Short Communication

Differential Impact of P-Glycoprotein (ABCB1) and Breast Cancer Resistance Protein (ABCG2) on Axitinib Brain Accumulation and Oral Plasma Pharmacokinetics

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ABSTRACT:
The second-generation tyrosine kinase inhibitor and anticancer drug axitinib is a potent, orally active inhibitor of the vascular endothelial growth factor receptors 1, 2, and 3. Axitinib has clinical activity against solid tumors such as metastatic renal cell carcinoma and advanced pancreatic cancer. We studied axitinib transport using Madin-Darby canine kidney II cells overexpressing human ABCB1 or ABCG2 or murine Abcg2. Axitinib was a good substrate of ABCB1 and Abcg2, whereas transport activity by ABCG2 was moderate. These transporters may therefore contribute to axitinib resistance in tumor cells. Upon oral administration of axitinib, Abcg2(−/−) and Abcb1a1/b;Abcg2(−/−) mice displayed 1.7- and 1.8-fold increased axitinib areas under the plasma concentration-time curve from 0 to 4 compared with those of wild-type mice. Plasma concentrations in Abcb1a1/b;Abcg2(−/−) mice were not significantly increased. In contrast, relative brain accumulation of axitinib in Abcb1a1/b;Abcg2(−/−) mice was, respectively, 6.8- and 13.9-fold higher than that in wild-type mice at 1 h and 4.9- and 20.7-fold at 4 h after axitinib administration. In Abcg2(−/−) mice, we found no significant differences in brain accumulation compared with those in wild-type mice. Thus, Abcb1 strongly restricts axitinib brain accumulation and completely compensates for the loss of Abcg2 at the blood-brain barrier, whereas Abcg2 can only partially take over Abcb1-mediated axitinib efflux. Hence, Abcg2 has a stronger impact on axitinib oral plasma pharmacokinetics, whereas Abcb1 is the more important transporter at the blood-brain barrier. These findings illustrate that in vitro transport data for ABCB1 and ABCG2 cannot always be simply extrapolated to the prediction of the relative impact of these transporters on oral availability versus brain penetration.

Introduction

The ATP-binding cassette (ABC) transporters P-glycoprotein (P-gp/ABCB1) and breast cancer resistance protein (BCRP/ABCG2) affect the disposition of a variety of endogenous and exogenous compounds, including many anticancer drugs. Both transporters are expressed at the apical membranes of enterocytes, hepatocytes, and renal tubular epithelial cells, where they potentially limit gastrointestinal absorption or mediate direct intestinal, hepatic, or renal excretion of their substrates. Moreover, ABCB1- and ABCG2-mediated efflux activity in brain endothelial capillary cells of the blood-brain barrier (BBB) is crucial for the protection of the central nervous system from harmful compounds (Schinkel and Jonker, 2003; Vlaming et al., 2009). In addition, ABC transporters are expressed in many tumor types, mediating multidrug resistance against anticancer drugs (Borst and Oude Elferink, 2002).

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ABBREVIATIONS: ABC/Abc, ATP-binding cassette; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; BBB, blood-brain barrier; TKI, tyrosine kinase inhibitor; AG013736, N-methyl-2-[3-[3-(3-hydroxy-2-isquinolinyl)ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; AUC, area under the plasma concentration-time curve; ANOVA, analysis of variance.
against various tumor types, such as metastatic renal cell carcinoma (RCC) (Rixe et al., 2007; Rini et al., 2009), metastatic breast cancer (Rugo et al., 2005), thyroid cancer (Cohen et al., 2008), advanced non–small-cell lung cancer (Schiller et al., 2009), and pancreatic cancer (Spano et al., 2008). Phase III studies testing the effect of axitinib in advanced pancreatic cancer and in metastatic RCC and pancreatic cancer are ongoing (ClinicalTrials.gov numbers NCT00920816, NCT00678392, and NCT00471146).

Animal studies and early-stage clinical trials have shown beneficial effects of VEGFR-targeting agents including the TKIs cediranib, sorafenib, sunitinib, and dasatinib for the treatment of malignant glioma (summarized in Rahmen et al., 2010). A crucial characteristic for an anticancer drug in treating brain tumors or brain metastases is its ability to reach all the tumor cells and therefore often its ability to cross the BBB. Many anticancer drugs are subject to ABCB1- and ABCG2-mediated efflux at the BBB, resulting in significantly reduced brain concentrations (Gottesman et al., 2002; Vlaming et al., 2009). Although therapeutic efficacity of axitinib against brain tumors has not yet been assessed, it is relevant to know the impact of ABC transporters on the axitinib brain accumulation with respect to possible future clinical applications.

To our knowledge no data are currently available regarding interactions of axitinib with ABCB1 and ABCG2. Therefore, the aim of this study was to investigate whether axitinib is a substrate of one or both of these transporters and how this would affect oral plasma pharmacokinetics and brain penetration of the drug. To assess the transport of axitinib in vitro we used MDCKII cells overexpressing human ABCB1 and ABCG2 as well as murine Abcg2. We next measured axitinib plasma concentration profiles and brain accumulation in WT, Abcb1a/1b(−/−), Abcg2(−/−), and Abcb1a/1b; Abcg2(−/−) mice upon oral administration.

Materials and Methods

Chemicals. Axitinib and elacridar (N-(4-(2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl)-phenyl)-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide, GFI20918) were purchased from Sequoia Research Products (Pangbourne, UK). [3H]Naludin (5.6 Ci/mol) was from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Zosuquidar (Eli Lilly, Indianapolis, IN) was a kind gift from Dr. O. van Tellingen (The Netherlands Cancer Institute, Amsterdam, The Netherlands). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise.

Transport Assays. For transepithelial transport assays we used the polarized Madin-Darby canine kidney (MDCKII) cell line and subclones transduced (using retroviral vectors) with human ABCB1, mouse Abcg2 (Bakos et al., 1998; Jonker et al., 2000), and a newly derived human ABCG2-overexpressing clone, recently generated by B. Poller, E. Wagenaar, S. C. Tang, and A. H. Schinkel (manuscript submitted for publication). Transport assays were performed as described previously with minor modifications (Lagas et al., 2009). Two hours before the experiment was started, cells were washed with phosphate-buffered saline and preincubated with Opti-MEM (Invitrogen, Carlsbad, CA) alone or containing either elacridar (5 μM) or zosuquidar (5 μM). At t = 0 h, the medium in the donor compartment was replaced with Opti-MEM containing axitinib (1 μM) alone or in combination with an inhibitor. Aliquots of 100 μl were taken at 2 and 4 h. The percentage of axitinib appearing in the acceptor compartment relative to the total amount added to the donor compartment at the beginning of the experiment was calculated. All data are means (n = 3) ± S.D. Transport ratios (r) were calculated by dividing apically directed by basolaterally directed axitinib translocation. Paracellular [3H]Clinulin leakage (0.09 μCi/well), which had to remain below 1% per hour, was measured in parallel in the same cells seeded and cultured in the same way to assure monolayer integrity. Because axitinib is sensitive to light-induced isomerization (Sparidans et al., 2009), adequate precautions were taken throughout all experimental procedures to limit light exposure as much as possible (e.g., reduced lighting during sample handling and collection of samples in amber tubes).

Animals. All mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used for this study were male WT, Abcb1a/1b(−/−) (Schinkel et al., 1997), Abcg2(−/−) (Jonker et al., 2002), and Abcb1a/1b; Abcg2(−/−) (Jonker et al., 2005) knockout mice of a >99% FVB genetic background, between 8 and 12 weeks of age. Animals were kept in a temperature-controlled environment with a 12-h light/dark cycle and received a standard diet (AM-III; Hope Farms, Woerden, The Netherlands) and acidified water ad libitum.

Plasma Pharmacokinetics and Brain Accumulation of Axitinib. Axitinib was dissolved in polysorbate 80-ethanol (1:1, v/v) (3.3 mg/l) and 3.3-fold diluted with NaCl 0.9% (w/v). Axitinib was administered orally at 10 mg/kg (10 ml/kg). To reduce variation in absorption, mice (n = 4–5 per group) were fasted at least 3 h before axitinib was given by gavage into the stomach using a blunt-ended needle. Multiple blood samples (30 μl) were collected from the tail vein either at 15, 30, and 60 min or at 15, 30, 60, 120, and 240 min using heparinized capillary tubes (Oxford Labware, St. Louis, MO). At the last time points (60 or 240 min), mice were sacrificed by cardiac puncture under isoflurane anesthesia followed by cervical dislocation. Brains were rapidly removed and homogenized on ice in 1 ml of 4% (w/v) bovine serum albumin. Plasma was obtained by centrifugation of blood samples at 5200g for 6 min at 4°C. All procedures were performed under limited light exposure of axitinib-containing samples.

Axitinib Analysis. Axitinib concentrations in Opti-MEM, plasma, and brain homogenate samples were analyzed by a sensitive and specific liquid chromatography-tandem mass spectrometry assay as described previously (Sparidans et al., 2009). Axitinib-containing solutions and samples were protected from light throughout all experimental procedures.

Calculation of AUC, Relative Brain Accumulation, and Statistical Analysis. The AUC was calculated using the trapezoidal rule, without extrapolating to infinity. One-way analysis of variance (ANOVA) was used for statistical analysis, and data obtained from knockout mice were compared with data from WT mice. Axitinib brain concentrations were corrected by the amount of drug in the brain vasculature, corresponding to 1.4% of the plasma concentration at the last time point (Dai et al., 2003). Relative brain accumulation (Pbrain) was calculated by dividing brain concentrations (Cbrain) at either t = 0 h or t = 4 h by the area under the plasma concentration-time curve from 0 to 1 h (AUC0–1) or 0 to 4 h (AUC0–4), respectively. For statistical analysis of brain accumulation data, the individual values were log-transformed to obtain normal distribution, and one-way ANOVA was performed. Differences were considered statistically significant when P < 0.05. All data are given as means ± S.D.

Results and Discussion

We first studied the interaction between axitinib and ABC transporters in vitro by measuring axitinib (1 μM) translocation through polarized monolayers of the MDCKII parental cell line and subclones overexpressing human ABCB1 or ABCG2 or mouse Abcg2. As shown in Fig. 1A, we observed moderate apically directed axitinib transport in the parental cell line (transport ratio r = 1.4), which was abrogated by treatment with the ABCB1/ABCG2 inhibitor elacridar and the relatively ABCB1-specific inhibitor zosuquidar (Fig. 1B). This finding suggests that this background transport was mediated by a substrate of human ABCB1. In subsequent transport experiments using MDCKII cells overexpressing human or mouse ABCG2, zosuquidar was included to block the background transport mediated by endogenous canine ABCB1 (Fig. 1B). Whereas axitinib was moderately transported by human ABCG2 (r = 1.3), we observed substantially apically directed translocation by the mouse Abcg2 (r = 3.4). Axitinib transport by human and mouse ABCG2 was efficiently blocked by elacridar. Axitinib was not significantly transported by...
human ABCC2 or mouse Abcc2 expressed in MDCKII cells (data not shown). To the best of our knowledge, this is the first report demonstrating active transport of axitinib by ABCB1 and ABCG2/Abcg2. We subsequently studied the single and combined effects of Abcb1 and Abcg2 on axitinib plasma pharmacokinetics and brain accumulation using WT, Abcb1a/1b (H11002/H11002), Abcg2 (H11002/H11002), and Abcb1a/1b; Abcg2 (H11002/H11002) mice. Because axitinib is given orally to patients, we administered axitinib orally at a dose of 10 mg/kg. In Abcg2(−/−) and Abcb1a/1b;Abcg2(−/−) mice we found 1.7- and 1.8-fold, statistically significant increases in AUC0–4 h values compared with those in WT mice (Fig. 2; Table 1). In contrast, the AUC0–4 h in Abcb1a/1b(−/−) mice was not significantly increased (P = 0.12). Qualitatively similar results were obtained by measuring the axitinib plasma concentration-time curves in Abcg2(−/−) and Abcb1a/1b;Abcg2(−/−) mice, but no increase in Abcb1a/1b(−/−) mice compared with that in WT mice (Table 1). The virtually identical axitinib plasma concentration-time curves in Abcg2(−/−) and Abcb1a/1b;Abcg2(−/−) mice as shown in Fig. 2 also suggest a substantial impact of Abcg2 on axitinib plasma pharmacokinetics, whereas the effect of Abcb1 on axitinib plasma concentrations appears to be minor or negligible. This finding seems to be at odds with the observed efficient axitinib transport
activity by ABCB1 in vitro. However, it is a fairly common observation that many good in vitro ABCB1 substrates show little or no activity by ABCB1 in vitro. However, it is a fairly common observation.

FIG. 2. Plasma concentration-time curves of axitinib in male WT (○), Abcg2(−/−) (□), Abcb1a/1b(−/−) (▲), and Abcb1a/1b;Abcg2(−/−) (▲) mice after oral administration of 10 mg/kg axitinib. Data are given as means ± S.D. (n = 5).

We next studied the impact of Abcb1 and Abcg2 on axitinib brain accumulation in different mouse strains. Oral administration of 10 mg/kg axitinib to Abcb1(−/−) mice did not result in significantly altered brain concentrations, either at 1 or at 4 h, compared with that in WT animals (Fig. 3, A and B; Table 1). In contrast, brain concentrations in Abcb1a/1b(−/−) mice showed statistically significant increases by 6.3- and 7.9-fold at 1 and 4 h, respectively, compared with those in WT mice. We further found markedly (and significantly) higher axitinib brain concentrations (20- and 42-fold at 1 and 4 h, respectively) in Abcb1a/1b;Abcg2(−/−) mice than in WT mice. Correcting the axitinib brain concentrations for the corresponding plasma AUCs also failed to reveal increased axitinib accumulation in brains of Abcg2(−/−) compared with those in WT mice, whereas brain accumulation in Abcb1a/1b(−/−) mice at 1 h was 6.8-fold higher than that in WT mice (P < 0.001) and 4.9-fold higher at 4 h (Fig. 3, C and D; Table 1). In Abcb1a/1b;Abcg2(−/−) mice, we found highly significant 14- and 21-fold higher brain accumulation at 1 and 4 h, respectively (Fig. 3, C and D; Table 1). In general, axitinib brain concentrations and brain accumulation were roughly 10- and 50-fold lower, respectively, at 4 h than at 1 h. This pattern was consistently observed among all mouse strains. These data suggest that in all strains during the 1st h of exposure a more or less steady-state situation in axitinib brain/plasma ratios has been established that is in part determined by Abcb1 and/or Abcg2 activity and that does not dramatically change between 1 and 4 h, as the plasma levels of axitinib drop. Thus, from 1 h on, the disappearance of axitinib from the brain was only modestly delayed compared with the disappearance from plasma. Brain/plasma ratios, which were quite similar between 1 and 4 h, further supported this observation (Fig. 3, E and F). Our results demonstrate that Abcb1 strongly reduces axitinib brain accumulation and can fully compensate for the loss of Abcg2 at the mouse BBB, because axitinib brain levels in Abcg2(−/−) mice were virtually identical to those in WT mice. On the other hand, Abcg2 can only partially compensate for the loss of the Abcb1-mediated efflux activity, in view of the increased axitinib brain accumulation in the absence of Abcb1. The 2- to 4-fold further increase in brain accumulation in Abcb1a/1b;Abcg2(−/−) mice compared with that in Abcb1a/1b(−/−) mice demonstrates that Abcg2 can contribute to the BBB for axitinib, but this is only clearly detectable in the absence of Abcb1.

### TABLE 1

**Plasma AUC, brain concentrations, and relative brain accumulation of axitinib in mice at 1 h and 4 h after oral administration at 10 mg/kg**

Data are means (n = 4–5) ± S.D. One-way ANOVA was performed for all AUCs and log-transformed brain penetration data obtained for WT and knockout mice. Part of these data are also presented in Figures 2 and 3.

<table>
<thead>
<tr>
<th>Strain</th>
<th>1 h</th>
<th>4 h</th>
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<tbody>
<tr>
<td></td>
<td>AUC(0–1 h) (µg/ml·h)</td>
<td>C_{brain} (µg/g)</td>
</tr>
<tr>
<td>WT</td>
<td>1.09 ± 0.58</td>
<td>0.10 ± 0.07</td>
</tr>
<tr>
<td>Abcg2(−/−)</td>
<td>1.76 ± 0.58*</td>
<td>0.08 ± 0.03</td>
</tr>
<tr>
<td>Abcb1a/1b(−/−)</td>
<td>0.95 ± 0.35</td>
<td>6.3</td>
</tr>
<tr>
<td>Abcb1a/1b;Abcg2(−/−)</td>
<td>0.35 ± 0.34***,†††,‡‡</td>
<td>0.07</td>
</tr>
</tbody>
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|        | AUC(0–4 h) (µg/ml·h) | C_{brain} (µg/g) | P_{trans} (× 10^{-3}·h^{-1}) | Fold increase |
| WT     | 2.68 ± 1.36 | 0.006 ± 0.004 | 2.3 ± 1.0 | 1.0 |
| Abcg2(−/−) | 4.67 ± 1.83** | 0.007 ± 0.003 | 1.5 ± 0.7 | 1.1 |
| Abcb1a/1b(−/−) | 3.95 ± 1.81 | 0.047 ± 0.042** | 1.2 ± 10.5* | 1.0 |
| Abcb1a/1b;Abcg2(−/−) | 4.95 ± 1.96** | 0.25 ± 0.20** | 9.7 | 42.4 |

* P < 0.05, compared with WT mice.
*** P < 0.001.
† P < 0.05, compared with Abcg2(−/−) mice.
†† P < 0.01.
††† P < 0.001.
‡ P < 0.05, compared with Abcb1a/1b(−/−) mice.
‡‡ P < 0.01.


Our brain accumulation data for axitinib are similar to published results in Abcb1 and Abcg2 knockout mice for TKIs such as imatinib, dasatinib, lapatinib, gefitinib, and erlotinib, all shared substrates of both efflux transporters (Lagas et al., 2009; Oostendorp et al., 2009; Polli et al., 2009; Agarwal et al., 2010; Kodaira et al., 2010). Abcb1 and Abcg2 limit the brain accumulation of these TKIs in concert, but with Abcb1 providing the major contribution. In contrast, for sorafenib, which is only a very moderate ABCB1 substrate in vitro, Abcg2 was found to be the major determinant for limiting brain accumulation (Lagas et al., 2010; Agarwal et al., 2011). The disproportionately high brain penetration of the indicated TKIs found in Abcb1a/1b;Abcg2(-/-) mice compared with that in mice deficient for only one transporter raised the question of the underlying mechanisms. Various explanations have been proposed, including adaptive increases in the complementary transporter expression in the BBB of single Abcg2 or Abcb1a/1b knockout strains (Zhou et al., 2009) or potential synergistic interactions between ABCB1 and ABCG2 (Polli et al., 2009). However, we have found that Abcg2 RNA expression in brain and small intestine of FVB background Abcb1a/1b knockout mice was not different from that of WT mice and that Abcb1 RNA expression in brain and small intestine was also not altered in FVB background Abcg2(-/-) mice (Jonker et al., 2000; de Vries et al., 2002).

**FIG. 3.** Brain concentrations (A and B), relative brain accumulation (C and D), and brain/plasma ratios (E and F) of axitinib in male WT, Abcg2(-/-), Abcb1a/1b(-/-), and Abcb1a/1b;Abcg2(-/-) mice after oral administration of 10 mg/kg axitinib at t = 1 h and τ = 4 h. Relative brain accumulation was calculated by dividing brain concentrations by the axitinib plasma AUC_{0-1h} and AUC_{0-4h}, respectively. Data are means ± S.D. [n = 5, except n = 4 for Abcg2(-/-) and Abcb1a/1b;Abcg2(-/-) at t = 1 h]. *: P < 0.05; **: P < 0.001, compared with WT mice; †: P < 0.05; ††: P < 0.01; †††: P < 0.001, compared with Abcg2(-/-) mice; ‡: P < 0.05; ‡‡: P < 0.01, compared with Abcb1a/1b(-/-) mice, using log-transformed data to normalize the S.D.s between study groups.
creased drug concentrations in the brains of forward kinetic analysis of brain penetration data of a wide selection of drugs. These authors concluded that the disproportionately increased drug concentrations in the brains of Abcb1a/1b;Abcg2(−/−) mice could be readily explained solely by the additive ABCB1- and ABCG2-mediated net efflux at the BBB, primarily because each in itself is considerably larger than the remaining clearance from the brain in the absence of these transporters. Hence, there is no need to postulate a synergistic interaction between ABCB1 and ABCG2 at the BBB to explain the observed brain accumulation data.

Several drugs targeting VEGFRs demonstrated promising results in early-stage clinical trials or animal experiments (summarized in Rahman et al., 2010). However, after an initial response, tumors often quickly became drug-resistant, potentially because of poor tumor cell exposure (Trédan et al., 2007). Preclinical coadministration of various TKIs such as dasatinib, gefitinib, and sorafenib to WT mice with the dual ABCB1 and ABCG2 inhibitor elacridar has resulted in significantly enhanced brain accumulation to levels similar to those observed in Abcb1a/1b;Abcg2(−/−) mice (Lagas et al., 2009, 2010). Furthermore, using the ABCB1- and ABCG2-inhibiting drug gefitinib, enhanced topotecan tumor penetration was demonstrated in mice bearing orthotopic human gliomas (Carcaboso et al., 2010). Clinical activity of axitinib was observed in a phase II study in patients with cytokine-refractory metastatic RCC with a response rate of 44.2% (Rixe et al., 2007). However, in 52% of the initial responders, the disease progressed for unknown reasons. Independently studying ABCB1 expression in tumors of patients with RCC revealed expression of ABCB1 at different levels in 100% of the tumors analyzed (Mignogna et al., 2006; Walsh et al., 2009). In addition, ABCB1 was suggested as a prognostic marker for RCC because an association between high ABCB1 expression and poor survival was described (Mignogna et al., 2006). Because the intracellular ATP-binding site of the VEGFR is the molecular target for axitinib (Hu-Lowe et al., 2008), high ABCB1 levels in tumor cells might prevent axitinib from crossing the plasma membrane, resulting in insufficient intracellular concentrations and thus resistance to axitinib. Concomitant administration of axitinib with inhibitors of ABCB1 and/or ABCG2 might thus be a promising approach to enhancing axitinib brain and brain tumor cell accumulation or to overcoming ABCB1-mediated drug resistance in RCC therapy.

Of interest, our experiments revealed a major role of Abcb1 in limiting axitinib brain accumulation compared with that of Abcg2, whereas in contrast only Abcg2, and not Abcb1, had a significant (albeit modest) impact on oral axitinib plasma concentrations. A possible explanation for this discrepancy might be different relative expression levels of Abcb1 and Abcg2 in intestinal enterocytes and brain endothelial capillary cells at the BBB. Indeed, several studies suggest higher expression of Abcb1 than Abcg2 in the mouse BBB (Zhou et al., 2009), which might explain why brain penetration data of drugs that are not extremely good Abcg2 substrates (dasatinib, lapatinib, and axitinib) generally show a major role of Abcb1 at the BBB. Regarding (oral) plasma AUCs, effects of ABC transporters are generally small compared with the effects observed at the BBB. This might be caused by higher background permeability for drugs at the intestinal wall, most likely also involving protein-mediated uptake processes, which could considerably reduce or even completely offset the impact of ABC transporter-mediated efflux. This may be the case for TKIs such as sorafenib, gefitinib, and lapatinib, for which neither Abcb1 nor Abcg2 has an impact on plasma kinetics. In contrast, dasatinib oral plasma AUCs are increased in Abcb1-deficient mice, whereas topotecan and axitinib oral AUCs are mainly influenced by Abcg2. The relative efficiency of the uptake processes may differ between individual TKIs and between intestine and BBB, which could also contribute to differential effects of Abcb1 and Abcg2 in intestine and BBB. Differential saturation conditions (intestinal drug concentrations are generally much higher than systemic concentrations upon oral drug administration) for both drug transporters with a specific drug might further play an important role. Our results illustrate that in vitro transport data for ABCB1 and ABCG2 cannot yet be simply extrapolated to the prediction of the relative impact of these transporters on both oral availability and brain penetration, with oral availability in particular being complicated. Therefore, we are still far from fully understanding all factors determining permeation of drugs across the important biological and pharmacological barriers involved.

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Wrote or contributed to the writing of the manuscript: Poller, Iusuf, Sparidans, Beijnen, and Schinkel.

References


