. Etoposide was extracted with 2 mL of 1,2-dichloroethane, and after thorough shaking for 5 min the mixture was centrifuged at 2,000 rpm × 3 min. The aqueous layer was removed, and the organic extract was transferred to a clean glass tube and evaporated to dryness under a constant nitrogen flow. The residue was reconstituted in 200 μL with drug-free human plasma and processed as described above. The etoposide glucuronide concentration was calculated by subtracting the original etoposide concentration from the etoposide concentration after deglucuronidation. Etoposide glucuronide concentrations are expressed in etoposide mass equivalents.

RNA isolation, cDNA synthesis, and real-time reverse transcriptase-PCR. Livers of male mice between 9 and 11 wk of age (n = 4 per genotype) were excised and immediately placed in an appropriate volume of RNAlater (QIAGEN). They were stored at 4°C until RNA was extracted using the RNeasy mini kit (QIAGEN) according to the manufacturer’s protocol. Subsequently, cDNA was generated using 5 μg of total RNA in a synthesis reaction using random hexamers (Applied Biosystems) and SuperScript II reverse transcriptase (Invitrogen) according to the supplier’s protocols. The reverse transcription reaction was done for 60 min at 42°C with a deactivation step of 15 min at 70°C. cDNA was stored at -20°C until use. Real-time reverse transcriptase-PCR was done using specific primers (QIAGEN) for Abcb1a, Abcc2, Abcc3, and Ugt1a1 on an Applied Biosystems 7500 real-time cycler system as previously described (14). Analysis of the results was done by the comparative Ct method as described (15) and statistical analysis was carried out on ΔCt values as previously described (16).

Pharmacokinetic calculations and statistical analysis. Pharmacokinetic parameters were calculated by noncompartmental methods using the software package WinNonlin Professional version 5.0. The area under the plasma concentration-time curve (AUC) was calculated using the trapezoidal rule, with extrapolating to infinity. Elimination half-lives ($t_{1/2}$) were calculated by linear regression analysis of the log-linear part of the plasma concentration-time curves. The peak plasma concentration ($C_{\text{max}}$), and the time of maximum plasma concentration ($T_{\text{max}}$) were estimated from the original data. Plasma clearance (CL) after i.v. administration was calculated by the formula $\text{CL} = \frac{\text{Dose}}{\text{AUC}_{i.v.}}$ and the oral bioavailability (F) was calculated by the formula $F = \frac{\text{AUC}_{\text{oral}}}{\text{AUC}_{i.v.}} \times 100\%$. The two-sided unpaired Student’s t-test was used for statistical analysis. Data obtained with single and combination knockout mice were compared with data obtained with WT mice, unless stated otherwise. Differences were considered statistically significant when $P < 0.05$. Data are presented as means ± SD.

Results

Impact of Abcc2 and P-gp on plasma pharmacokinetics of etoposide and its glucuronide. To investigate the separate and combined impact of Abcc2 and P-gp on absorption, distribution, and elimination of etoposide, we studied plasma pharmacokinetics in WT, Abcc2$^{-/-}$, Abcb1a1/1b$^{-/-}$, and Abcb1a1/1b;Abcc2$^{-/-}$ mice. After oral administration of etoposide (30 mg/kg body weight) the plasma concentrations and the area under the oral plasma concentration-time curve ($\text{AUC}_{\text{oral}}$) were not different between WT and Abcc2$^{-/-}$ mice (Fig. 1A and Table 1). Abcb1a1/1b$^{-/-}$ mice had a

37°C for 24 h. After this incubation, samples were completed to 500 μL with drug-free human plasma and processed as described above. The etoposide glucuronide concentration was calculated by subtracting the original etoposide concentration from the etoposide concentration after deglucuronidation. Etoposide glucuronide concentrations are expressed in etoposide mass equivalents.
2.7-fold greater AUCoral and the Cmax was 3.3-fold increased (Table 1). Combined deficiencies of Abcc2 and P-gp resulted in another 1.3-fold higher AUCoral compared with Abcb1a/1b-/− mice (and 3.5-fold compared with WT mice) and Cmax was 1.3-fold greater than for Abcb1a/1b-/− mice (P < 0.05 for both parameters; Table 1 and Fig. 1A). These results suggest that P-gp restricts the (re)uptake of etoposide from the gut, whereas single Abcc2 deficiency does not affect the oral etoposide plasma pharmacokinetics. However, when P-gp is absent, Abcc2 can also have an impact on oral etoposide plasma pharmacokinetics.

After i.v. administration of 30 mg/kg etoposide, Abcc2 deficiency had a modest effect on the AUCi.v. (Fig. 1B and Table 1). In contrast, the absence of P-gp resulted in a 1.4-fold higher AUCi.v. (P < 0.001), and combined deficiencies led to a 1.6-fold increased AUCi.v. (P < 0.001; Table 1).

Conjugation of etoposide to glucuronic acid plays a major role in its elimination in humans, rats, and rabbits (13, 17, 18). We therefore determined the etoposide glucuronide concentrations in plasma after i.v. administration of etoposide (Fig. 1C and Table 1). Abcc2 deficiency resulted in a 1.9-fold higher AUCi.v. for etoposide glucuronide compared with WT mice (P < 0.001), whereas mice lacking P-gp had only a 1.2-fold higher AUCi.v. (P < 0.01; Table 1). The absence of both transporters had an additive effect on the etoposide glucuronide AUCi.v., which was 2.2-fold higher than in WT mice (P < 0.001). Our data indicate that upon i.v. administration, P-gp is mainly important for the parent compound, whereas Abcc2 deficiency primarily affects the plasma pharmacokinetics of the glucuronide metabolite.

To compare the impact of P-gp and Abcc2 on the total etoposide plasma pharmacokinetics (etoposide plus etoposide glucuronide), we also plotted the etoposide plasma concentration-time curves after deglucuronidation (Fig. 1D) and calculated the total AUCi.v. (Table 1). The total AUCi.v. was 1.3-fold elevated in Abcb1a/1b-/− mice (P < 0.01) and 1.5-fold increased in Abcc2-/− mice (P < 0.01). Combination knockout animals had a 1.9-fold higher total AUCi.v., which is additive compared with both single knockout strains (P < 0.001; Table 1). These results indicate that upon i.v. administration, P-gp and Abcc2 equally contribute to the overall disposition and elimination of etoposide, P-gp primarily through etoposide itself and Abcc2 primarily through etoposide glucuronide.

![Fig. 1.](image-url) Plasma concentration-time curves in male FVB WT (■), Abcc2-/- (▵), Abcb1a/1b-/- (□), and Abcb1a/1b;Abcc2-/- (▲) mice, for etoposide (A and B), etoposide glucuronide (C), and for total etoposide after deglucuronidation (D), determined after oral (A) and i.v. (B, C, and D) administration of 30 mg/kg etoposide. Etoposide glucuronide concentrations are expressed in etoposide mass equivalents. Data are means ± SD; n = 4 for both oral and i.v. administration. Insets, a semilogarithmic representation of the data (B and D). Note the difference in concentration scales between the panels.
Effect of Abcc2 and P-gp on the urinary and fecal excretion of etoposide and its glucuronide. In humans, rats, and rabbits, elimination of etoposide mainly occurs via urinary excretion of unchanged etoposide and etoposide glucuronide (13, 17, 18). Because P-gp and Abcc2 markedly affect the plasma pharmacokinetics of etoposide and its glucuronide, we next assessed the overall excretion of these compounds in the same panel of knockout mice. Urine and feces were collected for 24 hours after i.v. administration of 30 mg/kg [3H]etoposide, and the cumulative excretion of unchanged etoposide and total radioactivity, representing etoposide and its metabolites, was determined (Fig. 2). For P-gp–deficient animals, excretion of unchanged etoposide and total radioactivity in urine and feces was not different from WT mice (Fig. 2A and B). Urinary excretion of unchanged etoposide in mice lacking Abcc2 and/or P-gp was not different compared with WT mice, but the amount of total radioactivity in urine was highly increased in Abcc2−/− and Abcb1a/1b−/−;Abcc2−/− mice. Given the higher plasma concentrations of etoposide glucuronide in Abcc2−/− and Abcb1a/1b−/−;Abcc2−/− mice (Fig. 1C) we investigated the urinary output of this metabolite. Indeed, etoposide glucuronide accounted for an important part (21-23% of the given dose) of urinary radioactivity in these strains, versus 3% to 6% in WT and Abcb1a/1b−/− mice (Fig. 2A and C). Roughly a third of the total radioactivity in the Abcc2-deficient strains could not be directly ascribed to either unchanged etoposide or its glucuronide, suggesting that additional etoposide metabolite(s) are excreted in the urine when Abcc2 is absent. In contrast to the urinary excretion, the fecal excretion of unchanged etoposide and total radioactivity was strongly reduced in Abcc2−/− mice, and especially in Abcb1a/1b−/−;Abcc2−/− mice, but not in Abcb1a/1b−/− mice (Fig. 2B). Most of the decreases in total radioactivity excreted seemed to be due to the reduced excretion of unchanged etoposide. These results indicate that Abcc2, but not P-gp, has an important impact on the fecal and urinary excretion of etoposide and its glucuronide, respectively.

Impact of Abcc2 and P-gp on biliary and direct intestinal excretion of etoposide. To investigate the roles of Abcc2 and P-gp in biliary and direct intestinal excretion we carried out gall bladder cannulation experiments. Anesthetized mice with a cannulated gall bladder and a ligated common bile duct were i.v. injected with 30 mg/kg [3H]etoposide and the biliary output was measured for 1 hour. WT mice excreted 12.0 ± 1.3% of the dose as unchanged etoposide in their bile (Fig. 3A), although the amounts of etoposide in the livers of the various strains were unchanged (Fig. 3D). This shows that Abcc2 dominates the biliary excretion of unchanged etoposide, whereas P-gp contributes very little, if anything, to this excretion. Supplementary Fig. S1 shows a schematic overview of these results.

In WT mice the biliary excretion of unchanged etoposide was not different from total radioactivity output, indicating that virtually only etoposide was excreted. However, in Abcb1a/1b−/− mice, the biliary output of total

### Table 1. Plasma pharmacokinetic parameters after oral or i.v. administration of etoposide at 30 mg/kg

<table>
<thead>
<tr>
<th>Strain</th>
<th>Etoposide AUC(0–∞), h.mg/L</th>
<th>Etoposide Cmax, mg/L</th>
<th>Etoposide tmax, h</th>
<th>Etoposide F, %</th>
<th>Etoposide glucuronide AUC(0–∞), h.mg/L</th>
<th>Etoposide glucuronide Cmax, mg/L</th>
<th>Etoposide glucuronide t1/2, el, h</th>
<th>Etoposide glucuronide CL, L/h.kg</th>
<th>Etoposide glucuronide h.mg/L (0–∞)</th>
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<tr>
<td>WT</td>
<td>4.19 ± 0.38</td>
<td>1.92 ± 0.16</td>
<td>0.5</td>
<td>26.0 ± 2.5</td>
<td>31.0 ± 1.21</td>
<td>6.38 ± 0.61</td>
<td>0.35 ± 0.02</td>
<td>0.5</td>
<td>31.0 ± 1.21</td>
</tr>
<tr>
<td>Abcb1a/1b−/−</td>
<td>11.3 ± 0.75*</td>
<td>6.38 ± 0.61†</td>
<td>0.5</td>
<td>49.4 ± 5.4*</td>
<td>18.5 ± 0.74*</td>
<td>0.5</td>
<td>0.5</td>
<td>23.0 ± 1.00</td>
<td>28.3 ± 0.75§</td>
</tr>
<tr>
<td>Abcc2−/−</td>
<td>4.17 ± 0.14</td>
<td>2.17 ± 0.50</td>
<td>0.5</td>
<td>1.31 ± 0.10*</td>
<td>1.60 ± 0.08†</td>
<td>0.5</td>
<td>0.41 ± 0.04</td>
<td>0.5</td>
<td>1.67 ± 0.09†</td>
</tr>
<tr>
<td>Abcb1a/1b−/−;Abcc2−/−</td>
<td>14.8 ± 0.45†‡</td>
<td>8.35 ± 1.08†‡</td>
<td>0.5</td>
<td>14.8 ± 0.45†‡</td>
<td>33.0 ± 1.80</td>
<td>14.8 ± 0.45†‡</td>
<td>5.2 ± 0.04†</td>
<td>33.0 ± 1.80</td>
<td>57.3 ± 3.01‡</td>
</tr>
</tbody>
</table>

NOTE: Etoposide glucuronide concentrations are expressed in etoposide mass equivalents. Data are means ± SD, n = 4 for both oral and i.v. administration.

*P < 0.01, compared with WT mice
†P < 0.001, compared with WT mice.
‡P < 0.001, compared with Abcb1a/1b−/− mice.
§P < 0.01, compared with Abcb1a/1b−/− mice.
∥P < 0.05, compared with WT mice.
radioactivity, representing etoposide and its metabolites, was markedly higher than that of unchanged etoposide (the difference being ∼12% of the dose), indicating that etoposide metabolite(s) were present in the bile. Etoposide glucuronide in the bile of these mice was determined and accounted for 10.1 ± 2.4% of the dose. This means that the higher output of total radioactivity in this strain (Fig. 3A) represents mainly etoposide glucuronide. In Abcb1a/1b; Abcc2−/− mice, biliary excretion of radioactivity other than parent etoposide was again negligible (Fig. 3A), suggesting that Abcc2 is primarily responsible for biliary excretion of etoposide glucuronide in the Abcb1a/1b−/− mice.

Because in gall bladder cannulation experiments the common bile duct is ligated, drugs found in the gut contents can be solely attributed to direct excretion across the intestinal wall. In the small intestinal contents of WT mice, ∼10% of the dose was found as unchanged etoposide, and levels of total radioactivity (also ∼10% of the dose) revealed that little metabolite was present (Fig. 3B). Both Abcb1a/1b−/− and Abcb1a/1b; Abcc2−/− mice had markedly less unchanged etoposide in their gut, indicating that P-gp mediates direct intestinal excretion of etoposide across the gut wall. The decrease in direct intestinal excretion of etoposide from ∼19% of the dose in Abcc2−/− mice to ∼2% of the dose in Abcb1a/1b; Abcc2−/− mice illustrates the profound impact of P-gp in this process. These observations are in line with the data presented in Fig. 1A, which showed that P-gp drastically restricts the intestinal uptake of etoposide. Interestingly, Abcc2−/− mice had highly increased levels of radioactivity in their gut contents (∼20% of the dose), which could entirely be attributed to unchanged etoposide (Fig. 3B). We note that the decrease in biliary output as well as the increase in direct intestinal excretion of etoposide in Abcc2−/− mice was ∼10% of the dose (Fig. 3A and B). It is therefore tempting to speculate that impaired biliary excretion in Abcc2−/− mice results in higher levels of etoposide in the systemic circulation and subsequent excretion of etoposide across the intestinal wall into the gut, presumably via P-gp. If this is indeed the case, this process must occur quite efficiently and shortly after i.v. injection while plasma concentrations of etoposide are still high, because at 60 min after i.v. injection plasma concentrations were not elevated in cannulated Abcc2−/− mice (Fig. 3C).

The total radioactivity, but not the unchanged etoposide concentration, in the liver of P-gp–deficient mice was significantly higher than in WT mice (Fig. 3D). Together with the increased biliary excretion of etoposide glucuronide, these results suggest that Abcb1a/1b−/− mice have an increased hepatic formation and subsequently a higher biliary excretion of etoposide glucuronide.

**Accumulation of etoposide glucuronide in livers of Abcc2; Abcc3−/− mice.** ABCC3 was previously found to efficiently transport etoposide glucuronide in vitro (19), whereas the affinity of ABCC2 towards this metabolite was not reported. To investigate the impact of Abcc2 and Abcc3 on etoposide glucuronide transport in vivo, we studied liver accumulation in conscious, freely moving WT, Abcc2−/−, Abcc3−/−, and Abcc2; Abcc3−/− mice 1 hour after i.v. administration of 30 mg/kg etoposide. We also included Abcb1a/1b−/− and Abcb1a/1b; Abcc2−/− mice to obtain a complete picture. Importantly, in contrast to single Abcc2−/− or Abcc3−/− mice, in the livers of Abcc2; Abcc3−/− mice a highly increased amount of etoposide glucuronide was found (Fig. 4). This indicates that excretion of etoposide glucuronide from the liver to the systemic blood circulation is primarily dependent on basolateral

![Fig. 2. Urinary and fecal excretion of etoposide in male FVB WT, Abcc2−/−, Abcb1a/1b−/−, and Abcb1a/1b; Abcc2−/− mice, over 24 h after i.v. administration of 30 mg/kg [3H]etoposide. In feces and urine (A and B), total radioactivity ([3H]label), representing etoposide and its metabolites, and unchanged etoposide were measured, whereas etoposide glucuronide was determined only in urine (C). Etoposide glucuronide concentrations are expressed in etoposide mass equivalents. Data are means ± SD; n = 5. *, P < 0.05; **, P < 0.01; and ***, P < 0.001, compared with WT mice. Note the difference in concentration scales among the panels.](www.aacrjournals.org)
Liver accumulation of etoposide glucuronide in Abcc2-/- and Abcb1a/1b;Abcc2-/- mice was markedly lower than in WT mice (Fig. 4B). We previously observed that hepatic protein expression of Abcc3 is upregulated in male Abcc2-/- mice (9), and in the present study we also found higher Abcc3 mRNA expression in livers of male Abcc2-/- and Abcb1a/1b;Abcc2-/- mice (Fig. 5, described below). Higher hepatic expression and activity of Abcc3 likely results in an elevated efflux of etoposide glucuronide to the blood circulation and accordingly lower liver accumulation in these strains. The markedly increased etoposide glucuronide plasma concentrations in Abcc2-/- and Abcb1a/1b;Abcc2-/- mice (Fig. 4A) are consistent with this idea.

**Hepatic mRNA expression of Ugt1a1, Abcc3, Abcb1a, and Abcc2.** The highly elevated concentrations of etoposide glucuronide in the bile of Abcb1a/1b-/- mice (Fig. 3A) may be the result of increased hepatic formation of etoposide glucuronide. A possible cause for increased etoposide glucuronide formation would be upregulation of UDP-glucuronosyltransferase (UGT) enzyme(s) in the liver. In human liver microsomes, conjugation of etoposide to glucuronic acid is mainly mediated by UGT1A1 (20, 21). We therefore evaluated hepatic mRNA levels of Ugt1a1 in WT, Abcc2-/-, Abcb1a/1b-/-, and Abcb1a/1b;Abcc2-/- mice. Indeed, Ugt1a1 mRNA was 2- to 3-fold increased in single and combination P-gp–deficient mice (Fig. 5). This may explain the higher output of etoposide glucuronide in the bile of Abcb1a/1b-/- mice. In contrast, the highly increased plasma concentration and urinary output of etoposide glucuronide in Abcc2-/- mice (Figs. 1C and 2C) were not associated with elevated hepatic Ugt1a1 expression (Fig. 5). Presumably, there is little biliary excretion of etoposide glucuronide in Abcc2-/- mice.
from the Abcc2<sup>-/-</sup> liver, there is more opportunity for conversion to etoposide glucuronide. The finding that Abcb1a<sub>1b</sub>;Abcc2<sup>-/-</sup> mice had similarly elevated Ugt1a1 expression, but strongly reduced biliary excretion of etoposide glucuronide (≤0.5% of the dose) compared with Abcb1a<sub>1b</sub>;Abcc2<sup>-/-</sup> mice (10% of the dose; Fig. 3A), indicates that Abcc2 must be responsible for the biliary output of the glucuronide metabolite.

We previously reported that Abcc3 protein is upregulated in the livers of male Abcc2<sup>-/-</sup> mice compared with WT mice (9). Because Abcc3 plays an important role in the efflux of etoposide glucuronide from the liver to the blood, we evaluated hepatic mRNA expression in the same panel of knockout mice. Abcc3 was upregulated in the livers of P-gp-deficient (2.2-fold) and Abcc2-deficient (3.2-fold) animals, with the highest mRNA expression in Abcb1a<sub>1b</sub>; Abcc2<sup>-/-</sup> mice (5.3-fold; Fig. 5). Note that Abcb1a and Abcc2 were not differentially expressed in the livers of Abcc2<sup>−/−</sup> and Abcb1a<sub>1b</sub>/<sup>−/−</sup> mice (Fig. 5), indicating that upregulation or downregulation of these genes does not confound our data.

Discussion

In the present study we show that the hepatobiliary output of both etoposide and etoposide glucuronide is almost entirely dependent on Abcc2, and not on P-gp. Furthermore, P-gp was found to restrict the oral (re)uptake of unchanged etoposide (as shown before; ref. 3) and to mediate substantial direct intestinal excretion of etoposide across the gut wall. We also show that Abcc3 is responsible for the efflux of etoposide glucuronide from the liver to the systemic blood circulation, especially when Abcc2 is absent. Supplementary Fig. S1 shows a schematic overview of these results.

Although Abcc2 almost exclusively mediates the biliary excretion of parent drug, the liver and plasma concentrations of unchanged etoposide upon i.v. administration were not markedly elevated in Abcc2-deficient mice (Fig. 3A). This may be explained by increased hepatic formation of the main metabolite etoposide glucuronide. Yet, Abcc2<sup>-/-</sup> mice did not display higher levels of etoposide glucuronide in their livers than WT mice (Fig. 3D), whereas plasma levels were highly increased (Fig. 1C). Apparently, when the canalicular transporter Abcc2 is absent, the (up-regulated) basolateral Abcc3 takes over and efficiently extrudes etoposide glucuronide from the liver to the blood circulation. The highly elevated glucuronide levels in the livers of Abcc2;Abcc3<sup>-/-</sup> mice confirm this idea (Fig. 4B).

We previously observed that protein expression of Abcc3 is upregulated in the livers of male Abcc2<sup>-/-</sup> mice (9). In the present study we show that Abcc3 mRNA is increased in the livers of male Abcc2<sup>-/-</sup> as well as Abcb1a<sub>1b</sub>; Abcc2<sup>-/-</sup> mice. Elevated hepatic Abcc3 expression in Abcc2-deficient animals likely contributes to efficient extrusion of etoposide glucuronide from the liver to the blood. Subsequently, the glucuronide metabolite can be eliminated in the urine. Indeed, Abcc2-deficient mice had up to 7-fold increased urinary output of etoposide glucuronide compared with their WT counterparts (Fig. 2C). Our observation in mice that disrupted hepatobiliary excretion of etoposide results in increased hepatic formation and subsequent urinary excretion of etoposide metabolites, may also apply to etoposide pharmacokinetics in humans. In fact, this may explain why many pharmacokinetic studies in patients with elevated plasma levels of bilirubin or even with obstructive jaundice failed to show differences in total clearance, elimination half-life, or volume of distribution of unchanged etoposide (17, 22–25). Compared with these patients, Abcc2<sup>-/-</sup> mice show very similar results when only unchanged etoposide is considered (Figs. 1A and B, 2A, and 3C). This existence of an efficient backup detoxification system for etoposide is of course favorable during patient treatment, as it reduces the risk of increased etoposide exposure due to variable hepatobiliary clearance.

Although Abcc3 is also more highly expressed in Abcb1a<sub>1b</sub>/<sup>−/−</sup> mice (Fig. 5), there is no marked increase in urinary etoposide glucuronide excretion (Fig. 2A and C).
This suggests that Abcc2 is considerably more efficient than Abcc3 in removing etoposide glucuronide from the liver, allowing very little etoposide glucuronide to leave the liver over the sinusoidal membrane.

Ugt1a1 was not more highly expressed in livers of Abcc2-/- mice, despite the highly increased plasma and urine levels of etoposide glucuronide in this strain (Figs. 1C and 2C). The formation of etoposide glucuronide in the liver of Abcc2-/- mice thus seems not to be limited by the capacity of the UGT enzyme, but rather by the hepatic residence time of etoposide, which is presumably increased in the absence of hepatobiliary excretion of etoposide by Abcc2. Interestingly, in humans receiving 80 to 120 mg/m² etoposide, approximately 8% of the dose was excreted as etoposide glucuronide in the urine within 24 hours (17). Another study revealed that the urinary excretion of etoposide glucuronide in patients receiving up to 3,500 mg/m² etoposide (~35-fold higher dose) was still 8.3% to 17.3% of the dose (18). Assuming that the patients in these two studies had comparable UGT1A1 expression, UGT1A1-mediated glucuronidation of etoposide seems not to be easily saturated in humans as well.

Both human ABCC2 and ABCC3 were previously shown to moderately transport etoposide in vitro (5, 6). However, Abcc3-/- mice did not display an enhanced lethality when exposed to etoposide (7). Furthermore, etoposide glucuronide was reported to be an excellent substrate for human ABCC3 (19), whereas information on the transport of etoposide glucuronide by ABCC2 is lacking. Our data indicate that etoposide is an excellent substrate of Abcc2 in vivo. In fact, biliary excretion of etoposide, which plays an important role in the elimination of etoposide in WT mice, is ~6-fold decreased in Abcc2-/- mice. We further found that mice deficient for both Abcc2 and Abcc3 had highly increased levels of etoposide glucuronide in their livers. Additionally, in single Abcc3-/- mice the hepatic etoposide glucuronide concentrations were not different from WT mice, whereas these concentrations in single Abcc2-/- and combination Abcb1a/1b;Abcc2-/- mice were even somewhat lower than in WT mice (Fig. 4B). The latter might well be...
explained by the upregulated Abcc3 in the livers of Abcc2−/− and Abcb1a1b/Abcc2−/− mice (ref. 9 and Fig. 5). Collectively, these results indicate that etoposide glucuronide is a good substrate for both Abcc2 and Abcc3 and that the elimination of this metabolite from the liver is almost completely dependent on these efflux pumps. However, when Abcc2 and Abcc3 are both present, as in the Abcb1a1b/ mice, Abcc2 seems to be more efficient than Abcc3 and etoposide glucuronide is directed towards bile rather than blood (Fig. 3A and C).

In this study we show that Abcc2, and not P-gp, dominates the biliary excretion of etoposide. Previously, we found that the biliary excretion of the anticancer agent paclitaxel was also dominated by Abcc2, whereas P-gp played only a minor role (11). In addition, ABCC2 was shown to be overexpressed in hepatocellular carcinoma (26, 27), and silencing of ABC2 in HepG2 tumor cells (hepatoma cells) with an ABCC2 antisense construct resulted in a highly increased sensitivity towards etoposide (25-fold), doxorubicin (12-fold), vincristine (50-fold), and cisplatin (25-fold; ref. 28). ABCC2 thus seems to play an important role in the liver, under physiologic and pathologic conditions. On the one hand ABCC2 often dominates the hepatobiliary drug excretion, and on the other hand ABCC2 can confer multidrug resistance in hepatocellular carcinoma.

Although it is difficult to assign an exact number, we estimate that with P-gp, Abcc2, and Abcc3, we can explain about half or more of the overall plasma pharmacokinetic behavior of etoposide in WT mice. We base this estimate on the often >2-fold quantitative shifts that we observed in the various knockout strains for oral availability, plasma levels, biliary and direct intestinal excretion, and overall urinary and fecal elimination. Our findings may be relevant for patients treated with etoposide, because for P-gp, ABCC2, and ABC3 polymorphisms have been described that affect their transport activity. On the one hand, variation in transporter expression might explain why the oral availability of etoposide varies widely among patients (25-80%), and we think that this could be a major factor in variable etoposide toxicity. On the other hand, the direct and indirect redundancies in etoposide (glucuronide) detoxifying functions among these three transporters will to some extent mitigate the toxicologic and therapeutic impact of variation in activity of just one of the transporters. Knowledge of factors that affect the pharmacokinetics may help to improve pharmacotherapy with etoposide and possibly other related drugs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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P-glycoprotein (P-gp/Abcb1), Abcc2, and Abcc3 Determine the Pharmacokinetics of Etoposide

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