Fluids and Barriers of the CNS

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ATP-binding cassette transporter inhibitor potency and substrate drug affinity are critical determinants of successful drug delivery enhancement to the brain

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Abstract

Background Pharmacotherapy for brain diseases is severely compromised by the blood-brain barrier (BBB). ABCB1 and ABCG2 are drug transporters that restrict drug entry into the brain and their inhibition can be used as a strategy to boost drug delivery and pharmacotherapy for brain diseases.

Methods We employed elacridar and tariquidar in mice to explore the conditions for effective inhibition at the BBB. *Abcg2;Abcb1a/b* knockout (KO), *Abcb1a/b* KO, *Abcg2* KO and wild-type (WT) mice received a 3 h i.p. infusion of a cocktail of 8 typical substrate drugs in combination with elacridar or tariquidar at a range of doses. *Abcg2;Abcb1a/b* KO mice were used as the reference for complete inhibition, while single KO mice were used to assess the potency to inhibit the remaining transporter. Brain and plasma drug levels were measured by LC-MS/MS.

Results Complete inhibition of ABCB1 at the BBB is achieved when the elacridar plasma level reaches 1200 nM, whereas tariquidar requires at least 4000 nM. Inhibition of ABCG2 is more difficult. Elacridar inhibits ABCG2-mediated efflux of weak but not strong ABCG2 substrates. Strikingly, tariquidar does not enhance the brain uptake of any ABCG2-subtrate drug. Similarly, elacridar, but not tariquidar, was able to inhibit its own brain efflux in ABCG2-proficient mice. The plasma protein binding of elacridar and tariquidar was very high but similar in mouse and human plasma, facilitating the translation of mouse data to humans.

Conclusions This work shows that elacridar is an effective pharmacokinetic-enhancer for the brain delivery of ABCB1 and weaker ABCG2 substrate drugs when a plasma concentration of 1200 nM is exceeded.

Keywords Blood-brain barrier, ABCB1, ABCG2, Elacridar, Tariquidar, Protein binding, Drug delivery, Mice

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Background

The blood-brain barrier (BBB) is a specialized barrier that separates the brain from the blood stream and plays a vital role in brain homeostasis. The BBB tightly regulates the uptake of essential nutrients and other useful components while limiting the uptake of potentially hazardous agents. However, this extraordinary defense mechanism also poses a formidable hurdle to adequate delivery of drugs for treatment of intracranial diseases [1, 2]. Unfortunately, there are only very few drugs that manage to attain therapeutic levels within the brain, even when their physico-chemical properties, such as lipophilicity, theoretically enable straightforward entry. Approaches to increase drug delivery to the brain that are currently being explored, include mechanical disruption of endothelial cell tight-junctions by focused ultrasound, delivery via nanoparticles armed with ligands for receptor-mediated transport and direct bypassing of the BBB via convection-enhanced delivery (all reviewed by Terstappen et al. [2]). Furthermore, certain pharmaceutical companies proactively engineer compounds with suitable characteristics for brain entry [3–5].

An alternative approach to improve BBB penetration of drugs is via inhibition of ABCB1 (P-glycoprotein, P-gp) and/or ABCG2 (breast cancer resistance protein, BCRP). These ATP-Binding Cassette (ABC) transporters are drug efflux pumps that are expressed at the BBB and are well-known to limit the brain distribution of many lipophilic small molecule drugs that may otherwise reach the brain in high concentrations [6–8]. For example, Parrish et al. showed that the Brain-to-Plasma (B/P) area under the curve (AUC) ratio of palbociclib in wild-type mice was less than 1/100th of the value observed in mice deficient for both *Abcb1a/b* and *Abcg2* ((B/P AUC = 0.064 vs. 7.36) rendering it inefficacious against intracranially implanted glioblastoma [9]. Hence, dual inhibition of these ABC-transporters at the BBB may serve as a foundational platform for improving the delivery of drugs that currently have limited utility in brain diseases.

The development of ABCB1 inhibitors garnered significant attention starting from the mid-1970s within the context of treating multidrug-resistant cancers. The idea of combating multidrug resistance of cancer by concomitant use of an ABCB1 inhibitor and a cytotoxic drug aroused great excitement and geared large investments for developing inhibitors by most major pharmaceutical companies. By the end of the previous century, several selective and non-competitive ABCB1 inhibitors with low-nanomolar potency in vitro, including elacridar and tariquidar, had been discovered [10]. Unfortunately, two decades and many failed clinical trials later, the disappointment was likely at least as big as the initial enthusiasm. As a consequence, this field has been largely abandoned and consensus shifted and none of the clinical trials using these inhibitors for reversing multidrug resistance of tumor cells have been unsuccessful, albeit that there may be good reasons for revisiting their use [8]. From the perspective of increasing drug delivery to the brain, however, tariquidar and elacridar are among the most attractive candidates as they are also inhibitors of ABCG2 [11]. In vitro cellular efflux studies using Calcein-AM or mitoxantrone revealed that elacridar and tariquidar are about equipotent inhibitors of ABCB1, but that tariquidar is less potent ABCG2 inhibitor than elacridar [12]. Preclinical studies have demonstrated that elacridar can increase the brain penetration of many substrate drugs to levels achieved in mice lacking both transporters (reviewed in [13]). These studies predominantly utilize mouse brain homogenates and plasma to determine total and/or unbound drug levels and only occasionally radiolabeled drug and positron emission tomography (PET) [14–16]. The clinical translation of this concept of improving the brain distribution by inhibition of ABC transporters relies solely on PET tracers and in most studies tariquidar was used as inhibitor. Tariquidar is not a registered drug, but a clinical grade intravenous i.v. formulation is available for human studies from AzaTrius Pharmaceuticals Pvt Ltd. Tariquidar has been given at dose levels up to 8 mg/kg [17, 18] and at this highest dose level, complete inhibition of ABCB1 at the human BBB was assumed [19].

Although an i.v. formulation of tariquidar is useful for proof-of-concept studies, the availability of an oral formulation would be more practical for chronic (daily) use, since oral medication allows self-administration at home. Oral dosing of tariquidar has been tried, but the oral bioavailability appeared to be very low [20]. In case of elacridar, only an oral drug formulation has been developed by GlaxoSmithKline. Initially, relatively low doses were used as the target plasma level of elacridar was set at 100 ng/ml (appr. 200 nM) [21, 22]. In later studies dose escalation has been tried, but the plasma level of elacridar in patients plateaus below 150 ng/ml (≤300 nM) following oral dosing between 100 and 1000 mg [23]. This result is in line with previous work of GlaxoSmithKline showing that plasma levels of elacridar hardly exceeded 200 ng/ml in rats, dogs and primates even when given at very high doses [24]. Notably, mice are the exception, with plasma levels of 600–1000 ng/ml being attainable, explaining why studies in mice are generally more successful. Efforts are in progress to improve the formulation of elacridar, with the aim to increase the plasma levels in humans [25]. However, it is unclear what plasma level of elacridar is actually needed for inhibition of ABCB1 and ABCG2 at the BBB.

The overarching concept is to improve drug access to the brain to enable effective pharmacotherapy of brain diseases. The immediate goal of this study is to determine

the target plasma level of elacridar necessary to enhance the brain distribution of therapeutic agents to achieve therapeutic levels. We hypothesized that the increase in drug distribution may depend on both inhibitor potency and the affinity of the substrate drug. Therefore, we have used a mixture of drugs, including weak and/or strong substrates of ABCB1 and/or ABCG2 as model compounds. These results enable translation of our concept to human subjects in two ways. First, to justify the initiation of clinical studies with novel elacridar formulations, we need to assess whether the elacridar plasma level is within a range that is expected to improve the brain distribution of investigational therapeutic agent. Secondly, this study will inform us which type of substrate drugs will be appropriate candidates. Subsequently, this may then be confirmed using PET-labeled compound in patients.

We used Friends Virus B (FVB) mice, deficient for both *Abcb1a/b* and *Abcg2* genes (the murine homologs of human *ABCB1* and *ABCG2*, respectively) as a reference for complete inhibition. Single *Abcb1a/b*-deficient mice are used to assess the potency of elacridar in inhibiting *Abcg2*, while vice versa single *Abcg2*-deficient mice are used to assess inhibition of Abcb1a/b. Wild-type (WT) mice, proficient for both ABC transporters act as reference for humans (Fig. 1A). Tariquidar is included in the study for benchmarking against elacridar, leveraging existing clinical data with tariquidar on ABC transporter inhibition at the BBB. Overall, we find that elacridar exhibits greater inhibitory potency compared to tariquidar. Our results suggest that elacridar could serve as a valuable pharmacokinetic-enhancer for enhancing the brain penetration of substrates for ABCB1 and weak to moderately potent substrates for ABCG2.

Materials and methods

Chemicals and drug solutions

Erlotinib was kindly provided by OSI Pharmaceuticals, Inc. (Melville, NY). Verapamil and loperamide originate from Sigma-Aldrich (St Louis, MO, USA), vemurafenib from Syncom (Groningen, The Netherlands), afatinib from ChemieTek (Indianapolis, IN, USA), palbociclib, dasatinib and ibrutinib from Medkoo Biosciences (Morrisville, NC, USA), Elacridar. HCl from GlaxoSmithKline (Research Triangle Park, NC, USA) and tariquidar was a generous gift of Azatrius (Mumbai, India). Elacridar-d9 was a gift of Izumi Biosciences (Lexington). Except for palbociclib (5 mM), all compounds were dissolved at 10 mM in dimethyl sulfoxide (DMSO). For in vitro studies, a substrate drug mixture of 100 µM of each compound was

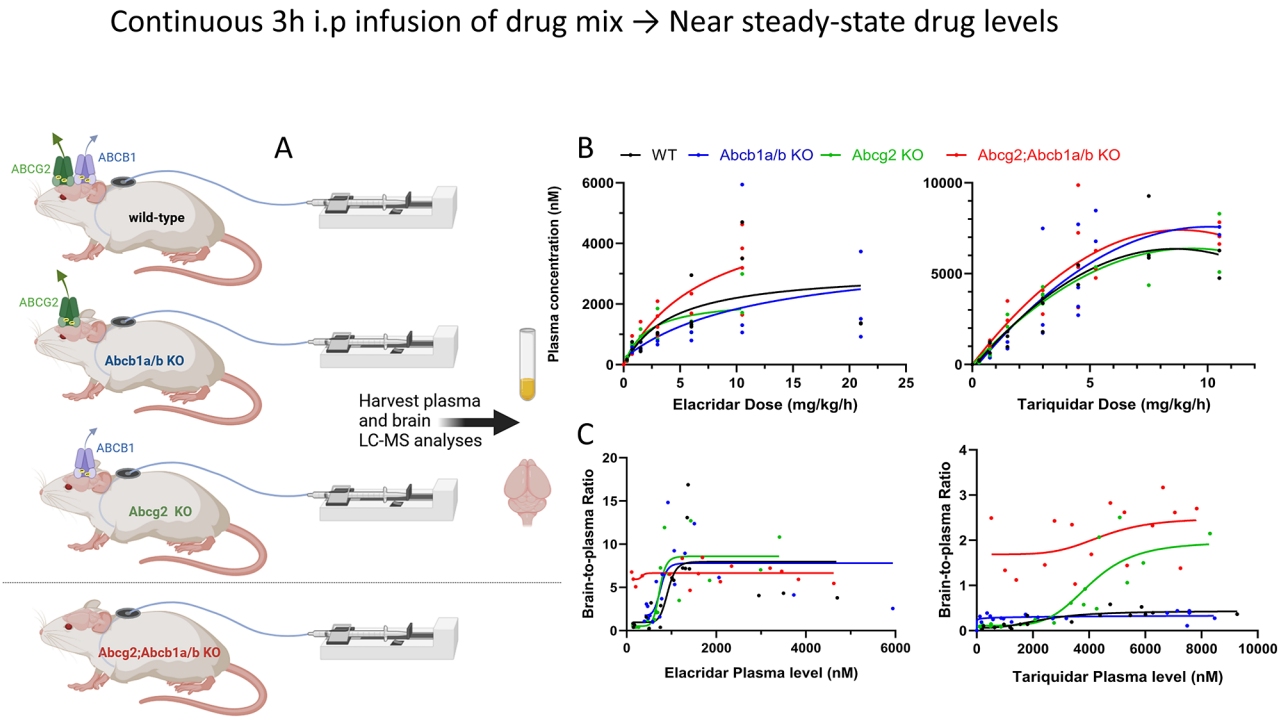

Fig. 1 Plasma concentration and brain-to-plasma ratio of elacridar and tariquidar. (**A**) Schematics of the study setup. Freely moving mice with an i.p catheter receive a 3 h continuous infusion of an 8-drug mixture supplemented with a dose range of elacridar or tariquidar. (**B**) Plasma concentration at the end of the infusion as a function of dose level. (**C**) Brain penetration (brain-to-plasma ratio) of elacridar or tariquidar as a function of the plasma concentration of the inhibitor. Created with BioRender.com

prepared in DMSO. For analytical purposes this mixture also contained 100 µM elacridar and tariquidar.

Stable isotope labeled compounds ibrutinib-d8, vemurafenib-^{13}C6 and afatinib-d8 were purchased from Alsachim (Illkirch Graffenstaden, France) and erlotinib-d6, palbociclib-d8, Ddasatinib-d8, loperamide-d6, verapamil-d6 and elacridar-d4 from Toronto Research Chemicals (Toronto, Canada). All compounds were dissolved at 1 mg/ml in DMSO and diluted 100-fold in DMSO to yield an internal standard (IS) stock mixture of 10 µg/ml of each.

Cell lines and drug accumulation assay

We used the parental Madine Darby Canine Kidney (MDCKII) cell line and its human ABCB1/MDR1 (Pgp) and ABCG2/BCRP overexpressing sublines to assess drug accumulation. Cells were cultured in Minimum Essential Medium (MEM), supplemented with L-glutamine, sodium pyruvate, MEM vitamins, penicillin/streptomycin, non-essential amino acids and 10% fetal calf serum (FCS) (all from Life Technologies, Carlsbad, CA) and maintained in 5% CO2 in humidified air at 37 °C.

At the day of the experiment, the MDCK-II cells and sublines were trypsinized and washed with medium to remove trypsin. The cell suspension were counted and portions containing 1×10^6 cells were aliquoted into 30 separate 1.5 ml Eppendorf vials. These tubes were centrifuged (800 rpm, 5 min) and the supernatants were aspirated. Each tube now contains a cell pellet of 1×10^6 cells. Before, a 100 nM substrate drug mix was already prepared by making a 1000-fold dilution of the aforementioned 100 µM DMSO stock mix in 10%FBS containing MEM medium. Next, elacridar or tariquidar was added to aliquots of this substrate drug mix in 10%FBS medium to create a range of increasing inhibitor concentrations (0, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5000 nM) mix. Next, the cell pellets of 1×10^6 cells were resuspended in 200 µl of the prepared drug solutions (each inhibitor concentration was assayed in triplicate) and placed in a Thermomixer using a shaking speed of 800 rpm for 15 min at 37 °C. Vials were centrifuged for 2 min at 10,000 rpm (4 °C), followed by two washing steps (resuspension and spinning) with 1-ml ice-cold PBS. Following the last wash, the samples were re-suspended in 50 µl of human plasma and were stored at -20 °C until LC-MS/MS measurement. The concentration in the cell lysate was calculated back to an intracellular drug level using the equation:

$$\text{Intracellular concentration} = \frac{drug\ amount\ in\ the\ sample\ (pmol)}{cell\ count * intracellular\ volume\ per\ cell\ (ml)}$$

The mean intracellular volume of MDCKII cells was assumed to be 2.15 pL [26]. The inhibitor concentration vs. intracellular drug level concentrations were fitted using the [Agonist] vs. response –Variable slope (four parameters) nonlinear fit in Graphpad Prism 10.1.2.

Relative plasma protein binding of elacridar and tariquidar in mouse versus human plasma

We used the Pierce rapid equilibrium dialysis (RED) device 8k MWCO (ThermoFisher Scientific) in a competition setup. Mouse and human plasma was diluted to 30% (v/v) in MEM. Diluted human plasma spiked with 1000 nM elacridar/ tariquidar was pipetted into the sample chamber, while diluted mouse plasma spiked with elacridar-d9 was added to the buffer chamber. Similarly, mouse plasma spiked with 1000 nM elacridar/ tariquidar was pipetted into the sample chamber, while human plasma spiked with elacridar-d9 was added to the buffer chamber. The plate was placed on a shaker in an incubator at 37'C, 5% CO2 and samples were taken from both sample and buffer chamber at 2, 5, 7 and 24 h.

Animals

The animal experiments were in accordance with 2010/63/EU and conducted under license AVD301002016595 and protocol 2.5.8776. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Male WT, *Abcb1a/b* KO, *Abcg2* KO and *Abcg2;Abcb1a/b* KO mice, backcrossed to 99% FVB were used between 9 and 20 weeks of age and maintained in InnoVive disposable cages in a temperature-controlled environment with a 12-hr light/12-hr dark cycle. They all received a standard diet and acidified water *ad libitum*. Expression data of the transport protein in these mice has been described [27].

Surgery and cannulation

Peri-operative analgesia involved carprofen (rimadyl: Pfizer) 0.067 mg/ml in non-acidified drinking water for 3 days and once 15 min pre-operative s.c. 0.1 mg/kg buprenorphine (Temgesic; Schering-Plough). Following anesthesia using isoflurane (4% and 2.5% for induction and maintenance), the peritoneal wall was exposed via a small incision in the abdominal skin. The peritoneum was lifted with a forceps and punctured with a 18 g needle. The polyurethane cannula tubing (Instech laboratories, Plymouth Meeting, PA, USA) was inserted into the peritoneal cavity and sutured to the peritoneum. Via a small incision in the neck, a subcutaneous tunnel was made towards the abdomen. The cannula was guided into the trocar, which was then gently withdrawn to guide the cannula to the neck. The cannula was then connected to a One Channel Vascular Access Button™ with protective cap (Instech), which was sutured to the skin. The mice were allowed to recover for at least four days.

Plasma pharmacokinetics and brain accumulation

The drug stock mix contained 400 µg/ml of loperamide and verapamil, 100 µg/ml of erlotinib, afatinib and palbociclib, 50 µg/ml dasatinib, vemurafenib and ibrutinib in DMSO and was supplemented with elacridar (35, 17.5, 10, 5, 2.5, 1.25 or 0 mg/ml) or tariquidar (17.5, 12.5, 7.5, 5, 2.5, 1.25, 0.5 or 0 mg/ml) in DMSO. The final formulation comprised this drug stock mix (in DMSO), Cremophor EL (Sigma-Aldrich) and saline (1:1:8, v/v/v).

The animals were placed solitary and connected to a BASi syringe pump (Bioanalytical systems, West Lafayette, IN, USA) via a catheter and swivel to allow free movement (Instech). The intraperitoneal infusion started at a flow rate of 10 µl/min for the first three minutes and then continued for 3 h at 3 µl/min. Normalized to a mean body weight of 30 g, the dose levels were appr. 240 µg/kg/h for loperamide and verapamil, 60 µg/kg/h for erlotinib, afatinib palbociclib and 30 µg/kg/h for vemurafenib, dasatinib and ibrutinib. For elacridar and tariquidar the highest dose levels were 21 and 10.5 mg/kg/h, respectively.

Mice were deeply anesthetized with isoflurane (4%) while cardiac puncture was performed with heparinized syringes for blood collection. Next, the animal was sacrificed by cervical dislocation and the brain was collected, weighed and stored at -20 °C until homogenization with 3 ml of bovine serum albumin 1% (w/v) in water. Blood was centrifuged 5 min at 5000 rpm and 4℃, then the plasma was separated and stored at -20 °C until analysis.

Drug analysis

Details of the method development and validation have been described in a separate methodology paper, now available as preprint [28]. In brief, drug levels were analyzed using liquid chromatography tandem mass spectrometry (LC-MS/MS). The system comprised an UltiMate 3000 Autosampler and HPLC pump (Thermo Scientific, Waltham, MA, USA) and API3000 MS/MS (Sciex, Framingham, MA, USA). Instrument control and data acquisition and quantification was done with Analyst 1.6.2 (Sciex) and Dionex Chromatography-MS (DC-MS) link 2.12 software (Thermo). Acquisition parameters of the mass spectrometer are listed in Table S1. Separation was performed at ambient temperature on a Zorbax Extend C18 column (2.1×100 mm, particle size 3 µM, Agilent, Santa Clara, CA, USA). Mobile phase A (0.1% formic acid in water) and B (methanol) was delivered at 0.4 ml/min. Between 0 and 5 min a linear gradient from 20 to 95%B was applied. Next, 95%B was maintained for 3 min followed by re-equilibration at 20%B. Total run time was 15 min per sample.

The concentrations in unknown mouse plasma samples were assessed using calibration samples prepared in blank human plasma. For mouse brain homogenates we used mouse brain homogenate calibration samples. Sample pretreatment involved liquid-liquid extraction. Samples (50 µl), IS work solution (50 µl of 10 ng/ml in methanol: water (60:40;v/v)) and 1 ml tert-butyl methyl ether in a 2 ml Eppendorf vial were mixed for 15 min and centrifuged 1 min (14,000 rpm. Vials were placed in dry-ice ethanol and when the aqueous layer was frozen the organic layer was decanted into a 1.5 ml Brand vial and dried by vacuum (Savant Speedvac, Thermo). Residues were reconstituted in 100 µl of methanol: water (60:40; v/v) and 25 µl was injected into the LC system. All samples were measured at least in duplicate in separate runs, together with calibration samples from 1 to 200 nM and Quality Control samples of 2, 5, 20 and 50 nM. Mouse plasma samples containing more than 200 nM elacridar or tariquidar were diluted 20-fold in human plasma prior to sample pretreatment.

Of note, we have included additional compounds in the substrate mix, next to the selection that was used during the method validation described in the aforementioned methodology paper [28]. The method was not changed, except for adding the compounds and adjusting the MS acquisition settings. Information about the method, the validation and quality performance is provided in the supplementary information (Additional data; File 1; Tables S1A-B; Fig. S1A-D).

Data analysis

Additional validation of the LC-MS/MS assay were done as described before [28]. Dose-response curves were fitted using the [Agonist] vs. response –Variable slope (four parameters) nonlinear fit in Graphpad Prism 10.1.2. and were used to calculate IC_{50} and EC_{50} values (90% confidence interval; CI90, between brackets). Comparisons between multiple groups were done by 0ne-way ANOVA.

Results

In vitro accumulation assay shows higher potency of elacridar over tariquidar

We selected a panel of drugs based on previous papers about their substrate affinities (Additional file 2: Table S2), as well as our own experience. We aimed to make a selection of drugs that were either weaker or stronger substrates of ABCB1 and/or ABCG2. The choice was verified by conducting in vitro drug accumulation experiments in MDCK cells using very low sub-toxic drug concentrations. The MDCKII parental and ABCB1 or ABCG2 overexpressing cell lines are often used in transwell assays as this is a sensitive way to determine whether compounds are substrates of these ABC-transporters. Here, we have used these cell lines for studying intracellular drug accumulation using a range of inhibitor concentrations (elacridar or tariquidar to compare their inhibitory potencies (50% inhibitory concentration;

IC_{50} values) under identical conditions. Expression levels of ABCB1 and ABCG2 in these overexpressing cell lines have been reported by Li et al. [29] and were suited for this purpose. Cell suspensions were incubated with the 8-drug mixture, each compound at about 100 nM, together with increasing concentrations of either elacridar or tariquidar (range: 0-5000 nM).

In MDCKII parent cells, the intracellular drug levels of most drugs increased slightly with increasing inhibitor concentrations (Additional Data File 2; Fig. S2). In cases of verapamil, erlotinib and ibrutinib fitting was unsuccessful. This finding aligns with the fact that these MDCKII parent cells express only low levels of endogenous canine ABCB1 and/or ABCG2 [30, 31]. The largest increase is observed for dasatinib, which is apparently more readily exported out of MDCKII cells by the baseline level of canine ABCB1 and/or ABCG2.

In absence of the inhibitor, the accumulation of most compounds is substantially less in MDCKII-MDR1 and/or MDCKII-BCRP cells compared to MDCKII parent cells. This finding confirms that the mix of substrate drugs at this low concentration were unable to inhibit the transporter (see also Table S2 for literature reported IC_{50} values for ABCB1 and ABCG2 inhibition). Based on the potency of elacridar and tariquidar to inhibit ABCG2 in vitro, we arbitrarily categorized these compounds into Class I (no substrate), Class II (intermediate substrate) and Class III (strong substrate). The intracellular concentration of all substrate drugs increased with increasing inhibitor concentrations, reaching a plateau that was in the same range as in the MDCKII parent cells. Both elacridar and tariquidar inhibit ABCB1 better than ABCG2, as the IC_{50} values of both inhibitors are about 10-fold higher for ABCG2 than for ABCB1. Moreover, for both ABCB1 and ABCG2 substrate drugs, the IC_{50} values of elacridar were consistently lower than those of tariquidar, indicating that elacridar has an about 2 to 4-fold higher molar potency compared to tariquidar (Table 1 and Fig. S2). In MDCK-BCRP cells, loperamide and verapamil followed an accumulation pattern similar to that of the MDCKII parental cell line, as expected for typical non-ABCG2 substrate drugs. For erlotinib and ibrutinib, we were unable to assess IC_{50} values in MDCK-MDR1 and parent cells, as the intracellular concentrations of both substrates did not increase with increasing concentrations of elacridar and/or tariquidar. Although this may indicate that these have very weak affinity for ABCB1, the relatively poor intracellular accumulation of erlotinib and ibrutinib may also hamper the accuracy of this in vitro potency assessment.

Data plots of each drug / cell line are depicted in Figure S2.

Approaching steady-state plasma levels of substrate drugs during a 3 h continuous infusion

Following this in vitro work, we assessed the potency of elacridar and tariquidar in vivo to inhibit ABCB1 and ABCG2 at the BBB. We use brain-to-plasma (B/P) ratios of the substrate drugs as readout of brain penetration. Following bolus injection, the B/P ratio may change in time when the clearance from brain differs from plasma. We therefore administered the 8-drug mixture plus the

Table 1 Potencies (IC_{50}) of elacridar and tariquidar to inhibit ABCB1 and ABCG2-mediated efflux of each compound in the 8-drug mixture in vitro (CI 90% (between brackets)

Class	Substrate drug	In vitro $IC_{50,\ plasma}$ (nM)				Fold difference Tariquidar over Elacridar	
		Elacridar		Tariquidar			
		ABCB1	ABCG2	ABCB1	ABCG2	ABCB1	ABCG2
I	Loperamide	14.0 (8.5–23.1)	NA	31.1 (23.4–42.0)	NA	2,2	NA
	Verapamil	9.8 (0-23.8)	NA	28.3 (15.4–55.1)	NA	2,9	NA
	Ibrutinib	NA	NA	NA	NA	NA	NA
II	Palbociclib	22.4 14.5–37.0)	33.9 (18.8–61.7)	39.8 (33.6–46.9)	245 (30.9-∞)	1,8	7,2
	Erlotinib	NA	155 (89–278)	NA	675 (341-∞)	NA	4,4
	Dasatinib	42.0 (32.7–53.4)	205 (163–265)	75.1 (59.7–95.6)	765 (555–1402)	1,8	3,7
III	Afatinib	23.8 (15.5–40.2)	388 (243–834)	37.5 (29.2–48.7)	807 (636–1155)	1,6	2,1
	Vemurafenib	22.8 (13.4–44.7)	435 (240–1518)	60.7 (37.0-101)	1595 (1310–2263)	2,7	3,7

ABCB1 was assessed using MDCK-MDR1 and ABCG2 using MDCKII-BCRP cell lines. Fold differences represent the difference in potency (IC_{50}) of tariquidar versus elacridar. The inhibitor concentration vs. intracellular drug level concentrations were fitted using the [Agonist] vs. response –variable slope (four parameters) nonlinear fit in Graphpad prism 10.1.2

inhibitor (elacridar or tariquidar) using a 3-hour continuous i.p. infusion in order to reach more or less steady-state plasma levels of substrate drugs. The dose levels of the substrate drugs were chosen from pilot studies. During these pilots, we collected serial blood samples from the tail vein to assess the plasma concentrations during the infusion (Fig. S3). Based on these pilots, we selected dose levels that were as low as possible in order to minimize/avoid drug-drug interaction, but did result in brain levels in WT mice that were sufficiently above the lower limit of quantification of the assay. The plasma levels at the end of the infusion were generally similar across genotypes and remained below 50 nM for most substrate drugs (Fig. S4). Only verapamil, erlotinib and vemurafenib were slightly higher (100–250 nM), but still far below therapeutic plasma levels in patients. The dose level of the inhibitor was varied to achieve a range of plasma concentrations between 0 and 6000 nM. Most likely due to solubility issues, elacridar demonstrated a non-proportional increase with dose, limiting the maximum achievable systemic exposure. Hence, we had relatively few animals with a plasma concentration above 2000 nM (Fig. 1B). Some non-proportional increase was also seen with tariquidar, but levels above 6000 nM were achieved.

Elacridar more profoundly enhances its own brain accumulation

Besides inhibitors, elacridar and tariquidar are also substrates of ABCB1 and ABCG2. Hence, they might be able to enhance their own brain accumulation. In *Abcg2;Abcb1a/b* KO mice the B/P ratios for elacridar and tariquidar are about 6 and 2, respectively (Fig. 1C). The B/P ratio of elacridar in WT and single *Abcg2* KO or *Abcb1a/b* KO mice increases with its plasma concentration, reaching a B/P ratio similar as in *Abcg2;Abcb1a/b* KO mice at about 1200 nM. The B/P ratio of tariquidar in *Abcg2* KO mice start to increase only at plasma levels above 4000 nM. Thus, the potency of tariquidar to inhibit its own ABCB1-mediated efflux at the BBB is considerably less than elacridar. Intriguingly, the B/P ratio did not increase in *Abcb1a/b* KO and WT mice, implying that tariquidar cannot enhance its own accumulation in mice that are proficient for *Abcg2*. The absolute brain concentration of tariquidar does increase, but just proportional to the plasma concentration.

Elacridar is also a more potent pharmacokinetic-enhancer for brain accumulation of other substrate drugs

Subsequently, we examined the B/P ratios of the eight substrate drugs in relation to the plasma levels of the inhibitors. The dosage and associated plasma levels of the substrates were similar across all genotypes (Fig. S4). At the BBB, verapamil and ibrutinib appear to be exclusive ABCB1 substrates (here designated Class I) (Fig. 2). They achieve similar B/P ratios in *Abcg2;Abcb1a/b* and *Abcb1* KO mice - both strains lacking ABCB1 transport protein - with no further increase as inhibitor plasma levels rise. Loperamide is also predominantly kept out of the brain by ABCB1, but there also appears to be some contribution by ABCG2, as we find consistently higher levels in *Abcg2;Abcb1a/b vs. Abcb1* KO mice. All other drugs are expelled from the brain by both ABCB1 and ABCG2 transport proteins.

Inhibition of ABCB1-mediated efflux at the BBB in *Abcg2* KO mice can be achieved (Figs. 2 and 3). The 50% effective inhibitory concentration (EC_{50}) in plasma of elacridar for ABCB1 is consistent for all drugs at about 600 nM (Table 2), while near complete inhibition is achieved when the elacridar plasma concentration is higher than 1200 nM. The EC_{50} of tariquidar for ABCB1 is much higher (3000 to 5000 nM). Near complete ABCB1 inhibition requires at least 4000 nM tariquidar for verapamil and erlotinib to 6000 nM for loperamide and afatinib. (Fig. 3). In some cases (e.g. dasatinib), tariquidar tends to increase the brain accumulation in *Abcg2;Abcb1a/b* KO mice, but only at very high plasma levels.

In contrast to ABCB1, inhibiting ABCG2-mediated efflux at the BBB is more difficult. For palbociclib, dasatinib, and erlotinib (i.e. Class II) the maximum inhibition of efflux in *Abcb1a/b* KO mice is achieved above 1200 nM of elacridar. However, despite approaching the B/P ratios observed in *Abcg2;Abcb1a/b* KO mice, erlotinib and palbociclib do not fully attain these. Moreover, the dual ABCB1/ABCG2 substrates vemurafenib and afatinib show very limited improvement in brain accumulation with elacridar in *Abcb1* KO and WT mice.

In the case of tariquidar, the situation is more unfavorable, as none of the ABCG2-substrate drugs exhibit significant enhancement in brain accumulation with tariquidar in *Abcb1a/b* KO mice. Consequently, the B/P ratio for all substrates remains low even at very high tariquidar plasma levels.

Some drugs accumulate poorly into the brain when ABCG2 and ABCB1 are inhibited or absent

Besides assessing the required plasma level of inhibitor for complete abrogation of efflux, it is also important to assess the gain in the B/P ratio. Some drugs can accumulate well into the brain. For example, the B/P ratio of verapamil increases from 0.33 to about 5.9 when ABC transporter mediated efflux is absent, thus gaining roughly 17-fold increase (Fig. 2). Similarly high B/P ratios are found for loperamide (0.25 to 5.6; 22-fold), palbociclib (0.26 to 7.8; 30-fold) and afatinib (0.18 to 3.7; 21-fold). In contrast, other drugs do gain in B/P ratio; dasatinib (0.10 to 0.26; 2.6-fold), vemurafenib (0.016 to 0.16; 9.7-fold) or

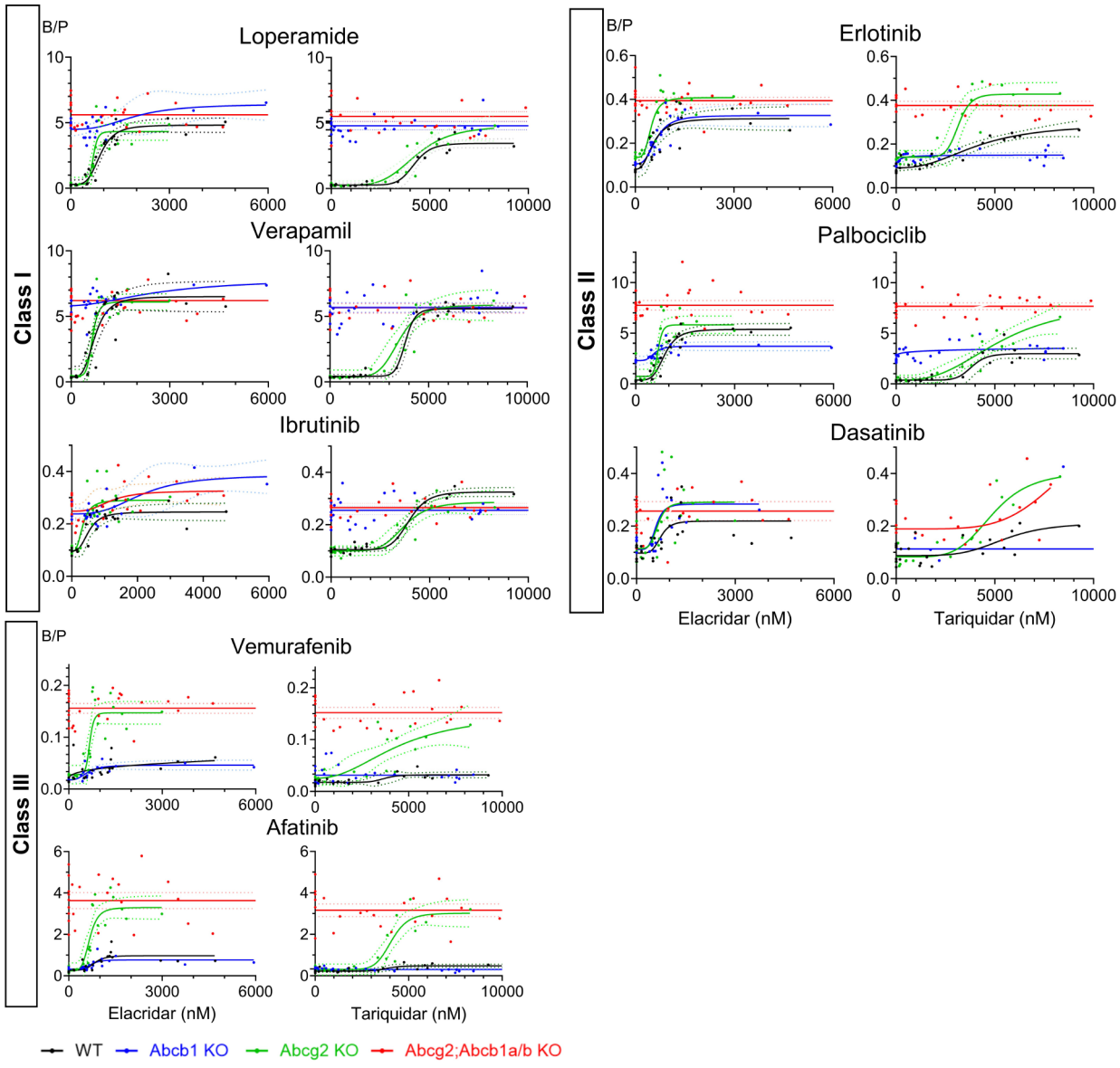

Fig. 2 Brain penetration (B/P-ratio) of each drug as a function of inhibitor plasma level. The drugs are classified as: (I) Abcb1 only substrates, (II) Abcb1 and weak Abcg2 substrates and (III) Abcb1 and strong Abcg2 substrates, as shown below. The inhibitor concentration vs. brain-to-plasma ratios curves were fitted using the [Agonist] vs. response –Variable slope (four parameters) nonlinear fit in Graphpad Prism 10.1.2. The dashed lines indicate the 90% CI around the curves

erlotinib (0.073 to 0.39; 5.4-fold), but the absolute B/P ratio remains very modest. The low brain penetrance of these compounds is apparently caused by other factors besides ABCB1 and ABCG2, such as chemical-physical properties, low binding to intracranial proteins relative to plasma proteins and the action of other efflux transporters. Although the underlying reason cannot be pinned by our studies, a low B/P ratio in *Abcg2;Abcb1a/b* KO mice is important to consider when selecting drugs that might benefit most from pharmacological inhibition of ABCB1 and ABCG2. For example, although elacridar is only able to increase the brain-to-plasma ratio of palbociclib to 75% in *Abcg2;Abcb1a/b* KO mice, the absolute B/P ratio (5.1) is still very high.

The relative plasma protein binding of elacridar and tariquidar is similar in mouse and human plasma

The potency to modulate efflux transport at the BBB has been linked to the free fraction of the inhibitors [32]. To enable comparing human and mouse inhibitor exposure at the BBB, we sought to evaluate the protein binding differences between elacridar and tariquidar in human *versus* mouse plasma using the rapid equilibrium dialysis (RED) devices. Previous studies reported that the free

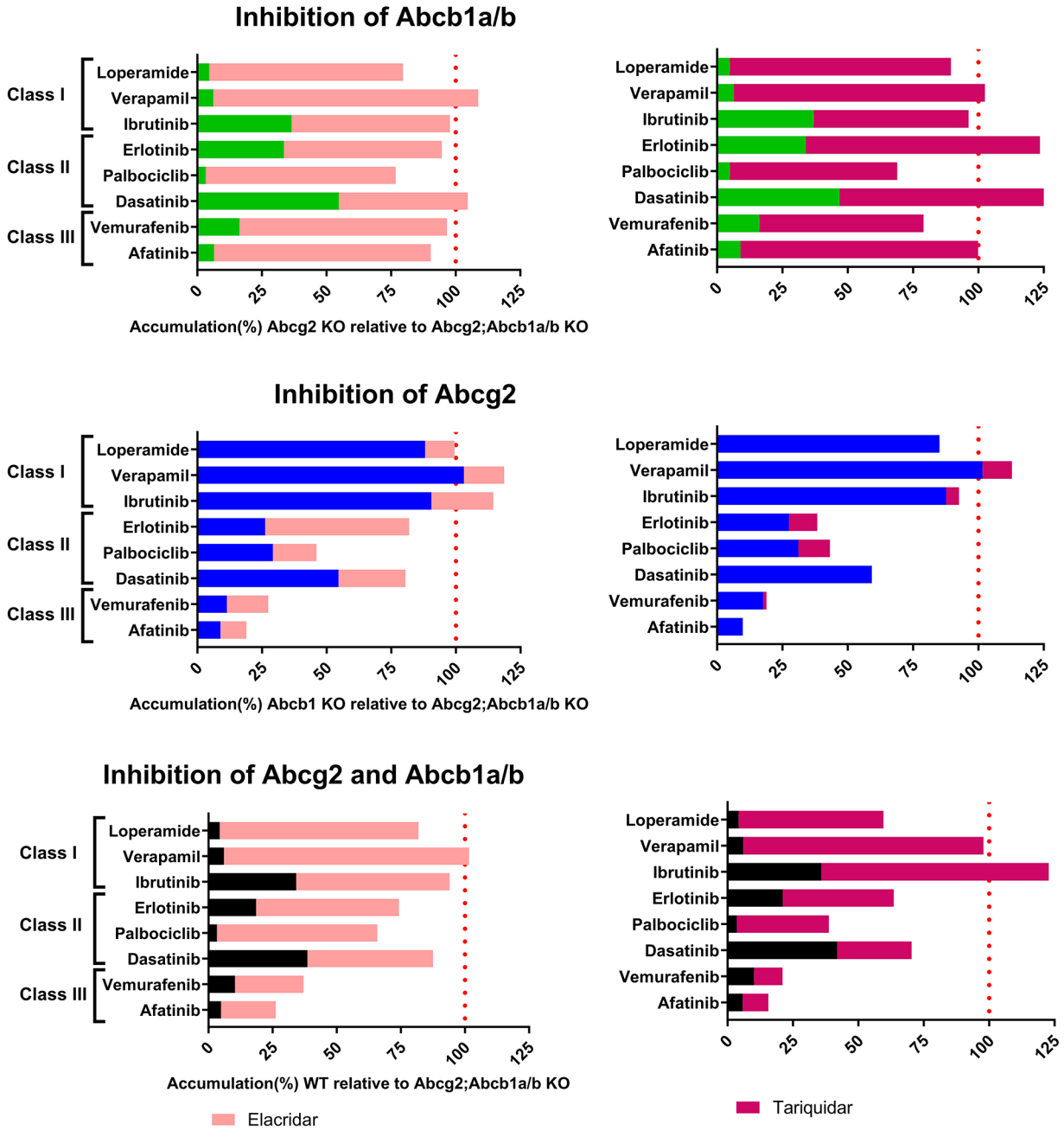

Fig. 3 Potency of elacridar and tariquidar to inhibit Abcg2 and Abcb1a/b. The drugs are classified as: (I) Abcb1 only substrates, (II) Abcb1 and weak Abcg2 substrates and (III) Abcb1 and strong Abcg2 substrates, as shown below. The accumulation of the substrates in Abcb1 KO mice (blue bars), Abcg2 KO mice (green bars) and WT mice (black bars). The color-filled bars show the maximum gain with elacridar or tariquidar. The red dotted line (100%) designates the accumulation of drugs in Abcg2;Abcb1a/b KO mice. The inhibitor concentration vs. brain-to-plasma ratio were fitted using the [Agonist] vs. response –Variable slope (four parameters) nonlinear fit in Graphpad Prism 10.1.2

fraction of elacridar and tariquidar was below 0.01 when using the standard procedure using buffer in the receiver compartment [24, 33] Given the challenges associated with the low aqueous solubility of elacridar, we designed a modified setup with mouse or human plasma in sample chamber (donor side) and human plasma in the "buffer" (receiver) chamber. By replacing the aqueous buffer by plasma we aimed to minimize loss due to container and membrane adsorption. Obviously, in this setup we do not assess free drug levels, but we anticipated that differences in total drug between compartments after equilibration would reflect relative protein binding in mouse *versus*

Table 2 50% effective inhibitory plasma concentrations (EC_{50}) of elacridar and tariquidar for Abcb1a/b and/or Abcg2-mediated efflux of substrate drugs from the brain of Abcg2 KO, Abcb1 KO and WT mice

		In vivo EC_{50} (nM)					
		Elacridar			**Tariquidar**		
Mouse strain		*Abcg2* KO	*Abcb1a/b* KO	WT	*Abcg2* KO	*Abcb1a/b* KO	WT
Inhibition of		ABCB1a	ABCG2	Both	ABCB1a	ABCG2	Both
Class	**Substrate drug**						
I	Loperamide	650 (479–943)	1929 (873-∞)	890 (746–1074)	4297 (3671–7691)	NA	4226 (3754–4695)
	Verapamil	586 (480–696)	(-)	720 (557–953)	3492 (2914–4742)	(-)	3888 (3557–4222)
	Ibrutinib	335 (219–503)	2137 (1435–6354)	519 (338–723)	3693 (3018–4607)	NA	3951 (3714–4182)
II	Erlotinib	482 (360–580)	631 (476–839)	545 (316–802)	3144 (2606–3506)	NA	4119 (2920–18538)
	Palbociclib	677 (618–731)	563 (361–841)	879 (772–989)	5320 (3670–∞)	NA	3824 (3294–4377)
	Dasatinib	643 (375–858)	590 (442–780)	732 (483–994)	4900 (**)	NA	5500 (**)
III	Afatinib	669 (603–723)	NA	NA	4489 (2128-∞)	NA	NA
	Vemurafenib	669 (578–762)	NA	NA	4067 (3642–4865)	NA	NA

Data plots of each drug are depicted in Fig. 2

(-) No value (no substrate), NA; not reached; CI 90% (between brackets)

human plasma. We assessed the equilibrium kinetics using human plasma or mouse plasma spiked with our drug mix and elacridar and tariquidar in the sample chamber *versus* blank human plasma in the buffer chamber. We also included a set of samples with complete medium (MEM+10% FCS; cMEM) containing the same drug mix in the sample chamber with the aim to investigate potential in vitro *versus* in vivo relationships.

To our surprise, less than 2–3% of elacridar and tariquidar was retrieved in the receiver chamber even after 24 h of incubation (Fig. S5). Moreover, elacridar was relatively unstable in this batch of mouse plasma. Thus, equilibrium was not at all achieved and the fraction of elacridar recovered in the receiver compartment was too low for an accurate assessment of the relative protein binding between human and mouse plasma. Notably, most other drugs also required at least 24 h of incubation in the RED plate to reach equilibrium, except for vemurafenib that behaved similar to elacridar. From our experiment with the drug mix in complete medium (10% FBS in medium), we noted that transfer from the sample to the receiver chamber was much more efficient, likely due to the lower protein content in complete medium. Based on this result, we decided to repeat the experiment using 30% diluted human and mouse plasma in medium spiked with elacridar and tariquidar. Opposite to the sample chamber containing diluted human plasma, the receiver compartment contained diluted mouse plasma and vice versa. The sample in the buffer chamber was spiked with elacridar-d9 at the same concentration. In this way, we can be sure that loss due to adsorption in the receiver buffer compartment cannot occur. We used a batch of mouse plasma wherein elacridar was relatively stable. Under these conditions, we observed a gradual transfer of elacridar to the receiver compartment reaching about 10% of the donor concentration after 24 h (Fig. 4). Notably, elacridar-d9 followed the same kinetics from the receiver to the donor compartment. Most importantly, there was no difference between human to mouse and mouse to human transfer, suggesting that the relative protein binding is similar. Similar values were also found for tariquidar.

Discussion

Numerous drugs with potential efficacy against brain diseases are substrates of ABCB1 and/or ABCG2 and the absence of these transporters in the BBB significantly increases the brain distribution of such drugs. Consequently, dual inhibition of ABCB1 and ABCG2 emerges as a viable platform to enhance drug delivery to the brain, as has been documented in human subjects with tariquidar. In this study, we demonstrate that elacridar is a considerably more potent pharmacokinetic-enhancer of brain entry, clearly outperforming tariquidar in its capacity to inhibit these transporters at the BBB.

Given that both ABC transporters are important for efflux of drugs at the BBB, dual inhibition of ABCB1/ABCG2 will be required for a more broad-spectrum drug delivery platform. Both elacridar and tariquidar were developed as ABCB1 inhibitors in the 1990s. At a later

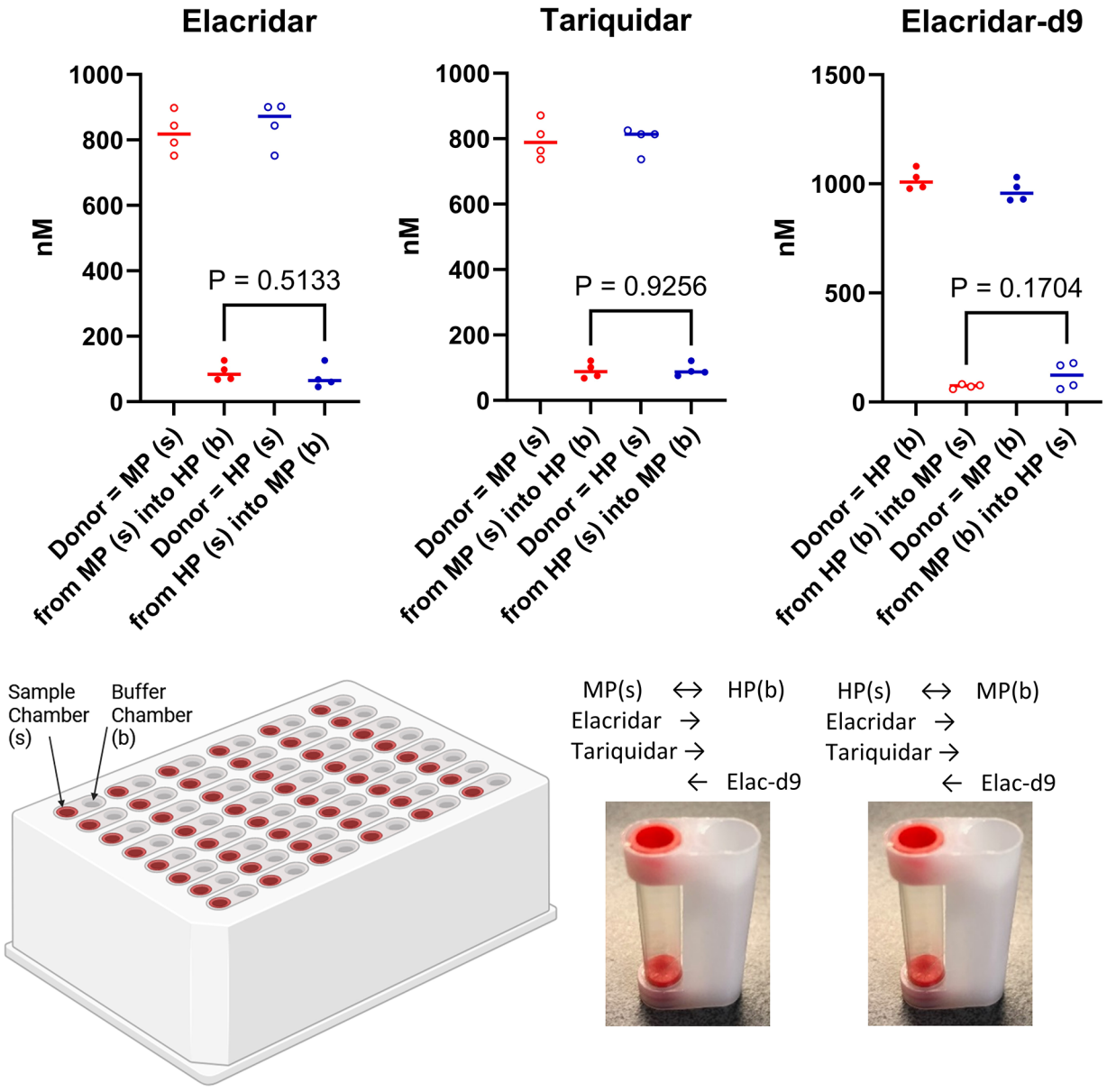

Fig. 4 Plasma protein binding of elacridar and tariquidar. Mouse plasma (MP) and human plasma (HP), each diluted 30% (v/v) in MEM and spiked with 1000 nM elacridar and tariquidar was added to the sample chamber (s), while the buffer compartment (b) was filled with 30% (v/v) diluted human or mouse plasma, respectively, containing 1000 nM of elacridar-d9. Depicted is the recovery after 24 h incubation at 37 °C. As an example: From MP(s) into HP(b) describes the drug concentration recovered in human plasma (HP) in the buffer (b) chamber (receiver) coming from mouse plasma (MP) spiked with drug in the sample (s) chamber (donor). Students t-test (Graphpad Prism 10.1.2) Created with BioRender.com

stage, they were also found to be inhibitors of ABCG2, albeit less potent. In line with previous studies, our in vitro data confirm that the in vitro molar-potency of both inhibitors is roughly 10-fold stronger for ABCB1 than for ABCG2.

Studies comparing drug levels in brain homogenates and plasma from *Abcg2;Abcb1a/b* KO mice vs. WT mice have demonstrated that the brain distribution of many drugs can be 10- to even 100-fold higher when both transporters are absent [6–9, 13]. Likewise, enhancement of drug distribution has been observed in WT mice receiving concomitant elacridar or tariquidar, albeit that tariquidar has been used less frequently in mice. Notably, however, tariquidar is predominantly used for pharmacologic inhibition of drug transport at the BBB in human studies using PET tracers, such as [^{11}C]verapamil [34, 35] and [^{11}C]desmethyl loperamide [17, 36]. Although the brain distribution of these PET probes increases when

tariquidar is also administered, the effect size of the enhancement in humans generally appears smaller than in mice.

Several factors may contribute to this apparent discrepancy between pre-clinical and clinical outcome. One likely contributing factor pertains to the technical differences between these methodologies. The sampling of brain tissue and subsequent analyses by selective (LC-MS/MS based) analytical methods allows the accurate quantification of very low levels. The B/P ratio of a compound with an extremely poor brain distribution can be as low as 0.02 (e.g. vemurafenib), effectively reflecting the quantity of drug present inside the vessel lumen of the brain specimen as was previously assessed by [^{14}C] inulin and [^{14}C]sucrose distribution [37]. When the brain distribution of such a drug in a KO mice increases to 1, this would imply a 50-fold increase. In case of PET, such ultralow brain-to-blood values are never reported, most likely because the sensitivity to detect such low signals above the background following radiotracer injection is inadequate. Illustrative examples of this phenomenon can be extracted from PET studies that have been conducted in rats and mice. For example, the brain uptake of [^{11}C] sorafenib using PET was 3-fold higher in *Abcg2;Abcb1a/b* KO mice vs. WT mice [38], but 9.3 fold higher when using LC-MS/MS on brain homogenates [39]. An even more extreme example is gefitinib, reported to have a 70-fold higher brain uptake in *Abcg2;Abcb1a/b* KO mice [37]. Based on PET studies with [^{11}C]gefitinib, the brain uptake was 8-fold higher [40] and only 2-fold higher with [^{18}F]gefitinib [15]. Interestingly, this latter study with [^{18}F]gefitinib also included validation cohorts receiving [^{14}C]gefitinib to measure radioactivity in homogenates. This resulted in an 8-fold difference between *Abcg2;Abcb1a/b* KO mice vs. WT mice, being more in line with [^{11}C]gefitinib [40], but still considerably different from LC-MS/MS results [37]. These differences are likely due to the fact that the unchanged tracer is just a fraction of the total radioactivity in the sample.

These examples demonstrate that the metabolic stability of the PET tracer is important as [^{11}C]-labeled degradation products may increase the background signal. Many PET studies in humans have been conducted with [^{11}C]verapamil [19, 35, 41]. In mouse studies [^{11}C]verapamil increased about 6-fold from 1.3 in WT to 7.9 in *Abcg2;Abcb1a/b* KO mice [16], whereas in this LC-MS/MS based study, we observed an 17-fold increase (0.33 to 5.9). In rats, unchanged verapamil contributed to 48% of the radioactivity in the brain at 60 min after dosing [42]. Next to *N*-dealkylation (yielding [^{11}C]D-617 and D-717) and *O*-demethylation (into D-702 and D-703), N-demethylation to [^{11}C]formaldehyde occurs. This latter low molecular weight gaseous compound may distribute relatively well into the brain [43, 44], thus enhancing background signals.

In the earliest human PET studies with [^{11}C]verapamil, tariquidar was administered at a 2 mg/kg dose by a 30 min i.v. infusion and this resulted in a maximum 2-fold increased brain radioactivity for just the duration of the infusion [41]. Later studies applied higher dose levels (4–8 mg/kg) and used extended infusion times (1.5–2.5 h) [19, 35]. In the latter case, the B/P ratio of radioactivity at steady-state ($V_{T, brain}$) increased from 0.72 to 2,63 (3.5-fold), which was considered to reflect nearly complete inhibition of ABCB1. The plasma level of tariquidar at the end of the infusion reached about 3000 to 4000 nM. Notably, plasma concentrations below 4000 nM were still insufficient for complete inhibition of verapamil efflux in our study (Fig. 2) as well as in a study in rats [45]. The fact that the fold-change in brain uptake of an ABCB1 substrate, like verapamil, in humans is less than achieved in mice is therefore likely because (1) of a lower sensitivity to detect low signals above the background following radiotracer injection and/or (2) because complete inhibition of ABCB1 at the BBB in humans was still not achieved with tariquidar. For practical reasons PET remains the most obvious choice to assess inhibition of drug transport at the human BBB, however, we need to keep in mind that PET may underestimate the fold change difference.

In contrast to elacridar, tariquidar was not able to inhibit its own ABCG2-mediated efflux from the brain in our study. This result fits with earlier work from Bankstahl et al. [46] showing that a high dose of cold tariquidar (15 mg/kg) given i.v. did increase the brain uptake of a tracer dose of [^{11}C]-tariquidar in *Abcg2*-KO mice, but not in *Abcg2* proficient mice. The ability of elacridar to inhibit its own ABCG2-mediated efflux already at about 1200 nM plasma levels may be an important reasons why elacridar is also more potent to inhibit the efflux of other ABCG2 substrates. In fact, tariquidar was ineffective in increasing the brain accumulation of any of the tested ABCG2 substrate drugs in this series.

Elacridar has been formulated for oral dosing but the standard formulation suffers from a poor and nonlinear oral bioavailability [23], hence an improved oral formulation will be required for successful application as a pharmacokinetic-enhancer of brain penetration. In this study, we addressed the pivotal question about the required plasma level needed to enhance brain entry and propose that 1200 nM (about 700 ng/ml) as target plasma level. Such plasma levels were not yet reached in a recent PET study with [^{11}C]erlotinib in patients receiving a novel elacridar solid dispersion formulation. This may be one of the reasons why the effect on the brain distribution of [^{11}C]erlotinib was insignificant in this study [47]. Moreover, the fold-change increase in brain distribution of

erlotinib when both ABCB1 and ABCG2 are absent is very modest, which may make it more challenging to find any differences using PET. A further reason might be that expression of ABCG2 appears to be higher at the human BBB than at the mouse BBB [48]. Hence, our study demonstrate that stronger ABCG2 substrate drugs (e.g. afatinib) are less appropriate candidates for pharmacokinetic-enhanced brain entry by elacridar.

Typically, the preference is for free drug levels over total drug levels when evaluating drug accumulation in the brain [32]. However, this approach wasn't employed in our study, as our primary aim was to gauge the effect size of drug accumulation between ABC transporter proficient and deficient animals. Given these conditions, we anticipate that free drug levels in plasma and brain tissue would remain consistent across the mouse genotypes used in this series. Moreover, we also show that the assessment of free drug levels using widely applied tools such as the RED device is impossible in case of elacridar and tariquidar as equilibrium cannot be achieved. Although this indicates that elacridar and tariquidar have a very high plasma protein binding, and thus very low free drug levels according to current assays, our results show they are able to inhibit ABCB1-mediated efflux at the BBB at clinically meaningful total plasma concentrations. Elacridar is also able to increase the brain penetration of weak to moderate ABCG2 substrates. Fortunately, the plasma protein binding of elacridar and tariquidar in human plasma appears to be similar as in mouse plasma. This, together with the knowledge that tariquidar can increase the brain penetration of specific ABCB1 substrates (e.g. verapamil) in humans, supports the translatability of these preclinical data to humans.

The objective of this work is to devise the conditions needed to enhance the brain distribution of therapeutic agents and with that hopefully improve the therapy of brain diseases. Obviously, enhancing brain drug distribution by using inhibitors of ABCB1 and/or ABCG2 also holds the risk of inducing neurotoxicity or increase the gravity of side effects in other peripheral organs. This cannot be discerned from this study, where we applied low sub-toxic, yet likely also sub-therapeutic dose levels. Safety of such combinations need to be established in well-designed follow-up (pre-)clinical studies.

In summary, elacridar emerges as a significantly more potent inhibitor of both ABCB1 and ABCG2 compared to tariquidar. This suggests that the effects observed in humans with tariquidar can likely be surpassed by using elacridar. Elacridar's superior inhibition of ABCG2 at the BBB expands the range of potential candidate drugs for treating diseases of the brain. For example, our data strongly suggest that elacridar may boost the brain penetration of palbociclib by at least 30-fold, potentially making this CDK4/6 inhibitor a more useful drug for treating glioblastoma.

Abbreviations

ABC	ATP-Binding Cassette
AUC	area under the curve
BBB	blood-brain barrier
BCRP	breast cancer resistance protein
B/P	brain-to-plasma
DMSO	dimethyl sulfoxide
EC_{50}	50% effective inhibitory concentration
FCS	fetal calf serum
FVB	Friends virus B
IC_{50}	50% inhibitory concentration
IS	internal standard
i.p.	intraperitoneal
i.v.	intravenous
KO	knockout
LC-MS/MS	liquid chromatography- tandem mass spectrometry
MDCK	Madine Darby Canine Kidney
MEM	Minimum Essential Medium
PET	positron emission tomography
PBS	phosphate buffered saline
P-gp	P-glycoprotein
RED	rapid equilibrium dialysis
s.c.	subcutaneous
WT	wildtype

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Author contributions
AL, SZ, AM, CHC, NV, OT conducted (animal) experiments and analyzed samples; AL, MG, AM, OT interpreted the data; AL, MG, JHB and OT wrote the manuscript; MCG and OT conceptualized the study setup, OT supervised all aspects. All authors read and approved the final manuscript.

Data availability
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval
The animal experiments were in accordance with 2010/63/EU and conducted under license AVD301002016595 and protocol 2.5.8776.

Consent for publication
Not Applicable.

Competing interests
OT is co-inventor on a patent application (Bunt and Van Tellingen, 2014) dealing with development of an improved oral formulation for elacridar. All other authors declare no competing interests.

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