Comparison of ATP-Binding Cassette Transporter Interactions with the Tyrosine Kinase Inhibitors Imatinib, Nilotinib, and Dasatinib

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ABSTRACT:

Although the development of tyrosine kinase inhibitors (TKIs) to control the unregulated activity of BCR-ABL revolutionized the therapy of chronic myeloid leukemia, resistance to TKIs is a clinical reality. Among the postulated mechanisms of resistance is the overexpression of ATP-binding cassette (ABC) transporters, such as P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2), which mediate reduced intracellular drug accumulation. We compared the interactions of the TKIs imatinib, nilotinib, and dasatinib with ABCB1 and ABCG2 in ex vivo and in vitro systems. The TKIs inhibited rhodamine 123 and Hoechst 33342 efflux mediated by endogenous expression of the transporters in murine and human hematopoietic stem cells with potency order nilotinib $\gg$ imatinib $\gg$ dasatinib. Studies with ABCB1-, ABCG2-, and ABCC1-transfected human embryonic kidney 293 cells verified that nilotinib was the most potent

inhibitor of ABCB1 and ABCG2. Cytotoxicity assays in stably transduced K562-ABCG2 and K562-ABCB1 cells confirmed that the TKIs were also substrates for the two transporters. Like imatinib, both nilotinib and dasatinib decreased ABCG2 surface expression in K562-ABCG2 cells. Finally, we found that all TKIs were able to compete labeling of ABCB1 and ABCG2 by the photo-cross-linkable prazosin analog [125 I]iodoarylazidoprazosin, suggesting interaction at the prazosin-binding site of both proteins. Our experiments support the hypothesis that all three TKIs are substrates of ABC transporters and that, at higher concentrations, TKIs overcome transporter function. Taken together, the results suggest that therapeutic doses of imatinib and nilotinib may diminish the potential of ABCB1 and ABCG2 to limit oral absorption or confer resistance. Clinical data are required to definitively answer the latter question.

Chronic myeloid leukemia (CML) is a hematopoietic stem cell (HSC) cancer initiated by the unregulated activity of the BCR-ABL tyrosine kinase. The development of small molecule tyrosine kinase inhibitors (TKIs), beginning with orally administered imatinib, revolutionized CML therapy. The second- and third-generation TKIs nilotinib and dasatinib are already in clinical application. The majority of patients with chronic-phase CML treated with imatinib maintain du-

rable responses; however, relapse can occur after drug withdrawal (Cortes et al., 2004) and drug resistance is observed in a subset of patients (Roumiantsev et al., 2002). Second- and third-generation TKIs are more potent and can overcome imatinib resistance in some instances but may fail to eradicate the malignant CML clone (Bhatia et al., 2003). Because CML is considered a stem cell disease, it is intriguing to postulate that inherent protective mechanisms such as the expression of ATP-binding cassette (ABC) transporters could contribute to relapse (Zhou et al., 2001; Scharenberg et al., 2002; Jordanides et al., 2006). ABCG2 and ABCB1 are also expressed in other tissues crucial for the pharmacokinetic profile of these drugs, including the blood-brain barrier and the gut (Robey et al., 2007). The question of whether imatinib interacts with these ABC transporters has been addressed in multiple publications with contradictory results, and

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ABBREVIATIONS: CML, chronic myeloid leukemia; HSC, hematopoietic stem cell; TKI, tyrosine kinase inhibitor; ABC, ATP-binding cassette; FTC, fumitremorgin C; MK571, 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoyl)ethylsulfanyl]methylsulfanyl] propionic acid; IAAP, iodoarylazidoprazosin; HEK, human embryonic kidney; FCS, fetal calf serum; PB, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; IMEM, Iscove's modified Eagle's medium; MES, 4-morpholineethanesulfonic acid; TBS, Tris-buffered saline; p, phosphorylated; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KSL, c-Kit⁺/Sca-1⁺/lineage[−].

limited data are available for the more potent second- and third-generation TKIs nilotinib and dasatinib.

Several reports have highlighted the interactions between TKIs and ABC transporters. Initial reports suggested that ABCB1 may play a role in resistance to imatinib, because overexpression of ABCB1 was noted in an imatinib-selected cell line (Mahon et al., 2000). Subsequent studies evaluating imatinib as an ABCB1 substrate both confirmed (Dai et al., 2003; Mahon et al., 2003; Illmer et al., 2004) and contradicted (Ferraro et al., 2003) these initial results. Imatinib was also found to act as an inhibitor of ABCB1 (Hamada et al., 2003). Likewise, imatinib was reported to be a substrate (Burger et al., 2004) or inhibitor (Ozvegy-Laczka et al., 2004) of ABCG2, with some reports suggesting that imatinib is only an inhibitor of ABCG2 (Houghton et al., 2004). Ozvegy-Laczka et al. (2004) contributed the fact that imatinib exhibits a high-affinity interaction with ABCG2, higher than that with ABCB1 or ABCC1. Shukla et al. (2008) provided biochemical evidence that imatinib behaves as a substrate of ABCG2 and ABCB1 in a narrow concentration range and that at high concentrations it might act exclusively as an inhibitor due to its high affinity, a finding that is emerging as a consensus in the literature.

These dual activities may also apply to nilotinib. Mahon et al. (2008) reported that ABCB1 was a mechanism of resistance to nilotinib. We found not only that the second-generation TKI nilotinib was a potent inhibitor of ABCG2 but also that ABCG2-transfected K562 cells were 2- to 3-fold resistant to nilotinib and demonstrated that ABCG2 can transport nilotinib at nanomolar concentrations (Brendel et al., 2007). Tiwari et al. (2009) also recently reported nilotinib to be an inhibitor of both ABCB1 and ABCG2. However, opposing views regarding the ability of ABCB1 or ABCG2 to transport nilotinib have been proposed (Davies et al., 2009). On the other hand, dasatinib has been described as a substrate of ABCB1 and ABCG2 but only as a weak modulator of ABCB1 (Giannoudis et al., 2008; Hiwase et al., 2008); no data regarding modulation of ABCG2 or ABCC1 are available. Nakanishi et al. (2006) noticed in BCR-ABL-positive K562 cells that inhibition of BCR-ABL by imatinib in turn inhibits the phosphatidylinositol 3-kinase/AKT pathway leading to post-transcriptional down-regulation of ABCG2, which could attenuate the ABCG2-mediated resistance to TKIs in BCR-ABL-dependent model systems and confound experiments characterizing the interaction of the TKIs.

Clinicians have with imatinib, nilotinib, and dasatinib three tools to effectively inhibit the BCR-ABL kinase. Thus, we sought to systematically describe a uniform profile of the interaction among the three major BCR-ABL inhibitors in clinical application and two of the best-studied ABC transporters in drug resistance, ABCG2 and ABCB1. For dasatinib, we provide biochemical evidence for its interaction with ABCG2 and ABCB1. These studies provide a frame of reference for pharmacokinetic and pharmacodynamic studies in patients needed to finally determine the impact of these transporters in the clinic.

Materials and Methods

Chemicals. Dasatinib was obtained from Bristol-Myers Squibb Co. (Stamford, CT), and imatinib and nilotinib (AMN107) were from Novartis (Basel, Switzerland). Fumitremorgin C (FTC) was synthesized by Thomas McCloud (Developmental Therapeutics Program, Natural Products Extraction Laboratory, National Institutes of Health, Bethesda, MD). Rhodamine 123, Hoechst 33342, and verapamil were obtained from Sigma-Aldrich (St. Louis, MO). Tariquidar was obtained from Xenova Research (Slough, Berkshire, UK). Valspodar was a gift from Novartis Pharmaceuticals (Cambridge, MA). MK571 was purchased from EMD Biosciences (San Diego, CA). Calcein AM and BODIPY-prazosin were obtained from Invitrogen (Carlsbad, CA). Pheophorbide a was purchased from Frontier Scientific (Logan, UT). [¹²⁵I]Iodoarylazidoprazosin (IAAP) (2200 Ci/

mmol) was acquired from PerkinElmer Life and Analytical Sciences (Waltham, MA).

Cell Culture. Human embryonic kidney (HEK) 293 cells transfected with empty pcDNA3.1 vector or with vector containing *ABCB1* or wild-type *ABCG2* were described previously (Robey et al., 2003) as were cells transfected with *ABCC1* (Müller et al., 2002). Stable transfectants were maintained in modified Eagle's medium containing 10% fetal calf serum (FCS), penicillin, and streptomycin with G418 at a concentration of 2 mg/ml. The CML cell line K562 [DSMZ (German National Resource Centre for Biological Material, Braunschweig, Germany)], K562 cells stably overexpressing wild-type ABCG2 (Yanase et al., 2004) (a kind gift from Dr. Yoshikazu Sugimoto, Department of Chemotherapy, Kyoritsu University of Pharmacy, Tokyo, Japan), or wild-type ABCB1 (Hafkemeyer et al., 2000) (a kind gift from Dr. Michael Gottesman, Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD) were maintained in RPMI 1640 medium supplemented with 10% FCS, penicillin, and streptomycin. All cells were grown at 37°C in 5% CO₂.

Functional Assays with Hoechst 33342 and Rhodamine 123 in Murine Bone Marrow. For studies using murine bone marrow, adult hematopoietic cells were obtained from C57BL/6 mice. Femora and tibiae were dissected and flushed with PBS, without calcium or magnesium but supplemented with 2% heat-inactivated calf serum (PBS+). After red cell lysis with 0.01 M NH₄Cl, cells were filtered through a nylon screen (BD Falcon, Heidelberg, Germany) and resuspended at 1×10^6 cells/ml in 2 ml of prewarmed DMEM containing 2% fetal bovine serum and 10 mM HEPES (DMEM+). We then used rhodamine 123 as a probe for Abcb1 function and Hoechst 33342 as a probe for Abcg2 function in murine hematopoietic stem cells. Murine bone marrow cells were incubated with 0.2 µg/ml rhodamine for 30 min at 37°C, spun down, and resuspended in medium containing 2.5 µg/ml Hoechst and incubated for an additional 90 min at 37°C. The effects of imatinib, nilotinib, and dasatinib on the stem cell efflux phenotype were analyzed by including these drugs at various concentrations during the Hoechst and rhodamine incubations. To compare the effect of imatinib, nilotinib, and dasatinib to known inhibitors, we used verapamil for ABCB1 (25 µM) (Sigma-Aldrich), FTC (10 µM) for ABCG2, and tariquidar (1 µM) and reserpine (5 µM) for ABCB1 and ABCG2 inhibition, respectively. The cells were subsequently washed twice with PBS+ and kept on ice for antibody staining. Cells were first incubated with a panel of biotinylated monoclonal antibodies to lineage markers [eBioscience Mouse Hematopoietic Lineage Flow Panel: CD3 (145-2C11), CD45R/B220 (RA3-6B2), CD11b (M1/70), B220 (6B2), TER-119, Ly-6G (RB6-8C5); eBioscience, San Diego, CA]. The cells were then washed, subsequently stained with directly conjugated antibodies to Sca-1 (D7-APC; eBioscience) and c-kit (2B8-PE-Cy7) (eBioscience), and streptavidin-Alexa Fluor 750 (Invitrogen) secondary antibody was used to visualize lineage markers (Challen et al., 2009). After these steps, cells were resuspended in PBS+ and analyzed on a triple laser instrument (BD LSR II; BD Biosciences, San Jose, CA) after propidium iodide staining (2 µg/ml) to exclude dead cells.

Rhodamine Staining in Human CD56⁺ Cells and Human Bone Marrow Stem Cells. Peripheral blood was collected from healthy volunteers according to a protocol approved by the National Cancer Institute Institutional Review Board. Human bone marrow was obtained, after written informed consent as approved by the ethics committee of Philipps University of Marburg. Fragments from human femora were obtained from patients undergoing hip replacement surgery. Isolation of mononuclear cells was performed as described previously (Robey et al., 1999; Scharenberg et al., 2002). Cells were washed in PBS and resuspended in IMEM containing 10% FCS (IMEM+). Rhodamine (0.2 µg/ml) with or without desired inhibitor was added, and cells were incubated for 30 min at 37°C (accumulation phase), washed in PBS+, resuspended in IMEM+, and incubated for an additional 60 min at 37°C in the presence or absence of tested inhibitor (efflux phase). After staining, cells were washed twice in ice-cold PBS+. Peripheral blood mononuclear cells were stained with phycoerythrin-conjugated monoclonal antibody against human CD56 (BD Biosciences) at 4°C, washed, resuspended in PBS, and analyzed on a FACSort cytometer. No significant toxicity was noted as evaluated with propidium iodide. Mononuclear cells from human bone marrow were stained with APC-Cy7-conjugated monoclonal antibodies against human lineage markers [CD2 (S5.2), CD3 (SK7), CD4 (SK3), CD8 (SK1), CD13 (MY7-RD1; Beckman Coulter, Fullerton, CA), CD14 (M_P9), CD15 (MMA), CD19 (SJ25C1), CD20 (L27), CD56 (NCAM16.2), and glycophorin A], CD34-PE-CY7, CD 38-APC,

and CD45-PERCP-Cy5.5 (Challen et al., 2009). Cells were subsequently washed, resuspended in PBS+ and analyzed on a BD LSR II cytometer. Dead cells were excluded from analysis using 4,6-diamidino-2-phenylindole.

Fluorescent Substrate Efflux Studies in Transfected Cells. Log-phase cultures of HEK293 cells transfected with vector containing wild-type *ABCB1*, *ABCG2*, or *ABCC1* were trypsinized and resuspended in IMEM containing 10% FCS. Cells were incubated with the desired fluorescent substrate for 30 min at 37°C in the presence or absence of TKI or inhibitor followed by a subsequent substrate-free 60-min efflux period in the presence or absence of inhibitor. For analysis of *ABCG2* function, 1 μ M pheophorbide a or 200 nM BODIPY-prazosin was added, and 0.5 μ g/ml rhodamine or 200 nM BODIPY-prazosin was used to study *ABCB1*. Calcein AM (200 nM) served as a fluorescent substrate for *ABCC1*. FTC at 10 μ M was used as a positive control for *ABCG2* inhibition, 3 μ g/ml valsopodar for *ABCB1*, and 50 μ M MK571 for *ABCC1* inhibition. Cells were then washed in ice-cold PBS and resuspended in ice-cold PBS containing 2 μ g/ml propidium iodide to exclude nonviable cells. To address the question of reversibility of inhibition, cells were incubated with concentrations of a TKI found to be inhibitory and a fluorescent substrate for 30 min. Cells were washed twice and allowed to efflux for 0, 30, 60, or 120 min in substrate and inhibitor-free medium.

Cytotoxicity Assays. Parental K562 cells or K562 cells stably transfected with *ABCG2* or *ABCB1* were seeded in 96-well plates at 25,000 cells/well and were grown for 60 h with increasing concentrations of imatinib, nilotinib, and dasatinib. Cells were stained with propidium iodide after incubation and analyzed by flow cytometry to determine percentage of viable cells.

Cytotoxicity assays with HEK293 cells were performed based on the sulforhodamine B assay (Robey et al., 2003). Cells were plated at a density of 10,000 cells/well in 96-well plates and allowed to attach overnight at 37°C in 5% CO₂. Cytotoxic agents were added at increasing concentrations and the TKIs were added concomitantly. The plates were allowed to incubate for 96 h at 37°C in 5% CO₂, after which cells were fixed with 50% trichloroacetic acid, washed, and allowed to dry. Plates were stained with sulforhodamine B solution (0.4% sulforhodamine B w/v in 1% acetic acid) for 30 min and washed 3 times in 1% acetic acid solution. Sulforhodamine was then solubilized with 10 mM Tris base, and optical densities were read on a plate reader at an absorbance of 570 nm. Each concentration was tested in quadruplicate, and controls were performed in replicates of eight. A two-tailed Student's *t* test was performed to determine statistical significance.

[¹²⁵I]IAAP Labeling Studies and ATPase Assay with Crude Membranes. Crude membranes from *ABCB1*-expressing High Five insect cells or *ABCG2*-expressing MCF-7 FLV1000 cells were incubated with the desired concentration of TKIs for 5 min at room temperature in 50 mM Tris-HCl, pH 7.5. Next, 3 to 6 nM [¹²⁵I]IAAP (2200 Ci/mmol) were added, and the samples were incubated for an additional 5 min under subdued light. The samples were then cross-linked by UV illumination (365 nm) on ice, electrophoresed, and analyzed. The labeled *ABCG2* was immunoprecipitated using 5 to 6 mg/ml BXP-21 antibody.

For the ATPase assay, crude membranes isolated from High Five insect cells expressing *ABCB1* or *ABCG2* (100 mg of protein/ml) were incubated at 37°C with various concentrations of imatinib, nilotinib, and dasatinib in ATPase assay buffer (0.05 mM KCl, 5 mM sodium azide, 2 mM EDTA, 10 mM MgCl₂, 1 mM dithiothreitol, and 50 mM Tris-MES, pH 6.8) for 5 min. The reaction was started by the addition of 5 mM ATP and was terminated by the addition of 0.1 ml of an SDS solution after 20 min; the amount of inorganic phosphate released was quantified by a colorimetric reaction as described previously (Ambudkar, 1998). The basal activity was subtracted to calculate percentage of stimulation in the presence of the TKIs.

Determination of ABC Transporter-Mediated Changes in TKI-Induced CKRL Dephosphorylation. In brief, K562-*ABCG2*, K562-*ABCB1*, and parental K562 cells (4 × 10⁵/well) were incubated with increasing doses of imatinib or dasatinib for 12 h or nilotinib for 24 h. These time points were selected to optimize differences in CKRL phosphorylation in the K562 cells. Cells were washed twice with ice-cold PBS and lysed with 50 μ l of lysis buffer containing protease inhibitors (PhosSTOP; Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitors (aprotinin, leupeptin, and phenylmethylsulfonyl fluoride) at 4°C. The cell lysate was sonicated and prepared in a desired final concentration in SDS sample buffer (62.5 mM Tris-HCl, 2% w/v

SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% w/v bromophenol blue). Denatured protein (10–20 μ g/sample) was separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked with TBS containing 5% milk for 30 min, washed three times with TBS and 0.1% Tween 20 (TBS-T) and incubated overnight with primary anti-pCKRL (1:1000; Cell Signaling Technology, Danvers, MA) and anti-glyceraldehyde-3-phosphate dehydrogenase antibody (1:2500; American Research Products, Belmont, MA) diluted in TBS-T with 5% bovine serum albumin (BSA). Membranes were washed three times with TBS-T and probed with secondary IRDye 680 anti-rabbit IgG and IRDye 800 anti-mouse IgG (1:10,000 in TBS-T with 5% BSA; LI-COR Biosciences, Lincoln, NE) for 1 h. Fluorescence was determined using an Odyssey infrared imaging system (LI-COR Biosciences). Expression of pCKRL was normalized to GAPDH expression, and pCKRL expression for cells treated with TKI was calculated versus untreated cells, which were assigned a value of 100%. A two-tailed Student's *t* test was performed to determine statistical significance of differences in pCKRL levels.

Analysis of TKI-Induced Changes in *ABCG2* Expression in K562-*ABCG2* Cells. In brief, K562-*ABCG2* cells (5 × 10⁵/well) were incubated in increasing concentrations of imatinib, nilotinib, and dasatinib. Cells were harvested after 14 h, washed twice in ice-cold PBS, and then incubated with phycoerythrin-conjugated anti-human *ABCG2* antibody or isotype control (both from eBioscience) in 2% BSA in PBS for 30 min. After staining, samples were subsequently analyzed by flow cytometry in ice-cold PBS containing 2 μ g/ml propidium iodide. Mean channel values of anti-*ABCG2* and isotype control histograms were subtracted and calculated relative to the untreated control.

Results

Inhibition of *Abcb1*- and *Abcg2*-Mediated Transport in Murine HSCs by the TKIs Imatinib, Nilotinib, and Dasatinib. We began comparative studies in the murine system, because HSCs are known to express ABC transporters and can be highly enriched using a combination of surface markers, such as c-Kit⁺/Sca-1⁺/lineage[−] cells (KSL cells) (Fig. 1A) (Goodell et al., 1996; Zhou et al., 2001). We combined surface marker characteristics with Hoechst and rhodamine dye efflux, allowing us to study the interaction of the TKIs with *Abcg2* and *Abcb1* simultaneously in the most primitive stem cell subset. As seen in Fig. 1B, KSL cells readily efflux rhodamine 123 (*x*-axis) as well as Hoechst 33342 (*y*-axis, top left panel), indicative of the fact that this subset of cells expresses both *Abcb1* and *Abcg2*. Tariquidar (Fig. 1B, top right panel), a dual *Abcb1* and *Abcg2* inhibitor, blocked transport of both dyes, as did reserpine (data not shown). Verapamil prevented Hoechst transport only slightly, but abolished rhodamine transport (Fig. 1B, bottom right panel). The *Abcg2* inhibitor FTC abolished Hoechst transport completely but did not inhibit rhodamine transport (Fig. 1B, bottom left panel). We next examined the *in vitro* interactions of the TKIs using this murine system. Imatinib (Fig. 1C) was able to abolish transport of Hoechst at concentrations less than 500 nM but significantly inhibited rhodamine transport only at higher concentrations. Although nilotinib abrogated *Abcg2*-mediated Hoechst efflux at concentrations less than 100 nM, *Abcb1*-mediated efflux of rhodamine was fully inhibited only at higher concentrations (Fig. 1D). Dasatinib inhibited *Abcg2* less potently than imatinib and significantly affected transport mediated by *Abcb1* at higher micromolar concentrations (Fig. 1, E and F). Taken together, these experiments demonstrate that all three TKIs interact with *Abcb1* and *Abcg2* expressed by primitive murine hematopoietic stem cells. Furthermore, the data suggest that nilotinib inhibits both transporters more potently than imatinib or dasatinib.

Inhibition of *ABCB1*-Mediated Rhodamine 123 Transport in Human CD34⁺ Bone Marrow Cells and CD56⁺ Cells by TKIs. We next sought to confirm our results with murine cells in human

FIG. 1. Inhibition of Abcb1- and Abcg2-mediated transport in murine KSL cells by the TKIs imatinib, nilotinib, and dasatinib. A, KSL cells in murine bone marrow were labeled and incubated with 0.2 μ g/ml rhodamine 123 for 30 min at 37°C and then with 2.5 μ g/ml Hoechst 33342 in the absence or presence of known inhibitors (B) or varying concentrations of imatinib (C), nilotinib (D), or dasatinib (E and F) as outlined under *Materials and Methods*. For plots B to F, the y-axis is Hoechst fluorescence and the x-axis is rhodamine fluorescence. Tariquidar (1 μ M) served as a positive control for inhibition of ABCB1 and ABCG2, FTC (10 μ M) for inhibition of ABCG2, and verapamil (15 μ M) for inhibition of ABCB1.

ex vivo models. Lineage-negative, CD38⁻, and CD34⁺ human bone marrow cells are enriched for repopulating stem cells and are known to express ABCB1. As shown in Fig. 2A, imatinib inhibited rhodamine transport minimally even at concentrations up to 10 μ M, nilotinib abrogated ABCB1-mediated efflux of rhodamine at concentrations of approximately 1 μ M, and dasatinib exhibited only a slight ability to block rhodamine transport at the highest concentration. Although ABCG2 is also expressed on human

cells, defining the side population fraction, the reported frequency of side population in total bone marrow is quite low at 0.04% and thus unsuitable for the comparisons in this article.

Differentiated human CD56⁺ natural killer cells also express high endogenous levels of ABCB1, and this property has been exploited in the past to determine the efficacy of ABCB1 modulators in clinical trials (Robey et al., 1999). Imatinib had a dose-dependent inhibitory effect but failed to inhibit rhodamine transport completely, even at concentrations

Fig. 2. Inhibition of ABCB1 in human ex vivo systems. A, mononuclear cells were isolated from human femora as described under *Materials and Methods* and incubated with 0.5 $\mu\text{g}/\text{ml}$ rhodamine 123 for 30 min at 37°C in the absence or presence of the desired concentration of imatinib, nilotinib, dasatinib, or verapamil. Cells were then washed and allowed to incubate in rhodamine-free medium for 60 min continuing without or with inhibitor. Cells were subsequently stained with APC-Cy7-conjugated lineage markers to isolate the CD38⁺ and CD34⁺ population. Verapamil (black line) at a concentration of 50 μM served as a positive control for ABCB1 inhibition. B, peripheral blood mononuclear cells were separated by density gradient and incubated with rhodamine 123 with or without inhibitors as in A above. Subsequent to the 60-min efflux period, cells were stained with anti-CD56 antibody. Valsopodar (3 $\mu\text{g}/\text{ml}$) served as a positive control for ABCB1 inhibition.

up to 10 μM . Nilotinib abrogated ABCB1-mediated efflux of rhodamine at concentrations of approximately 1 μM . With only slight inhibition at 10 μM , dasatinib exhibited the weakest effect on rhodamine transport of all tested TKIs (Fig. 2B).

Inhibition of ABCB1, ABCG2, and ABCC1 by the TKIs in Transfected Cells. The effects of the TKIs were then examined in transfected cell lines to carefully characterize their interactions with the three transporters associated with drug resistance. We studied HEK293 cells stably transfected with *ABCG2*, *ABCB1*, or *ABCC1*. In *ABCG2*-expressing HEK293 cells, 10 μM imatinib was as effective as 1 μM nilotinib in preventing ABCG2-mediated pheophorbide a transport, demonstrating that nilotinib is a more potent inhibitor of ABCG2 (Fig. 3). This is in agreement with our results from the murine system (Fig. 1) and consistent with our previous report (Brendel et al., 2007). Dasatinib did not inhibit pheophorbide a transport in HEK293-ABCG2 cells and reduced BODIPY-prazosin efflux to a lesser degree than did imatinib and nilotinib. Whereas imatinib and dasatinib had only minimal effects on ABCB1-mediated rhodamine efflux, even at a concentration of 10 μM , nilotinib was able to inhibit ABCB1-mediated rhodamine transport at a concentration of 1 μM (Fig. 3). In contrast, in ABCB1-overexpressing HEK293 cells, BODIPY-prazosin efflux was inhibited by both imatinib and nilotinib. Whereas increasing amounts of nilotinib and imatinib inhibited ABCC1 equipotently at higher concentrations, dasatinib did not affect transport of calcein AM. Washout experiments demonstrated that inhibition of ABC transporter functions by TKIs was reversible (data not shown). Our data suggest that nilotinib is the more potent inhibitor of both ABCB1 and ABCG2 transporter proteins and that imatinib might act as an inhibitor for certain ABCB1 substrates. Because trough plasma levels of 4 μM , 2 μM , and 100 nM have been reported for imatinib, nilotinib, and dasatinib, respectively (Bradeen et al., 2006), the concentrations needed to inhibit ABC transporter proteins may thus be clinically achievable for some TKIs.

TKIs Compete Photolabeling of ABCB1 and ABCG2 by [¹²⁵I]IAAP and Stimulate ATPase Activity. Photoaffinity labeling and ATPase assays have proven useful for identification of compounds that interact with ABC transporters. We next performed ex-

periments with the prazosin analog [¹²⁵I]IAAP in crude membranes overexpressing ABCB1 or ABCG2 to determine whether the TKIs directly interact with ABC transporter proteins. As seen in Fig. 4A, imatinib and nilotinib were both found to interact at the prazosin binding site of ABCB1, as the photolabeling of this protein by [¹²⁵I]IAAP was effectively inhibited by increasing concentrations of TKI. Dasatinib, even at 20 μM , interacted only weakly with ABCB1 (Fig. 4A). All three TKIs were shown to interact with the prazosin-binding site of ABCG2 (Fig. 4B). Whereas imatinib and nilotinib had a high affinity for ABCG2 with low nanomolar IC_{50} values, consistent with previous reports (Brendel et al., 2007; Shukla et al., 2008), the IC_{50} of dasatinib was approximately 3 μM .

Our measurements of ATPase activity showed that, although imatinib and nilotinib are known to effectively stimulate the ATPase activity of ABCG2 and ABCB1 at low nanomolar concentrations (Brendel et al., 2007; Shukla et al., 2008), dasatinib stimulated ABCB1 with a maximum of approximately 1.3-fold (Fig. 4C) and ABCG2 with a maximum 1.4-fold (Fig. 4D) over basal activity. Dasatinib was also found to effectively inhibit verapamil-mediated stimulation of ABCB1, confirming its interaction with ABCB1 (data not shown). Taken together, these results suggest that all TKIs tested directly interact with the ABC transporter proteins examined.

Inhibition of ABCB1- and ABCG2-Mediated Drug Resistance by Nilotinib and Imatinib. We next examined whether the TKIs could reverse drug resistance mediated by ABC transporters. Four-day cytotoxicity assays were performed on HEK293 cells transfected with empty vector or vector containing *ABCB1* or *ABCG2*. Cells were incubated with various concentrations of the ABCB1 substrate romidepsin or the ABCG2 substrate topotecan in the presence or absence of 1 μM nilotinib or 5 μM imatinib. Imatinib and nilotinib were not toxic at these concentrations (data not shown); nilotinib was tested at a lower concentration because of its greater potency, as observed in the foregoing assays. As seen in Table 1, although nilotinib was able to completely reverse the high levels of resistance to romidepsin in *ABCB1*-transfected cells, imatinib had only a partial effect. In the case of ABCG2, both imatinib and nilotinib were able to completely reverse ABCG2-mediated topotecan

FIG. 3. Interaction between TKIs and multidrug resistance proteins. Trypsinized HEK293 cells transfected with *ABCG2*, *ABCB1*, or *ABCC1* were incubated with the desired fluorescent substrate (1 μ M pheophorbide a or 200 nM BODIPY-prazosin for *ABCG2*, 0.5 μ g/ml rhodamine 123 or 200 nM BODIPY-prazosin for *ABCB1*, or 200 nM calcein AM for *ABCC1*) in the presence or absence of the desired inhibitor for 30 min at 37°C. Cells were subsequently washed and allowed to incubate in substrate-free medium for 60 min continuing in the absence or presence of inhibitor. FTC (10 μ M) served as a positive control for inhibition of *ABCG2*, valsopodar (3 μ g/ml) for *ABCB1*, and MK571 (100 μ M) for *ABCC1*.

FIG. 4. TKIs compete for photolabeling of *ABCB1* and *ABCG2* by [125 I]IAAP. We incubated crude membranes from High Five insect cells expressing *ABCB1* (A) or *ABCG2*-expressing MCF-7 FLV1000 cells (B) with [125 I]IAAP and increasing doses of TKIs. The samples were then cross-linked by UV illumination, subjected to electrophoresis, and analyzed as outlined under *Materials and Methods*. Autoradiograms from one of at least three independent experiments are shown. ATPase activity was determined in crude membranes from High Five insect cells expressing *ABCB1* (C) or *ABCG2* (D) incubated in the presence of varying concentrations of dasatinib as outlined under *Materials and Methods*. Results compiled from at least three independent experiments are shown in C and D.

resistance. Dasatinib was not found to inhibit *ABCB1*- or *ABCG2*-mediated resistance at concentrations that were nontoxic to cells (data not shown). These results were in agreement with those observed in the flow cytometry experiments, demonstrating that imatinib and nilotinib were superior inhibitors of *ABCB1* and *ABCG2*.

***ABCG2* and *ABCB1* Overexpression in K562 Cells Mediates Moderate Resistance to Imatinib, Nilotinib, and Dasatinib.** Having confirmed the ability of TKIs to act as inhibitors, we next sought to determine whether they were also transport substrates. Previous work with cells expressing ABC transporters has addressed the question of whether *ABCG2* and *ABCB1* can mediate

resistance to tyrosine kinase inhibitors (Mahon et al., 2000; Ferrao et al., 2003; Illmer et al., 2004; Jordanides et al., 2006; Davies et al., 2009). The results lack comparability because of differences in the model systems used. We thus performed cytotoxicity assays with imatinib, nilotinib, or dasatinib on BCR-ABL-positive parental K562 cells as well as transduced K562-*ABCG2* and K562-*ABCB1* cells, with results summarized in Table 2. We confirmed expression of *ABCB1* and *ABCG2* using Western blotting and functional assays and additionally found no *ABCC1* activity (data not shown). *ABCB1* expression conferred 9-fold resistance to imatinib but was less effective in preventing nilotinib-induced cell

TABLE 1

ABC transporter expression confers resistance to TKIs

IC₅₀ values (nanograms per milliliter for romidepsin, micromolar concentration for topotecan) were determined by the sulforhodamine B assay as described under *Materials and Methods*. In combination assays, imatinib was used at a concentration of 5 μ M and nilotinib at a concentration of 1 μ M.

	HEK pcDNA3.1	HEK ABCB1	HEK ABCG2	RR ^a
Romidepsin	0.0026 $\pm$ 0.0013	2.5 $\pm$ 1.1		961
Romidepsin + imatinib	0.0012 $\pm$ 0.00050	0.28 $\pm$ 0.29		108
Romidepsin + nilotinib	0.0017 $\pm$ 0.0011	0.0048 $\pm$ 0.0020		1.8
Topotecan	0.036 $\pm$ 0.016		0.72 $\pm$ 0.39	20
Topotecan + imatinib	0.027 $\pm$ 0.024		0.016 $\pm$ 0.0035	0.4
Topotecan + nilotinib	0.032 $\pm$ 0.0065		0.026 $\pm$ 0.0065	0.7

RR, relative resistance.

^a RR values were calculated by dividing the IC₅₀ of the transporter-expressing cells by the IC₅₀ of the empty vector-transfected cells.

TABLE 2

TKIs reverse ABCB1- and ABCG2-mediated drug resistance

IC₅₀ values (nanomolar concentration) were determined by propidium iodide exclusion as outlined under *Materials and Methods*. For combination assays, FTC was used at a concentration of 5 μ M and tariquidar at a concentration of 100 nM.

	K562 Parental	K562 ABCB1	RR ^a	K562 ABCG2	RR ^a
Imatinib	112 $\pm$ 1.0	1007.5 $\pm$ 6.6	9	210.3 $\pm$ 1.5	1.9
Imatinib + FTC	111 $\pm$ 1.0			109.7 $\pm$ 2.1	0.98
Imatinib + tariquidar	110.7 $\pm$ 1.2	128.0 $\pm$ 2.6	1.1		
Nilotinib	4.1 $\pm$ 1.0	10.3 $\pm$ 0.2	2.5	10.6 $\pm$ 0.4	2.6
Nilotinib + FTC	4.1 $\pm$ 0.3			2.8 $\pm$ 0.3	0.68
Nilotinib + tariquidar	4.1 $\pm$ 0.4	5.7 $\pm$ 0.6	1.4		
Dasatinib	0.4 $\pm$ 0.05	4.8 $\pm$ 0.29	12	1.0 $\pm$ 0.01	2.5
Dasatinib + FTC	0.4 $\pm$ 0.05			0.3 $\pm$ 0.01	0.75
Dasatinib + tariquidar	0.4 $\pm$ 0.05	0.5 $\pm$ 0.1	1.2		

RR, relative resistance.

^a RR values were calculated by dividing the IC₅₀ of the resistant line by the IC₅₀ of the parental line.

Fig. 5. ABC transporters prevent TKI-mediated dephosphorylation of pCRKL in K562 cells. A, total cell lysates were obtained from K562 parental, K562-ABCB1, and K562-ABCG2 cells incubated with various concentrations of imatinib or dasatinib for 12 h or nilotinib for 24 h. Lysates were subjected to SDS-polyacrylamide gel electrophoresis, and protein was transferred to nitrocellulose membranes. The membranes were incubated overnight with anti-pCRKL antibody and subsequently incubated with secondary antibody that was detected with the Odyssey infrared imaging system. B, expression of pCRKL obtained from at least two independent experiments described in part A was normalized to GAPDH expression. Expression of pCRKL is reported based on assigning control cells a value of 100. Error bars represent S.D. Pgp, ABCB1.

death (3-fold). ABCB1 expression mediated 11-fold resistance to dasatinib. For all TKIs, resistance was rapidly lost once cells were exposed to drug concentrations higher than the observed IC₅₀ values (data not shown). The ABCB1 inhibitor tariquidar reversed the observed resistance. K562-ABCG2 cells exhibited low-level resistance (2–3-fold) to all tested TKIs, relative to parental K562 cells, and this resistance could be reversed by the addition of FTC. The inhibitors had no effect on TKI sensitivity in K562 parental cells.

ABCB1 and ABCG2 Prevent pCRKL Dephosphorylation. To confirm that TKIs are indeed substrates of ABC transporters, we examined the effect of ABC transporter expression on CRKL phosphorylation, a surrogate marker of BCR-ABL activity, by immunoblotting. As seen in Fig. 5, K562 cells expressing ABCB1 or ABCG2 exhibited increased pCRKL levels compared with parental K562 cells when treated with any of the TKIs. This effect was inhibited when ABCB1- or ABCG2-expressing cells were incubated with the TKIs in the presence of inhibitors (data not

FIG. 6. TKIs decrease ABCG2 surface expression. A, ABCG2-transfected K562 cells were incubated for 14 h with 1 μ M imatinib, nilotinib, or dasatinib, after which time cells were incubated with anti-ABCG2 antibody (denoted by solid line) or a negative control antibody (denoted by black histogram). Fluorescence was then determined with a flow cytometer. Histograms from one of at least three independent experiments are shown. Untreated cells are represented by the gray histogram. B, effect of varying concentrations of imatinib, nilotinib, or dasatinib on ABCG2 surface expression as measured by 5D3 antibody staining of K562 cells. Results are relative to ABCG2 surface expression levels found on untreated cells and assigned a value of 100.

shown). In addition, mean expression of pCRKL from all experiments performed was normalized to GAPDH expression and plotted versus concentration of TKI (Fig. 5B). Expression of pCRKL in control cells was assigned a value of 100%. Parental cells are denoted by the solid line, ABCG2-overexpressing by the dashed line, and ABCB1-overexpressing cells by the dotted line. Normalized pCRKL levels between parental cells and both ABCB1-overexpressing and ABCG2-overexpressing cells were statistically significantly different ($p < 0.05$) for imatinib (concentrations 2.5 and 0.63 μ M), nilotinib (concentrations 50, 25, 12.5, and 6.25 nM), and dasatinib (concentrations 500, 100, 50, and 5 nM). These results are in agreement with the results of the cytotoxicity assays, again suggesting that TKIs are substrates of ABC transporters.

Tyrosine Kinase Inhibitors Decrease Surface Expression of ABCG2 in K562 Cells. Nakanishi et al. (2006) previously reported that imatinib decreases ABCG2 surface staining by inhibition of BCR-ABL in K562-ABCG2 transduced cells. Therefore, we sought to determine whether nilotinib and dasatinib would have a similar effect. In accordance with the previous report, not only was imatinib (1 μ M) able to decrease ABCG2 surface expression of K562 ABCG2 cells but nilotinib and dasatinib also had a similar effect (Fig. 6A). Of the three TKIs examined, dasatinib seemed to be the most potent because concentrations as low as 1 nM were able to decrease ABCG2 surface expression by an amount that required 1 μ M imatinib or 100 nM nilotinib (Fig. 6B).

Discussion

Imatinib therapy effectively induces and maintains complete hematological response in a majority of patients with chronic-phase CML

but fails to eradicate persisting malignant cells, and in some patients, resistant CML clones emerge. Although second- and third-generation tyrosine kinase inhibitors such as nilotinib and dasatinib have been proven to be advantageous, especially when BCR-ABL kinase mutations emerge (Hochhaus et al., 2002; O'Hare et al., 2005), they still fail to achieve cure of the disease, despite their increased potency (Bhatia et al., 2003; Copland et al., 2006). Because CML is considered to be a stem cell disease and stem cells are known to express ABC transporter proteins (Zhou et al., 2001; Scharenberg et al., 2002), we proposed that a better description of the interaction between tyrosine kinase inhibitors and ABC transporters could provide insight into resistance to these molecularly targeted agents. TKIs have been implicated as modulators of ABC transporters in both in vitro and ex vivo systems. Herein, we demonstrated that nilotinib is a more potent ABCB1 and ABCG2 inhibitor in ex vivo and in vitro models than imatinib and dasatinib and that the latter in turn interacts significantly less with both transporters. We also demonstrated that both ABCG2 and ABCB1 mediated low to moderate resistance to imatinib, nilotinib, and dasatinib in a K562 cell line model, indicating that all three drugs are substrates for ABCG2 and ABCB1. The ability of ABCB1 and ABCG2 to mediate resistance to TKIs was also supported by data showing increased pCRKL levels in K562 cells expressing ABCG2 or ABCB1 versus parental cells treated with TKIs. Taken together, our data support the conclusion that all three drugs are substrates and inhibitors, depending on the drug concentration.

Experiments with physiological transporter expression levels in primary mouse bone marrow stem cells identified imatinib, nilotinib, and dasatinib as modulators of both Abcb1 and Abcg2, affecting both Abcb1-mediated rhodamine transport and Abcg2-mediated Hoechst

34222 transport. Results with nilotinib parallel recent work demonstrating that nilotinib is a potent inhibitor of ABCB1 and ABCG2 (Tiwarei et al., 2009). However, dasatinib was not as effective as imatinib or nilotinib in inhibiting rhodamine transport in primary mouse bone marrow stem cells, nor did it significantly inhibit ABCB1 in human ex vivo systems even at high concentrations. In primary mouse bone marrow stem cells as well as in human CD56⁺ cells, we find that, in contrast to experiments with human CD34⁺/CD38[−] bone marrow and transfected HEK cells, imatinib effectively exerts blocking activity on rhodamine efflux at the tested concentrations. Imatinib was not found to influence ABCB1-mediated rhodamine transport in ABCB1-transfected HEK293 cells at concentrations up to 10 μ M, in agreement with the findings of Hamada et al. (2003). However, imatinib is able to inhibit transport of BODIPY-prazosin in this cell line at the same concentrations. The ability of imatinib to block BODIPY-prazosin efflux from this cell line, whereas rhodamine transport is hardly affected, suggests that imatinib could interfere with extrusion of some drugs by ABCB1, whereas transport of other drugs could be unaffected. This result is in agreement with a model proposing different binding sites for ABCB1 (Shapiro and Ling, 1998). Likewise, we found that, although dasatinib did not inhibit ABCG2-mediated pheophorbide a transport in cell lines, it was able to modulate BODIPY-prazosin efflux at high concentrations. This may also be due to multiple drug-binding sites reported for ABCG2 (Giri et al., 2009).

We demonstrate that imatinib and nilotinib are inhibitors of both ABCB1 and ABCG2 in vitro and that nilotinib is significantly more potent than imatinib. Our results with imatinib and nilotinib parallel previous reports with the tyrosine kinase inhibitor gefitinib. Gefitinib was first shown to act as an inhibitor of ABCG2- and ABCB1-mediated drug resistance. Much like the results presented herein, the ability of ABCG2 and ABCB1 to confer resistance to gefitinib decreased with increasing gefitinib concentration. This was postulated by Elkind et al. (2005) to be due to a decreased off-rate for gefitinib with increasing drug concentration, which may also be the case for imatinib and nilotinib. It should also be noted that the effect observed in Fig. 6—loss of cell surface expression due to TKI treatment—would phenotypically appear as inhibition of transporter function, because loss of surface expression would most likely result in decreased transport of substrates. This was postulated to be a BCR-ABL-specific effect as noted for imatinib by Nakanishi et al. (2006). However, further studies would be required to prove this hypothesis because other kinases are also inhibited by TKIs.

Given the role of ABCG2 and ABCB1 in oral drug absorption, in the blood-brain barrier, and in renal excretion, inhibition of these transporters by TKIs may affect pharmacokinetics or distribution of other drugs when given concurrently. Nilotinib, an inhibitor at lower concentrations, is a better candidate for influencing the pharmacokinetics of substrate drugs than imatinib or dasatinib. Because ABCG2 and ABCB1 comprise a major part of the blood-brain barrier, inhibition by TKIs could effect increased brain penetration of substrates. High local concentrations in the gut would probably render both nilotinib and imatinib effective inhibitors of transport in the gut endothelium. This effect could be exploited clinically.

ABCG2- and ABCB1-overexpressing K562 cells were found to be resistant to treatment with imatinib, nilotinib, and dasatinib, suggesting that ABC transporter function in stem cells could cause inherent resistance to these tyrosine kinase inhibitors and thus prevent eradication of the malignant clone. Although resistance was found to be within the range of clinically attainable concentrations for imatinib, resistance was observed only at very low drug concentrations for nilotinib and dasatinib, offering the hope that resistance to nilotinib

and dasatinib via ABCG2 and ABCB1 may not be observed at clinically relevant levels. The question of whether imatinib, nilotinib, and dasatinib may function as substrates or solely as inhibitory molecules is crucial for predicting the involvement of transporters in drug resistance. We previously reported that nilotinib is a substrate for ABCG2 at low concentrations (Brendel et al., 2007). Giannoudis et al. (2008) and Hiwase et al. (2008) demonstrated that dasatinib is a substrate for ABCG2 and ABCB1. Many groups have reported that ABCG2- and ABCB1-overexpressing K562 cells were resistant to imatinib compared with parental cells, and resistance could be reversed by specific inhibitors (Illmer et al., 2004; Nakanishi et al., 2006; Brendel et al., 2007). In the present report, we demonstrated that both ABCG2 and ABCB1 mediated low to moderate resistance to imatinib, nilotinib, and dasatinib in a K562 cell line model, indicating that all three drugs are substrates for ABCG2 and ABCB1. It is noteworthy that ABC transporter-mediated resistance to nilotinib and dasatinib occurs only at very low drug concentrations.

The data presented here explain why there has been significant controversy in the field regarding the potential of ABC transporters to confer drug resistance. There is clear concentration dependence determining whether imatinib or nilotinib assumes the phenotype of substrate or inhibitor. The variability that has been model-dependent may also be reflected in patients: which phenotype predominates could depend on location. In the gastrointestinal tract, at the point of oral absorption, it is very likely that concentrations are high and the inhibitor phenotype prevails and adequate drug is absorbed. In the stem cell niche, at the leukemic cell surface, concentrations may be low and the substrate phenotype could dominate. As one strategy to evaluate this question, clinical samples can be evaluated to determine whether ABC transporter levels increase with time and drug exposure. Other strategies are needed. Some clinical studies have suggested that a threshold exists for imatinib efficacy, approximately 1000 ng/ml (Picard et al., 2007), implying that any process that reduces intracellular concentrations, particularly at the leukemic stem cell niche, could have a deleterious impact. The work presented here shifts the burden of investigation from the laboratory back to the clinic.

Note Added in Proof

During the preparation of this article, Hegedus et al. (2009) reported interactions between ABCG1 and ABCG2 and the TKIs nilotinib, dasatinib, and bosutinib.

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