Abcc4 Together with Abcb1 and Abcg2 Form a Robust Cooperative Drug Efflux System That Restricts the Brain Entry of Camptothecin Analogues

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Abstract

Purpose: Multidrug resistance–associated protein 4 (ABCC4) shares many features with P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2), including broad substrate affinity and expression at the blood–brain barrier (BBB). However, the pharmacologic relevance of ABCC4 at the BBB is difficult to evaluate, as most drugs are also substrates of ABCB1 and/or ABCG2.

Experimental Design: We have created a mouse strain in which all these alleles are inactivated to assess their impact on brain delivery of camptothecin analogues, an important class of antineoplastic agents and substrates of these transporters. Wild-type (WT), Abcg2−/−, Abcb1a/b−/−, Abcc4−/−, Abcb1a/b;Abcg2−/−, Abcb2;Abcc4−/−, and Abcb1a/b;Abcg2;Abcc4−/− mice received i.v. topotecan, irinotecan, SN-38, or gimatecan alone or with concomitant oral elacridar. Drug levels were analyzed by high-performance liquid chromatography (HPLC).

Results: We found that additional deficiency of Abcc4 in Abcb1a/b;Abcg2−/− mice significantly increased the brain concentration of all camptothecin analogues by 1.2-fold (gimatecan) to 5.8-fold (SN-38). The presence of Abcb1a/b or Abcc4 alone was sufficient to reduce the brain concentration of SN-38 to the level in WT mice. Strikingly, the brain distribution of gimatecan in brain of WT mice was more than 220- and 40-fold higher than that of SN-38 and topotecan, respectively.

Conclusion: Abcc4 limits the brain penetration of camptothecin analogues and teams up with Abcb1 to form a robust cooperative drug efflux system. This concerted action limits the usefulness of selective ABC transport inhibitors to enhance drug entry for treatment of intracranial diseases. Our results also suggest that gimatecan might be a better candidate than irinotecan for clinical evaluation against intracranial tumors. Clin Cancer Res; 19(8); 2084–95. ©2013 AACR.

Introduction

The blood–brain barrier (BBB) is a complex well-organized structure that serves to protect the brain by limiting the entry of most exogenous compounds into brain (1). On the other hand, it is seen as a major obstacle for many therapeutic agents which might otherwise be effective against brain diseases including brain cancer (2–4). The BBB is formed by the brain endothelial cells, which are closely linked to each other by tight junctions, lacking fenestrae and having low pinocytic activity. As a consequence, the brain entry of substances requires transepithelial passage, thus allowing strict regulation of brain entry by a range of uptake and efflux transporters (5). ATP-binding cassette (ABC) drug efflux transporters expressed at the BBB restrict the entry of many compounds into the brain (6). The dramatic impact of P-glycoprotein (P-gp, ABCB1/Abcb1a) on the brain entry of substrate agents was first shown by Schinkel and colleagues (7) using Abcb1a-deficient mice and was later shown to be important for a plethora of agents. The cooperative action of breast cancer resistance protein (BCRP, ABCG2/Abcg2) in combination with Abcb1 became clear when we used compound Abcb1a/Abcg2-deficient mice (8). Until then, the functionality of Abcg2 at the BBB had not been convincingly shown by results obtained with single Abcg2-deficient mice because of the overlapping substrate affinities of Abcb1a/b and Abcg2 and because the presence of Abcb1a/b alone is sufficient to reduce the concentration of dual ABCB1/ABCG2 substrates in the brain to the level of that in wild-type (WT) mice.
**Translational Relevance**

The blood–brain barrier (BBB) is a major hurdle for drugs to reach into the brain, and a proper understanding of the pharmacologic relevance of the important drug efflux transporter proteins that are located at the BBB is critical for optimal treatment of intracranial malignancies. Many potentially useful drugs are substrates for one or several of these transporters and may for that reason target the tumor inadequately. We here identify ABCG4 as an important player next to ABCB1 and ABCG2. The mouse models described here will help in selecting those candidate drugs for further clinical trials that elicit a good brain entry because they lack affinity toward any of these transporters or, alternatively, they will be instrumental in establishing the feasibility of using drug transport inhibitor to improve brain entry of the candidate drug.

Camptothecin and its analogues are potent topoisomerase I inhibitors and represent an important class of antineoplastic agents with a wide spectrum of antitumor activity. Two camptothecin analogues, topotecan and irinotecan (CPT11), have already been approved for treatment of advanced ovarian cancer, small cell lung cancer, and colon cancer (9–11). Whether they could also be efficacious in advanced ovarian cancer, small cell lung cancer, and colon cancer (11). Whether they could also be efficacious against brain malignancies is speculative, but CPT11 is receiving considerable attention with more than 10 ongoing or planned clinical trials involving brain cancer (source: http://clinicaltrials.gov). However, whether these drugs can cross the BBB in sufficient amounts to be active against intracranial tumors remains uncertain. In *in vitro* studies have shown that CPT11 and its active metabolite SN-38 are also substrates of ABCB1 and/or ABCG2 (12–16), which may thus exclude these compounds from the brain, as was shown for topotecan (8). Gimatecan is another camptothecin analogue with different pharmaceutical properties. Relative to topotecan and CPT11/SN-38, it is a more lipophilic compound and a weaker substrate of ABCB1 (17, 18). It is also reported to have none or minimal affinity for ABCG2 (18, 19), although Marchetti and colleagues (17) reported that gimatecan is a substrate of Abcg2.

Besides ABCB1 and ABCG2, multidrug resistance protein 4 (MRP4, ABCC4/Abcc4) is also expressed at the BBB and the choroid plexus epithelium (20). ABCC4 transports a wide range of more polar endogenous molecules such as nucleotides, urate and folates, bile acids, and glutathione conjugates but is also reported to transport antiviral, antibacterial, cardiovascular, and anticancer agents including topotecan. CPT11/SN-38, and gimatecan (18, 20–24) in *in vitro*. The initial claim (20) that the brain accumulation of topotecan is higher in single Abcc4-deficient mice could not be replicated, presumably because topotecan is also a substrate of Abcb1 and Abcg2, which dominate the restriction of its brain entry (8). More recently, however, it was shown that Abcc4-deficient mice accumulate more oseltamivir carboxylate (Ro64-0802) in their brain (25). Importantly, this relatively hydrophilic metabolite of oseltamivir is not a substrate of Abcb1 (26), explaining why the substrate functionality of Abcc4 at the BBB could be shown in single Abcc4-deficient mice.

To allow more accurate assessment of the impact of Abcc4 on the BBB penetration without interference by Abcb1a/b and Abcg2, we conducted a comprehensive comparison between Abcb1a/b; Abcg2; Abcc4 versus Abcb1a/b; Abcg2-deficient mice and a range of other control strains. For this purpose, we used the camptothecin analogues topotecan, CPT11, SN-38, and gimatecan, which all differ to some extent in their lipophilicity and affinities toward these ABC transporters. Our results clearly show the profound impact that Abcc4 can have on the brain penetration of substrate drugs.

**Materials and Methods**

**Chemicals and drugs**

Topotecan and elacridar were kindly provided by GlaxoSmithKline. Irinotecan (Campto) was from Pfizer; SN-38 was from Sequoia Research Products; and gimatecan was provided by Novartis Pharmaceuticals Inc. Blank human plasma was obtained from healthy donors (Sanquin). All other chemicals were purchased from Merck.

**Animals**

Mice were housed and handled according to institutional guidelines complying with Dutch legislation. All experiments with animals were approved by the local animal experiment committee. The animals used in this study were WT, Abcg2<sup>−/−</sup>, Abcb1a/b<sup>−/−</sup>, Abcc4<sup>−/−</sup> (20), Abcb1a/b<sup>−/−</sup>, Abcg2<sup>−/−</sup>, (8), Abcg2<sup>−/−</sup>, Abcc4<sup>−/−</sup>, and Abcb1a/b<sup>−/−</sup> Abcg2<sup>−/−</sup> mice, all of more than 99% FVB genetic background, between 8 and 14 weeks of age. The animals were kept in a temperature-controlled environment with a 12-hour dark cycle and received a standard diet (AM-II, Hope Farm B.B.) and acidified water ad *libitum*.

**Plasma and brain pharmacokinetics**

Topotecan and CPT11 were diluted to 0.5 mg/mL in 5% (w/v) glucose, whereas gimatecan and SN-38 were dissolved (2 mg/mL) in dimethyl sulfoxide (DMSO). They were administered i.v. at a dose of 2 mg/kg (for topotecan, SN-38, and gimatecan) or 5 mg/kg (for CPT11). Elacridar prepared as described earlier (8) was given per os at a dose of 100 mg/kg 2 hours before camptothecins. Continuous infusion of topotecan was achieved with Alzet minipumps model 1003D (Durect Corp.) filled with 2 or 10 mg/mL of topotecan in 5%(w/v) glucose. Pumps were placed in the peritoneal cavity under isoflurane anesthesia, and mice were sacrificed 28 to 30 hours after placement.

Mice were anesthetized by isoflurane. Blood was collected by cardiac puncture and kept on ice. The mice were sacrificed and brains were dissected. Plasma was separated by centrifugation at 10,000 × *g* for 5 minutes at 4°C. Brains were homogenized in 3 mL 1% (w/v) bovine serum albumin and centrifuged for 10 minutes at 10,000 × *g*. The brain supernatants were then collected and centrifuged again for 10 minutes at 10,000 × *g* for plasma and brain pharmacokinetics.
albumpin (BSA). Both plasma and brain homogenates were stored at −20°C until analysis. The brain accumulation was corrected for the amount of drug in the brain vasculature (1.4%; ref. 27).

Drug analysis

Topotecan, CPT11, and SN-38 samples were analyzed by high-performance liquid chromatography (HPLC) as described previously (28, 29) but with minor modification. In brief, 100 μL of biological sample was mixed with 200 μL of ice-cold methanol and centrifuged at 4°C, 20,000 × g for 10 minutes. Next, 100 μL of the supernatant fraction was mixed with 200 μL of ice-cold perchloric acid (2% in water). After centrifugation at 4°C, 20,000 × g for 3 minutes, 100 μL of clear supernatant was analyzed by HPLC.

For gimatecan, 50 μL of sample was mixed with of ice-cold methanol and centrifuged at 4°C, 20,000 × g for 10 minutes. A volume of 200 μL of supernatant fraction was mixed with 300 μL 0.01 mol/L sodium borate, centrifuged at 4°C, 20,000 × g for 5 minutes and 100 μL of clear solution was injected into the HPLC system.

HPLC was conducted using a DGP-3600A pump with SRD-3600 Solvent Rack, a model WPS-3000TS autosampler ( Dionex), and a model FP-1520 fluorescence detector (Jasco) operating at 380/527 nm (excitation/emission). Separation of topotecan, CPT11, and SN-38 was conducted using a Zorbax SB-C18 column (75 × 4.6 mm i.d., Rockland Technologies Inc.) and for gimatecan a Symmetry C18 column (2.1 × 150 mm i.d., Waters). The mobile phase for the topotecan consisted of methanol, 0.1 mol/L hexane-1-sulfonic acid, and 0.01 mol/L TEMED adjusted to pH 6.0 with phosphoric acid (25:10:65, v/v/v). The mobile phase for CPT11 and SN-38 consisted of 0.1 mol/L ammonium acetate buffer pH 6.4 containing 5 mol/L tetrabutylammonium bromide, triethylamine, and acetonitrile (790:1:210, v/v/v). The mobile phase for gimatecan consisted of 0.1 mol/L hexane-1-sulfonic acid, and 0.01 mol/L TEMED adjusted to pH 6.8 (30:70, v/v).

Ex vivo carboxylesterase activity measurement

Carboxylesterase activity in mouse plasma was measured by monitoring ex vivo conversion of CPT11 into SN-38 using previously described methods with slight modification (30). In short, 20 μL 2.5 mmol/L CPT11 was mixed with 800 μL 20 mmol/L Tris-HCl buffer (pH 7.5) and incubated at 37°C for 30 minutes to reach equilibrium between the lactone and carboxylate forms of CPT11. Next, 200 μL fresh plasma collected from WT, Abcg2−/- or Abcb1a/b; Abcg2−/−; Abcb1a/b; Abcg2−/− mice (n = 3) was added (final CPT11 concentration is 50 μmol/L) and the mixtures were kept at 37°C with shaking. At 0 minutes, 1, 2, and 4 hours, 100 μL samples were collected for the determination of CPT11 and SN-38 concentrations.

Results

Role of Abcc4 in topotecan brain delivery

The novel Abcg2;Abcc4−/− and Abcb1a/b; Abcg2; Abcc4−/− strains were obtained by cross-breeding of the Abcc4−/−, Abcg2−/−, and Abcb1a/b; Abcg2−/− mice. These mice are viable, fertile, and do not display any overt phenotype.

To investigate the role of Abcc4 in the brain penetration of topotecan without the interference by Abcg2 and Abcb1a/b, we determined the topotecan concentrations in brain and plasma from WT, Abcg2−/−, Abcb1a/b; Abcg2−/−, and Abcb1a/b; Abcg2; Abcc4−/− mice after i.v. administration of 2 mg/kg topotecan. In line with our previous results (8), the absence of Abcg2 alone caused a marked 5-fold increased plasma concentration of topotecan in comparison with Abcg2-proficient WT mice (Fig. 1A), whereas it caused only a small (2-fold) and nonsignificant higher brain concentration. As a consequence, the brain-to-plasma ratio was lower in Abcg2−/− mice relative to WT controls. To understand this counterintuitive result, we also determined the brain and plasma levels at 5 minutes after drug administration and found that the differences between the strains were much smaller at this very early time point (Fig. 1D), implicating that the absence of Abcg2 alone has little effect on the distribution to the brain. At 1 hour, the plasma levels were reduced by about 20- and 6-fold in WT and Abcg2−/− mice, respectively, whereas the brain levels were reduced by only 5- and 3-fold. Thus, it appears that efflux from the brain cannot keep up with the much more rapid elimination from plasma and the reduced brain-to-plasma ratio in Abcg2−/− versus WT mice is a consequence of the more rapid decay in the plasma concentration in WT mice.

At both 1 and 4 hours, the plasma concentration of topotecan in Abcb1a/b; Abcg2−/− and Abcb1a/b; Abcg2; Abcc4−/− mice was similar, whereas the concentration in brains of Abcb1a/b; Abcg2; Abcc4−/− mice was significantly higher than in brains of Abcb1a/b; Abcg2−/− mice (Fig. 1B). Overall, this resulted in a 2.0- and 1.9-fold elevated brain-to-plasma ratio of topotecan at 1 and 4 hours, respectively, in Abcb1a/b; Abcg2; Abcc4−/− mice versus Abcb1a/b; Abcg2−/− mice (Fig. 1C).

Because topotecan is relatively good water soluble, we decided to carry out a similar experiment where topotecan (2 mg/kg/d) was delivered by Alzet minipumps to achieve steady-state plasma concentrations. The difference in systemic exposure in WT and Abcg2−/− strains at this dose was modest as steady-state plasma levels differed only by about 3-fold. The brain concentration was significantly higher in Abcb1a/b; Abcg2; Abcc4−/− mice than in Abcb1a/b; Abcg2−/− mice (Fig. 1E–G), confirming the impact of Abcc4. We also included a cohort of WT mice that received a 5-fold higher dose level (10 mg/kg/d) in an attempt to compensate for the higher clearance in WT mice; however, the plasma levels were not proportionally higher. We have no clear explanation for this finding.

Together these results show that Abcc4 restricts the brain penetration of topotecan in the absence of Abcb1a/b and Abcg2 but has no effect on the plasma level of topotecan.
Roles of Abcb1a/b, Abcg2, and Abcc4 in brain delivery of CPT11 and its active metabolite SN-38

Abcb1a/b;Abcg2<sup>−/−</sup> mice had significantly higher plasma levels of CPT11 and SN-38 than Abcg2<sup>−/−</sup> mice, whereas there was no difference between Abcb1a/b;Abcg2<sup>−/−</sup> and Abcb1a/b;Abcg2;Abcc4<sup>−/−</sup> mice receiving CPT11 (Fig. 2A).

Interestingly, the CPT11 level in plasma of all Abcg2<sup>−/−</sup> mice was markedly lower than that of WT mice, whereas the plasma level of SN-38 was significantly higher than that of WT mice. Given the fact that the sum of concentrations (SUM[CPT11 + SN-38]) in plasma of WT and Abcg2<sup>−/−</sup> mice were similar, the reduction in CPT11 levels are probably not

Figure 1. Topotecan brain and plasma pharmacokinetics. Plasma concentrations, brain concentrations, and brain-to-plasma ratios of topotecan in WT, Abcg2<sup>−/−</sup>, Abcb1a/b;Abcg2<sup>−/−</sup>, and Abcb1a/b;Abcg2;Abcc4<sup>−/−</sup> mice 1 and 4 hours (A–C) and 5 minutes (D) after i.v. administration of 2 mg/kg topotecan or 28 to 30 hours after implantation of an Alzet minipump delivering 2 or 10 (high-dose) mg/kg/d (E–G). Data are means ± SD. n = 8, 5, 10, 5 (1 hour) and 5, 5, 5, 5 (4 hours) for WT, Abcg2<sup>−/−</sup>, Abcb1a/b;Abcg2<sup>−/−</sup>, and Abcb1a/b;Abcg2;Abcc4<sup>−/−</sup> mice (A–C); n = 5 and 5 for WT and Abcg2<sup>−/−</sup> mice (D); n = 5, 4, 5, 5 and 5 for WT, WT (high-dose), Abcg2<sup>−/−</sup>, Abcb1a/b;Abcg2<sup>−/−</sup>, and Abcb1a/b;Abcg2;Abcc4<sup>−/−</sup> mice (E–G). **, P < 0.01; ***, P < 0.001, compared with WT mice. ###, P < 0.001, compared with Abcb1a/b;Abcg2<sup>−/−</sup> mice.
compared with P
Abcg2
collected plasma of WT, the accelerated conversion of CPT11 into SN-38. We evaluated active metabolite SN-38, an increased expression of carboxylesterase(s) principally involved in the conversion of CPT11 to its due to elimination (efflux) by Abcg2. As carboxylesterase(s) are principally involved in the conversion of CPT11 to its active metabolite SN-38, an increased expression of carboxylesterase(s) in Abcg2-deficient mice might underlie this accelerated conversion of CPT11 into SN-38. We evaluated the ex vivo conversion rate of CPT11 into SN-38 using freshly collected plasma of WT, Abcg2−/−, and Abcb1a/b;Abcg2−/− mice. Following the incubation of CPT11 in Abcg2−/− murine plasma for only 1 hour, more than 20% of the parent drug was already converted into SN-38, whereas only 1% of CPT11 was converted to SN-38 after 4-hour incubation in WT plasma. A similar conversion rate to that in Abcg2−/− mice was observed in plasma of Abcb1a/b; Abcg2−/− mice.

The marked elevation of the CPT-11 to SN-38 conversion in all strains that are deficient in Abcg2 relative to Abcg2-proficient strains makes it more difficult to interpret the role of the ABC transporters on the brain penetration. It is clear, however, that Abcb1a/b plays a pivotal role in brain penetration of CPT11 given the 10.2- and 15.9-fold higher brain CPT11 levels at 1 and 4 hours, respectively, in Abcb1a/b; Abcg2−/− mice versus Abcg2−/− mice (Fig. 2C). Abcb1a/b also limits the brain penetration of SN-38, although the difference between Abcg2−/− and Abcb1a/b;Abcg2−/− mice was smaller (about 2.5-fold). Importantly, however, the additional deletion of Abc4 in the absence of Abcb1a/b and Abcg2 resulted in a further 3.4-fold higher brain concentration of SN-38 at 1 hour. Similarly, the CPT11 levels were higher, but the difference was only 1.5-fold. Overall, the SUM[CPT-11 + SN-38] in brain and the brain-to-plasma ratio of Abcb1a/b;Abcg2−/− mice were significantly higher than those of Abcb1a/b;Abcg2 mice (Fig. 2F and Table 1)

**Roles of Abcb1a/b, Abcg2, and Abcc4 in brain delivery of gimatecan**

Gimatecan is a relatively new camptothecin analogue and little is known about the impact of drug efflux transporters of this analog in vivo. Therefore, we evaluated the roles of Abcb1a/b, Abcg2, and Abcc4 in gimatecan...
plasma and brain pharmacokinetics using our knockout mice. Unlike topotecan and CPT11, the plasma concentration of gimatecan was not different across all strains on i.v. administration of 2 mg/kg gimatecan, except there was a 1.5-fold elevation of the gimatecan plasma level in Abcb1a/b;Abcg2;Abcc4 mice and Abcb1a/b-proficient strains (WT, Abcc4; Abcg2;Abcc4; and Abcb1a/b;Abcg2;Abcc4) at 4 hours (Fig. 3A). Moreover, the plasma levels of all strains were much higher than those of topotecan or CPT11/SN-38.

Interestingly, there were also smaller differences in the brain concentration of gimatecan across all strains, relative to topotecan and CPT11/SN-38 (Fig. 3B). However, there were clear differences between Abcb1a/b-deficient strains (Abcb1a/b;Abcg2;Abcc4; and Abcb1a/b;Abcg2;Abcc4) and Abcb1a/b-proficient strains (WT, Abcg2; Abcc4; and Abcg2;Abcc4). Deletion of Abcb1a/b alone caused a small but significant 1.9-fold increase relative to WT mice. Vice versa, the brain penetration in Abcb1a/b-proficient Abcg2;Abcc4; and Abcg2;Abcc4 mice was similar to that in WT mice and much lower than in Abcb1a/b;Abcg2; and Abcb1a/b;Abcc4 mice. Consequently, Abcb1a/b appears to be the most important factor limiting the brain penetration of gimatecan. Although the difference in the brain concentration of gimatecan between Abcb1a/b;Abcg2; and Abcb1a/b;Abcc4; and Abcb1a/b;Abcc4; and Abcg2;Abcc4 mice was small, the brain-to-plasma ratio in the latter was significantly higher at both 1 and 4 hours, indicating that Abcc4 also contributes to limiting the brain penetration of gimatecan. The same was seen for Abcg2 by comparing Abcb1a/b; Abcb1a/b;Abcg2; and Abcb1a/b;Abcc4; and Abcb1a/b;Abcc4; and Abcg2;Abcc4 mice. Overall, however, the impact of these drug efflux transporters on gimatecan brain penetration is not as strong as for topotecan or CPT11.

Effect of the dual ABCB1 and ABCG2 inhibitor elacridar on brain penetration of SN-38 and gimatecan

We previously reported that co-administration of the dual ABCB1 and ABCG2 inhibitor elacridar together with topotecan markedly increased the brain penetration of topotecan (8). Because our present findings show that Abcc4 also impairs the brain penetration of camptothecin analogues, we investigated the effect of elacridar on the brain penetration of SN-38 in various Abcc4-deficient strains. SN-38 was used because the previous experiments clearly indicated that this compound was one of the best Abcc4 substrates. Co-administration of elacridar markedly enhanced the plasma SN-38 concentration across all strains, with the most dramatic increase in WT and Abcc4 mice (Fig. 4A). Presumably, the impact of elacridar on the plasma level of SN-38 is mainly due to the inhibition of Abcg2-mediated elimination because (i) the plasma level of SN-38 in Abcb1a/b;Abcg2; and Abcc4 mice was markedly higher than that of WT mice; (ii), the plasma level of SN-38 was only moderately increased in Abcb1a/b;Abcc4; and (iii) the SN-38 plasma level did not differ between Abcb1a/b; and Abcb1a/b; Abcg2;Abcc4; and Abcg2;Abcc4 mice, suggesting that Abcb1a/b is not actively involved in the elimination of SN-38. Intriguingly, elacridar also substantially increased the plasma level of SN-38 in Abcb1a/b;Abcg2;Abcc4 mages by a yet unknown cause.

Similar to what we found for topotecan, the brain-to-plasma ratio cannot be compared when the plasma elimination is very different, such as between Abcg2-proficient and -deficient strains.

Therefore, we focused on the actual brain concentration of SN-38. Abcb1a/b;Abcg2;Abcc4 mice lacking all 3 transporters had a 6.6-fold higher brain concentration of SN-38 relative to WT mice. Strikingly, there was no difference between the concentrations of SN-38 in brains of Abcb1a/b;Abcg2; and WT mice (Fig. 4B). In line with this result, administration of elacridar did not increase the SN-38 concentration in brain of WT mice as well. These results suggest that Abcc4 alone is sufficient to maintain a similar brain level of SN-38 as achieved in WT mice.
Similarly, the brain concentrations in Abcg2\(^{-/-}\), Abcc4\(^{-/-}\), and Abcg2;Abcc4\(^{-/-}\) mice were also not different compared with WT mice, suggesting that the presence of Abcb1a/b alone is also enough to reduce the brain concentration of SN-38 to the level of WT.

Because Abcb1a/b\(;/\)Abcc4\(^{-/-}\) mice do not exist, we could not evaluate the role of Abcg2 by a similar genetic analysis. However, with the help of elacridar to inhibit Abcb1a/b and (partially) Abcg2, we can make an estimation of the relative importance of Abcg2. The brains of Abcg2;Abcc4\(^{-/-}\) mice that receive elacridar accumulate to about 70% of the level observed in Abcb1a/b;Abcg2;Abcc4\(^{-/-}\) mice, indicating that Abcb1a/b is substantially, albeit not completely, inhibited by elacridar (Fig. 4). When we compare the brain penetration of SN-38 in Abcc4\(^{-/-}\) mice versus Abcg2;Abcc4\(^{-/-}\) mice both receiving elacridar and assume that Abcb1a/b is inhibited to the same extent in both strains, the difference in brain penetration between these 2 strains will be mainly due to the activity of Abcg2.

We also investigated the effect of elacridar on gimatecan, as gimatecan appears to be a weaker substrate of Abcc4. Surprisingly, co-administration of elacridar did not increase the brain-to-plasma ratio of gimatecan. The brain concentration of gimatecan in mice receiving elacridar did not differ at 1 hour and was 1.7-fold increased at 4 hours (\(P<0.05\)) relative to mice not receiving elacridar. However, when corrected for the plasma concentration, the brain penetration of gimatecan was not significantly different in WT mice in the presence or absence of elacridar (Fig. 4E and F), suggesting that the increased brain penetration at 4

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**Figure 4.** Effect of elacridar on the brain and plasma pharmacokinetics of SN-38 and gimatecan. Plasma concentrations (A), brain concentrations (B), and brain-to-plasma ratios (C) of SN-38 in WT, Abcg2\(^{-/-}\), Abcc4\(^{-/-}\), Abcg2;Abcc4\(^{-/-}\), Abcb1a/b;Abcg2;Abcc4\(^{-/-}\) mice 1 hour after i.v. administration of 2 mg/kg SN-38 with or without co-administration of elacridar (2 hours before the SN-38 administration, oral 100 mg/kg). Data are means ± SD. \(n=5, 5, 4, 5, 5, 4, 5\) and 5 for WT, WT + elacridar, Abcc4\(^{-/-}\), Abcc4\(^{-/-}\) + elacridar, Abcg2\(^{-/-}\), Abcg2;Abcc4\(^{-/-}\), Abcg2;Abcc4\(^{-/-}\) + elacridar, Abcb1a/b/Abcg2\(^{-/-}\), Abcb1a/b/Abcc4\(^{-/-}\), Abcb1a/b/Abcc4\(^{-/-}\) + Abcg2;Abcc4\(^{-/-}\) + elacridar groups (A–C). \(n=3, 3, 4\) (1 hour) and 3, 3, 3 (4 hours) for WT, WT + elacridar, Abcc4\(^{-/-}\), Abcc4\(^{-/-}\) + elacridar, Abcg2;Abcc4\(^{-/-}\), and Abcg2;Abcc4\(^{-/-}\) + elacridar groups (D–F). *, \(P<0.05\); **, \(P<0.01\); ###, \(P<0.001\), compared with the first column of the same subgroup. \&, \(P<0.05\); \#&, \(P<0.01\); ##&, \(P<0.001\), compared with the second column of the same subgroup.

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**Figure 4.** Effect of elacridar on the brain and plasma pharmacokinetics of SN-38 and gimatecan. Plasma concentrations (A), brain concentrations (B), and brain-to-plasma ratios (C) of SN-38 in WT, Abcg2\(^{-/-}\), Abcc4\(^{-/-}\), Abcg2;Abcc4\(^{-/-}\), Abcg2;Abcc4\(^{-/-}\) mice 1 hour after i.v. administration of 2 mg/kg SN-38 with or without co-administration of elacridar (2 hours before the SN-38 administration, oral 100 mg/kg). Data are means ± SD. \(n=5, 5, 4, 5, 5, 4, 5\) and 5 for WT, WT + elacridar, Abcc4\(^{-/-}\), Abcc4\(^{-/-}\) + elacridar, Abcg2\(^{-/-}\), Abcg2;Abcc4\(^{-/-}\), Abcg2;Abcc4\(^{-/-}\) + elacridar, Abcb1a/b/Abcg2\(^{-/-}\), Abcb1a/b/Abcc4\(^{-/-}\), Abcb1a/b/Abcc4\(^{-/-}\) + elacridar groups (A–C). \(n=3, 3, 4\) (1 hour) and 3, 3, 3 (4 hours) for WT, WT + elacridar, Abcc4\(^{-/-}\), Abcc4\(^{-/-}\) + elacridar, Abcg2;Abcc4\(^{-/-}\), and Abcg2;Abcc4\(^{-/-}\) + elacridar groups (D–F). *, \(P<0.05\); **, \(P<0.01\); ###, \(P<0.001\), compared with the first column of the same subgroup. \&, \(P<0.05\); \#&, \(P<0.01\); ##&, \(P<0.001\), compared with the second column of the same subgroup.
hours is mainly a reflection of the higher gimatecan plasma levels. Also, no significant additional effect of elacridar was found on brain concentration of gimatecan in Abcb1a/b; Abcg2/C0/C0 mice. Similar to SN-38, elacridar increased the plasma levels in Abcb1a/b; Abcg2/C0/C0 mice by a yet unidentified mechanism.

Impact of Abcb1a/b, Abcg2, and Abcc4 on brain distributions of camptothecins

For all tested camptothecin analogues, their brain distributions (rendered as the percentage of total dose present in brain) were significantly increased when Abcb1a/b, Abcg2, and Abcc4 were simultaneously deleted. Loss of these transporters caused a profound increase of percentage of total dose of topotecan in brain (22- and 84-fold at 1 and 4 hours, respectively), slightly less profound for CPT11 (16- and 23-fold) and only a moderate increase for gimatecan (3.8- and 3.9-fold, Table 1), suggesting that the brain distributions of all camptothecins are affected by the drug efflux transporters but to differing extents depending on their unique structures.

We also compared the brain distributions of all camptothecins (Fig. 5). The brain distributions of the camptothecin analogues show a wide variation between WT and Abcb1a/b; Abcg2; Abcc4−/− mice. Strikingly, even in WT mice in which all these efflux transporters are present, up to 0.40% of total gimatecan dose was present in the brain at 1 hour and 0.21% at 4 hours. These values are more than 40- and 220-fold higher than those found for topotecan and for SN-38 at 1 hour, respectively, and 160- and 140-fold at 4 hours, respectively. In contrast, the percentage of CPT11 that was converted to SN-38 and detected in brains of Abcb1a/b; Abcg2; Abcc4−/− mice was exceptionally low (0.026% and 0.010% at 1 and 4 hours, respectively) and was even lower in WT mice (0.002% and 0.001% at 1 and 4 hours, respectively). At 1 hour after drug administration, this was only 9% of the total SN-38 and CPT11 level in brain.

Discussion

The present study shows that Abcc4 is an important factor limiting the brain penetration of the camptothecin analogues topotecan, CPT11, SN-38, and gimatecan. However, because of the overlapping affinities of Abcb1a/b and Abcg2 (2 other dominant drug efflux transporters at the BBB), the actual contribution of Abcc4 to the elimination of camptothecins in brain can only be assessed when the 2 other transporters are absent. Together, this cooperative drug efflux system of functionally overlapping transporters constructs a robust protective barrier against molecules entering the brain. Unfortunately, however, this protection may also lead to inadequate exposure of brain malignancies when treated with camptothecin analogues or other triple-substrate drugs. Because of the presence of Abcc4, co-administration of the dual ABCB1 and ABCG2 inhibitor elacridar is unable to enhance the brain penetration of SN-38. In contrast to SN-38, the more lipophilic analogue gimatecan has itself much more favorable brain distribution properties and, from that perspective, may be a more useful candidate for treatment of intracranial tumors than any of the other camptothecin analogues.
Abcc4 is well known for its ability to transport a range of endogenous molecules and drugs including camptothecin analogues (18, 20–24). Abcc4 was first found by reverse transcriptase PCR in the microvessels-enriched fraction of bovine brain (31). Its presence in the apical membranes of mouse brain microvessels and in the basolateral membranes of choroid plexus epithelium was shown by Leggas and colleagues (20) using Abcc4-knockout mice. The absence of Abcc4 in the choroid plexus in Abcc4−/− mice resulted in about 10-fold higher topotecan levels in the cerebrospinal fluid. Similarly, they observed a higher brain concentration of topotecan in Abcc4−/− mice versus Abcc4-proficient WT mice (20). This latter result, however, could not be confirmed when we compared Abcc4−/− and WT mice, even when looking into multiple strain backgrounds, gender, dose levels, and sampling times (8). The lack of an increased brain penetration of topotecan in the Abcc4−/− mice can now be explained by the presence of Abcb1a/b and Abcg2 at the BBB. The relative importance of each of the transporters at the BBB is shown using our Abcb1a/b;Abcg2;Abcc4−/− mice as a reference. In most earlier studies comparing WT and ABC transporter knockout mice, the WT has been taken as reference (e.g., stating that absence results in an x-fold increase of compound y). This makes sense when looking at single knockouts but becomes much more complex when analyzing compound knockouts. Instead, by taking the mouse model in which all of the studied transporters have been deleted as reference, we can now add in one of each ABC transporter at a time. Thus, by comparing Abcb1a/b;Abcg2;Abcc4−/− mice versus Abcb1a/b;Abcg2−/− mice, we can assess the role of Abcc4 and it turns out that Abcc4 alone is sufficient to reduce the SN38 levels (following SN38 administration) to those achieved in WT mice. It also follows that Abcc4 is an important factor for topotecan, gimatecan, and CPT11, albeit in a decreasing order (Table 1). A similar analysis was done for SN-38 and Abcb1a/b by comparing Abcb1a/b;Abcg2;Abcc4−/− and Abcg2−/− mice, showing that Abcb1a/b alone is also sufficient to reduce the brain level of SN-38 to that of WT mice.

Taking the most extensive combination knockout mouse as reference also provides a different view on what is frequently referred to as synergistic interaction between Abcb1a/b and Abcg2 in restricting the brain penetration of substances (32, 33). This claim of synergy is based on the finding that the absence of both Abcb1a/b and Abcg2 together results in a much greater brain accumulation than the absence of only Abcb1a/b or Abcg2, as was first described for topotecan (8). However, synergy implicates that 2 or more factors (e.g., drug transporters) together are more efficient in their action (i.e., reducing brain entry) than each of these factors by themselves. Obviously, this is not the case, as the presence of Abcb1a/b or Abcc4 alone was sufficient to reduce the brain concentration of SN-38 to the level in WT mice. True synergy would have implied that the presence/action of a single transporter would have had only a very minor effect on the brain accumulation, whereas only the combined presence would result in a profound reduction in the brain accumulation. Therefore, it is more appropriate to use the term cooperative drug efflux or concerted efflux, rather than synergy, to describe the interaction of these ABC transporters at the BBB. Following the SN-38 example, this expression of multiple transporters with overlapping affinities for substrates probably not only limits the entry of camptothecins in brain but also serves as a general defense mechanism protecting brain from potentially harmful substances. Inactivation or inhibition of 1 or even 2 transporters may challenge the transport capacity of the remaining transporter(s) but would not jeopardize the cooperative protection that is offered by the combination. This should be kept in mind when trying to modulate ABC transporter-mediated efflux at the BBB for pharmacologic purposes. Moreover, it is also important to realize that besides the drug efflux transporters ABCC4, ABCB1, and ABCG2 examined here, the relevance of other drug transporters (such as ABCC5 and ABCC10) still needs to be addressed.

Camptothecin analogues, in particular CPT11, are frequently applied in clinical trials in patients with glioma (34–37). Our work here calls into question whether CPT11 would be the most appropriate candidate. CPT11 is a produg that needs conversion into the active metabolite SN-38, which is 100- to 1,000-fold more potent. However, the brain accumulation of SN-38 is the lowest of this panel of camptothecin analogues. The amount of SN-38 found in the brain was less than 0.002% of the total dose (after administration of CPT11) and this is only about 9% of the SUM[CPT11+SN-38] in brain. Importantly, mice express the carboxylesterases that are responsible for the CPT11 to SN-38 conversion more abundantly than humans (38, 39). As a result, humans have even lower plasma levels of SN38, which may further diminish its brain accumulation.

On the other hand, gimatecan has a much more favorable brain penetration. A key difference between gimatecan and topotecan and CPT11 is the substitution of a lipophilic chain in position 7 of the planar aromatic 5-ring structure, making this compound more lipophilic and therefore probably more cell membrane permeable (18, 19). Gimatecan has shown significant efficacy in a number of experimental tumor models, including orthotopic brain tumors (40). Moreover, it was shown that the in vitro cytotoxicity to gimatecan was not affected by the overexpression of ABCB1 or ABCG2, although Transwell experiments showed that this compound is a substrate of Abcg2 (17, 18). The negligible transport by ABCB1 in the in vitro Transwell assays is not in line with the significant effects of Abcb1a/b on the brain penetration. This may be due to species differences in substrate affinity, or the ABC transporter knockout model may be a more stringent test than the in vitro assays to establish whether a compound is an ABCB1/Abcb1a/b substrate. Taking Abcb1a/b;Abcg2;Abcc4−/− as reference (Fig. 3), Abcb1a/b alone appears to be capable of reducing the brain levels of gimatecan almost to those found in WT mice, whereas Abcg2 and Abcc4 together were less efficient. Importantly, however, together these 3 ABC transporters cooperatively reduce the brain penetration of gimatecan by about 4-fold (Table 1).
Elacridar is an inhibitor of ABCB1 and ABCG2 and has been successfully used to increase the brain penetration of many substrate drugs, for example, gefitinib and sunitinib (41, 42), and also to some extent topotecan (8). Unfortunately, co-administration of elacridar to WT mice did not improve the brain penetration of SN-38 or gimatecan. The lack of effect on SN-38 brain penetration can be explained by the fact that Abcc4 alone was already sufficient to achieve low brain levels similar to those in WT mice and that elacridar does not inhibit Abcc4. Because of the concerted action by these 3 ABC transporters, it will be a challenging task to increase the brain penetration of SN-38 by modulation of ABC transporter activity at the BBB.

On the other hand, the BBB penetration of gimatecan was much less affected by the ABC transporters, although also in this case, elacridar did not result in any improvement. A possible explanation could be that gimatecan is in fact a good substrate of Abcb1a/b and/or Abcg2, but the high cell membrane permeability of the gimatecan masks this property thus making the partial inhibition of Abcb1a/b and Abcg2 by elacridar unnoticeable.

In conclusion, using a collection of compound ABC transporter knockout mice, we have shown that the cooperative action by Abcc4 with Abcb1a/b and Abcg2 at the BBB restricts the brain penetration of triple-substrate drugs. This work underscores the importance of preclinical models, as such information cannot be obtained from clinical studies in patients. Importantly, patients may benefit from these animal models as they may assist in selecting the most appropriate BBB-penetrable candidates of drugs that have to act inside the brain for clinical trial.

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No potential conflicts of interest were disclosed.

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Abcc4 Together with Abcb1 and Abcg2 Form a Robust Cooperative Drug Efflux System That Restricts the Brain Entry of Camptothecin Analogues

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