P-Glycoprotein and Breast Cancer Resistance Protein: Two Dominant Transporters Working Together in Limiting the Brain Penetration of Topotecan

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Abstract

Purpose: The brain is a pharmacologic sanctuary site, due to the presence of the blood-brain barrier (BBB). Whereas the effect of P-glycoprotein (P-gp) at the BBB is well established, the role of breast cancer resistance protein (BCRP) that is also expressed at the BBB is not.

Experimental Design: We have studied the effect of BCRP by administering topotecan to wild-type (WT), single *Mdr1a/b*$^{(-/-)}$ and *Bcrp1*$^{(-/-)}$, and compound *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ knockout mice. Drug levels in plasma and tissues were determined by high-performance liquid chromatography.

Results: The area under the plasma and tissue concentration-time curve (AUC) of topotecan in brains of *Mdr1a/b*$^{(-/-)}$ and *Bcrp1*$^{(-/-)}$ mice was only 1.5-fold higher compared with WT mice, but in *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice, where both transporters are absent, the AUC increased by 12-fold. The AUC in plasma was ~0.75-, 2.4-, and 3.7-fold higher in *Mdr1a/b*$^{(-/-)}$, *Bcrp1*$^{(-/-)}$, and *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice, respectively, resulting in 2.0-fold ($P < 0.01$), 0.65-fold (P, not significant), and 3.2-fold ($P < 0.01$), respectively, higher brain-to-plasma AUC ratios. Results using *Mrp4*$^{(-/-)}$ mice showed that this transporter had no effect on the brain penetration of topotecan. The P-gp/BCRP inhibitor elacridar fully inhibited P-gp–mediated transport of topotecan, whereas inhibition of Bcrp1-mediated transport by elacridar was minimal.

Conclusions: Our results using *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice clearly show the effect of Bcrp1 at the BBB and also show how two drug transporters act in concert to limit the brain penetration of topotecan. We expect that this finding will also apply to other drugs that are substrates of both P-gp and BCRP. Consequently, to improve the brain penetration of such compounds for targeting intracranial malignancies in patients, it will be essential to use potent inhibitors of both drug transporters.

The blood-brain barrier (BBB) is of pivotal importance for the central nervous system as it strictly regulates the brain internal milieu. However, the BBB not only protects the brain against the influence of harmful toxic substances but also restricts the entry of potentially therapeutic agents. Inadequate drug delivery is generally considered to be a major cause of systemic chemotherapy failure in the treatment of brain malignancies (1). The principal components of the BBB are the brain endothelial cells, which are closely linked together by complex tight junctions that restrict the passage of most compounds through the intercellular space between the endothelial cells. Consequently, entry of most substances into the brain requires transcellular trafficking through the endothelial cells, which is limited, however, by a lack of fenestrae and low endocytic activity. Besides these more or less passive constraints, drug accumulation in the brain is further limited by the expression of membrane-associated drug transporters located at the apical (luminal) side of the endothelial cells that build the BBB. The best-studied example in this regard is the ATP-binding cassette (ABC) transporter P-glycoprotein (P-gp, ABCB1), which was initially discovered in 1976 for its ability to confer multidrug resistance in tumor cells (2). In particular by using P-gp knockout [*Mdr1a*$^{(-/-)}$] mice, it has become clear that this transporter is responsible for the limited brain penetration of a wide range of compounds (3–5). More recently, the ABC half-transporter breast cancer resistance protein (BCRP, ABCG2; ref. 6) has been identified in the BBB (7–9). However, the role of this transporter on the brain penetration of compounds has not yet been so clearly defined. Cisternino et al. (10) showed increased brain uptake of mitoxantrone and prazosin in wild-type (WT) and *Mdr1a*$^{(-/-)}$ mice when given together with the P-gp and BCRP inhibitor elacridar (GF120918) by using *in situ* brain perfusion. Whereas the results with mitoxantrone and elacridar were confirmed by Lee et al. (11), these authors also included Bcrp1 knockout [*Bcrp1*$^{(-/-)}$] mice in their study and

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showed that the brain accumulation of mitoxantrone and dehydroepiandrosterone sulfate (DHEAS) was not higher compared with WT mice. Moreover, as elacridar also increased the brain accumulation in *Bcrp1*$^{(-/-)}$ mice, they concluded that the effect of elacridar was independent from Bcrp1, resulting in a minor role of Bcrp1 in the efflux of these compounds. However, more recently, it was shown that *Bcrp1*$^{(-/-)}$ mice accumulated significantly more of the anticancer agent imatinib than WT mice (12). Thus, whereas it seems that Bcrp1 may limit the brain penetration of xenotoxins, just like P-gp, the overall contribution of this transporter protein still needs to be established.

To study the contribution of Bcrp1 in more detail, we selected topotecan as model drug being a good substrate of Bcrp1 and a weaker substrate of P-gp (13, 14). Topotecan is a semisynthetic, water-soluble derivative of camptothecin that undergoes a reversible pH-dependent hydrolysis of its lactone moiety, yielding a hydroxyl carboxylate form. Topotecan is a potent inhibitor of topoisomerase I (15), a nuclear enzyme involved in DNA replication, repair, and transcription (16). Whereas lactone is most likely the pharmacologically active species, separate analysis of lactone and carboxylate is not critical for pharmacokinetic studies. A clinical pharmacokinetic-pharmacodynamic study showed that total topotecan levels (lactone plus carboxylate) correlated even better than lactone with biological response (17), which may be due to greater robustness of the assay and because the conversion rate is relatively uniform between subjects due to the narrow window of the physiologic pH. Besides its well-established antitumor activity against recurrent small-cell lung and ovarian cancer, topotecan has also shown moderate but potentially useful antitumor activity in patients with brain metastases (18, 19) and in adults with primary malignant glioma (20, 21). Thus, besides that topotecan is an interesting model compound, understanding the importance of BCRP (and P-gp) in the BBB penetration of topotecan may also have direct clinical relevance for treating intracranial malignancies. Although a disruption of the BBB within brain tumors is a known phenomenon, it is still very plausible that the BBB restricts chemotherapy efficacy against intracranial malignancies. For example, in primary brain tumors, such as high-grade gliomas, areas of viable tumor cells that infiltrate (deeply) into the normal brain where the BBB is intact surrounds the central part that harbors the leaky vessels (1). Similarly, the BBB may still be functional in metastatic lesions when they are still small (micrometastases) or when proliferating alongside the existing vasculature (vessel cooptation; ref. 22). Only when metastases grow beyond a size that requires neoangiogenesis for supply of oxygen and nutrient, these new blood vessels may feature disrupted BBB properties (23).

To improve the chemotherapy of multidrug-resistant tumors, a range of P-gp inhibitors has been developed (24, 25). Obviously, such inhibitors can also be used to increase the BBB penetration of anticancer agents to improve the chemotherapeutic treatment of patients with brain tumors. Preclinical studies with concomitant administration of P-gp inhibitors, such as cyclosporin A, valspodar (PSC833; refs. 24, 26, 27), elacridar (GF120918; refs. 28, 29), and zosuquidar (30, 31), have shown significantly increased drug concentrations into brain tissue. However, by using paclitaxel as a model drug, it was shown that elacridar is the most potent inhibitor of P-gp at the BBB (4). Furthermore, as mentioned above, elacridar is also an efficient inhibitor of BCRP (12, 32, 33) and was able to reverse BCRP-mediated resistance to topotecan (34). Elacridar has a good safety profile in humans and coadministration to patients with solid tumors resulted in a significant increase of the oral bioavailability of topotecan from 40% without to 97.1% (35).

The present study was designed to investigate the role of Bcrp1 and P-gp on the brain penetration of topotecan *in vivo*. Experiments were conducted in wild-type (WT), *Mdr1a/b*$^{(-/-)}$ (P-gp knockout), *Bcrp1*$^{(-/-)}$ (Bcrp1 knockout), and *Mdr1a/b*$^{(-/-)}$ *Bcrp1*$^{(-/-)}$ (P-gp and Bcrp1 knockout) mice with or without concomitant administration of elacridar. Our results clearly show that Bcrp1 is an important factor that in concert with P-gp limits the brain penetration of topotecan.

Materials and Methods

Reagents. Topotecan and elacridar (GF120918) were kindly provided by GlaxoSmithKline. All other chemicals were purchased from E. Merck and were used as supplied. Water was purified by the Milli-Q Plus system (Millipore).

Preparation of drug solutions. Topotecan was dissolved in glucose (5%; w/v) to yield a solution of 0.5 mg/mL (active substance). Elacridar (GF120918) was prepared freshly the day before each experiment and suspended at 5 mg/mL in a mixture of hydroxypropyl methylcellulose (0.5 g/L)/1% polysorbate 80 (v/v) with the aid of a Polytron PT1200 homogenizer (Kinematica AG). The suspension was kept protected from light and stirred continuously during administration.

Animals. Animals used in this study were female WT, *Mdr1a/b*$^{(-/-)}$ (*P-gp* knockout; ref. 36), *Bcrp1*$^{(-/-)}$ (*Bcrp1* knockout; ref. 37), or *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ (*P-gp* and *Bcrp1* knockout; ref. 38) mice of a FVB genetic background within the age of 9 to 15 weeks. *Mrp4*$^{(-/-)}$ mice were generated previously (39) and further crossed into FVB genetic background (40). Animals were housed and handled according to institutional guidelines complying with Dutch law. The mice were kept in a temperature-controlled environment with a 12-h light–12-h dark cycle and were given a standard diet (AM-II; Hope Farms B.V.) and acidified water was provided *ad libitum*. The animal ethics committee of our institute approved all experiments involving animals.

Study design and drug administration. This study comprises eight cohorts of animals receiving 5 mg/kg topotecan by i.v. injection in the tail vein, with or without elacridar administered orally by gavage into the stomach at a dose of 100 mg/kg (t = 2 h before topotecan administration) and 50 mg/kg (t = 2 and 6 h after topotecan administration). Each cohort consisted of 25 animals in which five animals were used per time point (t = 1, 4, 8, 12 and 24 h after topotecan administration). At these different time points, mice were anesthetized with methoxyflurane and blood samples were obtained by cardiac puncture and collected in tubes containing potassium EDTA as anticoagulant, which were centrifuged (10 min, 4,000 × g, 4°C) to separate the plasma fraction. The supernatant plasma fractions were transferred into clean vials and stored at -20°C until analysis. Immediately after cardiac puncture, the mice were sacrificed by cervical dislocation and brain, liver, kidney, long, spleen, and heart tissues were dissected. Tissues were put in preweighted polyethylene vials and kept on dry ice. Vials were weighted again to determine the tissue weight and stored at -20°C until further analysis.

Bioanalysis of topotecan. Frozen mouse plasma and tissues were thawed on ice and tissues were thoroughly homogenized (Polytron PT1200) in 4% (w/v) bovine serum albumin (5, 3, and 2 mL for liver, brain, and other tissues, respectively). Total topotecan levels (lactone plus carboxylate form) in plasma and tissues were determined using a validated reversed-phase high performance liquid chromatographic

Table 1. AUC and tissue-to-plasma AUC ratios of topotecan

	Plasma	Brain	Liver
Single agent			
WT	475 ± 49	150 ± 19 (0.32)	4,807 ± 610 (10.1)
Mdr1a/b$^{(-/-)}$	352 ± 17	226 ± 21 (0.64, $P < 0.01$*)	3,884 ± 377 (11.0)
Bcrp1$^{(-/-)}$	1,118 ± 109	239 ± 20 (0.21, NS*)	10,143 ± 513 (9.1)
Mdr1a/b$^{(-/-)}$*Bcrp1*$^{(-/-)}$	1,759 ± 118	1,794 ± 58 (1.02, $P < 0.01$*)	14,573 ± 781 (8.4)
With elacridar			
WT	1,332 ± 93	680 ± 45 (0.51, $P < 0.05$ †)	16,111 ± 1,328 (12.1)
Mdr1a/b$^{(-/-)}$	1,395 ± 40	651 ± 33 (0.47 $P < 0.05$ †)	16,233 ± 699 (11.6)
Bcrp1$^{(-/-)}$	2,226 ± 179	2,209 ± 85 (0.99, $P < 0.01$ †)	22,106 ± 1,823 (9.9)
Mdr1a/b$^{(-/-)}$*Bcrp1*$^{(-/-)}$	2,264 ± 133	2,526 ± 137 (1.12, NS †)	22,152 ± 420 (9.8)

NOTE: Distribution study of topotecan administered i.v. (5 mg/kg) with or without the P-gp/BCRP inhibitor elacridar given orally [100 mg/kg (t = -2 h) and 50 mg/kg (t = 2 h, 6 h)] in WT, *Mdr1a/b*$^{(-/-)}$ (P-gp knockout), *Bcrp1*$^{(-/-)}$ (Bcrp1 knockout), and *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ (compound P-gp and Bcrp1 knockout) mice (n = 25 mice per cohort). AUC_{plasma} (ng/mL × h; mean ± SE) and AUC_{tissue} (ng/g × h). Tissue-to-plasma AUC ratios are presented inside parentheses.
Abbreviation: NS, not significant.
*Tissue-to-plasma AUC ratios of WT versus *Mdr1a/b*$^{(-/-)}$, *Bcrp1*$^{(-/-)}$ and *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice.
†Tissue-to-plasma AUC ratios within same genotype without elacridar versus with elacridar.

method with fluorescence detection (41). In short, 100 μL of biological sample were mixed with 200 μL of methanol for protein precipitation and centrifuged at 20,000 × g for 10 min. Next, 100 μL of the supernatant fraction were mixed with 200 μL of perchloric acid (2% in water). After centrifugation (3 min, 20,000 × g, 4°C), the tubes were transferred to the autosampler vials and 100 μL were injected into the high-performance liquid chromatography system. The lower limit of quantitation was 50 pg/mL in plasma and tissue homogenates.

Pharmacokinetic and statistical analysis. By standard mathematical equations (see formulas below), we have calculated the area under the plasma and tissue concentration-time curve (AUC) using the linear trapezoidal rule and the SE of the AUC (SE_{AUC}) by the law of propagation of error from time point 0 h to the last sampling point where the concentration was above the lower limit of quantitation using the software package Quattro Pro (Corel Corp, 1996; version 6.02).

$$\mathrm{AUC} = \sum_{i=2}^{n} \mathrm{Concentration}_i \cdot \frac{(\Delta\mathrm{time}_{i-1} + \Delta\mathrm{time}_i)}{2}$$

$$\mathrm{SE}_{\mathrm{AUC}} = \sqrt{\left(\sum_{i=2}^{n} \mathrm{SE}_i \cdot \frac{(\Delta\mathrm{time}_{i-1} + \Delta\mathrm{time}_i)^2}{4}\right)}$$

with $\Delta\ \mathrm{time}_n = 0$ and $\mathrm{Concentration}_i$ and SE_i being the mean concentration and SE at each time point, respectively.

Next, the tissue-to-plasma AUC ratios were calculated using the formula

$$\mathrm{Ratio} = \frac{\mathrm{AUC}_{\mathrm{tissue}}}{\mathrm{AUC}_{\mathrm{plasma}}}$$

The SE of the tissue-to-plasma AUC ratios were calculated using the formula

$$\mathrm{SE}_{\mathrm{ratio}} = \frac{\mathrm{AUC}_{\mathrm{tissue}}}{\mathrm{AUC}_{\mathrm{plasma}}} \sqrt{\left(\frac{\mathrm{SE}_{\mathrm{tissue}}}{\mathrm{AUC}_{\mathrm{tissue}}}\right)^2 + \left(\frac{\mathrm{SE}_{\mathrm{plasma}}}{\mathrm{AUC}_{\mathrm{plasma}}}\right)^2}$$

The two-sided unpaired Student's t test was used for statistical analysis, and $P < 0.05$ was regarded as statistically significant.

Protein analysis. Membrane fractions from brain tissue were prepared by homogenizing brain tissues at 0.1 g wet weight/mL in homogenization buffer (1 mol/L Tris, 1 mol/L sucrose, and 0.5 mol/L Na-EDTA), containing a standard cocktail of proteases inhibitors (Complete; Roche) and centrifuged at 5,000 × g for 15 min at 4°C. The supernatant was concentrated by ultracentrifugation (rotor SW40, 100,000 × g, 30 min, 4°C) and the pellet was resuspended in 0.5 mL homogenization buffer and stored at -80°C until analysis. Protein concentrations were determined with the Bradford protein assay (Bio-Rad Laboratories). Proteins were subjected to SDS-PAGE and transferred to nitrocellulose (Bio-Rad Laboratories). The membranes were blocked for 1 h at room temperature in 0.1 mol/L Tris, 0.2% (v/v) Tween 20, 5% (w/v) with 5% milk powder. Incubation with the primary antibody against Bcrp1/Abcg2 (BXP-53, 1:400, a gift from Dr. A.H. Schinkel, Department of Experimental Therapy, Netherlands Cancer Institute, Amsterdam, the Netherlands) was done overnight at 4°C. Bcrp1 was detected by incubation of the blot with horseradish peroxidase–conjugated rabbit anti-rat IgG for 1 h at room temperature (1:1,000, DAKO). The enhanced chemiluminescence signal (Amersham) was detected by film (exposure time 10 min). Equal protein loading was confirmed by Poncheau S staining of the membranes after transfer (data not shown).

Results

Effect of genotype on the brain penetration of topotecan. This study was essentially designed to establish the effect of P-gp and Bcrp1 on the brain penetration of topotecan. Topotecan concentrations were determined in mouse plasma and brain as well as a selection of other tissues (liver, kidney, spleen, lung, and heart) as reference. Plasma and tissues were sampled from WT, *Mdr1a/b*$^{(-/-)}$, *Bcrp1*$^{(-/-)}$, and *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice at five different time points (t = 1, 4, 8, 12, and 24 h), thus covering the most significant part of the concentration versus time curves, and measured by a validated high-performance liquid chromatography analysis (Figs. 1 and 2). By using this approach, we were able to achieve highly statistically significant results for the differences in brain penetration (Table 1) using an acceptable number of animals, despite the complex setup with four different genotypes.

As topotecan is a substrate of P-gp and BCRP, which are both present in the BBB, it was anticipated that these drug transporters might affect the brain penetration of this drug. In

Table 1. AUC and tissue-to-plasma AUC ratios of topotecan (Cont'd)

Kidney	Spleen	Lungs	Heart
16,197 ± 4,495 (34.1)	4,672 ± 214 (9.8)	1,951 ± 400 (4.1)	1,190 ± 107 (2.5)
10,985 ± 4,445 (31.2)	3,427 ± 159 (9.7)	1,277 ± 72 (3.6)	933 ± 66 (2.7)
10,367 ± 467 (9.3)	6,109 ± 365 (5.5)	4,456 ± 443 (4.0)	2,893 ± 209 (2.6)
19,506 ± 3,802 (11.1)	16,263 ± 1,240 (9.3)	6,936 ± 597 (3.9)	4,270 ± 281 (2.4)
17,500 ± 1,348 (13.1)	18,844 ± 1,025 (14.2)	5,698 ± 328 (4.3)	3,789 ± 257 (2.8)
17,234 ± 855 (12.4)	15,025 ± 668 (10.8)	5,101 ± 174 (3.7)	3,323 ± 91 (2.4)
21,768 ± 1,687 (9.7)	20,885 ± 750 (9.4)	8,807 ± 637 (4.0)	4,987 ± 408 (2.2)
23,687 ± 1,601 (10.5)	27,240 ± 2,164 (12.0)	11,204 ± 2,532 (5.0)	5,028 ± 374 (2.2)

line with these expectations, we found that the overall AUC_{brain} in *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice was increased by 12-fold compared with WT mice (Table 1). Interestingly, the AUC_{brain} in *Mdr1a/b*$^{(-/-)}$ and in *Bcrp1*$^{(-/-)}$ mice was only 1.5-fold and 1.6-fold higher, respectively, compared with the AUC_{brain} in WT mice. Based on previous experience with other agents (e.g., paclitaxel ref. 4), we had expected a greater effect of the absence of P-gp on the brain penetration of topotecan. We have, therefore, checked if this rather low brain penetration might be explained by a compensatory higher expression of Bcrp1 in *Mdr1a/b*$^{(-/-)}$ mice; however, results from protein analysis by Western blot did not show a higher expression of Bcrp1 in *Mdr1a/b*$^{(-/-)}$ mice compared with WT mice (Fig. 3).

Because the plasma level of topotecan is a potentially important factor driving the brain penetration, we have calculated the tissue-to-plasma AUC ratios for the brain to correct for the higher plasma levels of topotecan in *Bcrp1*$^{(-/-)}$ and *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice. In *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice that lack both transporters, this ratio was >3-fold higher than in WT mice (Table 1). In *Bcrp1*$^{(-/-)}$ mice where P-gp is present, this ratio is not different from WT mice showing that P-gp alone is sufficient to maintain a low brain penetration of topotecan. In *Mdr1a/b*$^{(-/-)}$ mice (where Bcrp1 is present), the ratio was ~2-fold higher compared with WT mice, but still significantly lower compared with *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice ($P < 0.01$). Thus, the presence of Bcrp1 alone is not sufficient to fully maintain the same brain penetration of topotecan as P-gp does, albeit that this transporter also offers significant protection. The tissue-to-plasma AUC ratios in a range of other tissues that were tested (liver, kidney, spleen, lung, and heart) and which are not protected by a barrier like the BBB show proportional accumulation of topotecan into these organs. The only exception seems to be the kidney, where the tissue-to-plasma AUC ratio in Bcrp1-proficient mice [WT and *Mdr1a/b*$^{(-/-)}$] is higher than in Bcrp1-deficient mice (Table 1).

We have also depicted the brain-to-plasma concentration ratios at the various time points of tissue sampling (Fig. 1B). This picture more or less conforms to the results of the brain-to plasma AUC ratios, where *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice present with the highest ratios. It should be noted, however, that although the differences in the ratios seem to be most pronounced at the later time points, these do not translate into big differences in AUC between the different genotypes (see Table 1), as the concentrations in brain and plasma at the later time points are relatively low.

Effect of elacridar on the brain penetration of topotecan. Concomitant administration of elacridar was done to investigate to what extent this potent P-gp/BCRP inhibitor could increase the brain penetration of topotecan. Furthermore, we wanted to investigate the selectivity and potency of elacridar on both transporters for topotecan. Concomitant administration of elacridar to WT mice increased the AUC_{brain} of topotecan significantly by ~4.5-fold. However, as the AUC_{brain} is ~17-fold higher in *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice, it is clear that the inhibition of P-gp and/or Bcrp1 at the BBB by elacridar is far from complete. Whereas essentially the same AUC_{brain} was achieved in WT and *Mdr1a/b*$^{(-/-)}$ mice, the AUC_{brain} of topotecan in *Bcrp1*$^{(-/-)}$ mice that received elacridar was significantly higher. Taking into account the AUC_{plasma} of topotecan, the brain-to-plasma AUC ratio in *Bcrp1*$^{(-/-)}$ mice (which are P-gp proficient) receiving elacridar was not significantly different from *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice (which are P-gp deficient) receiving topotecan with or without concomitant elacridar, showing that elacridar is sufficiently potent to (almost) fully inhibit P-gp–mediated transport of topotecan at the BBB. In contrast, however, elacridar is a much less potent inhibitor of Bcrp1-mediated transport of topotecan at the BBB because the brain-to-plasma AUC ratio in *Mdr1a/b*$^{(-/-)}$ mice receiving elacridar was not different from WT mice receiving elacridar. The brain-to-plasma AUC ratios in *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice that receive elacridar are not different from *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice receiving topotecan as single agent, suggesting that there are no other elacridar-sensitive transporters for topotecan present at the BBB.

Role of drug transporters in the excretion/elimination of topotecan. It is known that drug transporters can be involved in the excretion of unchanged drug from the body, thus changing the clearance of such a compound. This study was not designed to provide an in-depth analysis of the consequences of P-gp and/or Bcrp1 deficiency or inhibition on the disposition of topotecan, but primarily to establish the plasma AUCs in the various cohorts because this factor is important for the brain accumulation. The AUC_{plasma} in *Mdr1a/b*$^{(-/-)}$ mice was moderately, but significantly ($P = 0.04$), lower than in WT mice in this series. Although this would imply that the clearance (which is inversely related) would be higher in mice lacking P-gp, the overall higher plasma level of topotecan at the later time points suggests that P-gp does play an, albeit very minor, role in the elimination of this drug.

In contrast, the AUC_{plasma} was much higher in Bcrp1-deficient mice. *Bcrp1*$^{(-/-)}$ mice showed higher plasma concentrations than

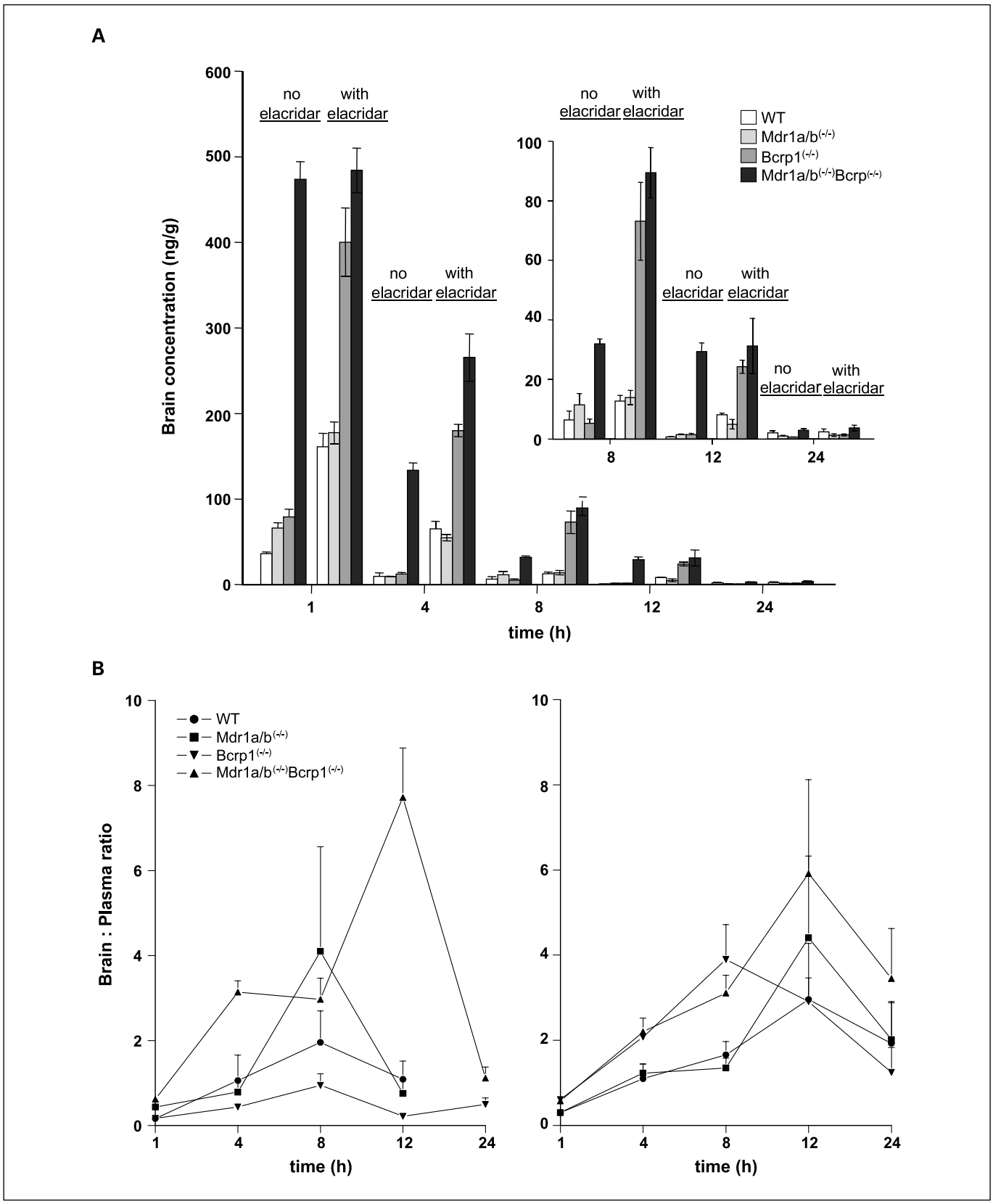

Fig. 1. *A,* brain concentrations of topotecan in WT, *Mdr1a/b*$^{(-/-)}$ (*P-gp* knockout), *Bcrp1*$^{(-/-)}$ (*Bcrp1* knockout), and *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ (compound *P-gp* and *Bcrp1* knockout) mice at t = 1, 4, 8, 12, and 24 h after dosing. All mice received topotecan (5 mg/kg) i.v. with or without concomitant administration of elacridar given orally [100 mg/kg (t = -2 h) and 50 mg/kg (t = 2 and 6 h)]. Columns, mean brain concentration in ng/g (n = 5 mice per time point); bars, SE. *B,* brain versus plasma concentration ratios in mice receiving topotecan (single agent; *left*) and in combination with elacridar (*right*).

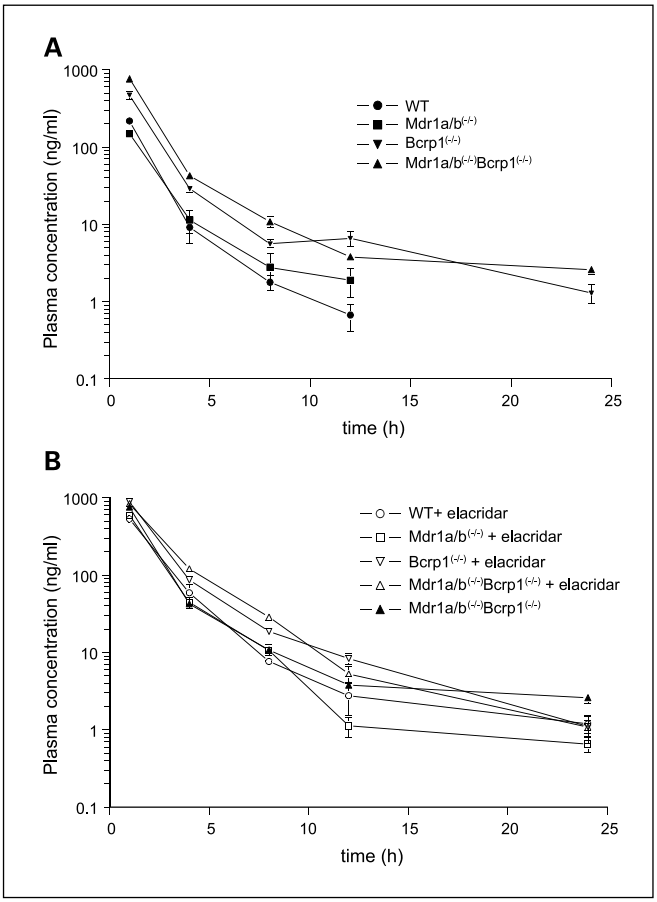

Fig. 2. *A,* plasma concentration-time profile of topotecan in WT, *Mdr1a/b*$^{(-/-)}$, *Bcrp1*$^{(-/-)}$, and *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice receiving topotecan as single agent. *B,* plasma concentration-time profile of topotecan in WT, *Mdr1a/b*$^{(-/-)}$, *Bcrp1*$^{(-/-)}$, and *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice receiving topotecan in combination with elacridar. As a reference, the concentration-time profile in *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice receiving topotecan alone has been included in this graph.

WT and *Mdr1a/b*$^{(-/-)}$ mice at all time points. When P-gp was also absent, the AUC_{plasma} increased further. Overall, the AUC_{plasma} in *Bcrp1*$^{(-/-)}$ and in *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice were 2.5- and 3.1-fold higher, compared with WT mice (Fig. 2A; Table 1). These results show that, in particular, Bcrp1 is an important factor in the excretion of topotecan from the body.

When elacridar was coadministered, the AUC_{plasma} in WT and *Mdr1a/b*$^{(-/-)}$ mice increased to values of ~60% of those in *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice, whereas the AUC_{plasma} in *Bcrp1*$^{(-/-)}$ mice was comparable with *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice (Fig. 2B). This result suggests that P-gp–mediated excretion was fully inhibited by elacridar, whereas Bcrp1 was not. Intriguingly, concomitant administration of elacridar increased the AUC_{plasma} in *Mdr1a/b*$^{(-/-)}$Bcrp1$^{(-/-)}$ mice significantly by another 30%, indicating that elacridar also inhibits other pathways involved in the elimination of topotecan.

The role of Mrp4 in the brain penetration of topotecan. Because it was recently reported that Mrp4 would be involved in the brain penetration of topotecan (39), we have also studied the brain accumulation of topotecan using *Mrp4*$^{(-/-)}$ mice of 99% pure (eight back-crosses) FVB background strain receiving 5 mg/kg of topotecan. In contrast to the previous claims, we could not confirm that *Mrp4*$^{(-/-)}$ mice accumulate more topotecan in the brain (Fig. 4). Because our conditions differed from those in the previous article (39), we have repeated this experiment by pair-wise analyses of cohorts of *Mrp4*$^{(-/-)}$ versus WT mice using a dose level of 2 mg/kg instead of 5 mg/kg, a sampling time point of 6 h after drug administration and by comparing gender and strain background influences. However, also under all these different conditions, we were unable to show a higher penetration of topotecan in *Mrp4*$^{(-/-)}$ mice (Fig. 4).

Discussion

This study clearly shows, for the first time, how the two transporter proteins P-gp and Bcrp1 in concert limit the brain penetration of a substrate agent (topotecan). Only by using compound P-gp and Bcrp1 knockout [*Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$] mice as a reference were we able to show that the presence of P-gp (in single Bcrp1 knockout mice) or Bcrp1 (in single P-gp knockout mice) could fully or partly return the brain-to-plasma AUC ratios to that in WT mice. Coadministration of elacridar, a dual P-gp/BCRP inhibitor in WT mice, resulted in improved topotecan penetration into brain tissue. However, based on results in the single knockout mice, inhibition of P-gp seems to be (almost) complete, whereas inhibition of Bcrp1 was minimal. These results highlight the great value of *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice in assessing the effect of these prominent transporters at the BBB.

Although the presence of BCRP in the BBB has been well established (7–9), the importance of this drug transporter in limiting the brain accumulation has not yet been clearly shown. In part, this may be due to the fact that many of the BCRP

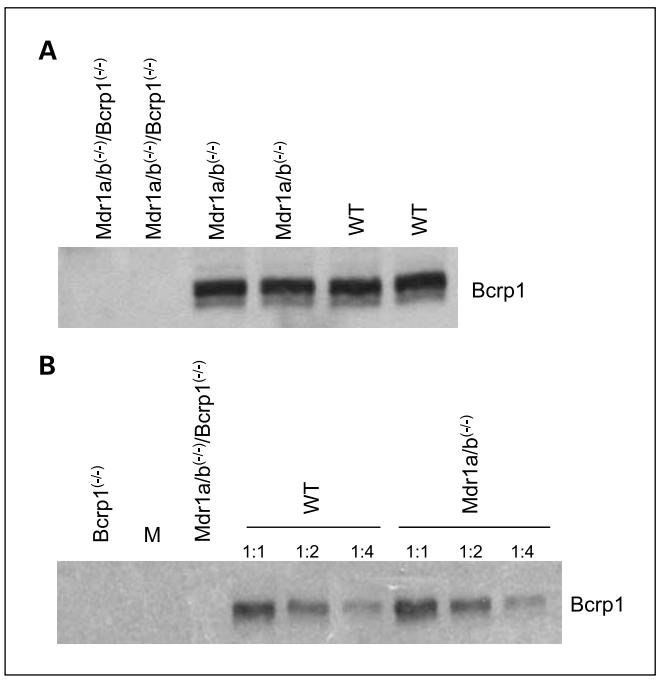

Fig. 3. Expression of Bcrp1 in the brain homogenates of WT, *Mdr1a/b*$^{(-/-)}$ (P-gp knockout), and *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ (P-gp and Bcrp1 knockout) mice using samples from two animals per genotype (*A*) and of serial dilutions (undiluted, 1:2, and 1:4) of samples from one other animal per genotype (*B*). Results did not indicate differences in expression of Bcrp1 between *Mdr1a/b*$^{(-/-)}$ versus WT mice.

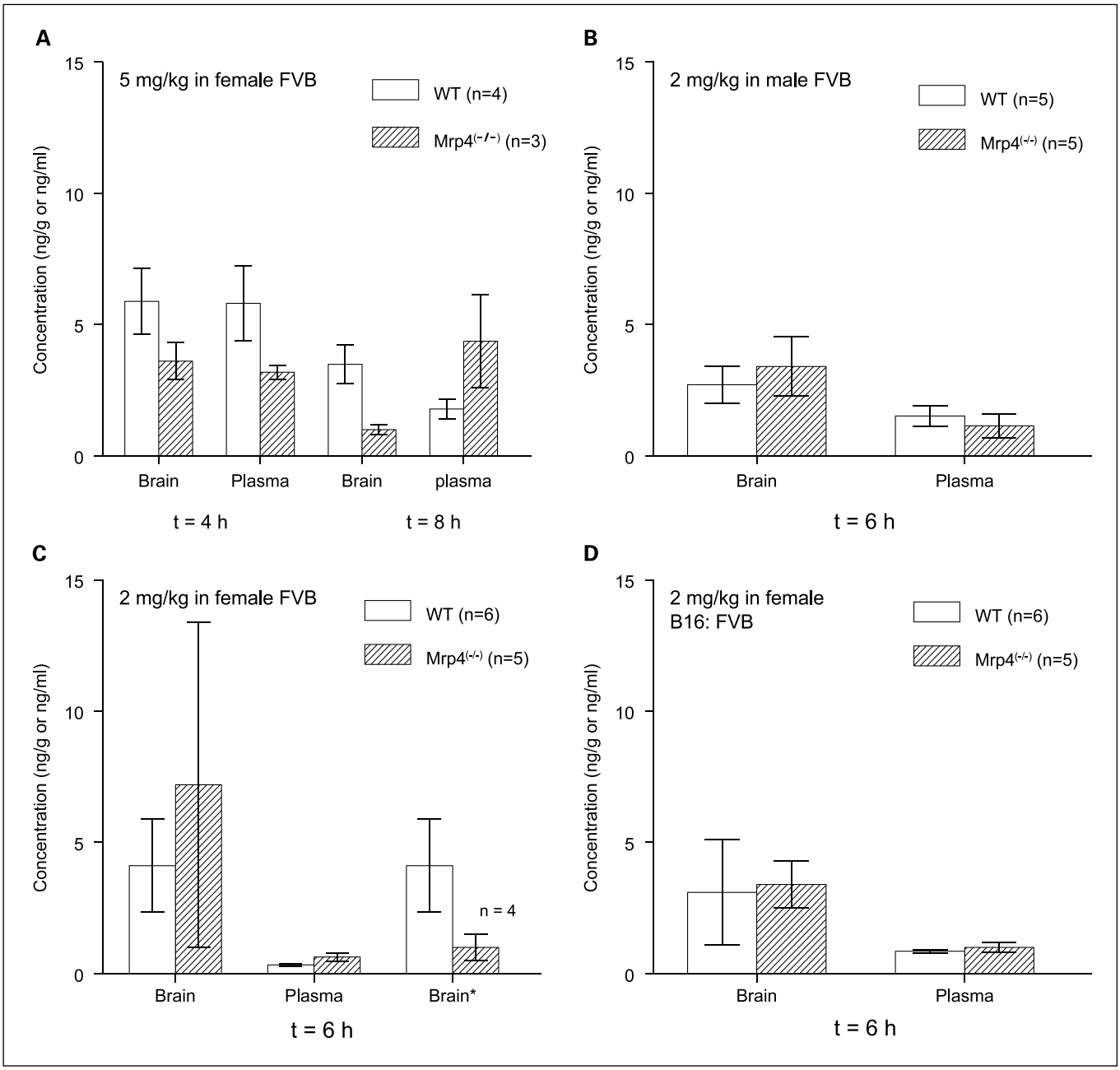

Fig. 4. Concentration of topotecan in brain and plasma from *Mrp4*$^{(-/-)}$ mice versus WT controls. First, topotecan was administered at 5 mg/kg to female *Mrp4*$^{(-/-)}$ mice of FVB background. Columns, mean values in brain and corresponding plasma samples of animals (n = group size) killed at 4 and 8 h (*A*); bars, SE. In subsequent experiments, topotecan was dosed at 2 mg/kg to female (*B*) or male (*C*) FVB mice and to female mice of B16:FVB mixed background (*D*) and brain and tissue samples were harvested at 6 h (*B* – *D*). The large error bar in *Mrp4*$^{(-/-)}$ mice in *C* was caused by one high value, which was omitted in the bar designated by Brain*.

substrates are also a substrate of P-gp, which may cause P-gp to conceal the effect of BCRP. The prominent role of P-gp in the BBB has clearly been shown by using P-gp knockout mice (3). By now, many articles using these P-gp knockout mice have been published and have shown that all substrates, even relatively weak ones, are efficiently extruded from the brain. Thus far, the more recently described *Bcrp1*$^{(-/-)}$ mice (37) have not yet been so widely used for studying the brain penetration of compounds. Lee et al. (11) concluded that Bcrp1 plays only a minor role based on their results with [^{3}H]DHEAS and [^{3}H]mitoxantrone using *Bcrp1*$^{(-/-)}$ mice. However, at the same time, they showed, by using *Mdr1ab*$^{(-/-)}$ mice, that P-gp also limits the brain uptake of these compounds, which may thus also explain why DHEAS and mitoxantrone levels were not higher in *Bcrp*$^{(-/-)}$ mice and increased significantly by concomitant elacridar. On the other hand, Breedveld et al. (12) have found 2.5-fold higher [^{14}C]imatinib levels in the brains of *Bcrp1*$^{(-/-)}$ mice, which, based on these current results, may come somewhat as a surprise because imatinib is also an excellent P-gp substrate (31). By using *Mdr1ab*$^{(-/-)}$ mice (Bcrp1 proficient)

versus *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice (Bcrp1 deficient), we are now able to show unambiguously the effect of Bcrp1 on the brain penetration of topotecan and we expect that this observation with topotecan will be broadly applicable to other BCRP substrate drugs that are also a substrate of P-gp. Importantly, however, this could only be shown in a P-gp–deficient background as single *Bcrp*$^{(-/-)}$ mice did not show a higher brain penetration of topotecan because of the action of P-gp. There may even be a compensatory higher expression of P-gp in *Bcrp*$^{(-/-)}$ mice, as there seems to be a trend to a lower brain-to-plasma AUC ratio in *Bcrp1*$^{(-/-)}$ versus WT mice.

Obviously, the effect of BCRP may differ from compound to compound as was previously found for P-gp, and thus the effects may be greater or lesser for any particular drug. For example, the differences in brain penetration between WT and *Mdr1a*$^{(-/-)}$ knockout mice of ivermectin, vinblastine, and paclitaxel (3–5) was much larger (10- to 50-fold) than for doxorubicin (2- to 3-fold; ref. 42), which may have been due to differences in affinity for P-gp, but also by differences in their affinity for brain tissue itself (tissue binding) and/or the involvement and affinity of other transporters. This latter reason seems to be important in case of topotecan, where we had also expected much higher topotecan levels in *Mdr1a/b*$^{(-/-)}$ mice. In particular, the observation that P-gp seems to have weak to moderate affinity for topotecan (13, 43), whereas the affinity of BCRP for topotecan is quite high (14), could explain the fairly limited increase of topotecan in the brain in *Mdr1a/b*$^{(-/-)}$ mice. Moreover, Cisternino et al. (10) reported that the mRNA levels of Bcrp1 in brain microvessels of *Mdr1a*$^{(-/-)}$ mice were 3-fold up-regulated in the BBB relative to WT mice and such a compensatory mechanism may also have a further effect on the drug accumulation in the brain. Based on our Western blot data, we were not able to confirm an overall higher expression of Bcrp1 protein in brain homogenates in our mice, suggesting that normal levels of Bcrp1 are already sufficient to limit the BBB penetration of topotecan. However, because we did not enrich our samples for brain microvessels, it is possible that a moderately increased expression of Bcrp1 in this compartment did occur.

Within this study, we have investigated the potency of elacridar to inhibit P-gp– and Bcrp1-mediated transport of topotecan *in vivo*. Obviously, this is of importance when using such inhibitors for improving the BBB penetration of chemotherapeutics for the treatment of intracranial tumors. By using single *Mdr1a/b*$^{(-/-)}$ and *Bcrp1*$^{(-/-)}$ mice versus *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice, we were able to show that elacridar could fully inhibit P-gp–mediated efflux of topotecan at the BBB but Bcrp1-mediated efflux of topotecan only partially. This incomplete inhibition of Bcrp1-mediated transport of topotecan by elacridar was not due to inadequate dose levels as we previously showed that the current dose-regimen of elacridar will result in plasma levels between 500 and 1,000 ng/mL for the 24 h period, which is in the higher range of what can be achieved in humans (44). Based on previous studies, elacridar seems to be ~10-fold more potent in inhibiting P-gp than BCRP. Hyafil et al. (29) reported that 0.05 to 0.10 μmol/L of elacridar was sufficient to fully reverse P-gp–mediated multidrug resistance in several drug-resistant cell lines exposed to vincristine or doxorubicin, whereas half-maximal inhibition (IC_{50}) was ~0.02 μmol/L. Similarly, Traunecker et al. (45) using Mes-Dx5 cells reported IC_{50} values between 0.007 and 0.091 μmol/L of elacridar, depending on the cytotoxic drug that was used. In contrast, a recent study from Boumendjel et al. (46) reported that the IC_{50} of elacridar for inhibition of mitoxantrone efflux from ABCG-2–transfected HEK-293 cells was 0.41 μmol/L. Thus, the higher potency of elacridar for inhibiting P-gp together with the fact that topotecan is a better substrate for Bcrp1 than P-gp provides a reasonable explanation why elacridar was hardly able to inhibit Bcrp1-mediated efflux of topotecan, whereas inhibition of P-gp–mediated efflux was virtually complete. As expected, elacridar also reduced the plasma clearance of topotecan, and also in this case full inhibition of Bcrp1-mediated transport of topotecan seems to be more difficult to achieve than inhibition of P-gp–mediated transport. Moreover, the further reduction in plasma clearance of topotecan in *Mdr1a/b*$^{(-/-)}$*Bcrp1*$^{(-/-)}$ mice when given in combination with elacridar suggests that this was also due to inhibition of other elimination routes besides Bcrp1- and P-gp–mediated excretion. Due to the higher plasma levels by elacridar, the gain in brain penetration of topotecan (tissue-to-plasma AUC ratio) in WT mice is only ~1.6-fold relative to topotecan given as single agent instead of the ~3-fold gain that might be obtained by full inhibition of both Bcrp1- and P-gp–mediated efflux at the BBB. This finding may limit the usefulness of combining topotecan with elacridar for the purpose of better treatment of intracranial malignancies.

Just recently, results on the brain penetration of topotecan when given with or without gefitinib also showed an ~1.6-fold higher brain extracellular fluid AUC versus plasma AUC ratio (47). Although this effect was considered to be due to inhibition of Bcrp1, it may well be that most of this gain is due to inhibition of P-gp as gefitinib is also an inhibitor of P-gp (48). Our results suggest that inhibition of P-gp–mediated efflux of topotecan at the BBB is more easily achieved than inhibition of Bcrp1-mediated efflux. Moreover, gefitinib may also elicit effects independent from P-gp and Bcrp1. By using our set of knockout mice, these possible interactions can easily be distinguished.

By using *Mrp4*$^{(-/-)}$ mice, it was previously reported that Mrp4 would be implicated in the BBB penetration of topotecan (39). However, taking into account our findings demonstrating that the presence of P-gp and Bcrp1 resulted in efficient exclusion of topotecan from the brain, this claim was in marked contrast to our results because both transporters are still present in *Mrp4*$^{(-/-)}$ mice. To clarify this discrepancy, we did additional experiments in *Mrp4*$^{(-/-)}$ mice using both the conditions (dose level, sampling times) of our study as well as those more similar as in the previous study. Clearly, our data in *Mrp4*$^{(-/-)}$ mice does not support the claim that this transporter has a significant effect on the penetration of topotecan into brain tissue, at least not when P-gp and/or Bcrp1 are present.

Besides their role in the BBB penetration, P-gp and BCRP are also involved in the excretion/elimination of many drugs. Although this study was not set up to provide a comprehensive analysis of the effect of these drug transporters on the disposition of topotecan, our results show that Bcrp1 has a much greater effect than P-gp on the plasma clearance of topotecan in mice. Both in mice and humans, a substantial fraction of the topotecan dose is excreted via the kidneys into the urine (49, 50). Bcrp1 is highly expressed in the kidney of mice (51, 52) and the markedly reduced plasma clearance of

topotecan may therefore be explained by a reduced renal excretion in the absence of Bcrp1. However, the lower tissue-to-plasma AUC ratio in kidney of Bcrp1-deficient mice relative to Bcrp1-proficient mice suggests that the absence of Bcrp1 has more effect on the influx of topotecan from the blood into the kidney than on the efflux from the kidney into the urine. In humans, the expression of Bcrp1 relative to other tissues in the body is much lower than in mouse kidney (6, 53) and, therefore, other drug transporters may be more important for the renal excretion in humans. Obviously, more research is warranted to better understand the role of BCRP on the plasma clearance of topotecan and the potential of drug interactions with BCRP inhibitors.

In conclusion, by using the present study setup, we were able to provide a definitive proof of the concept that Bcrp1 and P-gp are two dominant transporters at the BBB that work in concert in limiting brain penetration topotecan and we expect that this observation will also apply for other dual-substrate drugs. Unfortunately, inhibition of Bcrp1-mediated efflux of topotecan at the BBB by elacridar to improve the brain penetration of this drug was only minimal under the current conditions and this may limit the clinical usefulness of this combination for targeting brain malignancies. Consequently, more potent BCRP inhibitors are required. Our study underlines that BCRP is also an important component of the BBB that may act as a major hurdle in the delivery of therapeutic drugs into the brain for instance when targeting malignancies in the central nervous system.

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P-Glycoprotein and Breast Cancer Resistance Protein: Two Dominant Transporters Working Together in Limiting the Brain Penetration of Topotecan

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