Tariquidar and Elacridar Are Dose-Dependently Transported by P-Glycoprotein and Bcrp at the Blood-Brain Barrier: A Small-Animal Positron Emission Tomography and In Vitro Study

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ABSTRACT

Elacridar (ELC) and tariquidar (TQD) are generally thought to be nontransported inhibitors of P-glycoprotein (Pgp) and breast cancer resistance protein (BCRP), but recent data indicate that they may also be substrates of these multidrug transporters (MDTs). The present study was designed to investigate potential transport of ELC and TQD by MDTs at the blood-brain barrier at tracer doses as used in positron emission tomography (PET) studies. We performed PET scans with carbon-11-labeled ELC and TQD before and after MDT inhibition in wild-type and transporter-knockout mice as well as in in vitro transport assays in MDT-overexpressing cells. Brain entrance of \( ^{[11C]} \)ELC and \( ^{[11C]} \)TQD administered in nanomolar tracer doses was found to be limited by Pgp- and Bcrp1-mediated efflux at the mouse blood-brain barrier. At higher, MDT-inhibitory doses, i.e., 15 mg/kg for TQD and 5 mg/kg for ELC, brain activity uptake of \( ^{[11C]} \)ELC at 25 minutes after tracer injection was 5.8 ± 0.3, 2.1 ± 0.2, and 7.5 ± 1.0-fold higher in wild-type, \( ^{[11C]} \)Mdr1a/b(+/−) and \( ^{[11C]} \)Bcrp1(+/−) mice, respectively, but remained unchanged in \( ^{[11C]} \)Mdr1a/b(−/−) and \( ^{[11C]} \)Bcrp1(−/−) mice. Activity uptake of \( ^{[11C]} \)TQD was 2.8 ± 0.2 and 6.8 ± 0.4-fold higher in wild-type and \( ^{[11C]} \)Bcrp1(−/−) mice, but remained unchanged in \( ^{[11C]} \)Mdr1a/b(−/−) and \( ^{[11C]} \)Mdr1a/b(−/−)Bcrp1(−/−) mice. Consistent with the in vivo findings, in vitro uptake assays in Pgp- and Bcrp1-overexpressing cell lines confirmed low intracellular accumulation of ELC and TQD at nanomolar concentrations and increased uptake at micromolar concentrations. As this study shows that microdoses can behave pharmacoKinetically differently from MDT-inhibitory doses if a compound interacts with MDTs, conclusions from microdose studies should be drawn carefully.

Introduction

Multidrug transporters (MDTs) of the ATP binding cassette family, like P-glycoprotein (Pgp; MDR1, ABCB1), and breast cancer resistance protein (BCRP; ABCG2), are expressed in endothelial cells of the blood-brain barrier (BBB), limiting the entry of lipophilic xenobiotics, e.g., substrate drugs, into the brain by pumping them back into the blood. In addition to their extensively demonstrated function in drug-resistant cancer (Robey et al., 2010), regionally overexpressed MDTs at the BBB probably play a crucial role in mediating therapy refractoriness in neurologic diseases like epilepsy or depression by prohibiting the accumulation of therapeutically effective drug concentrations (Löscher and Potschka, 2005).

Positron emission tomography (PET) with tracers binding at or being transported by MDTs may be used for visualizing altered efflux transporter activity in vivo (Löscher and Langer, 2010; Mairinger et al., 2011). Additionally, PET has been proposed as a suitable method to perform clinical microdose studies to assess tissue distribution of radiolabeled drug candidates in humans, as only low nanomolar tracer concentrations are needed to perform PET scans (Wagner and Langer, 2011). Established Pgp-substrate tracers like \( [R]-[14C] \)verapamil or \( [14C] \)-N-desmethyl-loperamide are very efficiently transported by Pgp and consequently have very limited brain access (Liow et al., 2009; Kuntner et al., 2010). Therefore, to visualize regionally overexpressed Pgp in disease states, it is necessary to...
coadminister Pgp inhibitors like tariquidar (TQD) at nonmaximal inhibiting doses (Bankstahl et al., 2011). As an alternative strategy in several recent studies, we described the synthesis and in vivo pharmacokinetic properties of the radiolabeled MDT inhibitors \[ ^{1}C \] TQD and \[ ^{1}C \] ELC (elacridar) (Dörner et al., 2009; Bauer et al., 2010; Dörner et al., 2011; Wanek et al., 2012a, b).

TQD and ELC were long thought to be highly selective third-generation inhibitors of Pgp, but subsequent studies showed that both drugs also inhibit BCRP (Robey et al., 2004; Pick et al., 2008; Kühne et al., 2009). According to literature, both inhibitors are nontransported modulators of Pgp and BCRP, interacting with the transporters at other binding sites than substrates do (Hyafil et al., 1993; Martin et al., 1999), although a recent in vitro study indicated that, at low concentrations, TQD acts also as a substrate of BCRP (Kannan et al., 2011). Furthermore, with respect to the high lipophilicity of TQD (clog \( P = 6.1 \)) and ELC (clog \( P = 5.6 \)) (Egger et al., 2007; Padowski and Pollack, 2010), one would expect them to easily penetrate the BBB. Unexpectedly, our PET studies showed very low brain uptake of both tracers in wild-type mice, but uptake increased several times in Pgp/BCRP-deficient mice (Dörner et al., 2009; Bauer et al., 2010), indicating a possible substrate-like behavior. In parallel to our studies, Kawamura et al. (2010, 2011) reported similar findings. Nevertheless, until now it was still unclear if ELC and TQD are indeed MDT substrates in vivo.

To further investigate whether TQD and ELC act as transporter substrates, in the present study we performed paired PET scans with \[ ^{1}C \] TQD and \[ ^{1}C \] ELC in different types of transporter-knockout mice before and after transporter modulation with MDT-inhibitory doses of cold TQD or ELC, respectively. Additionally, in vitro transport assays using cells expressing murine or human Pgp or murine Bcrp1 were carried out.

This study illustrates that microdoses of ELC or TQD used in PET studies behave pharmacokinetically differently from MDT-inhibitory doses and that both tracers are indeed transported by Pgp and Bcrp1 in nanomolar doses.

### Materials and Methods

**Animals.** Female FVB/N (Friend Leukemia Virus, strain B; wild-type), \( Mdr1a/b^{-/-} \), \( Bcrp1^{-/-} \), and \( Mdr1a/b^{-/-} \), \( Bcrp1^{-/-} \) mice were obtained from Taconic Inc. (Germantown, NY). Mice were purchased and scanned at 6-8 weeks of age at a weight of 21.3 \( \pm \) 1.8 g and housed in groups of up to five individuals under controlled environmental conditions (22 \( \pm \) 1°C, 40-70% humidity) with a 12-hour light-dark cycle (lights on at 6:00) and ad libitum access to food and water. Before PET scanning, an adaptation period of at least 1 week was accorded to each animal. The study was authorized by the institutional animal care and use committees, and all study procedures were performed in accordance with the European Communities Council Directive of November 24, 1986 (86/609/EEC). Every effort was made to minimize both the suffering and the number of animals used in this study.

**Chemicals and Drugs.** If not stated otherwise, chemicals were purchased from Sigma-Aldrich (Schnelldorf, Germany) or Merck (Darmstadt, Germany) and were of analytical grade and used without further purification. TQD dimeylene was provided by Xenova (Slough, UK) and ELC hydrochloride by GlaxoSmithKline (Research Triangle Park, NC). TQD and ELC were freshly dissolved prior to each administration in 2.5% (w/v) aqueous dextrose solution or in 20% aqueous ethanol solution, respectively. Both inhibitors were injected at a volume of 4 ml/kg. \( K_{o}143 \) (3-(35S,6S,12A)-6-hydrobuthyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12a-octahydro-pyrazinol[1,2,3,1\'\,6\'j]pyridin[3,4-b]indol-3-y])-propionic acid tert-butyl ester was purchased from Axon Medchem BV (Groningen, The Netherlands) and PSC833 (6-(4\( \times \))2,4,6,8)-4-methyl-2-(methylamino)-3-oxo-6-octenoic acid)-7-L-valine-cyclosporin) was provided by Novartis (Basel, Switzerland). \[ ^{3}H \] TQD (specific activity, 2.6-3.2 GBq/\( m \)) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). For the in vitro assays ELC, TQD (1 \( \mu \)M final concentration), and \( K_{o}143 \) were dissolved in dimethylsulfoxide (DMSO (dimethylsulfoxide); <0.1% DMSO in final solution). PSC833 was dissolved in ethanol/Tween 80 (9:1; v/v; <0.1% ethanol/tween in final solution).

**Tracer Synthesis and Formulation.** \[ ^{1}C \] TQD and \[ ^{1}C \] ELC were synthesized as described previously (Dörner et al., 2009; Bauer et al., 2010). For i.v. injection, each radiotracer was formulated in a mixture of 0.9% aqueous saline/ethanol/polyethylene glycol 300 (50:15.3:5, v/v/v) to an approximate concentration of 370 MBq/ml. Radiochemical purity, as determined by radio high-performance liquid chromatography (HPLC), was greater than 98%, and specific activity at the end of synthesis was \( >100 \) GBq/\( m \).

**PET Experimental Procedure.** Isoflurane anesthesia (1-2% in oxygen) was induced and maintained during the whole experimental procedure. Mice were placed on a dual animal bed that was kept at 38°C to prevent hypothermia. A catheter, placed into a lateral tail vein, was used for i.v. administration of radiotracer and unlabeled inhibitor. At the beginning of tracer administration, dynamic PET imaging was initiated using a \( \mu \)PET Focus220 scanner (Siemens Medical Solutions, Knoxville, TN). Before the first PET scan, a transmission scan using a \( ^{57} \)Co point source was recorded over 10 minutes.

Four groups of mice (wild-type, \( Mdr1a/b^{-/-} \), \( Bcrp1^{-/-} \), and \( Mdr1a/b^{-/-} \), \( Bcrp1^{-/-} \), \( n = 5 \) each) underwent paired PET scans with either \[ ^{1}C \] TQD (31.0 \( \pm \) 8.3 MBq in a volume of 0.1 ml corresponding to 0.31 nmol of unlabeled TQD) or \[ ^{1}C \] ELC (35.5 \( \pm \) 7.5 MBq in a volume of 0.1 ml corresponding to 0.36 nmol of unlabeled ELC) before, during, and after administration of unlabeled inhibitor (TQD, 15 mg/kg; ELC, 5 mg/kg; equivalent to 17.9 and 8.3 \( \mu \))mol/kg, respectively; Fig. 1). For the high ELC and TQD doses we have shown complete inhibition of Pgp in an earlier study in rats (Kunnert et al., 2010), whereas no in vivo data are available for inhibition of Bcrp1. At low nanomolar tracer doses both inhibitors presumably do not inhibit Pgp or Bcrp1 function (see Table 1).

One hour after starting scan 1, unlabeled inhibitor was administered over a time period of about 60 seconds, followed by 90 minutes of PET data acquisition. Scan 2 was started at two hours after administration of unlabeled inhibitor (Fig. 1). An additional group of each mouse type (\( n = 3 \)) with or without preinjection of cold inhibitor underwent blood sampling using retro-orbital puncture at 25 minutes after tracer injection. Blood samples were weighed and measured for radioactivity in a gamma counter (PerkinElmer, Wellesley, MA). Blood radioactivity data were corrected for radioactive decay and expressed in units of percent injected dose per ml blood (%ID/ml).

**PET Data Analysis.** PET images were reconstructed by Fourier re-binning followed by two-dimensional filtered back projection with a ramp filter. A standard data correction protocol (normalization, attenuation, and decay correction) was applied. A calibration factor for converting units of PET images into absolute radioactivity concentration units was generated by imaging phantoms filled with a known concentration of \[ ^{1}C \] TQD or \[ ^{1}C \] ELC. Whole brain was manually outlined on PET images and time-activity curves in whole brain in units of percent injected dose per gram tissue (%ID/g) were calculated. Brain-to-blood ratios of activity were calculated by dividing activity concentrations measured with PET at 25 minutes after radiotracer injection by mean blood activity concentrations measured with a gamma counter in separate groups of mice.

**Synthesis of \[ ^{3}H \] ELC.** \[ ^{3}H \] Methyl-nosylate (74 MBq in 40 \( \mu \)l of toluene; PerkinElmer, Groningen, The Netherlands) was added to a reaction vial and the solvent was evaporated at 80°C under argon flow. Next, O-desmethyl-ELC (0.9 mg, 1.6 \( \mu \))mol (Dörner et al., 2009) and tetrabutylammonium hydroxide (1 \( \mu \)l of a 60% solution in water) in 100 \( \mu \)l of DMSO was dissolved and reacted for 60 minutes at 80°C. The reaction mixture was then cooled to 20°C and subjected to high-performance liquid chromatography (HPLC), was greater than 98%, and specific activity at the end of synthesis was \( >100 \) GBq/\( m \).

**Fig. 1.** PET study set-up. After 60 minutes of PET scan 1 (total duration: 150 minutes) with \[ ^{1}C \] ELC or \[ ^{1}C \] TQD, unlabeled ELC (5 mg/kg) or TQD (15 mg/kg) were intravenously administered to mice. Two hours later, \[ ^{1}C \] ELC or \[ ^{1}C \] TQD was injected again, and PET scan 2 (duration: 60 minutes) was acquired.
II-Bcrp cell line showed a distinctly decreased Bcrp expression already in the evaluation of human Bcrp, as the available Madin-Darby canine kidney (MDCK)-human Pgp-overexpressing cells in the in vitro assays. However, we could not overcome this counter (Wallac, Turku, Finland) at a counting efficiency of 40%. [3H]ELC (5.5 MBq, 7.4% radiochemical yield) was obtained with a purity of 98.7% and a specific activity of 2.7 GBq/μmol.

**Cell Lines.** To increase the translational value of our findings, we included human Pgp-overexpressing cells in the in vitro assays. However, we could not evaluate human Bcrp, as the available Madin-Darby canine kidney (MDCK)-II-Bcrp cell line showed a distinctly decreased Bcrp expression already in early passages (3–4) and a high expression of endogenous Pgp also (data not shown). Porcine kidney epithelial cells transfected with human MDRI or murine Mdr1a as well as MDCK-II cells transfected with murine Bcrp1 and respective wild-type cells were used for cell culture studies. All cell lines were kindly provided by P. Borst and A. Schinkel (The Netherlands Cancer Institute, Darmstadt, Germany) with a density of 0.4 × 10^6 cells/cm². In the transport experiments, where experiments were discarded in which TEER values were decreased by more than 15% compared with initial measurements. As another indicator of integrity of the monolayer, a [14C]-mannitol control was applied as described earlier (Luna-Tortós et al., 2008). Only monolayers with an apparent permeability of 10⁻⁶ cm/s were used. For 1 hour preincubation, culture medium was replaced with serum-free Opti-MEM, followed by 1 nM [3H]ELC in fresh Opti-MEM in the apical and basolateral chambers of the monolayer. Samples were taken from both compartments after 30, 60, 120, and 240 minutes.

**Statistical Analysis.** Data were statistically analyzed by one-way analysis of variance, followed by Bonferroni test for selected pairs of columns using Prism v.5 software (GraphPad Inc., La Jolla, CA). Tests were used two-tailed and a P < 0.05 was considered statistically significant. If not stated otherwise, all values are given as mean ± S.E.M.

**Results**

**Small-Animal PET.** For [11C]ELC, brain activity uptake in PET scan 1 was very low in wild-type mice at 25 minutes after tracer injection (0.84 ± 0.04%ID/g; Fig. 2, A and C). In Bcrp [11C]ELC mice, brain activity (1.06 ± 0.05%ID/g) was similar to wild-type mice, whereas in Mdr1a-b [11C]ELC mice a value of 2.30 ± 0.14%ID/g was reached (Fig. 2, A and C). The by-far highest brain uptake was observed in Mdr1a-b [11C]ELC mice (6.55 ± 0.41%ID/g). Administration of unlabeled ELC resulted in immediate increase of brain activity values in all mice types except for Mdr1a-b [11C]ELC mice (Fig. 2, A and C), reaching maximum levels after at least 60–90 minutes. In the second PET scan recorded at 120 minutes after...
administration of unlabeled ELC, activity uptake at 25 minutes after tracer injection was 5.8 ± 0.3, 2.1 ± 0.2, and 7.5 ± 1.0-fold higher compared with scan 1 in wild-type, Mdr1a/b<sup>−/−</sup>, and Bcrp1<sup>−/−</sup> mice, respectively, but remained unchanged in Mdr1a/b<sup>−/−</sup>Bcrp1<sup>−/−</sup> mice (Fig. 2, B and D).

Brain activity uptake of [11C]TQD in PET scan 1 was similarly low in wild-type mice at 25 minutes after tracer injection (0.64 ± 0.03% ID/g; Fig. 3, A and C) as for [11C]ELC. In Bcrp1<sup>−/−</sup> mice, brain activity of [11C]TQD (0.98 ± 0.07% ID/g) was slightly higher compared with wild-type mice, whereas in Mdr1a/b<sup>−/−</sup> mice the value was more than twice as high (1.48 ± 0.06% ID/g; Fig. 3, A and C). Again, the by far highest activity uptake was observed in Mdr1a/b<sup>−/−</sup>Bcrp1<sup>−/−</sup> mice (6.03 ± 0.42% ID/g). Administration of unlabeled TQD resulted in immediate increase of brain activity values only in wild-type and Bcrp1<sup>−/−</sup> mice, whereas brain activity remained nearly constant in Mdr1a/b<sup>−/−</sup> and Mdr1a/b<sup>−/−</sup>Bcrp1<sup>−/−</sup> mice (Fig. 3, A and C). In the second PET scan, activity uptake was 2.8 ± 0.2- and 6.8 ± 0.4-fold higher than in scan 1 in wild-type and Bcrp1<sup>−/−</sup> mice, but remained unchanged in Mdr1a/b<sup>−/−</sup> and Mdr1a/b<sup>−/−</sup>Bcrp1<sup>−/−</sup> mice (Fig. 3, B and D).

As blood activity levels of [11C]ELC and [11C]TQD showed differences between tested mice types and were increased after cold inhibitor administration in some animal groups (Table 2), brain-blood ratios of activity concentrations at 25 minutes after tracer injection were additionally calculated (Fig. 4). In scan 1, brain-blood-ratio of [11C]ELC was distinctly higher in wild-type, Mdr1a/b<sup>−/−</sup>, and Bcrp1<sup>−/−</sup> mice as well as in Bcrp1<sup>−/−</sup> and Mdr1a/b<sup>−/−</sup>Bcrp1<sup>−/−</sup> mice with and without Bcrp1 differed significantly from each other (Fig. 4B).

In Vitro Transport Assays. Accumulation of a low concentration (1 nM) of ELC in Mdr1a- and MDR1-overexpressing cells pronouncedly increased (2.2- and 1.6-fold, respectively) when Pgp was inhibited with PSC833 (Fig. 5A). In Bcrp1-overexpressing cells, the increase in ELC accumulation was 2.8-fold after transporter inhibition with Ko143 (Fig. 5C). Additionally, accumulation of ELC in Mdr1a- and Bcrp1-overexpressing cells was lower than in corresponding wild-type cells without transporter inhibition. When cells were incubated with a high concentration of ELC (1 μM), inhibition of murine Pgp by PSC833 or murine Bcrp1 by Ko143 did no longer alter the amount of intracellular ELC accumulation (Fig. 5, B and D). Furthermore, the difference between wild-type and murine transporter-overexpressing cells disappeared. For further evaluation, CETA performed with wild-type cells exposed to a low concentration of ELC (1 nM) resulted in comparable tracer concentrations in both compartments (Fig. 5E). In Bcrp1-overexpressing cells, however, ELC accumulated over time in the apical compartment (Fig. 5F), indicating transport by Bcrp1.

At low TQD concentration (1 nM), administration of PSC833 to Mdr1a- and MDR1-overexpressing cells led to a distinct (3.4- and 2.1-fold) increase of intracellular TQD accumulation (Fig. 6A). This was no longer observed when cells were exposed to high TQD concentration (1 μM; Fig. 6B). At 1 nM TQD, Bcrp1-overexpressing cells accumulated less TQD than wild-type cells (Fig. 6C). In Ko143-treated Bcrp1-overexpressing cells, an increase in TQD accumulation was seen at a low (1 nM) and also at a high

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**Fig. 2.** Representative sagittal PET summation images (0–60 minutes) of [11C]ELC for scan 1 (A) and scan 2 (B) in wild-type (WT), Mdr1a/b<sup>−/−</sup>, Bcrp1<sup>−/−</sup>, and Mdr1a/b<sup>−/−</sup>Bcrp1<sup>−/−</sup> mice. The radiation scale is set from 0.0 to 10.0% injected dose per gram (%ID/g). (C and D) Mean ± S.E.M. whole-brain time-activity curves of all mice types (WT, black squares; Mdr1a/b<sup>−/−</sup>, red circles; Bcrp1<sup>−/−</sup>, green triangles; Mdr1a/b<sup>−/−</sup>Bcrp1<sup>−/−</sup>, blue diamonds; n = 5 per type) are shown. Unlabeled ELC (5 mg/kg) was administered as an intravenous bolus at 60 minutes after start of scan 1 (C). For acquisition of scan 2 (D), a second [11C]ELC injection was performed at 2 hours after administration of unlabeled ELC. Application of cold ELC is indicated by an arrow.
(1 μM) concentration of TQD (4.4- and 2.2-fold, respectively; Fig. 6, C and D). Only at 5 μM TQD, tracer uptake did not differ anymore between untreated and inhibited Bcrp1-overexpressing cells (Fig. 6E).

**Discussion**

The present study was designed to investigate potential dose-dependent transport of two third-generation MDT modulators, ELC and TQD, at the BBB and to thereby further characterize their suitability as new PET tracers for assessing expression and function of Pgp and BCRP at the BBB. The major findings of this study are: First, brain entrance of [11C]ELC and [11C]TQD administered in low nanomolar tracer doses is limited by Pgp- and Bcrp1-mediated efflux in vivo at the mouse BBB. Second, at high, MDT-inhibitory doses, TQD, but not ELC, is still kept out of the brain by Bcrp1 transport, whereas Pgp transport is inhibited. Third, consistent with our in vivo findings, in vitro uptake assays show that both ELC and TQD are substrates of Pgp and Bcrp1 at nanomolar concentrations, but that transport is lost at micromolar concentrations. The concentrations of ELC and TQD at which both drugs were transported in vitro are clearly below those that inhibit the transporters (see Table 1).

In already published in vivo studies with radiolabeled TQD and ELC, both tracers were administered to mice lacking Pgp, Bcrp1, or both efflux transporters only in tracer doses (Dörner et al., 2009; Bauer et al., 2010; Kawamura et al., 2010, 2011). In this study, we could confirm the results of these experiments. In addition, we performed PET scans also after administration of therapeutically effective doses not only in wild-type but also in transporter-knockout mice. By using this approach, we substantiate that the efflux by Pgp and Bcrp1 in vitro are clearly below those that inhibit the transporters.

**TABLE 2**

Blood activity levels 25 minutes after tracer injection (n = 3 per mouse type)

<table>
<thead>
<tr>
<th>Mouse Type</th>
<th>FVB (Wild-Type)</th>
<th>Mdr1a/b&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Bcrp1&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>Mdr1a/b&lt;sup&gt;−/−&lt;/sup&gt;Bcrp1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean S.E.M.</td>
<td>Mean S.E.M.</td>
<td>Mean S.E.M.</td>
<td>Mean S.E.M.</td>
</tr>
<tr>
<td>[11C]Tariquidar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[%ID/ml] Scan 1</td>
<td>0.580 0.015</td>
<td>0.327 0.078</td>
<td>0.447 0.111</td>
<td>0.293 0.048</td>
</tr>
<tr>
<td>%ID/ml Scan 2</td>
<td>0.483 0.047</td>
<td>0.443 0.023</td>
<td>0.613 0.055</td>
<td>0.447 0.050</td>
</tr>
<tr>
<td>[11C]Elacridar</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[%ID/ml] Scan 1</td>
<td>0.220 0.042</td>
<td>0.457&lt;sup&gt;*&lt;/sup&gt;</td>
<td>0.064</td>
<td>0.587&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>%ID/ml Scan 2</td>
<td>0.480&lt;sup&gt;*&lt;/sup&gt; 0.015</td>
<td>0.453 0.069</td>
<td>0.547&lt;sup&gt;*&lt;/sup&gt; 0.030</td>
<td></td>
</tr>
</tbody>
</table>

Bcrp, breast cancer resistance protein; FVB, XXXX; ID, injected dose.
<sup>*</sup> Significant difference (P < 0.05) to wild-type mice.
<sup>*</sup> Significant difference (P < 0.05) to scan 1.

Fig. 3. Representative sagittal PET summation images (0–60 minutes) of [11C]TQD for (A) scan 1 and (B) scan 2 in wild-type (WT), Mdr1a/b<sup>−/−</sup>, Bcrp1<sup>−/−</sup>, and Mdr1a/b<sup>−/−</sup>Bcrp1<sup>−/−</sup> mice. The radiation scale is set from 0.0% to 10.0% injected dose per gram (%ID/g). (C and D) Mean (± S.E.M.) whole-brain time-activity curves of all mice types (WT, black squares; Mdr1a/b<sup>−/−</sup>, red circles; Bcrp1<sup>−/−</sup>, green triangles Mdr1a/b<sup>−/−</sup>Bcrp1<sup>−/−</sup>, blue diamonds; n = 5 per type) are shown. Unlabeled TQD (15 mg/kg) was administered as an intravenous bolus at 60 minutes after start of scan 1 (C). For acquisition of scan 2 (D), a second [11C]TQD injection was performed at 2 hours after administration of unlabeled TQD. Application of cold TQD is indicated by an arrow.
furthermore, demonstrate that substrate and inhibitory properties of TQD and ELC differ with regard to their interaction with Pgp and BCRP. Furthermore, we are the first reporting transport of ELC by Pgp and Bcrp as well as transport of TQD by Pgp in in vitro transport assays. We also reveal that this transport is concentration-dependent in vitro.

**Dose-Dependent Transport of ELC and TQD by Pgp and Bcrp In Vivo.** After administration of 5 mg/kg cold ELC, differences in [11C]ELC brain uptake between Mdr1a/b<sup>−/−</sup> and wild-type mice on the one hand, and between Bcrp1<sup>−/−</sup> and Mdr1a/b<sup>−/−</sup>Bcrp1<sup>−/−</sup> mice on the other hand disappeared, i.e., between animals expressing and lacking Pgp (Fig. 2, C and D). In contrast, a lower brain uptake was found in animals with Bcrp1 as compared with animals without Bcrp1 (Fig. 2, C and D). The latter differences were not present anymore if brain-blood ratios were calculated (Fig. 4A). These results indicate complete blocking of Pgp-mediated transport at the doses of TQD and ELC used, whereas there might still have been active transport by Bcrp1.

In principle, the in vivo behavior of [11C]TQD resembled that of [11C]ELC. Again, a high dose of cold TQD (15 mg/kg) completely abolished Pgp-mediated transport of [11C]TQD (Figs. 3, C and D, and 4B). In contrast to the [11C]ELC experiments, Bcrp1 activity was apparently only slightly inhibited at the used TQD dose. These data are in accordance with the literature reporting that ELC is a more potent BCRP inhibitor than TQD (Kühnle et al., 2009). The different behavior of low and high ELC and TQD doses or concentrations may be explained by the assumption that at least two binding sites exist: one binding site that mediates transport, and another one that induces inhibition. This would be consistent with an earlier study by Martin et al. (1999), who concluded from their experiments that TQD binds to Pgp at another position than substrates do. Concentration-dependent activity of MDT modulators was also found by Meier et al. (2006), who described transport of low verapamil concentrations by Pgp indicated by high ATPase activity. At higher concentrations transport activity did not reach a plateau, but decreased again to baseline levels indicating transporter inhibition. Driven by the observation that brain uptake of the Pgp inhibitor PSC833 was higher in Mdr1a/b<sup>−/−</sup> mice compared with wild-type mice (Mayer et al., 1997), Smith and colleagues performed bidirectional transport assays with [14C]PSC833. They found that PSC833 was dose-dependently transported by human Pgp, i.e., transport occurred at concentrations of 0.1 μM and 0.4 μM but no longer at 2 μM (Smith et al., 1998). Consistent with these in vitro results, Lemaire et al. (1996) observed dose-dependent brain penetration of PSC833 in vivo in rats. Interestingly, like for TQD and ELC tested in this study, earlier studies had proposed that PSC833 cannot be transported by Pgp (Archinal-Mattheis et al., 1995; Naito et al., 1996). One can conclude from our study results that TQD and ELC show similar properties like verapamil or PSC833.

An alternative explanation for the described data could be that TQD and ELC interact only with a substrate binding site and inhibit Pgp and BCRP competitively. They may be transported with very high affinity (K<sub>m</sub>) and slow velocity (V<sub>max</sub>) by Pgp and BCRP, leading to easily saturated transport capacity at micromolar concentrations. Nevertheless, if this behavior could be assumed for all MDT inhibitors, ATP activity should reach a plateau and not decrease again as described above for verapamil.

**Advantages of Including Combination–MDT-Knockout Mice and Different Doses of Drugs.** Since their development, TQD and ELC are generally considered to be noncompetitive and nontransported...
inhibitors (Hyafil et al., 1993; Martin et al., 1999; Thomas and Coley, 2003) studied mainly in vitro assays. Both were described as selective Pgp inhibitors at a time when BCRP had not yet been discovered (Hyafil et al., 1993; Martin et al., 1999). Later, inhibition of BCRP was described also (de Bruin et al., 1999; Maliepaard et al., 2001; Robey et al., 2004). Based on the assumption that both inhibitors only bind to the MDT, one would expect a higher brain PET signal of [11C]ELC and [11C]TQD in wild-type mice compared with animals lacking Pgp. However, in recent studies, we and others unexpectedly found about 2-fold higher brain concentrations of both tracers in Mdr1a/b<sup>−/−</sup> mice compared with the background strain (Dörner et al., 2009; Bauer et al., 2010; Kawamura et al., 2010, 2011). In contrast, no increased uptake was observed in mice lacking Bcrp1.

It has been proposed that it is not always sufficient to use different kinds of single MDT-knockout mice to elucidate whether a substance is transported by an MDT at the BBB at all, but that it can be crucial to use combination knockout models (Lagas et al., 2009). This is supported by our observation that in combination Mdr1a/b<sup>−/−</sup>/Bcrp1<sup>−/−</sup> mice, brain uptake of [11C]ELC and [11C]TQD was increased by 9- to 15-fold compared with wild-type animals (Bauer et al., 2010; Kawamura et al., 2010, 2011), confirming a concerted action of both transporters (de Vries et al., 2007; Lagas et al., 2009; Kodaira et al., 2010). Interpretation of results could easily have led to the wrong conclusions if only Mdr1a/b<sup>−/−</sup> and Bcrp1<sup>−/−</sup> mice had been used. Very recently, Liu et al. (2012) reported in vivo transport of ELC by BCRP and Pgp using a different approach supporting the findings of this study.

Fig. 5. Mean (± S.E.M.) uptake of 1 nM or 1 μM ELC in wild-type (WT), murine Mdr1a-, human MDR1- (A and B), or Bcrp1-overexpressing cells (C and D) with and without exposure to the Pgp inhibitor PSC833 (PSC) or the BCRP inhibitor Ko143 (Ko), respectively. Differences between wild-type and overexpressing cells as well as between overexpressing cells with or without transporter inhibition indicate transport activity. Additionally, transport of 1 nM ELC by Bcrp1-overexpressing cells was evaluated using the concentration equilibrium transport assay (CETA; E and F). Significant differences (P < 0.05) are indicated by asterisk.
Concentration-Dependent Transport of ELC and TQD by Pgp and Bcrp1 In Vitro. Consistent with our in vivo findings (see above), intracellular accumulation of [3H]ELC and [3H]TQD at low concentrations was impeded by Pgp and Bcrp1 transport. Blockage of Pgp and Bcrp1 led to increased intracellular accumulation of nanomolar [3H]ELC and [3H]TQD concentrations, demonstrating that both inhibitors are substrates of these MDTs. Such efflux transport was not seen at higher concentrations. At 1 nM, [3H]ELC intracellular accumulation was no longer prevented by Pgp or Bcrp1, whereas [3H]TQD was still efficiently kept out of cells by Bcrp1. At 5 μM, transport of [3H]TQD was no longer detectable.

In our study, it became evident that comparison of substance accumulation between wild-type cells and transporter-overexpressing cells is less sensitive for detection of transport in uptake assays than comparison between MDT-overexpressing cells with and without a selective MDT inhibitor (Figs. 5 and 6). This could explain why [3H]TQD uptake did not differ between Pgp- and non-Pgp-expressing cells in a comparable study performed by Martin et al. (1999). To further confirm Bcrp1 transport of ELC, we exemplarily performed a transport assay (CETA) with [3H]ELC in Bcrp1-overexpressing cells. This assay has been proven to be very sensitive, especially for lipophilic MDT substrates (Luna-Tortós et al., 2008; Löscher et al., 2011). Indeed, in consistency with the uptake assays, CETA also revealed ELC transport by Bcrp1 (Fig. 5, E and F).

In parallel to our study, Kannan et al. (2011) published an in vitro study in which they demonstrate transport of TQD by BCRP and are fully in line with our findings. Interestingly, in Pgp-overexpressing cells, Kannan and colleagues found an increased accumulation of [3H]TQD compared with parental cell line, which the authors interpreted as binding of [3H]TQD to Pgp. In contrast, we found lower [3H]TQD accumulation in Pgp-overexpressing as compared with wild-type cells (Fig. 6A). One reason for the failure of Kannan and co-workers to reveal Pgp transport of TQD could be that they did not add a Pgp inhibitor to their Pgp-overexpressing cells, which might have led to an increased uptake of TQD after inhibition.

Our experiments clearly show that the third-generation Pgp modulators ELC and TQD are concentration-dependently transported by Pgp and Bcrp1, both at the murine BBB in vivo as well as in vitro in cellular transport assays. Importantly, this study illustrates that microdoses can behave pharmacokinetically differently from MDT-inhibitory doses if a compound interacts with MDTs, indicating that conclusions from microdose studies should be drawn carefully given that the test compound could be a transporter modulator.

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