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Brain Accumulation of Ponatinib and Its Active Metabolite, *N*-Desmethyl Ponatinib, Is Limited by P-Glycoprotein (P-GP/ABCB1) and Breast Cancer Resistance Protein (BCRP/ABCG2)

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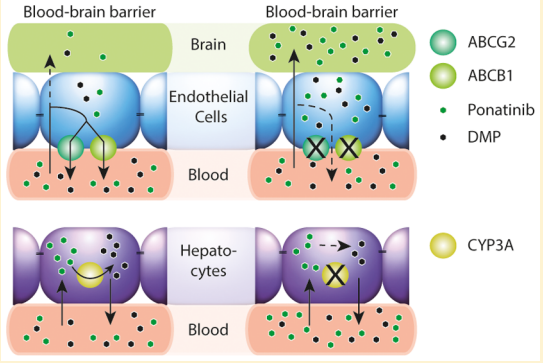
Supporting Information

ABSTRACT: Ponatinib is an oral BCR-ABL1 inhibitor for treatment of advanced leukemic diseases that carry the Philadelphia chromosome, specifically containing the T315I mutation yielding resistance to previously approved BCR-ABL1 inhibitors. Using *in vitro* transport assays and knockout mouse models, we investigated whether the multidrug efflux transporters ABCB1 and ABCG2 transport ponatinib and whether they, or the drug-metabolizing enzyme CYP3A, affect the oral availability and brain accumulation of ponatinib and its active *N*-desmethyl metabolite (DMP). *In vitro*, mouse Abcg2 and human ABCB1 modestly transported ponatinib. In mice, both Abcb1 and Abcg2 markedly restricted brain accumulation of ponatinib and DMP, but not ponatinib oral availability. Abcg2 deficiency increased DMP plasma levels ~3-fold. Cyp3a deficiency increased the ponatinib plasma AUC 1.4-fold. Our results suggest that pharmacological inhibition of ABCG2 and ABCB1 during ponatinib therapy might benefit patients with brain (micro)metastases positioned behind an intact blood-brain barrier, or with substantial expression of these transporters in the malignant cells. CYP3A inhibitors might increase ponatinib oral availability, enhancing efficacy but possibly also toxicity of this drug.



KEYWORDS: *ponatinib, ABCB1, ABCG2, CYP3A metabolism, brain accumulation*

INTRODUCTION

Ponatinib (Iclusig, previously AP24524) is a third-generation oral drug for treatment of chronic myeloid leukemia (CML) and Philadelphia chromosome positive (Ph+) acute lymphoblastic leukemia (ALL).[1,2] It was initially FDA approved in December 2012 and after a temporary suspension due to life-threatening risks, it was reapproved in December 2013.[3,4] Ponatinib is a tyrosine kinase inhibitor (TKI) designed to bind and inhibit BCR-ABL1 and especially its most prominent mutant form, T315I, which confers resistance to other tyrosine kinase inhibitors, such as dasatinib, imatinib, nilotinib, and bosutinib.[5] Ponatinib also inhibits some other kinases important for the pathogenesis of other malignancies, such as vascular endothelial growth factor receptors (VEGFR 1 and 2), platelet-derived growth factor receptor (PDGFR), fibroblast growth factor receptors 1−4 (FGFR1−4), RET, c-KIT, MEKK2, FLT3, RIPK1, and RIPK3.[5−11] This makes ponatinib a promising and interesting therapeutic drug.

The plasma protein binding of ponatinib is high (99.9%) and its half-life ($t_{1/2}$) is about 24 h in humans.[1] The drug is mainly excreted via feces after phase I and II biotransformation. In humans CYP3A4 is the predominant P450 isoenzyme involved in the microsomal biotransformation of TKIs, such as imatinib, nilotinib, bosutinib, dasatinib, and also ponatinib.[1,12−15] The main ponatinib metabolite, however, is the inactive carboxylic acid AP24600, formed by esterase- and/or amidase-mediated hydrolysis.[1,16] The pharmacodynamically active *N*-desmethyl (DMP, AP24567) and (minor) *N*-oxide (AP24734) metabolites are additionally formed by cytochrome P450 (mainly CYP3A4)-mediated biotransformation, as schematically shown in Figure S1.[1,17,18] DMP has a 4-fold reduced potency

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compared to ponatinib,[2] and its abundance in patients appears to be modest, with 8.3% of the ponatinib dose retrieved as DMP from feces.[18]

Multidrug efflux transporters of the ATP-binding cassette (ABC) protein family affect the disposition of a wide variety of endogenous and exogenous compounds, including numerous anticancer drugs. ABCB1 (P-glycoprotein) and ABCG2 (BCRP) are expressed in the apical membrane of epithelia in a number of organs which are essential for absorption and elimination of drugs like liver, small intestine, and kidney. They are also found in luminal membranes of barriers protecting sanctuary tissues like the blood-placenta, blood-testis, and blood−brain barriers (BBB). At these barriers ABCB1 and ABCG2 substrates are immediately pumped out of the epithelial or endothelial cells back into the blood. As a consequence, only small amounts of drug can accumulate in, for instance, the brain. This can compromise treatment of (micro)metastases that are present behind a functionally intact BBB.[19−21] Many anticancer drugs including TKIs are transported substrates of ABCG2 or ABCB1 or both. As a result, these transporters can significantly modulate the pharmacokinetic behavior, and hence the therapeutic efficacy and toxicity profile of these drugs.[22]

Some studies indicated that ponatinib could modestly interact with hABCB1 and more profoundly with hABCG2 *in vitro*, resulting in inhibition of these transporters, and possibly transport by hABCG2.[23] However, another study found that ponatinib was not noticeably transported by ABCB1 or ABCG2 in CML cells.[24] If these transporters could efficiently transport ponatinib *in vivo*, this might lead to decreased accumulation of ponatinib in transporter-expressing cancer cells, and thus pharmacotherapeutic resistance. Moreover, leukemic cells can spread outside the blood to other parts of the body, including the central nervous system (brain and spinal cord). About 7% of adult ALL patients have central nervous system (CNS) involvement at presentation.[25,26] Given the high ABCB1 and ABCG2 expression in the BBB, these transporters could potentially limit brain accumulation of ponatinib, which might reduce therapeutic efficiency against CML and ALL CNS metastases.

In this study we investigated whether ponatinib and DMP are transported substrates of ABCB1 and ABCG2 *in vitro* or *in vivo*, and how this might affect their oral plasma pharmacokinetics and brain penetration. Furthermore, ponatinib is substantially metabolized by cytochrome P450 (CYP3A4),[1,27] indicating that induction or inhibition of this enzyme may influence ponatinib exposure. We therefore also studied the influence of CYP3A on the (oral) systemic availability and tissue exposure of ponatinib and DMP.

MATERIALS AND METHODS

Chemicals. Ponatinib and zosuquidar were obtained from Sequoia Research Products (Pangbourne, UK). Ko143 was obtained from Tocris Bioscience (Bristol, UK). Isoflurane was purchased from Pharmachemie BV (Haarlem, The Netherlands), heparin (5000 IU ml^{-1}) was from Leo Pharma (Breda, The Netherlands), and Bovine Serum Albumin (BSA) Fraction V from Roche (Mannheim, Germany). Chemicals and reference standards used for the bioanalytical assay of ponatinib and its metabolite DMP were described previously.[28] All other chemicals and reagents were obtained from Sigma-Aldrich (Steinheim, Germany).

Transport Assays. Polarized Madin-Darby Canine Kidney (MDCK-II) cell lines transduced with either human (h)-ABCB1, murine (m)Abcg2, or hABCG2 cDNA were cultured in DMEM plus 10% FCS and 100 U/ml penicillin, 100 μg/mL streptomycin (complete medium), and used as described previously.[29] Transepithelial transport assays were performed in complete medium, but without antibiotics, in triplicate on 12-well microporous polycarbonate membrane filters (3.0-μm pore size, Transwell 3402, Corning Inc., Lowell, MA) as previously described.[29] In short, cells were allowed to grow an intact monolayer in 3 days, which was monitored with transepithelial electrical resistance (TEER) measurements. On the third day, cells were preincubated with the relevant inhibitors for 1 h, where 5 μM zosuquidar (ABCB1 inhibitor) and/or 5 μM Ko143 (ABCG2/Abcg2 inhibitor) were added to both apical and basolateral compartments. To inhibit endogenous canine ABCB1 in the MDCK-II Abcg2 and MDCK-II ABCG2 cell lines, we added 5 μM zosuquidar (ABCB1 inhibitor) to the culture medium throughout the experiment. The experiment was initiated by replacing the incubation medium from the donor compartment with freshly prepared medium containing 5 μM ponatinib alone or in combination with the appropriate inhibitors. At 4 and 8 h, 50 μL samples were collected from the acceptor compartment and stored at −30 °C until analysis. TEER was rechecked at the end of the transport experiment to confirm continuing intactness of the monolayer. The amount of transported drug was calculated after correction for volume loss due to sampling at each time point. Active transport was expressed by the transport ratio (r), which is defined as the amount of apically directed transport divided by the amount of basolaterally directed transport at a defined time point. Continual testing of transport of a range of other drugs (such as ceritinib and afatinib) and other compounds confirmed proper activity of hABCB1, mAbcg2, and hABCG2 in the MDCKII cell lines used.

Animals. Female wild-type, *Abcb1a/1b*$^{-/-}$,[30] *Abcg2*$^{-/-}$,[31] *Abcb1a/1b;Abcg2*$^{-/-}$,[32] and *Cyp3a*$^{-/-}$ mice,[33] all of a > 99% FVB genetic background, were used. Mice between 9 and 14 weeks of age were used in groups of 3−5 mice per strain. The mice were kept in a temperature-controlled environment with a 12 h light/dark cycle and received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*. Animals were housed and handled according to institutional guidelines in compliance with Dutch and EU legislation.

Drug Solutions. Ponatinib stock solution was prepared in DMSO at 20 mg/mL and stock aliquots were frozen at −80 °C. All working solutions were prepared freshly on the day of the experiment by allowing an aliquot to thaw and subsequent dilution of the aliquot 20-fold in 25 mM sodium citrate buffer in water (pH 2.75) resulting in a 1 mg/mL solution. This solution was immediately protected from light and vigorously shaken for 1 min to obtain a homogeneous and clear solution. Ponatinib was administered orally at a dose of 10 mg/kg (10 μL/g).

Plasma and Tissue Pharmacokinetics of Ponatinib. To minimize variation in absorption, mice were fasted for 2 h prior to oral administration of ponatinib, using a blunt-ended needle. Fifty μL blood samples were drawn from the tail vein using heparin-coated capillaries (Sarstedt, Germany). At the last time point mice were anesthetized using isoflurane inhalation and blood was collected via cardiac puncture. For the 24-h experiment, tail vein sampling took place at 0.5, 1, 2, 4, and

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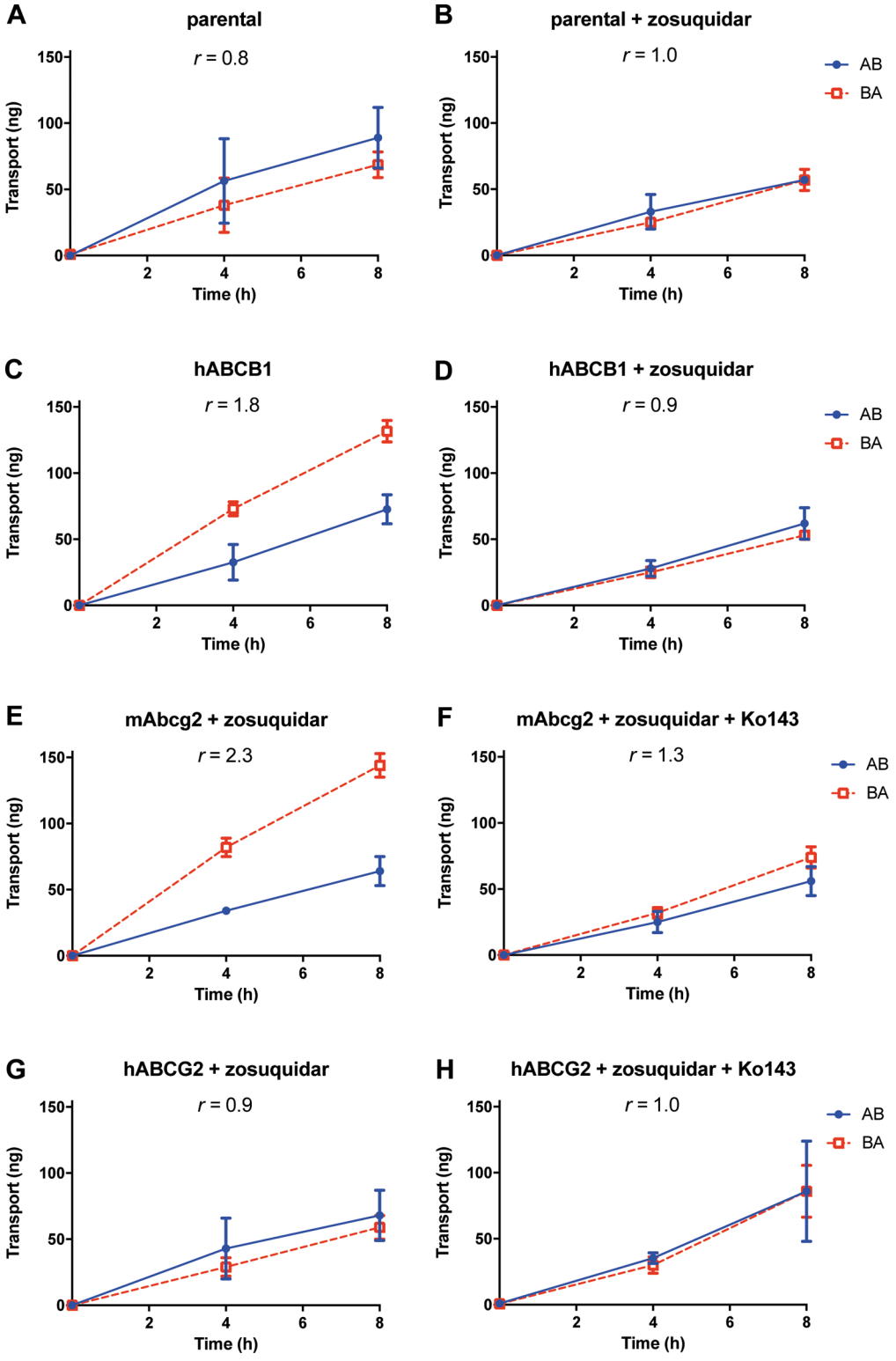

Figure 1. *In vitro* transport of ponatinib. Transepithelial transport of ponatinib (5 μM) was assessed in MDCK-II cells either nontransduced (A, B) or transduced with hABCB1 (C, D), mAbcg2 (E, F), or hABCG2 (G, H) cDNA. At t = 0 h ponatinib was added to the donor compartment; thereafter at t = 4 and 8 h the concentrations were measured and plotted as total amount (ng) of transport (n = 3). (B, D−H) Zosuquidar (5 μM) or Ko143 (5 μM) were added as indicated to inhibit hABCB1 or hABCG2 and mAbcg2, respectively. r, relative transport ratio. BA (red squares, dashed line) translocation from the basolateral to the apical compartment; AB (blue circles), translocation from the apical to the basolateral compartment. Data are presented as mean ± SD. The curves were forced through the origin, assuming 0 transport at t = 0.

8 h after oral administration. For the 2-h experiment, tail vein sampling took place at 0.25, 0.5, and 1 h after oral administration. At 24 or 2 h, respectively, mice were sacrificed by cervical dislocation and a set of organs was rapidly removed, weighed and subsequently frozen as whole organ at −30 °C. Prior to analysis, organs were allowed to thaw and

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homogenized in appropriate volumes of 4% (w/v) BSA in water using a FastPrep-24 device (MP Biomedicals, SA, California, USA). Homogenates were stored at −30 °C. Blood samples were immediately centrifuged at 2100 g for 6 min at 4 °C, and plasma was collected and stored at −30 °C until analysis. Ponatinib concentrations in brain tissue were corrected for the presence of plasma in the vascular space (1.4%).[34]

Drug Analysis. Ponatinib and *N*-desmethyl ponatinib (DMP) concentrations in culture medium, plasma, and tissue homogenates were analyzed with a previously reported liquid-chromatography tandem mass spectrometric (LC-MS/MS) assay, using deuterated internal standards.[28] Briefly, plasma and tissue homogenate samples were pretreated using liquid−liquid extraction with *tert*-butylmethyl ether, evaporated, and reconstituted before separation by reversed-phase liquid chromatography under alkaline conditions. The calibration curves of the linear assay ranged from 5 to 5000 ng/mL for ponatinib and 1−1000 ng/mL for DMP. Culture medium samples were pretreated using protein precipitation with acetonitrile and diluted before chromatographic injection. Ponatinib was quantified in the range 5 to 5000 ng/mL.

Statistics and Pharmacokinetic Calculations. The unpaired two-tailed Student's *t* test was used to determine significance in the transepithelial transport assays. The area under the curve (AUC) of the plasma concentration−time curve was calculated using the trapezoidal rule, without extrapolating to infinity. Individual concentration−time data were used to determine the peak plasma concentration (C_{max}) and the time to reach C_{max} (T_{max}). Relative organ accumulation (P_{organ}) was calculated by dividing organ concentrations (C_{organ}) at either t = 2 h or t = 24 h by the area under the plasma concentration−time curve from 0−2 h (AUC_{0-2h}) or 0−24 h (AUC_{0-24h}), respectively. Ordinary one-way analysis of variance (ANOVA) was used to determine significant differences between groups. Posthoc Tukey's multiple comparisons were used to compare significant differences between individual groups. When variances were not homogeneously distributed, data were log-transformed before applying statistical tests. Differences were considered statistically significant when $P < 0.05$. Data are presented as mean ± SD with each experimental group containing 3−5 mice.

RESULTS

Ponatinib Is Moderately Transported by hABCB1 and mAbcg2 *in Vitro*. We analyzed ponatinib (5 μM) transport across polarized monolayers of MDCKII cell lines stably transduced with hABCB1, mAbcg2, or hABCG2 cDNAs. The parental MDCKII cell line did not show apically directed transport (Figure 1A, r = 0.8) and addition of the potent ABCB1 inhibitor zosuquidar did not change this result (Figure 1B, r = 1.0). This indicates that endogenously present canine ABCB1 did not noticeably transport ponatinib in the parental cells. In the hABCB1 subclone, however, we observed modest apically directed transport of ponatinib (Figure 1C, r = 1.8), which was effectively inhibited by addition of zosuquidar (Figure 1D, r = 0.9). In MDCKII cells overexpressing mAbcg2 and hABCG2, we performed the transport assay in the presence of zosuquidar to exclude any transport contribution of endogenous canine ABCB1. We observed clear apically directed transport of ponatinib in mAbcg2 cells (r = 2.3), which was largely inhibited by the ABCG2 inhibitor Ko143 (Figure 1E and F). This indicates that ponatinib was moderately transported by mAbcg2. In the hABCG2 subclone we did not detect active transport of ponatinib and addition of Ko143 did not alter this profile (respective r = 0.9 and 1.0, Figure 1G and H). However, we note that the MDCKII-hABCG2 cell line consistently yields (much) lower transport rates for many drugs compared to the MDCKII-mAbcg2 line, which may relate to difficulty in expressing a high amount of hABCG2 in these cells.[35,36] These data demonstrate that ponatinib is moderately transported by hABCB1 and mAbcg2. Therefore, both transporters might play a role in the oral availability and brain penetration of ponatinib.

Availability of Oral Ponatinib Is Restricted by Cyp3a, but not by mAbcg2 or mAbcb1a/1b. Ponatinib is given orally to patients. We therefore investigated the single and combined effect of mAbcg2 and mAbcb1a/1b deficiency on ponatinib (10 mg/kg) oral availability and tissue disposition over 24 h in wild-type (WT), *Abcg2*$^{-/-}$, *Abcb1a/1b*$^{-/-}$, and *Abcb1a/1b;Abcg2*$^{-/-}$ mice (Figure 2A). We further investigated

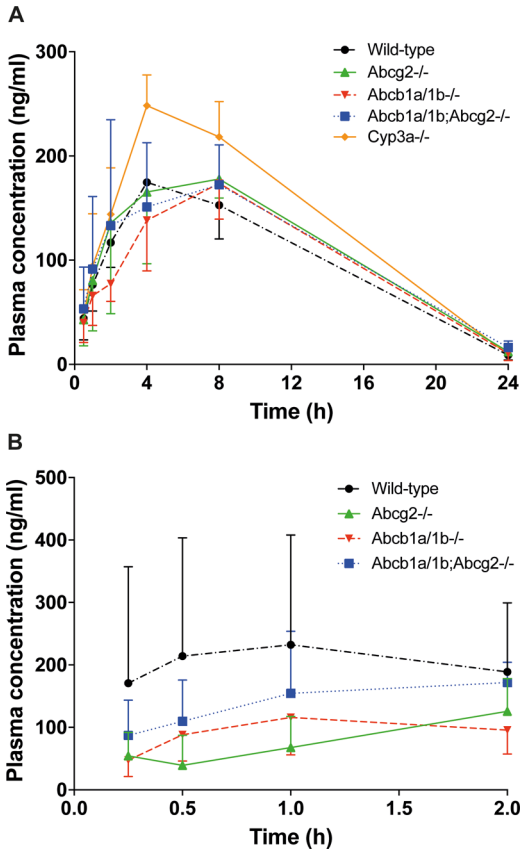

Figure 2. Plasma concentration−time curves of ponatinib in female WT (black circles), *Abcg2*$^{-/-}$ (green triangles), *Abcb1a/1b*$^{-/-}$ (red triangles), *Abcg2;Abcb1a/1b*$^{-/-}$ (blue squares), and *Cyp3a*$^{-/-}$ mice (yellow diamonds) over 24 h (A) or 2 h (B) after oral administration of 10 mg/kg ponatinib. Data are given as mean ± SD (SD rendered one-sided for improved clarity). n = 4−5 mice per group.

the impact of CYP3A-mediated metabolism on ponatinib kinetics using Cyp3a$^{-/-}$ mice, as the active ponatinib metabolite DMP is primarily formed by CYP3A4.[1,16] Neither single nor combined absence of Abcg2 and/or Abcb1a/1b resulted in a significant change in the oral availability of ponatinib during the first 24 h after oral intake (Figure 2A, Table 1). Absence of Cyp3a, however, did result in a significant 1.4-fold increase in plasma AUC_{0-24} relative to wild-type mice

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Table 1. Pharmacokinetic Parameters of Ponatinib and DMP at 24 h after Oral Administration of 10 mg/kg Ponatinib to Female Wild-Type, *Abcg2*$^{-/-}$, *Abcb1a/1b*$^{-/-}$, *Abcb1a/1b;Abcg2*$^{-/-}$, and *Cyp3a*$^{-/-}$ Mice[a]

			genotype			
parameter	compound	wild-type	*Abcg2* $^{-/-}$	*Abcb1a/1b* $^{-/-}$	*Abcb1a/1b;Abcg2* $^{-/-}$	*Cyp3a* $^{-/-}$
plasma $AUC_{(0-24)}$, h·ng/mL	ponatinib	2378 ± 293	2660 ± 383	2421 ± 395	2602 ± 188	3312 ± 486[c]
fold increase $AUC_{(0-24)}$		1.0	1.1	1.0	1.1	1.4
C_{max}, ng/mL		181 ± 26	201 ± 42	138 ± 49	210 ± 56	251 ± 69
T_{max}, h		4–8	2–8	4–8	2–8	4–8
C_{brain}, ng/g		22 ± 8	49 ± 7[c]	42 ± 19[b]	558 ± 109[d]	21 ± 4
fold increase C_{brain}		1.0	2.2	1.9	25.5	1.0
P_{brain} ($\times 10^{-3}$ h^{-1})		9.0 ± 2.5	19 ± 2.9[c]	17 ± 6.6[b]	215 ± 46[d]	6.9 ± 1.3
fold increase P_{brain}		1.0	2.2	1.9	24	0.8
plasma $AUC_{(0-24)}$, h·ng/mL	DMP	139 ± 15	445 ± 116[d]	199 ± 31	544 ± 85[d]	163 ± 44
fold increase $AUC_{(0-24)}$		1.0	3.2	1.4	3.9	1.2
C_{max}, ng/mL		11 ± 1.0	32 ± 7.4	13 ± 5.7	37 ± 6.8	13 ± 5.5
T_{max}, h		4–8	4–8	4–8	4–8	4–8
C_{brain}, ng/g		6.1 ± 2.8	13 ± 3.5[c]	10 ± 2.7	354 ± 26[d]	6.3 ± 1.6
fold increase C_{brain}		1.0	2.1	1.6	58	1.0
P_{brain} ($\times 10^{-3}$ h^{-1})		43 ± 18	29 ± 6.6[b]	51 ± 10	659 ± 83[d]	44 ± 5.8
fold increase P_{brain}		1.0	0.7	1.2	15	1.0

[a]AUC, area under the plasma concentration−time curve; C_{max} maximum concentration in plasma; T_{max}, the time after drug administration needed to reach maximum plasma concentration; C_{brain}, brain concentration; P_{brain}, brain accumulation (C_{brain} divided by plasma AUC); C_{liver}, liver concentration; P_{liver}, liver accumulation (C_{liver} divided by plasma AUC). [b], $P < 0.05$; [c], $P < 0.01$; [d], $P < 0.001$ compared to WT mice. Data are given as mean ± SD. All groups consisted of $n = 4–5$ mice.

Table 2. Pharmacokinetic Parameters of Ponatinib and DMP at 2 h after Oral Administration of 10 mg/kg Ponatinib to Female Wild-Type, *Abcg2*$^{-/-}$, *Abcb1a/1b*$^{-/-}$, and *Abcb1a/1b;Abcg2*$^{-/-}$ Mice[a]

			genotype		
parameter	compound	wild-type	*Abcg2* $^{-/-}$	*Abcb1a/1b* $^{-/-}$	*Abcb1a/1b;Abcg2* $^{-/-}$
plasma $AUC_{(0-2)}$, h·ng/mL	ponatinib	392 ± 303	142 ± 66	180 ± 85	265 ± 155
fold increase $AUC_{(0-2)}$		1.0	0.4	0.5	0.7
C_{brain}, ng/g		258 ± 195	142 ± 63	247 ± 163	2896 ± 588[d]
fold increase C_{brain}		1.0	0.5	1.0	11
P_{brain} (h^{-1})		0.68 ± 0.01	1.05 ± 0.28	1.28 ± 0.29[c]	12.3 ± 2.7[d]
fold increase P_{brain}		1.0	1.5	1.9	18
plasma $AUC_{(0-2)}$, h·ng/mL	DMP	21 ± 11	13 ± 7	20 ± 11	39 ± 22
fold increase $AUC_{(0-2)}$		1.0	0.6	0.9	1.9
C_{brain}, ng/g		7.2 ± 2.0	2.7 ± 0.5[b]	5.3 ± 3.3	55 ± 17[d]
fold increase C_{brain}		1.0	0.4	0.7	7.6
P_{brain} (h^{-1})		0.39 ± 0.15	0.23 ± 0.83	0.27 ± 0.58	1.51 ± 0.31[d]
fold increase P_{brain}		1.0	0.6	0.7	3.9

[a]AUC, area under the plasma concentration−time curve; C_{max} maximum concentration in plasma; T_{max}, the time after drug administration needed to reach maximum plasma concentration; C_{brain}, brain concentration; P_{brain}, brain accumulation (C_{brain} divided by plasma AUC); C_{liver}, liver concentration; P_{liver}, liver accumulation (C_{liver} divided by plasma AUC). [b], $P < 0.05$; [c], $P < 0.01$; [d], $P < 0.001$ compared to WT mice. Data are given as mean ± SD. All groups consisted of $n = 3–5$ mice

($P = 0.0026$) and *Abcb1a/1b*$^{-/-}$;*Abcg2*$^{-/-}$ mice ($P = 0.0168$) (Figure 2A, Table 1).

For the transporter knockout mice, qualitatively similar results were obtained in an independent experiment where mice were sacrificed 2 h after oral administration of 10 mg/kg ponatinib (Figure 2B, Table 2). There were no significant differences in plasma ponatinib levels between the mouse strains, although it is worth noting that there was high interindividual variation during the first 2 to 4 h after administration in all mouse strains (see also Figure 2A), possibly due to variable rates of stomach emptying for this drug. Collectively, our data indicate that neither Abcg2 nor Abcb1a/1b has a direct impact on the oral availability of ponatinib in mice, but that Cyp3a does limit ponatinib oral availability.

Abcg2 and Abcb1a/1b, but not Cyp3a, Limit Ponatinib Brain Accumulation in Mice. To investigate the impact of single and combined knockout of Abcg2 and Abcb1a/1b on ponatinib accumulation in the brain, we isolated mouse brains after termination of the 24-h and 2-h pharmacokinetic experiments. Figure 3A shows that only a low concentration of ponatinib accumulated in the brain of WT mice over 24 h after administration. Single absence of either Abcg2 or Abcb1a/1b resulted in a modestly increased brain concentration, respectively 2.2-fold ($P = 0.008$) and 1.9-fold ($P = 0.01$) relative to WT mice. However, when both ABC transporters were absent, ponatinib brain concentration was increased by a drastic 25.5-fold compared to WT brain (Figure 3A, $P < 0.0001$). Correcting for differences in plasma concentration, the brain-to-plasma ratio of *Abcb1a/*

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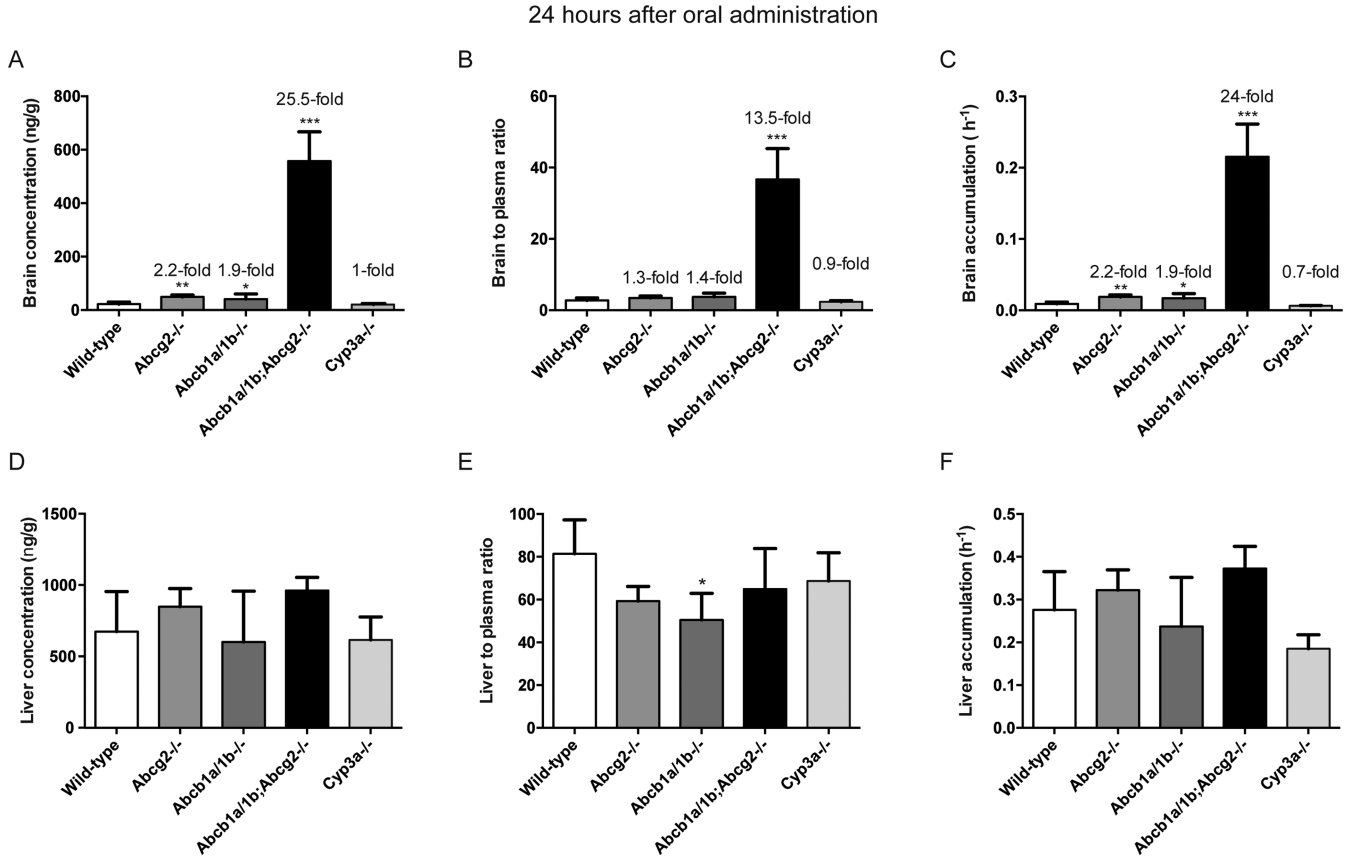

Figure 3. Brain and liver concentration (A, D), tissue-to-plasma ratio (B, E) and relative tissue accumulation (C, F) of ponatinib in female WT, *Abcg2*$^{-/-}$, *Abcb1a/1b*$^{-/-}$, and *Abcg2;Abcb1a/1b*$^{-/-}$ mice 24 h after oral administration of 10 mg/kg ponatinib. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to WT mice. Data are given as mean ± SD $n = 4–5$ mice per group.

1b;Abcg2$^{-/-}$ mice remained markedly higher compared to wild-type mice, while the single ABC transporter knockouts were also still somewhat higher, albeit not significantly (Figure 3B). Correcting for the plasma AUC, the brain accumulation of ponatinib 24 h after oral intake was again clearly and significantly increased by 24-fold in Abcb1a/1b$^{-/-}$;Abcg2$^{-/-}$ mice, and by 2.2- and 1.9-fold in the single *Abcg2*$^{-/-}$ and *Abcb1a/1b*$^{-/-}$ mice (Figure 3C). These data indicate that ponatinib is kept out of the brain by both Abcb1a/1b and Abcg2, and that these proteins are able to largely take over each other's transport function at the BBB. Only when both transporters were absent, ponatinib accumulated very substantially in the brain. At the same time, we observed no substantial impact of Abcg2 and Abcb1a/1b on the liver distribution of ponatinib (Figure 3D–F), illustrating the specific function of these transporters in the BBB.

Although the Cyp3a deficiency resulted in a modest increase in the ponatinib plasma AUC (Figure 2A, Table 1), the plasma concentration at 24 h was not significantly different from that in WT mice. The brain concentration and accumulation were also not significantly altered in *Cyp3a*$^{-/-}$ mice (Figure 3A–C). The same applied to the liver concentration and accumulation (Figure 3D–F). Collectively, these data suggest that Cyp3a did not impact brain (or liver) accumulation of ponatinib independent of its effect on the plasma concentration.

In general, the impact of transporter proteins on tissue accumulation of drugs is especially relevant when plasma concentrations are high. Mice were therefore also sacrificed 2 h after oral administration of 10 mg/kg ponatinib. Similar to the 24 h experiment, the brain concentration of ponatinib in *Abcb1a/1b;Abcg2*$^{-/-}$ mice showed a highly significant 11-fold increase ($P < 0.001$) compared to WT mice. No significant differences were found for the single knockout strains compared to WT mice (Figure 4A, Table 2). Correcting the ponatinib brain concentrations for the corresponding plasma AUCs (Figure 4C) showed a more pronounced difference of the single knockout strains compared to WT mice, especially for *Abcb1a/1b*$^{-/-}$ mice with a significant 1.9-fold increase. However, combined transporter deficiency resulted in an 18-fold increase in brain accumulation (Figure 4C). Thus, also at 2 h, both Abcg2 and Abcb1a/1b alone could still quite effectively restrict the brain accumulation of ponatinib, so that only the combination transporter knockout demonstrated a highly increased brain penetration of ponatinib. Similar to the 24 h experiment, we observed no substantial impact of Abcb1a/1b and/or Abcg2 deficiency on the liver distribution of ponatinib (Figure 4D–F). Accordingly, plotting of the brain-to-liver ratios of ponatinib at both 2 and 24 h resulted in qualitatively similar results, indicating a strong effect of the combination knockout on relative brain accumulation of ponatinib (Figure S2).

Effect of Cyp3a and Abcg2 and Abcb1a/1b on *N*-Desmethyl Ponatinib Disposition and Brain Accumulation. The ponatinib metabolite DMP is pharmacodynamically active, and can thus contribute to ponatinib treatment response. We therefore tested to what extent DMP pharmacokinetics was affected by Cyp3a and/or ABC transporter deficiency in mice. Conversion of ponatinib to DMP in

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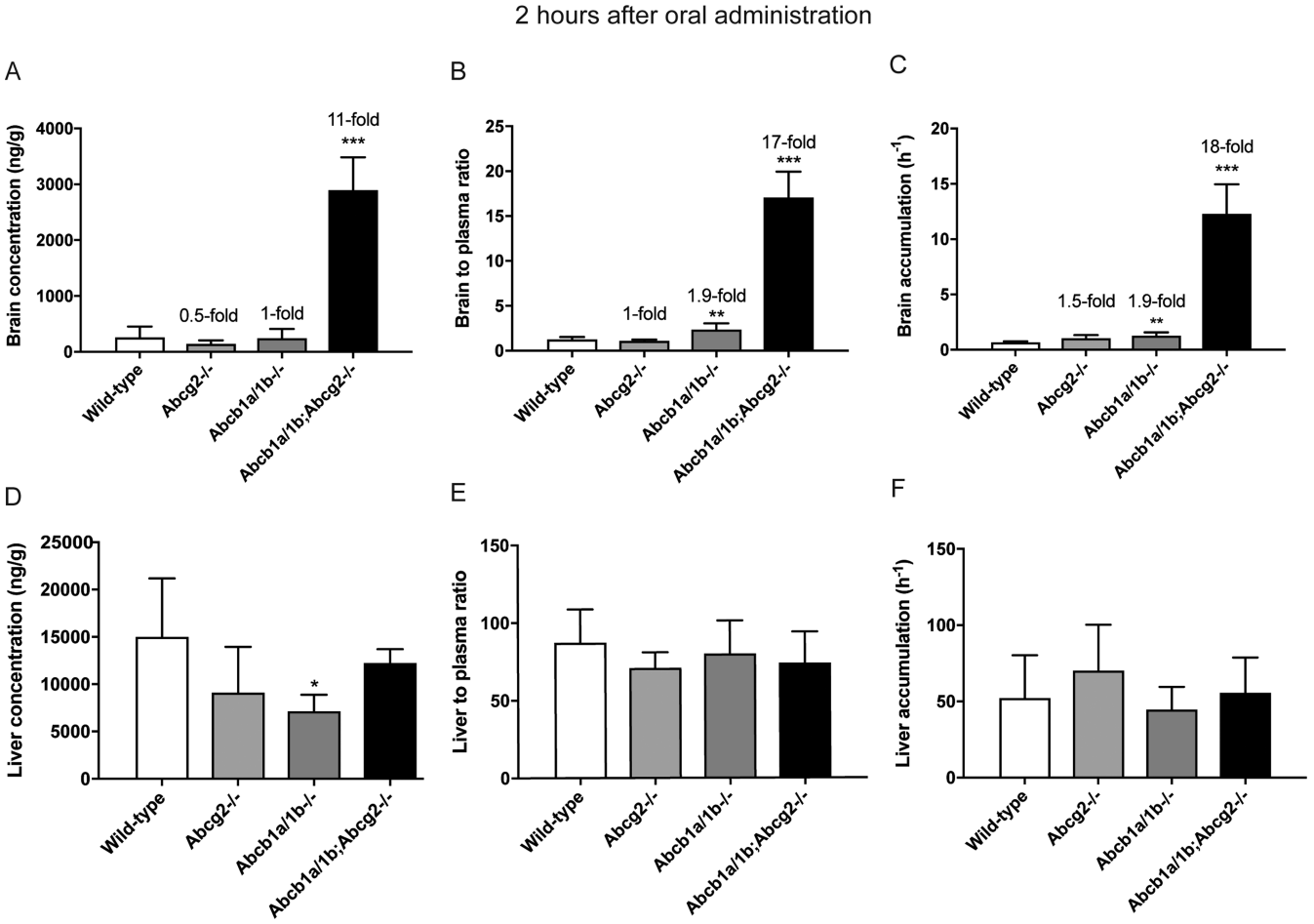

Figure 4. Brain and liver concentration (A, D), tissue-to-plasma ratio (B, E) and relative tissue accumulation (C, F) of ponatinib in female WT, *Abcg2*$^{-/-}$, *Abcb1a/1b*$^{-/-}$, and *Abcg2;Abcb1a/1b*$^{-/-}$ mice 2 h after oral administration of 10 mg/kg ponatinib. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to WT mice. Data are given as mean ± SD $n = 3–5$ mice per group.

humans is thought to be mediated primarily by CYP3A.[1] Indeed, the plasma AUC of ponatinib was modestly (1.4-fold) increased in *Cyp3a*$^{-/-}$ mice (Figure 2A, Table 1), but the plasma concentrations or AUC_{0-24} of DMP were not significantly decreased in *Cyp3a*$^{-/-}$ mice (Figure 5A, Table 1). However, the plasma DMP/ponatinib ratio was decreased, albeit not significantly, for most time points up to 8 h in *Cyp3a*$^{-/-}$ mice (Figure 5B, inset), which is in line with somewhat reduced (Cyp3a-mediated) formation of DMP in these mice. The brain concentration and accumulation of DMP was not significantly affected by Cyp3a deficiency, and the same applies for DMP liver concentration and accumulation (Table 1, Figure S2).

Interestingly, in striking contrast to the plasma levels of parental ponatinib, the plasma levels and AUC_{0-24} of DMP were increased by about 3- to 4-fold in both Abcg2-deficient strains (Figure 5A and Table 1). This suggests that Abcg2 is involved in the clearance of DMP from plasma. On the other hand, Abcb1a/1b deficiency had no marked effect on DMP plasma levels (Figure 5A, Table 1). We further investigated the impact of single and combined Abcg2 and Abcb1a/1b deficiency on DMP brain levels 24 h after oral intake of ponatinib. While the brain concentrations and accumulations in the single transporter knockout mice were not markedly different from those in WT mice, in the combined *Abcb1a/1b;Abcg2*$^{-/-}$ mice they were highly increased (Table 1, Figure S3A–C). Incidentally, the comparatively high DMP liver concentrations, liver-to-plasma ratios, and liver accumulations we observed in both the Abcg2-deficient strains (Figure S3D–F) could reflect diminished elimination of DMP from the liver at this late (24 h) phase, perhaps by reduced hepatobiliary excretion of DMP. When we looked at brain concentration and accumulation of DMP 2 h after oral administration of ponatinib, we obtained qualitatively similar results as in the 24 h experiment, although the quantitative effects were somewhat less pronounced (Figure S4A–C). Overall, these data indicate that both mAbcg2 and mAbcb1a/1b contribute to restricting the brain accumulation of DMP, and can largely take over each other's protective function at the BBB. On the other hand, Abcg2, but not Abcb1a/1b, appears to play a role in the elimination of DMP from plasma.

DISCUSSION

We found that ponatinib is transported by hABCB1 and mAbcg2 *in vitro*, and that this transport can be inhibited with specific inhibitors. *In vivo*, we did not observe a clear limiting effect of mAbcb1a/1b or Abcg2 on the oral availability of ponatinib in mice. However, the brain accumulation of ponatinib was modestly increased in both single transporter knockout strains, and highly increased in the *Abcb1a/1b;Abcg2*$^{-/-}$ combination knockout strain. No marked differences were observed for ponatinib pharmacokinetics in the liver

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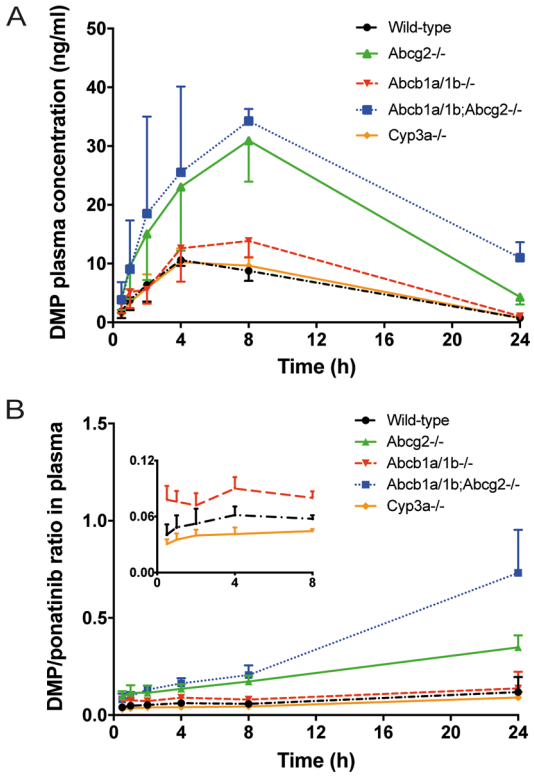

Figure 5. Plasma concentration−time curves of DMP in female WT (black circles), *Abcg2*$^{-/-}$ (green triangles), *Abcb1a/1b*$^{-/-}$ (red triangles), and *Abcg2;Abcb1a/1b*$^{-/-}$ mice (blue squares), and *Cyp3a*$^{-/-}$ mice (yellow diamonds) over 24 h (A) and the DMP:ponatinib plasma concentration ratio (B) after oral administration of 10 mg/kg ponatinib. Data are given as mean ± SD (SD rendered one-sided for improved clarity). n = 4−5 mice per group.

of the transporter knockout strains. Cyp3a deficiency resulted in a modest (1.4-fold) increase in plasma levels of ponatinib, suggesting increased oral availability of ponatinib. However, no changes were observed in the plasma levels of DMP, one of the active ponatinib metabolites formed by CYP3A in humans. Our data further indicate that DMP is substantially transported *in vivo* by mAbcg2 and mAbcb1a/1b, resulting in markedly (3- to 4-fold) increased plasma levels in both Abcg2-deficient strains, and strongly increased brain accumulation in combination (but not single) transporter knockout mice.

Our *in vitro* transport results are in line with the ATPase experiments of Sen et al., which showed the ability of ponatinib to stimulate hABCG2 and hABCB1 ATPase activity, and indicate that this reflected their ability to transport this drug.[23] Combining all available *in vitro* and *in vivo* data, including from the literature, it seems very likely that mAbcb1a/1b, mAbcg2, hABCB1, and probably also hABCG2, can transport ponatinib. However, it is possible that substantial expression of hABCG2 is needed in order to readily detect ponatinib transport. Expression of ABCG2 and ABCB1 has been associated with resistance to chemotherapy for a range of different drugs in several cancers, including leukemia.[37] Our data suggest that (over)expression of these transporters in cancer cells may also confer ponatinib resistance. This would suggest the possible usefulness of inhibiting these transporters when treating patients with ponatinib in order to reverse such tumor resistance. Moreover, possible drug−drug interactions affecting systemic effects of ABC transporters could also alter treatment efficacy.

Our results for the brain accumulation studies (Figures 3 and 4) clearly show that both mAbcg2 and mAbcb1a/1b can restrict brain accumulation of ponatinib in mice. Whereas single deficiency of Abcg2 or Abcb1a/1b results in only small increases in brain accumulation, the combined deficiency of both transporters causes a disproportionately large rise in brain accumulation. At the same time, ponatinib concentration and accumulation in for instance liver were not markedly altered between the strains (Figures 3 and 4), illustrating the unique behavior of brain in this respect. It appears that the liver primarily reflected the plasma concentration(s) of ponatinib, which suggests relatively easy translocation of this drug across the basolateral (sinusoidal) membrane of hepatocytes. The disproportionate increase in brain accumulation of ponatinib (and of its metabolite DMP, Figures S3 and S4) observed in the *Abcb1a/1b;Abcg2*$^{-/-}$ mice compared to single knockout strains was similar to that found for a range of other TKIs (e.g., axitinib, vemurafenib, lapatinib, gefitinib, erlotinib, sunitinib, dasatinib, regorafenib, and imatinib[29,35,38−43]), and can be explained by relatively straightforward pharmacokinetic models.[40,44] These models indicate that if two transporters each have a high contribution of efflux transport relative to the background efflux at the BBB in the absence of both transporters, the effect of single transporter ablation on brain accumulation will be far less than the effect of combined ablation. An interesting implication of these models and our findings on ponatinib (and DMP) brain accumulation is that next to Abcg2 and Abcb1a/1b, there can be no other efflux transporters in the BBB that are remotely as efficient in keeping ponatinib and DMP out of the brain.

The increase in treatment options and efficacy for patients with leukemia due to the advent of modern targeted drugs has increased the chance for isolated metastases behind the BBB to emerge, as effective control of peripheral malignancies greatly increases patient survival times. Malignancies in the brain are often hard to reach for drugs because of the presence of ABCB1 and ABCG2 in the continuous physical barrier between blood and brain tissue formed by the BBB. Pharmacological inhibition of these transporters during pharmacotherapy could therefore also improve treatment of metastases positioned in part or in whole behind the BBB. However, it should be noted that studies in mice can only provide qualitative information on the possible impact of these transporters in the blood-brain barrier of patients. In order to better assess the impact of such processes in patients, one can consider appropriate physiologically based pharmacokinetic modeling, or clinical positron-emission tomography (PET) studies with the drug of interest, in combination with highly efficacious ABCB1 and ABCG2 inhibitors, such as for instance elacridar.[45−47] Next to treatment of (metastatic) CML and ALL, a study by Whittle et al. has shown that ponatinib could potentially also be used for neuroblastoma therapy *in vivo*.[48] Also here pharmacological inhibition of the ABC efflux transporters in the BBB might possibly increase the therapeutic efficacy.

Our finding that Cyp3a deficiency in mice increases ponatinib oral availability (Figure 2A, Table 1) is in line with a significant role of CYP3A in metabolizing ponatinib. The fact that we did not observe obvious changes in the pharmacokinetics of DMP, one of its CYP3A-generated metabolites, could be explained by the possibility that other mouse enzymes, for instance one or more of the many mouse Cyp2c isoforms,

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might form this metabolite as well.[49] Additionally, it may be possible that CYP3A further metabolizes DMP. In this context it is worth noting that patients receiving ponatinib and the CYP3A inhibitor ketoconazole showed both an increase in plasma ponatinib levels, and a decrease in DMP levels.[27] The latter effect might indicate that in patients there are no efficient alternative pathways to form DMP from ponatinib, illustrating a difference between mice and man. Nonetheless, all data suggest that altered activity of CYP3A, which commonly occurs in humans, may affect the effective exposure of the body to ponatinib, and thus its therapeutic efficacy and toxicity risks.

Ponatinib is approved for treatment for CML and ALL. However, its use has to be carefully monitored, with revised prescribing information including a black box warning, and the need for risk evaluation and mitigation strategies mainly because of serious cardiovascular risks,[50] vascular occlusions and heart failure, but also hepatotoxicity. Ponatinib use therefore remains limited to second-generation drug-resistant and (T351I) mutated patients with Ph+ ALL,[51] or as third-line therapy in CML.[52] Since our data indicate that ABC efflux transporters and CYP3A4 could affect the biological availability of ponatinib and its active metabolite DMP at several levels, it will be important to also consider these factors as possible players in unexpected toxicity of ponatinib.

Based on our findings, it is likely that tumors substantially expressing ABCB1 and/or ABCG2 will demonstrate resistance to ponatinib. Thus, inhibiting these transporters with effective dual ABCG2 and ABCB1 inhibitors, such as elacridar during ponatinib therapy could potentially improve the tumor response. It may further be possible to increase the ponatinib concentration in the brain of patients with CNS tumors or metastases when ponatinib is coadministered with elacridar. Indeed, a recent case study suggested that ponatinib does not normally effectively enter the CNS.[53] Our results suggest that the oral availability, and hence the risk of toxicity, of ponatinib is not likely to be increased by such an elacridar coadministration treatment. In contrast, the oral availability of ponatinib could potentially be enhanced by coadministration of a CYP3A4 inhibitor, such as ketoconazole. However, caution should be exercised to prevent unexpected toxicity. Collectively, our findings suggest that coadministration of a dual inhibitor of ABCB1 and ABCG2 may increase the exposure to ponatinib and its active metabolite for brain (micro)metastases positioned behind a functionally intact blood-brain barrier, and could thus reduce the chance that patients will ultimately die of such normally poorly tractable lesions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.7b00257.

Schematic representation of the main metabolites formed from ponatinib in humans, brain to liver ratio at 2 and 24 h, brain and liver concentration, tissue-to-plasma ratio, and relative tissue accumulation of DMP (PDF)

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Notes

The authors declare the following competing financial interest(s): The research group of A.H.S. receives revenues from commercial distribution of some of the mouse strains used in this study.

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■ ABBREVIATIONS:

ABC, ATP-binding cassette; AUC, area under the plasma concentration−time curve; BBB, blood-brain barrier; BCRP/ABCG2, breast cancer resistance protein; BCR/ABL1 fusion gene, "breakpoint cluster region"/Abelson murine leukemia viral oncogene homologue 1; C_{max}, peak plasma concentration; DMP, *N*-desmethyl ponatinib; LLOQ, lower limit of quantitation; LOD, lower limit of detection; P-gp/ABCB1, P-glycoprotein; SD, standard deviation; TKI, tyrosine kinase inhibitor; T_{max}, time to reach peak plasma concentration

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