P-Glycoprotein (ABCB1) and Breast Cancer Resistance Protein (ABCG2) Restrict Brain Accumulation of the Active Sunitinib Metabolite N-Desethyl Sunitinib

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Received August 11, 2011; accepted January 9, 2012

ABSTRACT

N-desethyl sunitinib is a major and pharmacologically active metabolite of the tyrosine kinase inhibitor and anticancer drug sunitinib. Because the combination of N-desethyl sunitinib and sunitinib represents total active drug exposure, we investigated the impact of several multidrug efflux transporters on plasma pharmacokinetics and brain accumulation of N-desethyl sunitinib after sunitinib administration to wild-type and transporter knockout mice. In vitro, N-desethyl sunitinib was a good transport substrate of human ABCB1 and ABCG2 and murine Abc2, but not ABC2C or Abcc2. At 5 µM, ABCB1 and ABCG2 contributed almost equally to N-desethyl sunitinib transport. In vivo, the systemic exposure of N-desethyl sunitinib after oral dosing of sunitinib malate (10 mg/kg) was unchanged when Abcb1 and/or Abcg2 were absent. However, brain accumulation of N-desethyl sunitinib was markedly increased (13.7-fold) in Abcb1a1/1b1(−/−)-/Abcg2(−/−) mice, but not in Abcb1a1/1b1(−/−) or Abcg2(−/−) mice. In the absence of the ABCB1 and ABCG2 inhibitor elacridar, brain concentrations of N-desethyl sunitinib were detectable only in Abcb1a1/1b1(−/−)-/Abcg2(−/−) mice after sunitinib administration. Combined elacridar plus N-desethyl sunitinib treatment increased N-desethyl sunitinib plasma and brain exposures, but not brain-to-plasma ratios in wild-type mice. In conclusion, brain accumulation of N-desethyl sunitinib is effectively restricted by both Abcb1 and Abcg2. The effect of elacridar treatment in improving brain accumulation of N-desethyl sunitinib in wild-type mice was limited compared with its effect on sunitinib brain accumulation.

Introduction

ATP-binding cassette (ABC) efflux transporters, such as P-glycoprotein (ABC1), breast cancer resistance protein (ABCG2), and multidrug resistance protein 2 (ABC2), can have a significant impact on the absorption, distribution, excretion, and toxicity of xenobiotics (Glavinas et al., 2004). Several groups have shown that many tyrosine kinase inhibitors (TKIs) used in cancer therapy are substrates of both ABCB1 and ABCG2 (Dai et al., 2003; Burger et al., 2004; Bihorel et al., 2007; Chen et al., 2009; Lagas et al., 2009, 2010; Oostendorp et al., 2009) and found that the interaction with these ABC transporters may affect oral availability and brain accumulation of TKIs.

The TKI sunitinib malate (SU11248; Sutent, Pfizer, New York) is an orally active, small-molecule ATP-competitive multitargeted inhibitor of vascular endothelial growth factor receptors type 1 and 2, the platelet-derived growth factor receptors α and β, the stem cell factor receptor c-KIT, FMS-like TK-3 receptor, and the glial cell-line derived neurotrophic factor receptor (Chow and Eckhardt, 2007). Sunitinib is approved by the U.S. Food and Drug Administration for the treatment of advanced or metastatic renal cell carcinoma and imatinib-resistant gastrointestinal stromal tumors. Sunitinib displays an intrinsically high brain penetration among TKIs, and it is currently being tested in a...

After administration, sunitinib is metabolized primarily by cytochrome P450 3A4 to a major and pharmacologically active metabolite, N-desethyl sunitinib (Supplemental Fig. 1) (Houk et al., 2009). This is further metabolized by cytochrome P450 3A4 to an inactive compound (Adams and Leggas, 2007). In patients, plasma sunitinib and N-desethyl sunitinib accounted for 42 and 24%, respectively, of the total plasma radioactivity area under the plasma concentration-time curve (AUC)_∞ (Adams and Leggas, 2007). Given that N-desethyl sunitinib has a similar kinase inhibitory effect as sunitinib in vitro and similar plasma protein binding characteristics, the combination of sunitinib and N-desethyl sunitinib represents the total pharmacodynamically active drug in plasma. N-desethyl sunitinib may thus well account for one-third of the therapeutic effect of oral sunitinib. Previously, we have shown that sunitinib is transported in vitro by human ABCB1 and ABCG2 and murine Abcg2, but not by human ABCC2 or murine Abce2 (Tang et al., 2012). Simultaneous deficiency of Abcb1a1b and Abcg2, but not single Abcb1a1b or Abcg2 deficiency, resulted in highly increased brain levels of sunitinib in knockout mouse strains. We also demonstrated in wild-type mice that a clinically realistic oral coadministration of sunitinib and the dual ABCB1 and ABCG2 inhibitor elacridar could result in highly increased brain sunitinib levels (Tang et al., 2012). However, little is known so far about the interactions of N-desethyl sunitinib with ABC transporters in vitro or in vivo after sunitinib treatment.

In view of their expression in pharmacokinetically important organs and broad substrate specificity, we wanted to establish to what extent N-desethyl sunitinib is transported by human ABCB1, ABCG2, and ABCC2 and murine Abcg2 and Abce2 in vitro and what the consequences are for systemic availability and brain accumulation of N-desethyl sunitinib after oral administration of sunitinib as judged in knockout mouse models. We further tested whether these pharmacokinetic parameters of N-desethyl sunitinib could be improved by a clinically realistic coadministration of oral elacridar and oral sunitinib, with the ultimate aim of improving the overall therapeutic efficacy of sunitinib and its metabolite. We also aimed to obtain better insight into the factors that determine the relative impact of Abcb1 and Abcg2 on the brain accumulation of N-desethyl sunitinib after sunitinib intravenous administration. This may help efforts to overcome the blood-brain barrier (BBB) for therapeutic purposes.

Materials and Methods

Chemicals and Reagents. Sunitinib malate was purchased from Sequoia Research Products (Pangbourne, UK). N-desethyl sunitinib was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Elacridar [GF120918; N-4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isooquinolinyl)ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4- acridine carboxamide hydrochloride] (Evers et al., 2000) was kindly provided by GlaxoSmithKline (Stevenage, UK). Zosuquidar (LY-335979; (R)-4-[(1aR,6R,10b)-1,2,3,4-tetrahydro-1H-inden-6-yl]-α-(5-quinoxyloxy) methyl]-1-piperazine ethanol trihydrochloride] (El Lilly & Co., Indianapolis, IN) was a kind gift of Dr. O. van Tellingen (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Ko143 [(3S,8S,12aS)-1,2,3,4,6,7,12a-octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino[1’,2’:1,6]pyrido[3,4-b]indole-3-propanic acid 1,1-dimethyl-ethyl ester] was as described previously (Allen et al., 2002). [14C]Inulin was obtained from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Methoxyflurane (metofane) was supplied by Medical Developments Australia (Melbourne, Australia). Isoflurane (forane) was obtained from Abbott Laboratories Ltd (Queensbury, UK). Heparin (5000 IU/ml) was obtained from Leo Pharmaceuticals BV (Breda, The Netherlands). Bovine serum albumin (fraction V) was obtained from Sigma-Aldrich (Steinheim, Germany). All other chemicals and reagents were obtained from Sigma-Aldrich (Steinheim, Germany).

Cell Lines and Transport Assays. Polarized Madin-Darby canine kidney II (MDCKII) cells and their subclones transduced with human ABCB1, ABCG2, and ABCG2 or murine Abce2 and Abcg2 cDNA were used and cultured as described previously (Evers et al., 1998; Bakos et al., 2000; Jonker et al., 2000; Pavek et al., 2005; Zimmermann et al., 2008). Recently, Poller et al. (2011) generated a MDCKII cell line simultaneously overexpressing both ABCB1 and ABCG2 to better study the interplay of both transporters in vitro. Transepithelial transport assays were performed as described previously with minor modifications (Pavek et al., 2005). Two hours before starting the experiment, cells were washed with prewarmed phosphate-buffered saline and preincubated with 2 ml of Opti-MEM either alone or containing the dual ABCB1 and ABCG2 inhibitor elacridar (5 μM). The ABCB1 inhibitor zosuquidar (5 μM), the ABCG2 inhibitor Ko143 (1 μM), or a combination of zosuquidar and Ko143, which were present in both compartments during a 2-h preincubation period and during the transport experiment. Experiments were started (t = 0 h) by replacing the medium in one compartment (either basolateral or apical) with fresh Opti-MEM medium, either with or without inhibitor and containing 5 μM N-desethyl sunitinib. Cells were incubated at 37°C in 5% CO2, and 50-μl aliquots were taken at t = 2 and 4 h for determination of drug concentration. Transport was calculated as the fraction of drug found in the acceptor compartment relative to the total amount added to the donor compartment at the beginning of the experiment. Transport is given as mean percentage ± S.D. (n = 5). Membrane tightness was assessed in parallel by using the same cells seeded on the same day and at the same density, by analyzing transepithelial [14C]inulin (3.3 kBq/well) leakage. Leakage had to remain <1% of the total added radioactivity per hour. Active transport was expressed by the relative transport ratio (r), defined as r = percentage apically directed transport divided by basolaterally directed translocation after 4 h. Because of some interday variation in transport ratios, we digitally compared only transport ratios determined on the same day.

Animals. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Male wild-type, Abcb1a1b (-/-) (Schinkel et al., 1997), Abcg2(-/-) (Jonker et al., 2002), and Abcb1a1b (-/-) / Abcg2(-/-) (Jonker et al., 2005) mice, all of a >99% FVB genetic background, were used between 10 and 14 weeks of age. Animals were kept in a temperature-controlled environment with a 12-h light/dark cycle, and they received a standard diet (AM-II, Hope Farms, Woerden, The Netherlands) and acidicified water ad libitum.

Drug Solutions. Drug solutions for sunitinib and oral elacridar were prepared as described previously (Tang et al., 2012). For intravenous administration, elacridar hydrochloride was first dissolved in dimethyl sulfoxide at 150 mg/ml and further diluted with a mixture of ethanol, polyethylene glycol 200, and 5% glucose (2:6:2, v/v) to obtain a concentration of 4 mg/ml. N-desethyl sunitinib was dissolved in dimethyl sulfoxide at a concentration of 50 mg/ml and further diluted with 50 mM sodium acetate buffer, pH 4.6 to yield a concentration of 1 mg/ml. In all drug formulations drugs and/or...
modulators were completely dissolved, also during administration to the mice.

**Animal Experiments.** All of the animal experiments in which sunitinib was given either orally or intravenously were carried out as described previously (Tang et al., 2012). After oral sunitinib administration, multiple blood samples (~50 µl) were collected from the tail vein at 15 and 30 min and 1, 2, and 4 h. At 6 h, blood was collected by cardiac puncture under isoflurane anesthesia. This allowed determination of plasma concentration-time curves for each individual mouse.

**Brain Accumulation of N-Desethyl Sunitinib in Combination with Intravenous Elacridar Treatment.** Values of lower limit of quantification (LLQ) for N-desethyl sunitinib were 7.5 ng/g for the oral sunitinib in combination with oral elacridar for the brain accumulation experiment and 13.5 ng/g for the BBB efflux transport saturation experiment. This difference was caused by an improvement in the detection method to enhance sensitivity during the course of our studies. To circumvent detection problems of N-desethyl sunitinib in brain homogenates, we injected N-desethyl sunitinib (5 mg/kg) directly into the tail vein of wild-type and knock-out mice 15 min after an intravenous injection of either elacridar or vehicle in solution form. Blood and brain were isolated 60 min after N-desethyl sunitinib administration and processed as described previously (Tang et al., 2012).

**Drug Analysis.** Determination of N-desethyl sunitinib and the internal standard sunitinib-<sup>3</sup>H<sub>10</sub> was performed on a sensitive and specific liquid chromatography coupled with tandem mass spectrometry assay. The analytical method that was described previously (Lankheet et al., 2011; Tang et al., 2012) was used for the detection of sunitinib and N-desethyl sunitinib in plasma and brain homogenates. N-desethyl sunitinib was detected at the transition from m/z 371 to 283 with a retention time of 3.8 min. In case the (usually minor) E-isomer of desethyl sunitinib in plasma and brain homogenates, we injected N-desethyl sunitinib-<sup>3</sup>H<sub>10</sub> was performed on a sensitive and specific liquid chromatography coupled with tandem mass spectrometry to enhance sensitivity during the saturation experiment. This difference was caused by an improvement in the detection method to enhance sensitivity during the course of our studies. To circumvent detection problems of N-desethyl sunitinib in brain homogenates, we injected N-desethyl sunitinib (5 mg/kg) directly into the tail vein of wild-type and knock-out mice 15 min after an intravenous injection of either elacridar or vehicle in solution form. Blood and brain were isolated 60 min after N-desethyl sunitinib administration and processed as described previously (Tang et al., 2012).

**Pharmacokinetic Calculations and Statistical Analysis.** Pharmacokinetic parameters were calculated by noncompartmental methods using the software package PK Solutions 2.0.2 (Summit Research Services, Ashland, OH). The AUC was calculated by using the trapezoidal rule, without extrapolating to infinity. The AUC and maximum drug concentration in plasma (C<sub>max</sub>) were determined directly from individual concentration-time data. Data are presented as means ± S.D. For parametric statistical analysis, the individual values of Figs. 6, C to F and 7, A and D were log-transformed to obtain equality in variances. One-way analysis of variance (ANOVA) was used to determine significance between groups, after which post hoc tests with Bonferroni correction were performed for comparison between individual groups. Between-group comparisons of genotype or elacridar effect were made by using the two-tailed unpaired Student’s t test. Differences were considered statistically significant at p < 0.05.

**Results**

**In Vitro Transport of N-Desethyl Sunitinib.** Transepithelial drug transport was tested by using polarized monolayers of MDCKII parental cells and various ABC transporter-overexpressing derivative cell lines. N-desethyl sunitinib was modestly transported in the apical direction in the parental MDCKII cell line (transport ratio r of 2.1; Fig. 1A), presumably by the low-level endogenous canine ABCB1. In cells overexpressing human ABCB1, there was clear apically directed transport of N-desethyl sunitinib, with an r of 15.1 (Fig. 1B). N-desethyl sunitinib was also actively transported by human ABCG2 or murine Abcg2, with transport ratios of 6.0 and 6.6, respectively (Fig. 1, C and D). Addition of elacridar, a dual inhibitor of ABCB1 and ABCG2, completely inhibited polarized transport in MDCKII parental and MDCKII-ABCB1 cells (Fig. 1, E and F). However, elacridar at 5 µM did not completely inhibit the apical transport of N-desethyl sunitinib in MDCKII-ABCG2 cells (r reduced from 6.0 to 1.8) (Fig. 1G).
and had only a minimal effect on MDCKII cells overexpressing mouse Abcg2 (Fig. 1H). Elacridar at 5 μM does not modulate ABCC2/Abcc2 activity (Evers et al., 2000) and was therefore used to suppress any transport by endogenous canine ABCB1 in cells overexpressing human ABCG2 or murine Abcc2. Under these conditions, we did not observe polarized transport of N-desethyl sunitinib by either ABCC2 or Abcc2 (Fig. 1, I and J).

Because we did not observe complete inhibition of ABCG2- and Abcg2-mediated N-desethyl sunitinib transport by elacridar, we investigated whether transport of N-desethyl sunitinib in these cell lines could be completely inhibited with the specific and high-affinity ABCG2 inhibitor Ko143. The specific ABCB1 inhibitor zosuquidar was added to suppress any contribution of endogenous canine ABCB1 (Fig. 2A). N-desethyl sunitinib was actively transported in cells overexpressing human ABCG2 and murine Abcg2, with transport ratios of 13.5 and 20.3 versus 1.0 in parental cells (Fig. 2, B and C). The addition of Ko143 resulted in extensive inhibition of polarized transport in all of these cell lines (Fig. 2, D and E). Collectively, N-desethyl sunitinib was a good transport substrate of human ABCB1 and ABCG2 and murine Abcg2, but not human ABCB2 and murine Abcc2. Elacridar at 5 μM could only partially inhibit N-desethyl sunitinib transport by human ABCG2 and especially mouse Abcg2, a rather unusual observation, because in our experience in vitro transport of most Abcg2 substrates can be completely inhibited by 5 μM elacridar.

N-Desethyl Sunitinib Transport in MDCKII-ABCB1/ABCG2 Cells. We tested the relative contributions of human ABCB1 and ABCG2 to N-desethyl sunitinib transport at 5 and 20 μM in the absence or presence of Ko143 and zosuquidar in MDCKII cells simultaneously overexpressing ABCB1 and ABCG2 (Poller et al., 2011). Without inhibitor, we measured transport ratios of 13.3 and 16.5 at 5 and 20 μM, respectively (Supplemental Fig. 2, A and B). The ABCG2-mediated N-desethyl sunitinib transport in the presence of zosuquidar was reduced from an r of 9.7 at 5 μM to 5.8 at 20 μM (Supplemental Fig. 2, C and D). Upon blocking ABCG2 with Ko143, we observed ABCB1-mediated N-desethyl sunitinib transport ratios of 6.7 and 6.0 at 5 and 20 μM, respectively (Supplemental Fig. 2, E and F). Active transport of N-desethyl sunitinib at both 5 and 20 μM was completely abolished when both Ko143 and zosuquidar were present (Supplemental Fig. 3, G and H). Taken together, the contribution of ABCG2 to N-desethyl sunitinib transport is almost equal to that of ABCB1 at 5 and 20 μM, although some initial ABCG2 saturation may occur at 20 μM.

Impact of Abcb1 and Abcg2 on Plasma Pharmacokinetics of N-Desethyl Sunitinib after Oral Sunitinib Treatment. Because the parent compound sunitinib is given orally to cancer patients, we first studied the plasma concentration of N-desethyl sunitinib over time after oral sunitinib malate administration at 10 mg/kg to wild-type, Abcb1a1b1b1(−/−), Abcg2a(−/−), and Abcb1a1b1b1(−/−)/Abcg2a(−/−) mice. As shown in Fig. 3A and Table 1, there were no statistically significant differences in oral AUC or Cmax of N-desethyl sunitinib between the strains. The metabolite/parent drug AUC ratios ranged between 33 and 40% (Table 1) and also did not differ significantly between the strains. These ratios are similar to those observed in humans (Shirao et al., 2010). These results indicate that Abcb1 and Abcg2 do not have a substantial role in, or effect on, the availability, metabolism, or elimination of N-desethyl sunitinib after oral sunitinib administration.

Impact of Abcb1 and Abcg2 on Brain Accumulation of N-Desethyl Sunitinib after Oral Sunitinib Administration. As shown in Fig. 3B, the relative brain accumulation of N-desethyl sunitinib, determined 6 h after oral administration of sunitinib and corrected for the AUC0–6h, was not significantly different in Abcb1a1b1b1(−/−) and Abcg2a(−/−) mice compared with wild-type mice. In contrast, Abcb1a1b1b1b1(−/−)/Abcg2a(−/−) mice had a 13.7-fold increased brain accumulation (p < 0.05; see also Table 1). This indi-

Fig. 2. Transepithelial transport of N-desethyl sunitinib (5 μM) assessed by using MDCKII parental cells (A) or MDCKII cells transduced with human ABCG2 (B and D) or murine Abcg2 (C and E) cDNA. N-desethyl sunitinib application and measurement was as described in the legend of Fig. 1. B and C, the ABCB1 inhibitor zosuquidar (5 μM) was applied to specifically measure ABCG2/Abcg2-mediated transport. D and E, the ABCB2 inhibitor Ko143 (1 μM) was additionally applied to verify ABCG2/Abcg2-mediated transport. ▲, tranlocation from basolateral to apical compartment; □, tranlocation from apical to basolateral compartment. Points, means (n = 3); bars, S.D. At t = 4 h, 1% of transport is approximately equal to an apparent permeability coefficient (Papp) of 0.30 × 10−6 cm/s.
cates that brain accumulation of N-desethyl sunitinib was primarily restricted by both Abcb1 and Abcg2. Apparently, each of these transporters can largely take over the function of the other transporter at the BBB when knocked out. Only when both transporters are simultaneously absent can a large increase in brain accumulation of N-desethyl sunitinib occur.

**Influence of Elacridar on N-Desethyl Sunitinib Brain Accumulation after Oral Sunitinib Administration.** We wanted to assess the effect of elacridar on plasma pharmacokinetics and brain accumulation of N-desethyl sunitinib after oral sunitinib administration, given that elacridar markedly increased the brain accumulation of sunitinib, although not its oral bioavailability (Tang et al., 2012). In view of the potential clinical importance of oral application for both sunitinib and elacridar, we administered elacridar (100 mg/kg) orally 2 h before oral sunitinib malate (10 mg/kg) to the wild-type and Abcb1a1b/−/−/Abcg2/−/− strains and assessed plasma and brain N-desethyl sunitinib levels 1 h later, i.e., around the sunitinib time to reach maximum drug concentration in plasma (Supplemental Table 1). As shown in Fig. 4A, N-desethyl sunitinib plasma concentrations were not significantly different among the strains, regardless of administration with or without elacridar. The plasma metabolite-to-sunitinib ratios were not significantly different either (Supplemental Fig. 3). In vehicle-treated mice, brain concentrations of N-desethyl sunitinib in wild-type, Abcb1a1b/−/−, and Abcg2/−/− mice were below the LLQ (~7.5 ng/g). However, brain concentrations of N-desethyl sunitinib in Abcb1a1b/−/−/Abcg2/−/− mice were just above this limit. Elacridar treatment increased brain concentrations in wild-type mice to levels equal to those in Abcb1a1b/−/−/Abcg2/−/− mice (Fig. 4B). The N-desethyl sunitinib brain concentrations in Abcb1a1b/−/−/Abcg2/−/− mice were not significantly affected by elacridar treatment. These data suggest that oral elacridar treatment could inhibit the activity of Abcb1 and Abcg2 in the BBB, leading to increased N-desethyl sunitinib concentrations in the brain. However, the size of this effect could not be assessed in the absence of quantifiable brain values for vehicle-treated wild-type mice.

**Influence of Elacridar on Plasma and Brain Exposure of Intravenous N-Desethyl Sunitinib.** To circumvent these quantification problems, we injected N-desethyl sunitinib directly at an intravenous dose of 5 mg/kg to wild-type and Abcb1a1b/−/−/Abcg2/−/− mice 15 min after intravenous vehicle or elacridar administration, and we measured plasma and brain concentrations 1 h later. In
the absence of elacridar, the plasma concentrations of N-desethyl sunitinib in Abcg2(-/-) and Abcb1a1b(-/-)/Abcg2(-/-) mice, but not in Abcb1a1b(-/-) mice, were 1.9-fold higher than in wild-type mice (Fig. 5A). Elacridar treatment increased the N-desethyl sunitinib plasma levels in wild-type mice to levels equal to those in Abcb1a1b(-/-)/Abcg2(-/-) mice and significantly higher than in vehicle-treated wild-type mice (Fig. 5B). Collectively, these data suggest that Abcg2, but not Abcb1a1b, may contribute to the plasma elimination of intravenous N-desethyl sunitinib. In the absence of elacridar, brain concentrations of N-desethyl sunitinib were not significantly different in wild-type, Abcb1a1b(-/-), and Abcg2(-/-) mice, but they were increased 13.2-fold in Abcb1a1b(-/-)/Abcg2(-/-) mice compared with wild-type mice (Fig. 5C). Intravenous elacridar increased N-desethyl sunitinib brain concentrations (3.3-fold) in wild-type mice compared with wild-type mice without elacridar, but this level was still 4-fold lower than seen in Abcb1a1b(-/-)/Abcg2(-/-) mice without elacridar (Fig. 5D). We were surprised to find that the N-desethyl sunitinib brain levels found in Abcb1a1b(-/-)/Abcg2(-/-) mice pretreated with elacridar were 35% lower than in these mice given N-desethyl sunitinib alone (Fig. 5D). This suggests some inhibition by elacridar of N-desethyl sunitinib brain uptake. In the absence of elacridar, brain-to-plasma ratios in Abcb1a1b(-/-)/Abcg2(-/-) mice, but not Abcb1a1b(-/-) and Abcg2(-/-) mice, were markedly increased compared with wild-type mice (Fig. 5E). When looking at brain-to-plasma ratios, the difference between wild-type mice treated with elacridar or vehicle even lost statistical significance (Fig. 5F, compare white bars). However, the brain-to-plasma ratios of Abcb1a1b(-/-)/Abcg2(-/-) mice treated either with elacridar or vehicle were still significantly increased compared with wild-type mice without elacridar (Fig. 5F). The results indicate that the effect of intravenous elacridar in enhancing brain accumulation of N-desethyl sunitinib is quite limited, and there may even be some inhibition of N-desethyl sunitinib uptake into the brain. This results in somewhat higher brain concentrations compared with wild-type mice given N-desethyl sunitinib alone, but far less than the concentrations seen in mice genetically lacking both transporters.

Partial Saturation of Abcg2-Mediated N-Desethyl Sunitinib Transport in the BBB of Mice after High-Dose Sunitinib. We wanted to study the impact of Abcb1 and Abcg2 on brain accumulation of N-desethyl sunitinib after a high intravenous sunitinib administration (20 mg/kg) in knockout models. Plasma concentrations of N-desethyl sunitinib in Abcg2(-/-) and Abcb1a1b(-/-)/Abcg2(-/-) mice, but not in Abcb1a1b(-/-) mice, were modestly but significantly higher compared with wild-type mice at t = 10 min (Fig. 6A). At 1 h, plasma levels of this metabolite had increased relative to 10 min in all strains and the plasma levels in Abcg2(-/-) mice were still significantly higher than those in wild-type mice (Fig. 6B). Ten minutes after intravenous sunitinib administration, brain levels of N-desethyl sunitinib were not detectable in wild-type, Abcg2(-/-), and Abcb1a1b(-/-) mice, but marginally detectable in Abcb1a1b(-/-)/Abcg2(-/-) mice (Fig. 6C). At 1 h, N-desethyl sunitinib brain concentrations were detectable in all strains, and brain concentrations in Abcb1a1b(-/-) and Abcb1a1b(-/-)/Abcg2(-/-) mice, but not Abcg2(-/-) mice, were significantly higher than those in wild-type mice (Fig. 6D). Brain-to-plasma ratios were also significantly increased in Abcb1a1b(-/-)/Abcg2(-/-) mice, but not in Abcb1a1b(-/-) mice (Supplemental Fig. 4A). Upon high-dose intravenous sunitinib administration, the plasma N-desethyl sunitinib-to-sunitinib ratios were lower than after oral administration of sunitinib (10 mg/kg), but not significantly different among all strains except in Abcg2(-/-) mice, which displayed a 1.4-fold increase relative to wild-type mice (Supplemental Fig. 4B).

Discussion

In this study, we show that N-desethyl sunitinib is actively transported in vitro by human ABCB1 and ABCG2 and murine Abcg2, but not by human ABCC2 or murine Abce2. We also demonstrate in MDCKII cells stably coexpressing human ABCB1 and ABCG2 that the contribution of ABCG2 to N-desethyl sunitinib transport at 5 µM is nearly equal to
that of ABCB1. Upon oral sunitinib administration, the AUC$_{0–6h}$ of N-desethyl sunitinib did not differ among wild-type, Abcb1a$^{-/−}$/Abcg2$^{-/−}$, and Abcb1a$^{-/−}$/Abcg2$^{-/−}$/Abcg2$^{-/−}$ strains. However, we found a profound effect (13.7-fold) on N-desethyl sunitinib brain accumulation when both transport systems were absent from the BBB. In addition, brain concentrations of N-desethyl sunitinib were increased to just above the LLQ by concomitant oral elacridar treatment in wild-type mice. After intravenous administration of N-desethyl sunitinib, Abcg2 deficiency was found to

Fig. 5. A, C, and E, effect of genetic deletion on plasma (A) and brain (C) concentrations and brain-to-plasma ratios (E) of N-desethyl sunitinib in male wild-type, Abcb1a$^{-/−}$/Abcg2$^{-/−}$, and Abcb1a$^{-/−}$/Abcg2$^{-/−}$/Abcg2$^{-/−}$ mice 1 h after intravenous injection of 5 mg/kg of N-desethyl sunitinib. B, D, and F, effect of elacridar on plasma (B) and brain (D) concentrations and brain-to-plasma ratios (F) in wild-type and Abcb1a$^{-/−}$/Abcg2$^{-/−}$/Abcg2$^{-/−}$ mice. N-desethyl sunitinib was administered 15 min after intravenous administration of either elacridar (10 mg/kg) or vehicle. Columns, means ($n$ = 3–4); bars, S.D. *, $p < 0.05$; ***, $p < 0.001$, compared with wild-type mice receiving vehicle (one-way ANOVA). †, $p < 0.05$; ††, $p < 0.01$; †††, $p < 0.001$ compared with wild-type mice receiving vehicle (Student’s $t$ test). #, $p < 0.05$; ##, $p < 0.01$ compared with Abcb1a$^{-/−}$/Abcg2$^{-/−}$/Abcg2$^{-/−}$ receiving vehicle (Student’s $t$ test).
modestly enhance its systemic plasma levels, which may suggest a role in N-desethyl sunitinib elimination. In Abcb1a/1b(−/−)Abcg2(−/−) mice, but not in Abcb1a/1b(−/−) and Abcg2(−/−) mice, the brain levels of intravenously administered N-desethyl sunitinib were highly increased compared with wild-type mice. Thus, the brain accumulation of N-desethyl sunitinib, similar to that of its parent compound sunitinib, critically depends on the efflux activity of both Abcb1 and Abcg2. Intravenous elacridar increased N-desethyl sunitinib plasma and brain levels, but had no significant impact on the brain-to-plasma ratios of N-desethyl sunitinib in wild-type mice.

Our data suggest that there was no substantial role of Abcb1a/1b and Abcg2 in plasma pharmacokinetics of N-desethyl sunitinib after oral sunitinib administration. Accordingly, plasma levels of N-desethyl sunitinib were not substantially changed upon oral coadministration of elacridar and sunitinib (Fig. 4A). Upon direct intravenous administration, however, plasma levels of N-desethyl sunitinib were 1.9-fold increased in Abcg2(−/−) and Abcb1a/1b(−/−)/Abcg2(−/−) mice compared with vehicle-treated wild-type mice, but not in Abcb1a/1b(−/−) mice (Fig. 6A). These results suggest that Abcg2 might play a role in the systemic elimination of N-desethyl sunitinib after intravenous administration. In the presence of elacridar, plasma concentrations of N-desethyl sunitinib in wild-type and Abcb1a/1b(−/−)/Abcg2(−/−) mice were not different, suggesting that intravenous elacridar reduces the plasma elimination of N-desethyl sunitinib in wild-type mice, presumably via Abcg2. Likewise, Lagas et al. (2009) demonstrated previously that elacridar can inhibit the systemic elimination of the TKI dasatinib via Abcb1 and/or Abcg2. Our data suggest that a possible systemic elimination contribution of Abcg2 only became apparent at higher plasma levels (which would occur especially shortly after intravenous N-desethyl sunitinib administration), but not at lower plasma levels (after oral sunitinib administration). One could speculate that at low plasma levels alternative (non-Abcg2) N-desethyl sunitinib elimination processes predominate, but that these become saturated at higher plasma levels. If Abcg2 is not yet saturated at these levels, it will have a much more marked impact on N-desethyl sunitinib plasma concentrations than at lower plasma levels.

Like for many other shared Abcb1a/1b and Abcg2 substrates, the single disruption of Abcb1a/1b or Abcg2 in mice has only little effect on the brain accumulation of N-desethyl sunitinib, whereas simultaneous disruption of the two transporters results in a dramatic increase in N-desethyl sunitinib brain accumulation. This disproportionate effect has led some researchers to envisage a compensatory change with up-regulation of one transporter in the BBB of single knockout strains for the other transporter. However, extensive analyses of Abcg2 or Abcb1a expression in brain homogenates of, respectively, Abcb1a/1b or Abcg2 knockout mice as used by us revealed no significant change in the RNA and/or protein levels in either strain (Jonker et al., 2000; de Vries et al., 2007; Lagas et al., 2010). Moreover, Kodaira et al. (2010), using specific transport substrates for either transporter, found negligible shifts in brain accumulation of these substrates in the single knockout strains of the complementary

**Fig. 6.** Plasma concentrations (A and B) and brain concentrations (C and D) of N-desethyl sunitinib in male wild-type, Abcb1a/1b(−/−), Abcg2(−/−), and Abcb1a/1b(−/−)/Abcg2(−/−) mice 10 min (A and C) or 1 h (B and D) after intravenous injection of 20 mg/kg sunitinib malate. Columns, means (n = 5); bars, S.D. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with wild-type mice (one-way ANOVA). ND, not detectable.
transporter. This suggests that there is no substantial change in Abcb1a or Abcg2 activity in the BBB of the FVB background Abcg2 or Abcb1a/1b knockout mice, respectively. Kodaira et al. (2010) further showed that a (physiologically based) pharmacokinetic model of Abcb1a and Abcg2 activity in the BBB could readily describe the seemingly disproportionate effect of the combined disruption of these transporters compared with the single disruptions, without invoking changes in activity of the remaining transporter.

Our data show that the brain accumulation of N-desethyl sunitinib was highly increased in Abcb1a/1b(−/−)/Abcg2(−/−) mice 6 h after oral sunitinib administration. Therefore, we studied the brain accumulation of N-desethyl sunitinib after an oral coadministration schedule of elacridar and sunitinib that might also be feasible in a clinical setting. In this context it is important to note that the plasma exposure level of elacridar used in our study is also achievable in humans, as demonstrated by Kemper et al. (2001), who showed that a patient receiving 1000 mg of elacridar orally had almost the same elacridar plasma concentrations as mice treated with 100 mg/kg of elacridar orally. Somewhat disappointingly, brain levels of N-desethyl sunitinib were only just above the LLQ under this coadministration scheme (Fig. 4B). Direct intravenous administration of N-desethyl sunitinib at a dose of 5 mg/kg to wild-type mice resulted in higher brain levels of N-desethyl sunitinib, but still far lower than those in Abcb1a/1b(−/−)/Abcg2(−/−) mice (Fig. 5, A, C, and E). The results of Fig. 5, D and F suggest that elacridar could not fully inhibit the BBB efflux transporters with respect to N-desethyl sunitinib, resulting in lower brain accumulation compared with genetic deletion of Abcb1a/1b and Abcg2. Unexpectedly, elacridar treatment in Abcb1a/1b(−/−)/Abcg2(−/−) mice also lowered brain N-desethyl sunitinib levels compared with vehicle-treated mice, whereas plasma levels were not significantly affected (Fig. 5, B, D, and F). One speculative explanation is that this reduction effect might be caused by the inhibition of putative uptake transporters for N-desethyl sunitinib into the brain. Note that such a reduction was not observed for the parent drug sunitinib (Tang et al., 2012).

Even though elacridar is generally an effective inhibitor for ABCB1 and ABCG2 in vitro and in vivo, elacridar has been shown to be a better inhibitor of Abcb1 than Abcg2 in vitro (Allen et al., 2002; Matsson et al., 2009). We observed complete inhibition of ABCB1-mediated N-desethyl sunitinib transport, but only partial inhibition of human ABCG2- and especially murine Abcg2-mediated N-desethyl sunitinib transport (Fig. 1, C, D, G, and H). Note that, given the apparent difference in sensitivity to elacridar between mouse Abcg2 and human ABCG2, it could be that the effects of elacridar would be more pronounced in humans than in mice. In vivo, we were still able to observe a significant increase in brain concentration in wild-type mice treated with elacridar in combination with N-desethyl sunitinib, but the change in brain-to-plasma ratio was not statistically significant. The increase in the brain concentrations of N-desethyl sunitinib in wild-type mice could therefore be partly caused by the increased plasma concentrations in wild-type mice treated with elacridar (Fig. 5B). Our in vitro finding of poor Abcg2 inhibition by elacridar thus seems to be in agreement with our in vivo findings. Apparently, elacridar is overall not an efficient enough inhibitor of Abcg2-mediated transport of N-desethyl sunitinib. One could speculate that its affinity for Abcg2 is not high enough, because the high-affinity ABCG2/Abcg2 inhibitor Ko143 did completely inhibit transport in vitro. Thus, higher concentrations of elacridar may be needed to fully inhibit Abcg2 than to inhibit Abcb1 in the BBB. For N-desethyl sunitinib, however, increasing elacridar dosage might be counterproductive because we cannot exclude that it might also inhibit brain uptake more strongly.

Our in vitro data with ABCB1- and ABCG2-overexpressing MDCKII cells suggest that the transport contribution of ABCG2 in these cells is almost equal to that of ABCB1 at 5 μM N-desethyl sunitinib. Possibly beginning saturation of ABCG2-mediated, but not of ABCB1-mediated, N-desethyl sunitinib transport occurs at 20 μM. We have previously observed that plotting inverse transport ratios (AB/BA, or 1/r) obtained with this cell line yields good qualitative correlations with brain penetration data, especially for topotecan and sorafenib, but somewhat less for sunitinib (Puller et al., 2011). For its metabolite N-desethyl sunitinib, however, we again found a very good in vitro-in vivo correlation between brain concentration in the various wild-type and knockout strains and transcellular transport data obtained at 5 and 20 μM (Supplemental Fig. 5, compare C–F).

To obtain a better understanding of the relative ability of sunitinib and N-desethyl sunitinib to penetrate the brain, we compared the data obtained in this study with our previous findings for sunitinib (compare Table 1 and Supplemental Table 1). In wild-type mice, sunitinib has a 2.4-fold higher brain penetration than N-desethyl sunitinib, with a $P_{\text{brain}}$ of 1.6 and 0.67 observed for sunitinib and N-desethyl sunitinib, respectively. Six hours after oral sunitinib administration (10 mg/kg), sunitinib brain concentrations were 5.7-fold higher than N-desethyl sunitinib brain concentrations seen in wild-type mice. In addition, elacridar did not completely inhibit transport activity of human ABCG2 and murine Abcg2-mediated N-desethyl sunitinib transport in vitro, suggesting that N-desethyl sunitinib is a more avid human ABCG2 and murine Abcg2 substrate than sunitinib. Elacridar increased the brain-to-plasma ratio of sunitinib 12-fold (Tang et al., 2012), but that of its metabolite only 1.6-fold in wild-type mice (Fig. 5F). Therefore, elacridar had a much greater effect on the brain concentration of sunitinib than on that of N-desethyl sunitinib.

To the best of our knowledge, this is the first study demonstrating that Abcb1 and Abcg2 together restrict N-desethyl sunitinib brain accumulation, but not plasma pharmacokinetics of N-desethyl sunitinib upon oral sunitinib administration. Abcg2 may modestly enhance systemic elimination of N-desethyl sunitinib administered intravenously. Despite its effect on plasma and brain concentrations, intravenous elacridar had no significant impact on the brain-to-plasma ratios of N-desethyl sunitinib in wild-type mice. Together with our previous findings on sunitinib, the effect of elacridar is more pronounced for the brain accumulation of sunitinib than for that of N-desethyl sunitinib. Therefore, coadministration of elacridar seems a more attractive strategy for sunitinib than for N-desethyl sunitinib in improving the clinical efficacy against brain metastases and brain tumors positioned behind a functionally intact BBB.

Acknowledgments

We thank Dilek Iusuf and Selvi Durmus for critical reading of the manuscript.
ABC1 and ABCG2 Limit N-Desethyl Sunitinib Brain Disposition

ABCB1 and ABCG2 Limit N-Desethyl Sunitinib Brain Disposition

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