Impact of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) gene dosage on plasma pharmacokinetics and brain accumulation of dasatinib, sorafenib and sunitinib

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Running title: Impact of P-gp and Bcrp gene dosage on TKI brain penetration

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ABBREVIATIONS: ABC, ATP-binding cassette; ANOVA, analysis of variance; AUC, area under the plasma concentration-time curve; BBB, blood-brain barrier; BCRP, breast cancer resistance protein; Cbrain, brain concentration; Cmax, maximum drug concentration in plasma; DMSO, dimethyl sulfoxide; RT-PCR, real time-polymerase chain reaction; TKI, tyrosine kinase inhibitor.
Abstract

Low brain accumulation of anticancer drugs due to efflux transporters may limit chemotherapeutic efficacy, necessitating better understanding of the underlying mechanisms. P-glycoprotein (Abcb1a/1b) and breast cancer resistance protein (Abcg2) combination knockout mice often display disproportionately increased brain accumulation of shared drug substrates compared to single transporter knockout mice. Recently developed pharmacokinetic models could explain this phenomenon. To experimentally test these models and their wider relevance for tyrosine kinase inhibitors (TKIs) and other drugs, we selected dasatinib, sorafenib and sunitinib because of their divergent oral availability and brain accumulation profiles: brain accumulation of dasatinib is mainly restricted by Abcb1, that of sorafenib mainly by Abcg2, and that of sunitinib equally by Abcb1 and Abcg2. We analyzed the effect of halving the efflux activity of these transporters at the blood-brain barrier (BBB) by generating heterozygous Abcb1a/1b;Abcg2 knockout mice, and testing plasma and brain levels of the drugs after oral administration at 10 mg/kg. RT-PCR analysis confirmed ~2-fold decreased expression of both transporters in brain. Interestingly, whereas complete knockout of the transporters caused 24- to 36-fold increases in brain accumulation of the drugs, the heterozygous mice only displayed 1.6- to 1.9-fold increases of brain accumulation relative to wild-type mice. These results are well in line with the predictions of the pharmacokinetic models, and provide strong support for their validity for a wider range of drugs. Moreover, retrospective analysis of fetal accumulation of drugs across the placenta in Abcb1a/1b heterozygous knockout pups suggests that these models equally apply to the maternal-fetal barrier.
Introduction

Dasatinib, sorafenib and sunitinib are orally active, small-molecule multi-targeted tyrosine kinase inhibitors (TKIs) used for the treatment of cancer. Dasatinib (Sprycel; BMS-354825), a potent second generation BCR-ABL kinase inhibitor (Lombardo et al., 2004), used as first-line treatment for adult patients newly diagnosed with Philadelphia chromosome-positive chronic myelogenous leukemia in chronic phase (Kantarjian et al., 2010). Sorafenib (Nexavar, BAY43-9006), a Raf kinase and vascular endothelial growth factor receptor inhibitor, is currently used for the treatment of patients with unresectable hepatocellular carcinoma and advanced renal cell carcinoma (Escudier et al., 2007; Llovet et al., 2008). Sunitinib (Sutent, SU11248) is a receptor tyrosine kinase inhibitor that is used in the therapy of progressive, well-differentiated pancreatic neuroendocrine tumors, metastatic renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumor (Goodman et al., 2007; Raymond et al., 2011; Rock et al., 2007).

ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp; ABCB1) and breast cancer resistance protein (BCRP; ABCG2) are highly expressed in small intestinal epithelium and at the blood-brain barrier (BBB), where they can limit the oral availability, but especially the brain accumulation of many clinically used TKIs (see also Supplemental Figure 1) (Agarwal et al., 2010; Chen et al., 2009; Durmus et al., 2012; Lagas et al., 2009; Lagas et al., 2010; Mittapalli et al., 2012; Polli et al., 2009; Tang et al., 2012). There is considerable overlap in the substrate specificity between Abcb1 and Abcg2, and many TKIs, including dasatinib, sorafenib, and sunitinib, are dual substrates of these transporters (Agarwal et al., 2010; Chen et al., 2009; Lagas et al., 2009; Lagas et al., 2010; Tang et al., 2012). Dasatinib, sorafenib, and sunitinib can be classified into three different groups based on their known in vivo characteristics with regard to brain accumulation profiles (Supplemental Figure 1). For instance, brain accumulation of dasatinib is mainly restricted by Abcb1, whereas Abcg2 plays a more important role in limiting brain accumulation of sorafenib. For sunitinib, Abcb1 and Abcg2 contribute equally to its restricted brain accumulation. With respect to oral availability, dasatinib is primarily
restricted by Abcb1, whereas plasma pharmacokinetics of sorafenib and sunitinib are not affected by Abcb1 and/or Abcg2.

A striking finding from brain accumulation studies with shared Abcb1 and Abcg2 substrates is that the single disruption of Abcb1a/b or Abcg2 in mice often has little or no detectable effect on brain accumulation, whereas simultaneous disruption of these two transporters results in a dramatic increase of brain accumulation of many TKIs (see also Supplemental Figure 1) (Agarwal et al., 2010; Chen et al., 2009; Kodaira et al., 2010; Lagas et al., 2009; Lagas et al., 2010; Polli et al., 2009; Tang et al., 2012; Zhou et al., 2009). These findings have prompted researchers to envisage a synergistic or cooperative role of ABCB1 and ABCG2 in the efflux of dual substrates at the BBB. However, Kodaira et al. (2010) applied a relatively simple pharmacokinetic model that describes that the disproportionate effect of simultaneous removal of both transporters can simply result from the fact that the intrinsic efflux transport activities at the BBB of Abcb1 and Abcg2 are each considerably larger than the remaining (passive, or lowly active) efflux activity at the BBB. Therefore, the seemingly synergistic effect of the removal of both Abcb1 and Abcg2 on the accumulation of their shared substrates in the brain can be explained by their separate contributions to the net efflux at the BBB, without postulating any direct or indirect interaction between Abcb1 and Abcg2. Interestingly, similar predictions are made by the more sophisticated pharmacokinetic models developed by Kalvass and Pollack (2007) and Zamek-Gliszczynski et al. (2009), which aimed to resolve complications with describing transepithelial active transport as for instance identified by Bentz et al. (2005).

Each of these theoretical models also predicts that halving the amount of active transporter-mediated drug efflux activity at the BBB should result in only a minor increase of drug accumulation into the brain (never more than 2-fold), even if complete removal of the active transporter-mediated efflux results in a very large increase in brain accumulation. In order to test these predictions, we aimed to analyze the effect of halving the active efflux transporter activity at the BBB by using wild-type, heterozygous Abcb1a/b(+/−);Abcg2(+/−) and homozygous Abcb1a/b(−/−);Abcg2(−/−) mice, with 2, 1, and 0 active gene copies of each of the active transporters, respectively, and study the effect on TKI brain accumulation. We chose dasatinib,
sorafenib, and sunitinib as TKIs in view of their widely different behavior with respect to impact of the individual transporters on brain accumulation, their different intrinsic capacity to accumulate into the brain, and their different plasma levels upon oral administration (Supplemental Figure 1). This functional diversity makes it more likely that consistent results obtained for these three drugs can be extrapolated to a much wider range of drugs affected by Abcb1 and Abcg2.
Materials and Methods

Chemicals and reagents
Dasatinib, sorafenib and sunitinib were purchased from Sequoia Research Products (Pangbourne, UK). Heparin (5000 IU/ml) was obtained from Leo Pharma BV (Breda, The Netherlands). Lithium-heparinized microvetttes and dipotassium-EDTA microvetttes were obtained from Sarstedt (Numbrecht, Germany). EDTA disodium salt pH 8.0 was from Cambrex BioScience Inc. (Rockland, ME). Bovine serum albumin (BSA), fraction V, was purchased from Roche (Mannheim, Germany). Isoflurane (Forane) was from Abbott Laboratories (Queenborough, Kent, UK). All other chemicals and reagents were obtained from Sigma-Aldrich (Steinheim, Germany).

Animals
Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Male wild-type, Abcb1a/1b(+/-);Abcg2(+/-) and Abcb1a/1b(-/-);Abcg2(-/-) (Jonker et al., 2005) mice, all of a >99% FVB genetic background, were used between 8 and 14 weeks of age. Animals were kept in a temperature-controlled environment with a 12-hr light/12-hr dark cycle and received a standard diet (AM-II, Hope Farms B.V., Woerden, The Netherlands) and acidified water ad libitum. Abcb1a/1b(+/-);Abcg2(+/-) mice were the F1 of a cross between FVB wild-type and Abcb1a/1b(-/-);Abcg2(-/-) mice.

Drug solutions
Dasatinib was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 25 mg/ml and 25-fold diluted with 50 mM sodium acetate buffer (pH 4.6) to obtain a concentration of 1 mg/ml. Sorafenib tosylate was dissolved in DMSO (25 mg/ml) and 25-fold diluted with Cremophor EL/ethanol/water (1:1:6, v/v/v). Sunitinib malate was dissolved in DMSO at a concentration of 25 mg/ml and further diluted with 50 mM sodium acetate buffer (pH 4.6) to yield a concentration of 1 mg/ml. Dasatinib, sorafenib and sunitinib were administered orally at 10 mg/kg body weight (10 ml/kg).
Plasma pharmacokinetics and relative brain accumulation of TKIs in mice

To minimize variation in absorption upon oral administration, mice were fasted for 3 hr before dasatinib, sorafenib or sunitinib were administered by gavage into the stomach, using a blunt-ended needle. To prevent blood from coagulating, heparin was used for the dasatinib and sunitinib pharmacokinetic experiments, whereas EDTA was used for the sorafenib pharmacokinetic experiment. Tail vein blood sampling was performed at 0.25, 0.5, 1, 2 and 4 hr time-points after oral administration, using either microvettles containing dipotassium-EDTA or lithium heparin. Six hours after oral administration, mice were anesthetized with isoflurane and blood was collected by cardiac puncture, in which 0.5 M disodium-EDTA or 5000 IU/ml heparin were used as anticoagulants. Immediately thereafter, mice were sacrificed by cervical dislocation and brains were rapidly removed. Plasma was isolated from the blood by centrifugation at 2,100 g for 6 min at 4°C, and the plasma fraction was collected and stored at -20°C until analysis. Brains were homogenized with 1 ml of 4% BSA and stored at -20°C until analysis. Relative brain accumulation after oral administration was calculated by determining the drug brain concentration at 6 hr relative to the plasma AUC0-6 hr, as the AUC better reflects the overall drug exposure of the brain over time than the plasma concentration at 6 hr after oral administration.

Drug analyses
Dasatinib, sorafenib and sunitinib concentrations in plasma and brain homogenates were analyzed by liquid chromatography coupled to tandem mass spectrometry as described previously (Lagas et al., 2009; Sparidans et al., 2009; Tang et al., 2012, respectively). Lower limit of quantification (LLQ) values for dasatinib and sunitinib were 5 ng/ml and 15.6 ng/g for the plasma and brain homogenates, respectively. LLQ values for sorafenib were 10 ng/ml and 31.2 ng/g for the plasma and brain homogenates, respectively.

RNA isolation, cDNA synthesis and real-time RT-PCR
RNA isolation from mouse brain and small intestine and subsequent cDNA synthesis and RT-PCR were performed as described (Lagas et al., 2012). To circumvent detection of non-functional...
RNA which is transcribed from the Abcb1a and Abcg2 knockout alleles (Schinkel et al., 1994 and data not shown), we used RT-PCR probes positioned within the deleted exons of both genes. Forward 5’-CCCGGCTCACAGATGATGTT-3’ (F1) and reverse 5’-TTCCAGCCACGGGTAAATCC-3’ (R1) specific primers (Invitrogen Life Technologies) were used for the detection of Abcb1a in the wild-type alleles, which resulted in a 121-bp band. Forward 5’-CAGCAAGGAAAGATCCAAAGGG-3’ (F4) and reverse 5’-CACAACGTCATCTTGAACCACA-3’ (R4) specific primers (Invitrogen Life Technologies) were used for the detection of Abcg2 in the wild-type alleles, which resulted in a 110-bp band.

Statistical analysis
Pharmacokinetic parameters were calculated by non-compartmental methods using the software package PK Solutions 2.0.2 (Summit Research Services, Ashland, OH). The area under the plasma concentration-time curve was calculated using the trapezoidal rule, without extrapolating to infinity. The maximum drug concentration in plasma (C_{max}) and the time to reach maximum drug concentration in plasma (T_{max}) were determined directly from mean concentration-time data. Data are presented as means ± SD. For parametric statistical analysis, all the data except for plasma concentrations and AUC_{0-6hr} values were log-transformed to obtain equality in variances. One-way analysis of variance (ANOVA) was used to determine statistical significance of differences between groups, after which post-hoc tests with Bonferroni correction were performed for comparison between individual groups. Differences were considered statistically significant when P < 0.05.
Results

Expression levels of Abcb1a and Abcg2 in small intestine and brain of wild-type, Abcb1a/1b(+/−);Abcg2(+−) and Abcb1a/1b(−−);Abcg2(−−) mice

Since Abcb1b is not substantially expressed in wild-type mouse brain and small intestine (data not shown), we tested only Abcb1a and Abcg2 expression in these tissues of wild-type, Abcb1a/1b(+/−);Abcg2(+−) and Abcb1a/1b(−−);Abcg2(−−) mice using RT-PCR. To circumvent spurious detection of non-functional RNA which is still transcribed from the Abcb1a and Abcg2 knockout alleles (Schinkel et al., 1994) (data not shown), we used RT-PCR probes positioned within the deleted exons of both genes. We expected that the expression levels of wild-type Abcb1a and Abcg2 alleles in the small intestine and brain of heterozygous mice would be about half of the expression levels observed in wild-type mice. However, the small intestinal expression levels of Abcb1a and Abcg2 were not significantly different from those in wild-type mice, although experimental variation was quite substantial (Figure 1A and B). As expected, there was no significant expression of Abcb1a and Abcg2 in the small intestine of Abcb1a/1b(−−);Abcg2(−−) mice. The results suggest that, for small intestine, expression levels of Abcb1a and Abcg2 were not halved upon halving the gene copy number of the genes, but instead remained similar to the wild-type expression levels.

In contrast, Abcb1a RNA was 3.4-fold, and Abcg2 RNA 2.3-fold lower in the brain of male heterozygous mice as compared with wild-type mice, albeit with substantial variation in both wild-type and heterozygous values (Figure 1C and D). There was no significant expression of Abcb1a and Abcg2 in the homozygous knockout mice. In vivo brain accumulation studies have also been performed using female mice (Durmus et al., 2012), and we now found that in female heterozygous mice, the brain expression levels of Abcb1a and Abcg2 were also about half of the levels observed in wild-type mice (Figure 1E and F). Of note, relative expression levels of either Abcb1a or Abcg2 in brain were not significantly different between heterozygous males and females, and pooled results for both genders indicated roughly half the wild-type expression levels of Abcb1a and Abcg2 in brain of heterozygous mice (Figure 1G and H). This 2-fold
reduction (or perhaps slightly more for Abcb1a in males) of transporter expression in brains of heterozygous compared to wild-type mice allowed the intended analysis of transporter activity effects on brain accumulation of the TKIs.

Plasma pharmacokinetics of dasatinib, sorafenib and sunitinib in heterozygous Abcb1a/1b(+/-);Abcg2(+/-) mice
To assess the impact of heterozygous Abcb1 and Abcg2 on oral bioavailability of dasatinib, sorafenib and sunitinib, we orally administered these TKIs at 10 mg/kg to wild-type, Abcb1a/1b(+/-);Abcg2(-/-) and Abcb1a/1b(-/-);Abcg2(-/-) mice, and measured plasma concentrations over 6 hrs by LC-MS/MS. Upon dasatinib oral administration, the plasma AUC_0-6hr in heterozygous Abcb1a/1b(+/-);Abcg2(+/-) mice was 1.6-fold increased, but not significantly compared to wild-type mice. In contrast, a statistically significant 2.1-fold (P < 0.01) higher plasma AUC_0-6hr of dasatinib was observed in homozygous Abcb1a/1b(-/-);Abcg2(-/-) compared to wild-type mice (Figure 2A, D and Table 1), in line with the results of Lagas et al. (2009).

Upon sorafenib oral administration, although there were a few significant differences at individual time points, there was no significant difference in the overall plasma AUC_0-6hr among the 3 tested strains. This suggests that Abcb1 and Abcg2 did not play a role in the overall AUC_0-6hr of sorafenib (Figure 2B, E and Table 2), consistent with the data of Lagas et al. (2010).

Heterozygous and homozygous knockout mice also showed oral sunitinib plasma AUC_0-6hr values that were not significantly different from wild-type values (Figure 2C), although experimental variation was substantial. Probably related to that, the plasma AUC_0-6hr of Abcb1a/1b(+/-);Abcg2(+/-) mice was 1.9-fold and significantly higher (P < 0.01) than that of Abcb1a/1b(-/-);Abcg2(-/-) mice (Figure 2C and F, Table 3). The homozygous data were in agreement with the results of Tang et al. (2012). Taken together, heterozygosity for Abcb1 and Abcg2 knockout alleles had no significant effect on the plasma AUC_0-6hr of orally administered dasatinib, sorafenib and sunitinib.
Brain accumulation of dasatinib, sorafenib and sunitinib in \textit{Abcb1a/1b(+/-);Abcg2(+/-)} and \textit{Abcb1a/1b(-/-);Abcg2(-/-)} mice

In the same set of experiments, we also measured the brain concentrations at 6 hr after oral administration of dasatinib, sorafenib and sunitinib (10 mg/kg) in wild-type, \textit{Abcb1a/1b(+/-);Abcg2(+/-)} and \textit{Abcb1a/1b(-/-);Abcg2(-/-)} mice. Brain concentrations of all drugs were modestly increased (1.5- to 2.9-fold) in heterozygous \textit{Abcb1a/1b(+/-);Abcg2(+/-)} compared to wild-type mice, albeit not significantly for sorafenib (Tables 1-3, Figure 3A-C). The homozygous \textit{Abcb1a/1b(-/-);Abcg2(-/-)} mice had 58.4-, 27.8- and 19.7-fold higher brain concentrations of dasatinib, sorafenib and sunitinib than wild-type mice, respectively (Figure 3A-C; Tables 1-3). Brain accumulations of all drugs at 6 hr were also modestly increased (1.6- to 1.9-fold) in the heterozygous strain as compared to wild-type, albeit only statistically significant (\(P < 0.01\)) for dasatinib (Figure 3D-F; Tables 1-3). In contrast, \textit{Abcb1a/1b(-/-);Abcg2(-/-)} mice had 29.2-, 35.9- and 23.7-fold increased brain accumulations (\(P < 0.001\)) of dasatinib, sorafenib and sunitinib, respectively, relative to wild-type mice (Figure 3D-F; Table 1-3). Taken together, these results show that halving the amount of active efflux by Abcb1 and Abcg2 at the BBB had only a small impact (<2-fold) on the brain accumulation of dasatinib, sorafenib and sunitinib. Even without knowing the exact contribution of each individual transporter, substitution in the equations developed by Kodaira et al. (2010) allows prediction of the effects of halving the total amount of active Abcb1- and Abcg2-mediated transport. In this case, based on the homozygous knockout values, brain accumulation values in the heterozygous mice were predicted to be 1.93-, 1.95-, and 1.92-fold increased relative to wild-type levels for dasatinib, sorafenib, and sunitinib, respectively. These values fall well within the range of the experimental values obtained for these drugs (1.6- to 1.9-fold increases, Figure 3D-F, Tables 1-3). Similar results (i.e., just below 2-fold effects) are predicted by the models of Kalvass and Pollack (2007) and Zamek-Gliszczynski et al. (2009).
Discussion

The present study shows that halving the amount of active drug efflux transport by Abcb1 and Abcg2 at the BBB results in less than 2-fold increases in brain accumulation of several TKIs, even when complete removal of these active drug transporters results in 24- to 36-fold increases in brain accumulation of these drugs. These observations are fully in line with predictions of the theoretical pharmacokinetic models of Kodaira et al. (2010), Kalvass and Pollack (2007) and Zamek-Gliszcynski et al. (2009), and thus provide further support for their validity. Moreover, the diversity in properties of the tested TKIs in terms of plasma levels obtained (i.e., oral availability), intrinsic capacity to accumulate into the brain, and extent to which brain accumulation is relatively affected by Abcb1 and Abcg2, suggests that many more drugs transported by ABCB1 and/or ABCG2 will be subject to the same behavior. It is worth noting that the model will also apply to any other active drug efflux transporters present in the endothelial luminal membrane of the BBB, and can in principle, with some modification, be used for any number of these transporters.

An important feature of the models is that they explain the counterintuitive disproportionate increase in drug accumulation into the brain seen when two active BBB drug efflux transporters of a drug are simultaneously knocked out (or inhibited), relative to the situation when only one is knocked out. This turns out to be simply a consequence of the fact that the active efflux transport by each of the transporters is considerably larger than the remaining (passive, or lowly active) efflux transport at the BBB in the absence of both the efflux transporters. Thus, the apparently “synergistic” effect of simultaneously removing the activity of both transporters on brain accumulation of a drug can be explained without postulating any direct or indirect interaction between the transporters that would somehow modulate the transport activity of each of the individual (remaining) transporters when the other is knocked out. As shown and summarized before (Agarwal et al., 2012; Durmus et al., 2012), single homozygous knockout of Abcb1 or Abcg2 in FVB mice also does not result in significant expression changes of the remaining transporter in the brain. Although conceptually somewhat different, the lack of change
in brain expression per gene copy that we observed in the heterozygous $\text{Abcb1a/1b}(\pm/\pm)$; $\text{Abcg2}(\pm/\pm)$ is in line with this relative stability in brain expression of $\text{Abcb1a}$ and $\text{Abcg2}$.

Unlike the brain expression of $\text{Abcb1a}$ and $\text{Abcg2}$, in the intestine heterozygosity for the encoding genes does not result in halving of the RNA levels, but rather in levels that are similar to those in the wild-type mice (compare Figure 1A-B vs. 1C-H). Possibly the intestine, as a primary flexible protective barrier directly exposed to numerous xenobiotics, is more adapted to upregulating detoxifying proteins depending on effective exposure than the endothelial cells of the BBB. Indeed, we previously found that several detoxifying genes are readily up- or down-regulated in the intestine upon knockout of the detoxifying $\text{Cyp3a}$ gene (van Waterschoot et al., 2009). It may well be that there are more, and more highly active, xenobiotic nuclear receptors present in intestinal epithelial cells than in brain capillary endothelial cells, and certainly the exposure to potentially regulating xenobiotic factors is far higher in the intestinal cells than in the BBB cells. Both factors can contribute to a more effective compensatory upregulation of $\text{Abcb1a}$ and $\text{Abcg2}$ in intestine than in brain upon halving of the gene dosage.

Also a number of other studies (recently reviewed in Kalvass et al., 2013) suggest that expression and/or activity of ABCB1 and ABCG2 in the blood-brain barrier is not easily induced in either rodents or humans. This is important, as marked changes in BBB expression of active efflux transporters due to drug-drug interactions might present a risk for altered susceptibility to various CNS-active drugs. The relatively stable and apparently high expression of ABCB1 and ABCG2 in the BBB observed by others and us suggests that this risk is quite limited, and unlikely to be a major concern during routine pharmacotherapy in humans.

The three TKIs we tested at the same oral dosage (10 mg/kg) showed highly divergent oral availability and brain accumulation characteristics, in both wild-type and knockout mice (Tables 1-3). For instance, in wild-type mice the AUC$_{0-6\text{hr}}$ of sunitinib was nearly two orders of magnitude (75-fold) lower than that of sorafenib, whereas that of dasatinib was in between (16.7-fold lower than that of sorafenib). The relative AUC$_{0-6\text{hr}}$ results between the drugs in the knockout strain showed a similar profile. At the same time, the relative brain accumulation ($K_p$) of sunitinib in wild-type mice was 117- and 82-fold higher than that of sorafenib and dasatinib, respectively.
Again, the profile of relative results for the $K_p$s in the knockout strain was similar, although at a ~70-fold higher absolute level. Thus, the drug with the lowest plasma levels, sunitinib, had the highest relative brain accumulation. Clearly there can be many factors that contribute to these differences between the drugs, including differential impact of drug uptake and drug efflux transporters in the various epithelial and endothelial barriers, drug-metabolizing enzymes, saturation phenomena, hydrophobicity of the drugs and other physicochemical properties that define e.g. binding to plasma and tissue proteins and lipids. Nonetheless, despite the profound differences in intrinsic properties between the three TKIs tested here, they all adhere similarly to the models of Kodaira et al. (2010), Kalvass and Pollack (2007) and Zamek-Gliszcynski et al. (2009) with respect to impact of Abcb1a and Abcg2 on brain accumulation. Moreover, a recent pilot analysis of the effect of Abcb1a/1b;Abcg2 heterozygosity on brain accumulation of oral vemurafenib, another TKI, yielded very similar results (Durmus et al., 2012). These observations make it very likely that the same principle will apply to many other drugs with a wide range of divergent intrinsic properties, as long as they are substantially transported by Abcb1 and/or Abcg2.

The model of Kodaira et al. (2010) was developed for describing the impact of drug efflux transporters on drug accumulation across the BBB and the testis-blood barrier. However, this model, like that of Kalvass and Pollack (2007) and Zamek-Gliszcynski et al. (2009) can also be applied to any other functionally similar blood-tissue barrier. In this respect it is interesting to retrospectively consider the results we obtained with transplacental accumulation of drugs into fetuses that were wild-type, heterozygous, or homozygous for Abcb1a/1b (Smit et al., 1999). As each mouse fetus forms its own placenta, and the placenta has the genotype of the fetus, the individual placental barriers are also either wild-type, heterozygous, or homozygous for Abcb1a/1b. By crossing a heterozygous mother with a heterozygous father, placental barriers of each genotype can be obtained within one pregnant mother. As shown in Supplemental Figure 2, the transplacental fetal accumulation of the maternally administered drugs digoxin, saquinavir and paclitaxel was ~2.5-fold, ~7.5-fold, and ~16-fold increased in the homozygous knockout fetuses relative to wild-type, whereas accumulation in the heterozygous fetuses was 1.6-, 1.3-, and 1.3-
fold increased, respectively. Also here we observed the pattern (for saquinavir and paclitaxel) that a profound accumulation in full knockouts is associated with a small (and even statistically insignificant) increase in the heterozygous situation. Considering the experimental variation, these results are consistent with the model of Kodaira et al.: assuming about half of the wild-type efflux activity in the heterozygous placenta, predicted values were 1.43-, 1.76-, and 1.88-fold increase relative to wild-type, respectively. Qualitatively similar results are predicted with the pharmacokinetic models of Kalvass and Pollack (2007) and Zamek-Gliszczynski et al. (2009). The strong increase in homozygous fetuses indicates that the Abcb1a/1b transport activity in the placenta is considerably greater than any alternative remaining placental drug efflux activity. Also consistent with the models, the more modest impact of full knockout on digoxin fetal accumulation (~2.5-fold increase) was associated with a more or less intermediate (and statistically significant) ~1.6-fold increase in the heterozygous fetuses. We infer that the impact of efflux transporters on transplacental fetal accumulation of drugs can also be adequately described by the cited pharmacokinetic models, and that for saquinavir and paclitaxel Abcb1 is by far the most important drug efflux transporter limiting fetal drug accumulation.

Our results in mice confirm that a very substantial reduction of active efflux transporter activity in the blood-brain and placental barriers (>2-fold) would be needed to achieve a >2-fold increased penetration of drug substrates into brain or fetus, respectively, even if the transporters by themselves give a very high level of protection. As argued by Kalvass et al. (2013), it is not easy to achieve this level of inhibition of ABCB1 and ABCG2 in humans with therapeutic blood levels of currently available drugs. As the principles as described here in mice undoubtedly also apply to the human blood-tissue barriers, it seems very likely that also in humans substantial changes in drug penetration into brain and fetus due to drug-drug interactions inhibiting ABCB1 and ABCG2 are unlikely to occur. Still, one should always keep in mind that there may be species-specific differences in substrate specificity and inhibitor sensitivity of ABCB1 and ABCG2, and absolute transporter expression levels in the blood-tissue barriers may differ between man and mouse. Extrapolation of these preclinical results to humans should therefore, as always, be done with caution, and carefully tested before being applied in a clinical setting.
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Footnotes
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Reprint Requests
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Figure 1. Small intestinal (A and B) and brain (C – H) RNA expression levels of Abcb1a (left panels) and Abcg2 (right panels) in male (A – D), female (E and F) or pooled male and female (G and H) wild-type, Abcb1a/1b(+/−);Abcg2(+/-) and Abcb1a/1b(−/−);Abcg2(−/-) mice (+/+, +/- and −/−, respectively), as determined by real-time RT-PCR. Data are normalized to GAPDH expression. Values represent mean fold change ± SD, compared to wild-type mice (n = 4). *, ** and *** indicate P < 0.05, P < 0.01 and P < 0.001 compared with wild-type mice, respectively.

Figure 2. Plasma concentration-time curves (upper panels) and plasma AUC$_{0-6hr}$ (lower panels) of dasatinib (A and D), sorafenib (B and E) and sunitinib (C and F) in male wild-type, Abcb1a/1b(+/-);Abcg2(+/-) and Abcb1a/1b(−/−);Abcg2(−/-) mice receiving oral dasatinib, sorafenib or sunitinib at 10 mg/kg, respectively. Data represent mean ± SD (n = 5). *, ** and *** indicate P < 0.05, P < 0.01 and P < 0.001 compared with wild-type mice, respectively; †† and ††† indicates P < 0.01 and P < 0.001 compared with Abcb1a/1b(+/-);Abcg2(+/-) mice. Note the differences in Y-axis scales for the different drugs.

Figure 3. Brain concentration (upper panels) and relative brain accumulation (lower panels) of dasatinib (A and D), sorafenib (B and E) and sunitinib (C and F) at 6 hr in male wild-type, Abcb1a/1b(+/-);Abcg2(+/-) and Abcb1a/1b(−/−);Abcg2(−/-) mice (+/+, +/- and −/−, respectively) receiving oral dasatinib, sorafenib or sunitinib at 10 mg/kg, respectively. Data represent mean ± SD (n = 5). *, ** and *** indicate P < 0.05, P < 0.01 and P < 0.001 compared with wild-type mice, respectively; ††† indicates P < 0.001 compared with Abcb1a/1b(+/-);Abcg2(+/-) mice. Note the differences in Y-axis scales for the different drugs.
Table 1  Pharmacokinetic parameters, brain concentrations and relative brain accumulation of dasatinib in male wild-type, Abcb1a/1b(+/−);Abcg2(+/−) and Abcb1a/1b(−/−);Abcg2(−/−) mice receiving oral dasatinib at a dose of 10 mg/kg

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>Abcb1a/1b(+/−);Abcg2(+/−)</th>
<th>Abcb1a/1b(−/−);Abcg2(−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC\textsubscript{0−6hr}, ng/ml.hr</td>
<td>1070.8 ± 595.7</td>
<td>1705.9 ± 224.6</td>
<td>2286.9 ± 344.6 **</td>
</tr>
<tr>
<td>Fold change AUC\textsubscript{0−6hr}</td>
<td>1.0</td>
<td>1.6</td>
<td>2.1</td>
</tr>
<tr>
<td>C\textsubscript{max}, ng/ml</td>
<td>265.7 ± 202.6</td>
<td>468.5 ± 360.6</td>
<td>690.5 ± 194.8</td>
</tr>
<tr>
<td>T\textsubscript{max}, hr</td>
<td>4.00</td>
<td>0.25</td>
<td>0.50</td>
</tr>
<tr>
<td>C\textsubscript{brain}, ng/g</td>
<td>2.4 ± 1.6</td>
<td>6.8 ± 1.4 **</td>
<td>138.2 ± 22.9 ***/†††</td>
</tr>
<tr>
<td>Fold increase C\textsubscript{brain}</td>
<td>1.0</td>
<td>2.9</td>
<td>58.4</td>
</tr>
<tr>
<td>P\textsubscript{brain}, (×10\textsuperscript{2} hr\textsuperscript{−1})</td>
<td>0.21 ± 0.08</td>
<td>0.40 ± 0.09 **</td>
<td>6.1 ± 0.8 ***/†††</td>
</tr>
<tr>
<td>Fold increase P\textsubscript{brain}</td>
<td>1.0</td>
<td>1.9</td>
<td>29.2</td>
</tr>
</tbody>
</table>

Data represent mean ± SD (n = 5). ** and *** indicate P < 0.01 and P < 0.001 compared with wild-type mice, respectively; ††† indicates P < 0.001 compared with Abcb1a/1b(+/−);Abcg2(+/−) mice. Abbreviations: AUC\textsubscript{0−6hr}: area under the plasma concentration-time curve from 0 to 6 hr; C\textsubscript{max}, maximum plasma concentration; T\textsubscript{max}, time to reach maximum drug concentration in plasma; C\textsubscript{brain}, brain concentration of drug at 6 hr after oral administration; P\textsubscript{brain}, relative brain accumulation of drug at 6 hr after oral administration, calculated by determining the drug brain concentration relative to the AUC\textsubscript{0−6hr}. 
Table 2 Pharmacokinetic parameters, brain concentrations and relative brain accumulation of sorafenib in male wild-type, Abcb1a/1b(+/−);Abcg2(+/−) and Abcb1a/1b(−/−);Abcg2(−/−) mice receiving oral sorafenib at a dose of 10 mg/kg

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>Abcb1a/1b(+/−);Abcg2(+/−)</th>
<th>Abcb1a/1b(−/−);Abcg2(−/−)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUCₐ₀₋₆hr, ng/ml.hr</td>
<td>17933.9 ± 2029.0</td>
<td>14464.7 ± 833.1</td>
<td>14746.4 ± 3879.8</td>
</tr>
<tr>
<td>Fold change AUCₐ₀₋₆hr</td>
<td>1.0</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>C_max, ng/ml</td>
<td>5051.9 ± 532.4</td>
<td>4061.9 ± 626.8</td>
<td>3220.8 ± 1480.2</td>
</tr>
<tr>
<td>T_max, hr</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>C_brain, ng/g</td>
<td>25.7 ± 25.1</td>
<td>38.8 ± 23.8</td>
<td>714.0 ± 214.2 ***</td>
</tr>
<tr>
<td>Fold increase C_brain</td>
<td>1.0</td>
<td>1.5</td>
<td>27.8</td>
</tr>
<tr>
<td>P_brain, (×10⁻² hr⁻¹)</td>
<td>0.14 ± 0.15</td>
<td>0.27 ± 0.16</td>
<td>5.1 ± 2.0 ***</td>
</tr>
<tr>
<td>Fold increase P_brain</td>
<td>1.0</td>
<td>1.8</td>
<td>35.9</td>
</tr>
</tbody>
</table>

Data represent mean ± SD (n = 5). *** indicates P < 0.001 compared with wild-type mice, respectively; ††† indicates P < 0.001 compared with Abcb1a/1b(+/−);Abcg2(+/−) mice. Abbreviations: AUCₐ₀₋₆hr: area under the plasma concentration-time curve from 0 to 6 hr; C_max: maximum plasma concentration; T_max: time to reach maximum drug concentration in plasma; C_brain: brain concentration of drug at 6 hr after oral administration; P_brain: relative brain accumulation of drug at 6 hr after oral administration, calculated by determining the drug brain concentration relative to the AUCₐ₀₋₆hr.
Table 3  Pharmacokinetic parameters, brain concentrations and relative brain accumulation of sunitinib in male wild-type, *Abcb1a/1b(+/-);Abcg2(+/-)* and *Abcb1a/1b(-/-);Abcg2(-/-)* mice receiving oral sunitinib at a dose of 10 mg/kg

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>Abcb1a/1b(+/-);Abcg2(+/-)</th>
<th>Abcb1a/1b(-/-);Abcg2(-/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AUC</strong>&lt;sub&gt;0-6hr&lt;/sub&gt;, ng/ml.hr</td>
<td>238.9 ± 88.7</td>
<td>357.4 ± 80.4</td>
<td>183.7 ± 41.2 ††</td>
</tr>
<tr>
<td>Fold change AUC&lt;sub&gt;0-6hr&lt;/sub&gt;</td>
<td>1.0</td>
<td>1.5</td>
<td>0.8</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt;, ng/ml</td>
<td>55.5 ± 33.4</td>
<td>77.2 ± 16.0</td>
<td>37.6 ± 11.2</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt;, hr</td>
<td>2.00</td>
<td>4.00</td>
<td>1.00</td>
</tr>
<tr>
<td>C&lt;sub&gt;brain&lt;/sub&gt;, ng/g</td>
<td>36.5 ± 17.5</td>
<td>88.4 ± 11.7 *</td>
<td>721.3 ± 337.8 ***†††</td>
</tr>
<tr>
<td>Fold increase C&lt;sub&gt;brain&lt;/sub&gt;</td>
<td>1.0</td>
<td>2.4</td>
<td>19.7</td>
</tr>
<tr>
<td>P&lt;sub&gt;brain&lt;/sub&gt;, (&quot;10&lt;sup&gt;-2&lt;/sup&gt; hr&quot;&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>16.4 ± 10.1</td>
<td>25.8 ± 6.6</td>
<td>389.1 ± 153.4 ***†††</td>
</tr>
<tr>
<td>Fold increase P&lt;sub&gt;brain&lt;/sub&gt;</td>
<td>1.0</td>
<td>1.6</td>
<td>23.7</td>
</tr>
</tbody>
</table>

Data represent mean ± SD (n = 5). * and *** indicate P < 0.05 and P < 0.001 compared with wild-type mice, respectively; †† and ††† indicate P < 0.01 and P < 0.001 compared with *Abcb1a/1b(+/-);Abcg2(+/-)* mice, respectively. Abbreviations: AUC<sub>0-6hr</sub>: area under the plasma concentration-time curve from 0 to 6 hr; C<sub>max</sub>: maximum plasma concentration; T<sub>max</sub>: time to reach maximum drug concentration in plasma; C<sub>brain</sub>: brain concentration of drug at 6 hr after oral administration; P<sub>brain</sub>: relative brain accumulation of drug at 6 hr after oral administration, calculated by determining the drug brain concentration relative to the AUC<sub>0-6hr</sub>.
Figure 1

A. Abcb1a

B. Abcg2

C. Male Small intestine

D. Male Brain

E. Female Brain

F. Male + Female Brain
Figure 2

A

Wild-type

$\text{Abcb1a/1b}(+/-):\text{Abcg2}(+/-)$

$\text{Abcb1a/1b}(-/-):\text{Abcg2}(-/-)$

B

Sorafenib concentration in plasma

C

Sunitinib concentration in plasma

D

Dasatinib concentration in plasma

E

Sorafenib plasma AUC$_{0-4}$hr

F

Sunitinib plasma AUC$_{0-4}$hr

Wild-type

$\text{Abcb1a/1b}(+/-):\text{Abcg2}(+/-)$

$\text{Abcb1a/1b}(-/-):\text{Abcg2}(-/-)$