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Department of Pharmaceutical Sciences, St. Jude Children's Research Hospital, Memphis, Tennessee

Abstract

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Experimental Design: Transport of dasatinib was studied in cells transfected with human ABCC4 or the ortholog mouse transporter, Abcc4. Pharmacokinetic studies were done in wild-type and Abcc4-null mice. The influence of Abcc4 deficiency on dasatinib efficacy was evaluated in a model of Ph⁺ acute lymphoblastic leukemia by injection of luciferase-positive, p185(BCR-ABL)-expressing Arf(−/−) pre-B cells.

Results: Dasatinib accumulation was significantly changed in cells overexpressing ABCC4 or Abcc4 compared with control cells (P < 0.001). Deficiency of Abcc4 in vivo was associated with a 1.75-fold decrease in systemic exposure to oral dasatinib, but had no influence on the pharmacokinetics of intravenous dasatinib. Abcc4 was found to be highly expressed in the stomach, and dasatinib efflux from isolated mouse stomachs ex vivo was impaired by Abcc4 deficiency (P < 0.01), without any detectable changes in gastric pH. Abcc4-null mice receiving dasatinib had an increase in leukemic burden, based on bioluminescence imaging, and decreased overall survival compared with wild-type mice (P = 0.048).

Conclusions: This study suggests that Abcc4 in the stomach facilitates the oral absorption of dasatinib, and it possibly plays a similar role for other orally administered substrates, such as acetylsalicylic acid. This phenomenon also provides a mechanistic explanation for the malabsorption of certain drugs following gastric resection. Clin Cancer Res; 19(16); 4359–70. ©2013 AACR.

Introduction

Dasatinib, an orally administered inhibitor of Bcr/Abl and Src kinases, has been approved for the treatment of imatinib-resistant or imatinib-intolerant forms of chronic myeloid leukemia (CML) and Philadelphia chromosome-positive (Ph⁺) acute lymphoblastic leukemia (ALL; ref. 1). A mass balance study has indicated that, following oral administration, dasatinib accounts for only less than 1% and 19% of the dose recovered in urine and feces, respectively, suggesting that dasatinib is extensively metabolized before being eliminated from the body (2). The primary pathways of dasatinib metabolism in humans include N-dealkylation (M4) and N-oxidation (M5) by CYP3A4 and flavin-containing monoxygenase 3, respectively (3).

As with other tyrosine kinase inhibitors, dasatinib is subject to extensive interindividual pharmacokinetic variability. For example, the coefficient of variation in the area under the curve (AUC) of dasatinib is more than 50% in adult patients (4), and as high as 104% in a pediatric patient population (5). The high degree of pharmacokinetic variability observed with dasatinib has potentially important toxicologic and therapeutic ramifications. In particular, it was previously shown that the steady-state trough concentration of dasatinib in patients with CML is strongly associated with treatment-related toxicities as well as dose reductions and interruptions (6). Furthermore, pharmacokinetic studies conducted in mice bearing CML xenografts have suggested that the time course and extent of inhibition of tumoral biomarkers of efficacy are correlated with the plasma levels of dasatinib (7).

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Dasatinib is an inhibitor of the Bcr/Abl and Src kinases and is used in the treatment of chronic myeloid leukemia and Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia (ALL). The interindividual pharmacokinetic variability for dasatinib treatment is extensive and largely unexplained, and this phenomenon may have important ramifications for the agent’s clinical activity. We speculated that differential expression of the ATP-binding cassette transporter ABC4 (MRP4) might play a role in explaining this pharmacologic variability. We investigated the contribution of ABC4 to the pharmacokinetics and efficacy of dasatinib using an array of in vitro and in vivo model systems. Our results indicate that ABC4 is abundantly expressed in the stomach, where it facilitates the gastric absorption of dasatinib. Moreover, Abcc4 deficiency in mice was associated with reduced activity of dasatinib against a model of Ph+ALL. These results identified ABC4 as an important, previously unrecognized contributor to the oral absorption of dasatinib and this process may be relevant for other substrate drugs, such as acetylsalicylic acid.

Model-based simulations have indicated that a critical determinant of dasatinib’s pharmacokinetic variability is associated with absorption-related processes and to a lesser extent with clearance (8). This supposition is consistent with studies indicating that deficiency of certain ATP-binding cassette (ABC) transporters normally expressed on the apical membrane of intestinal enterocytes, such as ABCB1 (P-glycoprotein), is associated with an increased AUC of dasatinib after oral (9) but not intravenous administration (10). Because dasatinib is poorly permeable (11), yet very rapidly absorbed in humans compared with other tyrosine kinase inhibitors (12), its transport across both of the enterocytic membranes after oral administration is likely transporter mediated. One candidate transporter involved in the basolateral transport process is the multidrug resistance–associated protein 4 (MRP4; ABC4), which is known to interact with a number of tyrosine kinase inhibitors (13) and, on the basis of functional in vitro data obtained with cefadroxil (14) and in vitro expression data in Caco-2 cells (15), may be localized on the basolateral membrane of enterocytes. In the current study, we tested the hypothesis that dasatinib is a transported substrate of ABC4 in vitro and that deficiency of the ortholog mouse transporter Abcc4 is associated with an altered pharmacokinetic and efficacy profile of dasatinib in vivo.

In vitro transport studies

Dasatinib was obtained from LC Laboratories and imatinib was kindly provided by Novartis Pharmaceuticals. [3H]Dasatinib, [3H]Imatinib, [3H]Adefovir dipivoxil [bis(POM)]-PMEA; hereafter referred to as PMEA, and unlabeled PMEA were purchased from Moravek Biochemicals. [3H]Estradiol-17β-D-glucuronide (E2G), [14C]Estradiol-17β-acetate (TEA), and [acetylethyl-14C]Salicylic acid (aspirin) were obtained from American Radiolabeled Chemicals, and [7-14C]Salicylic acid from PerkinElmer. Inside-out vesicles of SF9 cells expressing ABC3 or ABC4 (Genomembrane) were used to determine uptake of either [3H]E2G, [3H]Imatinib, or [3H]Dasatinib using 5-minute incubations, as described (16), in the presence or absence of glutathione (GSH; Life Technologies). The efflux transport of [3H]PMEA, [3H]Imatinib, or [3H]Dasatinib was also evaluated in Saos-2 cells transfected with an empty pcDNA or Abcc4 using 4-hour incubations, as described (17). The uptake of [14C]E2G or [3H]Dasatinib was also evaluated in HEK293 cells containing an empty PMIG II vector or a flag-tagged cDNA of mOct1 or mOct2. The results from the in vitro transport studies were normalized to uptake values in cells transfected with an empty vector, after normalization to total protein content as measured by a Pierce BCA Protein Assay Kit (Thermo Scientific).

In vivo pharmacokinetic studies

Male mice knockout for Abcc4 [Abcc4(−/−)] were bred in-house and age-matched wild-type mice, all on a C57BL/6 background, were obtained from The Jackson Laboratory. Mice were housed in a temperature-controlled environment with a 12-hour light cycle and given a standard diet and water ad libitum. All in vivo experiments were approved by the Institutional Animal Care and Use Committee at St. Jude Children’s Research Hospital (Memphis, TN).

Dasatinib was formulated in DMSO (25 mg/mL) and diluted 25-fold in 50 mmol/L sodium acetate buffer (pH 4.6) to make a 1 mg/mL dosing solution immediately before drug administration by oral gavage, or in propylene glycol:deionized water (1:1, v/v) before intravenous administration (18). Imatinib was dissolved in sterile water to make a 10 mg/mL dosing solution. Mice were fasted for 3 hours before and during the study, with unrestricted access to drinking water. Dasatinib was administered by oral gavage or by tail vein injection at a dose of 10 mg/kg (18) and imatinib was given at a dose of 50 mg/kg (19). At selected time points after drug administration, blood samples (30 μL each) were taken from individual mice at 0.25, 0.5, or 0.67, and 1 hour from the submandibular vein using a lancet, and at 2 and 4 hours from the retro-orbital venous plexus using a heparinized capillary (Oxford Labware). A final blood draw was obtained at 6 hours by a cardiac puncture after anesthesia with isoflurane using a syringe and needle. Plasma samples were analyzed by liquid chromatography/tandem mass spectrometry (LC/MS-MS), using a Waters ACQUITY separation system coupled to a TQD detector (see below). Pharmacokinetic parameters were calculated using WinNonlin 6.2 (Pharsight).

The extent of binding of dasatinib to serum proteins (20) and blood cell partitioning of dasatinib were evaluated...
according to published methods (18). Intrinsic intestinal permeability was evaluated by determining the recovery of D-[2-3H]mannitol (American Radiolabeled Chemicals) in plasma, kidney, and liver at 2 hours after oral dosing (1 mg/kg), as described (21). In a separate experiment, the urinary bladder, gall bladder, and spleens, after perfusion, were removed from wild-type or Abcc4(−/−) mice 5 minutes after oral administration of dasatinib and analyzed for the presence of dasatinib by LC/MS-MS.

Quantitative determination of dasatinib and metabolites by LC/MS-MS

Frozen samples were thawed at room temperature and 10 μL aliquots of standard, quality control, or mouse sample were transferred to polypropylene microcentrifuge tubes containing 60 μL of a methanolic internal standards solution. The tubes were vortex-mixed for 60 seconds, followed by centrifugation at 10,000 rpm for 8 minutes at 4°C. Next, the supernatants were transferred to autosampler vials and 3 μL volumes were injected into a Waters ACQUITY separation system coupled to a TQD triple-quadrupole MS/MS detector. Separation was achieved on a BEH C18 column (1.7 μm; 50 × 2.1 mm; Waters) using a column heater operating at 40°C with an in-line filter. The autosampler temperature was maintained at 15 ± 5°C. The flow rate of a gradient mobile phase, composed of 0.1% formic acid in acetonitrile and 10 mmol/L ammonium acetate in 0.5% formic acid water, was set at 0.7 mL per minute, and separation was completed within 5 minutes. The instrument was equipped with an electrospray interface and was controlled by Masslynx 4.1 software. The analysis was conducted in MRM mode, as follows: m/z 488.16 > 401.11 for dasatinib, m/z 444.17 > 275.11 for N-deshydroxy dasatinib (M4), and m/z 504.17 > 387.12 for dasatinib N-oxide (M5). Dasatinib-d8, N-deshydroxy-d8 dasatinib, and dasatinib N-oxide-d8 (Toronto Research Chemicals) were used as internal standards (final concentration, 15 ng/mL). The MS-MS conditions included: capillary voltage, 0.6 kV; source temperature, 150°C; desolvation temperature, 450°C; cone gas flow, 10 L per hour; and desolvation gas flow, 900 L per hour.

Calibration curves of dasatinib, M4, and M5 were created by plotting the peak area ratios of analyte to the internal standard against the internal standard concentrations in the spiked matrix. The within-run and between-run precisions for dasatinib and its metabolites were less than 6.9% and the measured concentrations were all less than 5.3% of the nominal value.

Ex vivo microsomal incubations

Mouse liver and intestinal microsomes were prepared as described (22). Assays for cytochrome P450 enzymes were conducted as described by the manufacturer (BD Biosciences). Briefly, dasatinib (2 μmol/L) was incubated with liver or intestinal microsomes (1 mg/mL) for 55 minutes at 37°C (3) and the reaction was terminated by 100 μL ice-cold acetonitrile. Dasatinib and its main rodent metabolites M4 and M5 (23) were determined by LC/MS-MS.

Gastric transport studies

The ex vivo mucosal-to-serosal transport of dasatinib in isolated stomachs from wild-type or Abcc4(−/−) mice was determined on the basis of a published method (24), as was the in vivo gastric secretion of dasatinib (25). Briefly, after anesthetizing mice with isoflurane, the abdominal wall was opened longitudinally and a ligature was tightened around the cardia, to prevent reflux into the esophagus. Next, a tube was introduced into the duodenum and gently forced through the pylorus into the stomach cavity. To ensure tightness of the system and to avoid blood reflux into the stomach, 2 ligatures were fastened upstream and downstream of the pyloric sphincter. The tube was connected to a syringe to introduce dasatinib (1 mg/mL) in 50 mmol/L sodium acetate buffer (pH 4.6) or vehicle alone into the gastric lumen. At 5 minutes after the administration of dasatinib, a sodium heparin solution (0.1 mL; 50 IU) was injected into the inferior cava vein and immediately thereafter, livers were obtained after cervical dislocation to ensure euthanasia. Body temperature, pulse, respiration rate, and anesthetic administration were monitored throughout the procedure. Gastric pH was determined using an IQ150 pH Meter with a PH37-SS piercing-tip microprobe (Hach).

Transporter gene and protein expression

Gene expression in the small intestine and stomach of wild-type or Abcc4(−/−) mice was conducted on RNA extracted from whole organs using the RNeasy Mini Kit (Qiagen) and analyzed using the Mouse Transporter RT2 Profiles PCR array system (SA Biosciences). Relative gene expression was determined using the ΔΔCt method and normalized to the housekeeping gene, Gapdh.

Western blotting of Abcc4 (26) or Abcb1 and Abcg2 (27) was done according to published methods. The primary rat anti-human ABCC4 antibody, which also reacts with mouse Abcc4, was obtained from Abcam, and used at 1:50. The secondary antibody, rabbit anti-rat (Vector), was used at 1:600. Slides of 4 μm sections were cut from formalin-fixed paraffin-embedded tissues. All assay steps, including deparaffinization, rehydration, and epitope retrieval, were conducted on the Bond Max with Bond wash buffer (Leica) rinses between steps. Heat-induced epitope retrieval was conducted by heating slides in ER2 (Leica) for 30 minutes. Background Punisher (BioCare Chemical) was incubated on slides for 10 minutes. The Refine (Leica) detection system was used. Briefly, slides were incubated sequentially with hydrogen peroxide (5 minutes), primary antibody (15 minutes), secondary antibody (10 minutes), anti-rabbit horseradish peroxidase–conjugated polymer (8 minutes), 3,3’ diaminobenzidine (10 minutes), and hematoxylin (8 minutes). Normal human stomach samples were obtained from ProSci.

Activity of dasatinib against a model of Ph1 ALL

Details of the mouse model have been reported previously (28). Briefly, 2 × 105 luciferase-positive, p185(BCR-ABL)-expressing Arf(−/−) leukemia-initiating pre-B cells...
(LIC) were administered by tail vein injection into cohorts of healthy, nonconditioned, immunocompetent 10- to 12-week-old male wild-type or Abcc4(+/−) mice on a C57BL/6 background. Dasatinib formulated in 80 mmol/L citric acid (pH 3.1) or vehicle alone was administered by oral gavage to recipient mice at 10 mg/kg per dose once daily, 5 days per week for 4 weeks, starting on day 10 after LIC injection. Whole-animal luminescent imaging was conducted in all mice to monitor tumor engraftment and data analysis was conducted using a Xenogen IVIS-200 system and Living Image Version 3.01 software (Caliper Life Sciences), as described (29). Mice were observed daily and the experiment was terminated when animals showed signs of terminal illness, including hind-leg paralysis, inability to eat or drink, and/or were moribund.

LIC viability was determined using a MTT, Cell Proliferation Kit I (Roche), as previously described (30). The cells were treated with dasatinib at increasing concentrations (0.001–30 μmol/L) for 48 hours (2 independent experiments were carried out with 8 replicates at each concentration). The concentration inhibiting cell viability by 50% compared with vehicle-treated control cells (IC50) was determined using the software program GraphPad Prism version 5.0 (GraphPad Software). To evaluate growth inhibitory properties of dasatinib against LICs at average concentrations observed in vivo in wild-type and Abcc4(−/−) mice, a correction factor was applied to account for anticipated differences in the fraction unbound dasatinib in plasma of wild-type mice, Abcc4(−/−) mice, and cell culture medium, as described (31).

Statistical analysis
All data are presented as mean values with SE. Group differences as a function of cell-type or mouse genotype were evaluated using a Student t test. Two-tailed P values of less than 0.05 were considered as statistically significant. Overall survival was assessed by the Kaplan–Meier method, followed by a Mantel–Cox test. Statistical calculations were conducted using NCSS version 2004 (Number Cruncher Statistical System).

Results
In vitro transport of dasatinib by ABC4
Results from ABC4-overexpressing inside-out vesicles indicated that dasatinib was actively transported by ABC4 (Fig. 1A). When compared with control vesicles, dasatinib accumulation in the ABC4-overexpressing vesicles increased by 3.0-fold (P = 0.0014). Similarly, the...
accumulation of the prototypical ABCC4 substrate E2G was increased by 3.2-fold ($P = 0.0016$), although this was not observed for imatinib (1.3-fold; $P = 0.098$). The transport of dasatinib into vesicles-expressing ABCC4 did not reach a plateau within a clinically relevant range of concentrations (Supplementary Fig. S1). This observation is consistent with the relatively high Michaelis–Menten constants ($K_m$) reported for several other xenobiotic substrates of ABCC4, including 220 to 1,300 μmol/L for methotrexate, 640 μmol/L for leucovorin, and >1,000 μmol/L for adefovir and tenofovir (32). As several compounds underwent transport by ABCC4 only in the presence of physiologic concentrations of GSH (33), we also evaluated ATP-dependent cotransport of reduced GSH. While GSH (2 mmol/L) increased ABCC4-mediated transport of E2G by about two-fold, this phenomenon was not observed for dasatinib (Supplementary Fig. S2A). Under the same experimental conditions, dasatinib uptake was also facilitated by the related transporter ABCC3 (MRP3), although the extent of accumulation (1.3-fold; $P = 0.028$) was less compared with that observed for ABCC4 (Supplementary Fig. S2B).

Dasatinib was also found to be transported by mouse Abcc4, as exemplified by a significantly reduced accumulation in Saos-2 cells overexpressing Abcc4 compared with control cells (Fig. 1B). In this model system, no noticeable efflux transport by Abcc4 was noted with imatinib (Fig. 1B).

**Dasatinib pharmacokinetics in Abcc4-knockout mice**

We next evaluated the possible importance of this transporter for dasatinib in mice with a genetic deletion of Abcc4. The AUC for dasatinib in these animals after oral administration was significantly decreased by 1.75-fold compared with that observed in wild-type mice (Table 1). The respective concentration–time profiles of dasatinib suggest that the low systemic exposure in the Abcc4(−/−) mice is possibly due to an absorption defect rather than an event occurring in the terminal phase (Fig. 1C). This is consistent with the notion that the absorption rate constant is dramatically reduced in Abcc4(−/−) mice, leading to a delayed time-to-peak concentration, whereas the terminal half-lives of dasatinib were not significantly different between mouse genotypes (Table 1). As anticipated on the basis of in vitro transport data, the pharmacokinetic profile of oral imatinib was not substantially affected by Abcc4 deficiency.

To obtain further insights into the mechanism underlying the pharmacokinetic changes observed with dasatinib in the Abcc4(−/−) mice, the drug was also administered via intravenous bolus injection. The resulting concentration–time profiles (Supplementary Fig. S3) and pharmacokinetic parameter estimates (Table 1) were not significantly affected by Abcc4 deficiency. Moreover, the binding of dasatinib to serum proteins and its extent of blood cell partitioning (18) were independent of mouse genotype (Table 1).

To rule out potentially altered, compensatory changes in the expression of enzymes in the liver and intestine of Abcc4(−/−) mice at baseline, differential expression profiles of Cyp1a1, Cyp1b1, and Cyp3a11, the 3 isoforms with the highest dasatinib turnover (18), were evaluated. Compared with levels in liver and intestine of wild-type mice, transcripts of these enzymes were not increased in the Abcc4(−/−) mice (Fig. 2A and B). Furthermore, there were no potentially compensatory changes in enzyme activity, because Abcc4 knockout had no influence on the hepatic or intestinal microsomal metabolism of dasatinib to M4 (Fig. 2C) or M5 (Fig. 2D). This finding is consistent with

**Table 1.** Dasatinib pharmacokinetic parameters in wild-type mice and Abcc4(−/−) mice after oral or intravenous administration of dasatinib (10 mg/kg)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>Abcc4(−/−)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$K_a$ (1/h)</td>
<td>34.3 ± 6.85</td>
<td>2.49 ± 0.92</td>
<td>0.022</td>
</tr>
<tr>
<td>$T_{max}$ (h)</td>
<td>0.306 ± 0.223</td>
<td>2.19 ± 1.06</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$C_{max}$ (ng/mL)</td>
<td>182 ± 93.8</td>
<td>48.1 ± 16.5</td>
<td>0.0014</td>
</tr>
<tr>
<td>AUC (ng h/mL)</td>
<td>442 ± 152</td>
<td>253 ± 86.8</td>
<td>0.0041</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>4.05 ± 2.44</td>
<td>5.10 ± 2.34</td>
<td>0.54</td>
</tr>
<tr>
<td>F (%)</td>
<td>13.2</td>
<td>6.36</td>
<td></td>
</tr>
<tr>
<td>Intravenous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{max}$ (ng/mL)</td>
<td>3.387 ± 305</td>
<td>3.458 ± 323</td>
<td>0.73</td>
</tr>
<tr>
<td>AUC (ng h/mL)</td>
<td>3.361 ± 314</td>
<td>3.975 ± 371</td>
<td>0.93</td>
</tr>
<tr>
<td>$T_{1/2}$ (h)</td>
<td>3.96 ± 2.33</td>
<td>3.81 ± 2.49</td>
<td>0.90</td>
</tr>
<tr>
<td>CL (mL/min/kg)</td>
<td>49.6</td>
<td>41.9</td>
<td></td>
</tr>
<tr>
<td>$C_{blood}/C_{plasma}$</td>
<td>0.896 ± 0.013</td>
<td>0.940 ± 0.04</td>
<td>0.17</td>
</tr>
<tr>
<td>Blood cell partitioning (%)</td>
<td>33.0 ± 1.04</td>
<td>36.1 ± 2.86</td>
<td>0.16</td>
</tr>
<tr>
<td>Fraction unbound (%)</td>
<td>8.70 ± 0.440</td>
<td>8.12 ± 0.493</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Abbreviations: $C_{blood}/C_{plasma}$, ratio of concentration in blood to concentration in plasma; $C_{max}$, peak plasma concentration; F, apparent oral bioavailability; $K_a$, absorption rate constant; $T_{max}$, time-to-peak plasma concentration; $T_{1/2}$, half-life of the terminal phase.
recent data indicating that hepatic Cyp3a11 protein expression is not altered in adult male Abcc4(+/−) mice (34).

As dasatinib is a substrate for Abcb1 and Abcg2 (35) and these transporters have been implicated in restricting dasatinib absorption (9), we confirmed that their protein expression levels in the small intestine were not altered in Abcc4(+/−) mice (Supplementary Fig. S4A and S4B). In addition, there were no differences in intrinsic intestinal permeability between wild-type and Abcc4(+/−) mice, as determined from the recovery of mannitol in plasma, kidney, and liver after oral mannitol dosing (Supplementary Fig. S4C). Likewise, the altered systemic levels of dasatinib in Abcc4(+/−) mice cannot be explained by an intrinsically altered ability to excrete dasatinib because drug levels remained undetectable (<10 ng/mL) in the urinary bladder and gall bladder at 5 minutes after oral administration.

Gastric transport as a mechanism for dasatinib absorption

The notion that, in wild-type mice receiving oral dasatinib, peak concentrations in plasma were already observed at the first collection time point, prompted us to consider the possibility that dasatinib absorption occurs through the stomach. Consistent with this hypothesis, the Abcc4 protein was readily detectable in stomach samples from wild-type mice (Fig. 3A). In stomachs of wild-type mice, Abcc4 was detected in the mucosa of the fundus (Fig. 3B) with basal labeling of gastric pit mucosal cells and basolateral labeling of the parietal and chief cells; in the parietal cells, the basal labeling was faint-to-weak and the lateral labeling was strong. Labeling was absent in Abcc4(−/−) mice (Fig. 3C). In the human stomach, ABCC4 was detected in the gastric pit mucous cells, and basolateral labeling was present in parietal and chief cells (Fig. 3D).

We next evaluated the ex vivo transport of dasatinib in sacs prepared from mouse stomachs and found that Abcc4 deficiency was associated with up to 3.9-fold (P = 0.0025) reduced drug transport to the serosal side (Fig. 4A). To test whether the stomach is a site of absorption of dasatinib in vivo, a surgical procedure was implemented on anesthetized mice, allowing us to introduce a dasatinib dosing solution directly into the stomach, ligated at both the cardia and the pylorus. Analysis of liver samples, as a surrogate for portal and systemic plasma, obtained 5 minutes after introducing the drug confirmed that dasatinib can permeate the gastric mucosa through an Abcc4-mediated mechanism (Fig. 4B). During this experiment, the gastric pH remained on average 4.36 ± 0.32 and 4.31 ± 0.23 (P = 0.81) in wild-type and Abcc4(−/−) mice, respectively. This suggests that Abcc4 deficiency is unlikely to cause changes in gastric pH that could subsequently cause altered dasatinib solubility and lead to impaired absorption (36).

Analysis of stomachs from Abcc4(+/−) mice revealed that the expression of 84 ATP-binding cassette transporter and solute carrier genes was not substantially changed compared with stomachs obtained from wild-type animals, with the exception of a downregulation of Slc22a1 (4.85-fold) and Slc22a2 (4.35-fold; Fig. 4C). These genes encode the organic cation transporters Oct1 and Oct2 that have been implicated in xenobiotic transport (37). However, the
altered expression of Slc22a1 and Slc22a2 in the stomach of Abcc4(-/-) mice is unlikely to affect the pharmacokinetic profile of dasatinib because overexpression of Oct1 or Oct2 in mammalian cells did not alter intracellular accumulation of dasatinib (Fig. 4D), which suggests that the drug is not a substrate of these transporters.

As the stomach drains either directly or indirectly into the portal vein through short gastric veins from the fundus to the splenic vein, we also measured dasatinib concentrations in the spleen, an organ that expresses high levels of Abcc4, which in turn can restrict splenic accumulation of substrates such as topotecan (26) and PMEA (38). At 5 minutes after oral dasatinib administration, however, the drug levels were still higher in the spleen of wild-type mice (46.5 ± 11.8 ng/g) than in Abcc4(-/-) mice (26.7 ± 7.51 ng/g). This suggests that splenic trapping of dasatinib after oral administration is not substantially contributing to the low systemic plasma levels in Abcc4(-/-) mice.

**Influence of Abcc4 deficiency on dasatinib efficacy**

In view of the altered systemic exposure caused by Abcc4 deficiency and the established pharmacokinetic–pharmaco-dynamic relationships for dasatinib (7), we hypothesized that the antileukemic properties of dasatinib are dependent on host Abcc4 function. This hypothesis was evaluated in paired wild-type and Abcc4(-/-) mice receiving daily oral dasatinib starting on day 10 following the injection of luciferase-positive, p185(BCR-ABL)-expressing Arf(-/-) leukemia initiating pre-B cells (28). We found that these cells express low levels of Abcc4, as detected by real-time reverse transcriptase PCR, with an Abcc4 to Gapdh expression ratio of 0.002653. However, despite detectable transcripts, the intracellular accumulation or in vitro cell growth inhibitory properties of dasatinib in p185(BCR-ABL)-expressing Arf(-/-) leukemia initiating pre-B cells were not influenced by coinuculation with the ABC transporter inhibitor, MK571 (Supplementary Fig. S5A and S5B). Additional in vitro studies
indicated that exposure of these cells to dasatinib at a concentration equivalent to the average in vivo levels of dasatinib in plasma of wild-type mice was associated with a 5.5-fold increase (\(P < 0.001\)) in cell growth inhibition compared with levels of dasatinib in plasma of Abcc4\((-/-)\) mice (Supplementary Fig. S5C).

In subsequent in vivo experiments, whole-animal luminescent imaging was conducted to monitor tumor engraftment (Fig. 5A). Consistent with the prediction from the in vitro experiments, 2 weeks after start of treatment with dasatinib (day 24), the luminescent signal was increased by 1.6-fold in wild-type mice and by more than 5-fold in Abcc4\((-/-)\) mice (\(P = 0.016\); Fig. 5B). This suggests that the initial antileukemic activity of dasatinib given at the applied dose and schedule against this p185(BCR-ABL)-expressing Arf\((-/-)\) leukemia is dependent on host Abcc4 genotype. While all mice receiving vehicle only died within 2 weeks irrespective of genotype, there was a statistically significant survival advantage for wild-type mice receiving dasatinib compared with Abcc4\((-/-)\) mice (median survival, 35 vs. 31 days; \(P = 0.048\); Fig. 5C). Importantly, the observed systemic concentrations of dasatinib in these tumor-bearing mice receiving multiple daily doses of the drug were similarly dependent on Abcc4 genotype compared with results obtained in non tumor-bearing mice receiving a single dose (Fig. 5D).

Discussion

In this study, we found that dasatinib is a substrate for the ATP-binding cassette transporter ABCC4, that the oral absorption of dasatinib is at least partially dependent on ABCC4-mediated gastric transport, and that this process influences the drugs’ antitumor efficacy. The current data complement previous knowledge on the interaction of tyrosine kinase inhibitors with ATP-binding cassette transporters and may have important practical implications for their optimal use.

The oral absorption of small molecules is believed to be primarily occurring in the small intestine of the gastrointestinal tract, with the stomach playing a supporting...
role in the solubilization of weakly basic compounds (39). Indeed, only a handful of molecules, including ethanol, acetylsalicylic acid (aspirin), and certain anthocyanin-flavonoids in the *Vitis vinifera* grape (Cabernet Sauvignon), have been shown to be absorbed from the gastric compartment (25). Conventionally, for small molecules to cross cellular membranes by simple diffusion they are thought to possess certain physicochemical properties, most notably those defined by Lipinski’s rule of five (40). However, mounting recent evidence points to drug transport proteins playing a more significant role in the transfer of small molecules across cellular membranes than held previously (41).

In the case of aspirin, for example, the drug is rapidly absorbed from the stomach by crossing the luminal membrane in a nonionized form, which is favored by the highly acidic environment (42). The resulting change in pH caused by crossing the gastric mucosa would switch aspirin to a predominately ionized form that requires the assistance of a transporter to facilitate its movement across the basal side of the lumen before entering the bloodstream. Interestingly, aspirin was recently identified as a transported substrate of human ABCC4 (43) and we found that both aspirin and its metabolite salicylic acid are also substrates of mouse Abcc4 (Supplementary Fig. S6). This finding supports the possibility that aspirin, like dasatinib, may be entering the circulation through a process that is dependent, at least in part, on a gastric ATP-binding cassette transporter.

The ability of dasatinib to permeate the gastric mucosa through an ABCC4-mediated mechanism could be at the basis of the fast absorption kinetics of dasatinib in rodents as well as in humans (12). Although absorption is likely to also occur in the upper intestine, the relative contribution of the stomach in the process of dasatinib absorption remains to be determined. In this context, it is worthwhile pointing out that the fate of dasatinib in lower segments of the digestive tract is severely complicated by its strong pH-dependent aqueous solubility, which ranges from 18.4 mg/mL at pH 2.6 to only 0.008 mg/mL at pH 6.0 (36). As the intraluminal pH in humans acutely changes from highly acidic in the stomach to about pH 6 in the duodenum, and then gradually to pH 7.4 in the terminal ileum (44), it is very likely that the stomach contributes substantially to the absorption of oral dasatinib in patients. This possibility is consistent with previous studies indicating that dasatinib is exquisitely sensitive to agents that modulate the luminal pH of the stomach by suppressing gastric acid secretion.
such as H$_2$-receptor antagonists and proton pump inhibitors (45, 46).

It is possible that gastric transport also contributes to the oral absorption of other clinically important tyrosine kinase inhibitors. Although direct evidence of this is lacking, some recent studies have shown that major gastrectomy, but not small-bowel resection, is associated with significantly decreased plasma levels of imatinib in patients with gastrointestinal stromal tumors (47, 48). Similar findings have been reported for nilotinib (49), and in some patients, major gastrectomy was resulting in rapid disease progression. The observed decreases in exposure of these drugs have been attributed to impaired pH-dependent drug dissolution and solubility and/or an altered gastric motility resulting from the surgery. However, these explanations seem inadequate in view of the fact that the pharmacokinetics of imatinib (50) and nilotinib (51) are not substantially affected by omeprazole or its S-enantiomer esomeprazole, agents that both increase the pH of the gut as well as delay gastric emptying (52).

It is interesting to note that previous studies showed that, after oral administration, the pharmacokinetic profile of the ABCB4 substrates methotrexate (24) and cefadroxil (14) was unchanged in Abcc4(−/−) mice. The reasons underlying the apparent differences in outcome of these previous studies and our current results for dasatinib are not entirely clear, but it is likely that differential mechanisms regulating drug entry on the apical side of the gut lumen contribute. In addition, unlike dasatinib, methotrexate and cefadroxil are relatively high-affinity substrates of Abcc3 and this transporter may compensate for the loss of Abcc4 in the knockout mice. Indeed, concentrations of cefadroxil in portal and peripheral blood, after injection of the drug directly into a ligated jejunum, were significantly decreased in mice deficient for both Abcc3 and Abcc4, compared with wild-type mice or animals lacking only one of the transporters (14).

Regardless of the exact mechanism, our current findings provide further evidence that ABCC4 can affect the pharmacokinetic properties of a remarkably broad range of substrates.

Collectively, our results indicate that in humans and mice ABCC4 is abundantly expressed in the stomach, where it facilitates the gastric absorption of dasatinib. Moreover, Abcc4 deficiency in mice was associated with reduced activity of dasatinib against a model of Ph$^+$-ALL. These results identified ABCC4 as an important, previously unrecognized, contributor to the oral absorption of dasatinib and this process may be relevant for other substrate drugs, such as aspirin. In view of the established exposure–efficacy relationships for dasatinib (53), we suggest that caution is warranted when dasatinib has to be administered together with agents that potently inhibit ABCBC4.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


Supplementary Figure Legends

Contribution of Abcc4-Mediated Gastric Transport to the Absorption and Efficacy of Dasatinib

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Supplementary Fig. S1
Concentration-dependence of dasatinib transport by ABCC4. Experiments were performed in control inside-out vesicles or vesicles expressing human ABCC4. Data represent the mean of 2 independent experiments, and error bars represent the standard error. The dotted line represents the net transport by ABCC4.

Supplementary Fig. S2
Influence of reduced glutathione (GSH) and ABCC3 function on dasatinib transport. Uptake of [³H]estradiol-17β-D-glucuronide (E2G) (1 µM), a positive control substrate, or [³H]dasatinib (1 or 16 µM) was evaluated in control inside-out vesicles of Sf9 cells or vesicles expressing human ABCC4 (A) or ABCC3 (B). Data represent the mean 2 independent experiments, and are expressed as the average percent of uptake values in control cells. Error bars represent the standard error. The star (*) denotes a significant difference from control (P < 0.05).

Supplementary Fig. S3
Influence of Abcc4 function on dasatinib disposition. (A) Plasma concentration-time profile of dasatinib (i.v. dose, 10 mg/kg) in wildtype mice and Abcc4(-/-) mice. (B) The same data presented on a logarithmic Y-axis. Data represent the mean of 5 observations per time point, and error bars represent the standard error.

Supplementary Fig. S4
Expression of Abcb1 and Abcg2 in mouse intestine. (A) Protein expression of Abcb1 and Abcg2 normalized to Gapdh in the small intestine of wildtype mice and Abcc4(-/-) mice. (B) Relative expression of Abcb1 and Abcg2 in the small intestine of wildtype mice and Abcc4(-/-) mice. Data represent the mean (bars) and standard error (error bars) of 3 animals per group.
(C) Intrinsic intestinal permeability of wildtype and Abcc4(-/-) mice as determined by the recovery of D-[2-^3^H]mannitol in plasma, kidney, and liver at 2-h after an oral dose of 1 mg/kg. Data represent the mean (bars) and standard error (error bars) of 4 animals per group.

**Supplementary Fig. S5**

*In vitro* transport and activity of dasatinib against p185(BCR-ABL)-expressing Arf(-/-) leukemia initiating pre-B cells (LIC). (A) Uptake of [^3^H]dasatinib in LIC in the presence and absence of the ABC transporter inhibitor MK571 (1 µM). (B) Growth inhibitory properties of dasatinib against LIC in the presence (IC$_{50}$, 0.23 nM) and absence of MK571 (IC$_{50}$, 0.25 nM), as determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using 48-hour continuous exposure. (C) Growth inhibitory properties of dasatinib against LIC at concentrations equivalent to the average unbound plasma levels in Abcc4(-/-) mice (0.3 nM) and wildtype mice (1 nM). All data represent the mean 2 to 4 independent experiments, and error bars represent the standard error.

**Supplementary Fig. S6**

Influence of Abcc4 on aspirin transport. Accumulation of [^3^H]PMEA (10 µM), a positive control substrate, [acetyl-1^4^C]salicylic acid (aspirin) (20 µM), or [7-^1^4^C]salicylic acid (20 µM) was evaluated in Saos-2 cells transfected with an empty pcDNA vector (control) or mouse Abcc4. Data represent the mean 3-4 independent experiments, and are expressed as the average percent of uptake values in control cells. Error bars represent the standard error. The star (*) denotes a significant difference from control ($P < 0.05$).
Supplemental Figure S1

**Suppl Figure S1**

Uptake (pmol/mg/5 min) vs. Dasatinib (μM)

- ABCC4
- Control
- ABCC4-Control
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Clinical Cancer Research 19(16) 2013

Supplemental Figure S2

Suppl Figure S2
Supplemental Figure S3

(A) Abcc4(-/-) vs. Wildtype

(B) Abcc4(-/-) vs. Wildtype

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Clinical Cancer Research 19(16) 2013

Supplemental Figure S4

Suppl Figure S4

A

Wildtype  Abcc4(-/-)

Abcb1

Gapdh

Abcg2

Gapdh

B

Relative intensity (vs Gapdh)

Abcb1  Abcg2

Wildtype

C

Mannitol (% dose)

Plasma  Kidney  Liver

Abcc4(-/-)  Wildtype
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Supplemental Figure S5
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Clinical Cancer Research 19(16) 2013

Supplemental Figure S6