ABCB1 haplotypes do not influence transport or efficacy of tyrosine kinase inhibitors in vitro

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Abstract: Single-nucleotide polymorphisms (SNPs) in the gene coding for the efflux-transport protein ABCB1 (P-glycoprotein) are commonly inherited as haplotypes. ABCB1 SNPs and haplotypes have been suggested to influence the pharmacokinetics and therapeutic outcome of the tyrosine kinase inhibitor (TKI) imatinib, used for treatment of chronic myeloid leukemia (CML). However, no consensus has yet been reached with respect to the significance of variant ABCB1 in CML treatment. Functional studies of variant ABCB1 transport of imatinib as well as other TKIs might aid the interpretation of results from in vivo association studies, but are currently lacking. The aim of this study was to investigate the consequences of ABCB1 variant haplotypes for transport and efficacy of TKIs (imatinib, its major metabolite N-desmethyl imatinib [CGP74588], dasatinib, nilotinib, and bosutinib) in CML cells. Variant haplotypes – including the 61A>G, 1199G>A, 1236C>T, 1795G>A, 2677G>T/A, and 3435T>C SNPs – were constructed in ABCB1 complementary DNA and transduced to K562 cells using retroviral gene transfer. The ability of variant cells to express ABCB1 protein and protect against TKI cytotoxicity was investigated. It was found that dasatinib and the imatinib metabolite CGP74588 are effectively transported by ABCB1, while imatinib, nilotinib, and bosutinib are comparatively weaker ABCB1 substrates. None of the investigated haplotypes altered the protective effect of ABCB1 expression against TKI cytotoxicity. These findings imply that the ABCB1 haplotypes investigated here are not likely to influence TKI pharmacokinetics or therapeutic efficacy in vivo.

Keywords: imatinib, CGP74588, chronic myeloid leukemia, pharmacogenetics, N-desmethyl imatinib

Introduction

The tyrosine kinase inhibitors (TKIs) imatinib, dasatinib, and nilotinib are indicated for first-line treatment of chronic myeloid leukemia (CML). Moreover, dasatinib and nilotinib together with bosutinib are indicated for the treatment of patients with resistance or intolerance to first-line therapy. In general, most patients respond well to therapy, although a significant proportion of CML patients have to dose-adjust or switch therapies due to adverse events or suboptimal response.¹² In order to ensure a more personalized CML treatment strategy, a deeper understanding of the factors that determine response and resistance to the individual TKIs is required.

Imatinib, dasatinib, and nilotinib have previously been reported as substrates for the efflux-transport protein ABCB1 (P-glycoprotein).³⁻⁴ Less is known about bosutinib efflux, although a single report indicates that bosutinib is unlikely to be transported by ABCB1.⁴ In addition, the major imatinib metabolite N-desmethyl imatinib (CGP74588)
was also shown to be an ABCB1 substrate. This metabolite is pharmacologically active in vitro, although less potent than imatinib.

ABCB1 is expressed in tissues that are involved in the absorption and elimination of TKIs, including the intestine, liver, and kidneys. ABCB1 expression has also been found in CML stem cells and in the circulating leukocytes of CML patients. Consequently, in addition to influencing the influx–efflux of active drug into target cells, ABCB1-transport activity might influence the systemically circulating TKI concentration that reaches target cells. The influx-transport activity of the organic cation transporter 1 (OCT-1) as well as imatinib plasma concentration have previously been correlated with the outcome of CML therapy. These findings indicate that analysis of drug-transport activity, or markers thereof, might be useful predictors of response to imatinib, and perhaps to the second-generation TKIs as well.

The ABCB1 gene is highly polymorphic, with about 100 identified single-nucleotide polymorphisms (SNPs) located in the coding regions, some of which have been associated with the efflux, pharmacokinetics, or therapeutic outcome of several drug classes. Moreover, at least 28 coding and noncoding ABCB1 SNPs are frequently inherited together, defining distinct haplotypes. The most commonly studied haplotype consists of the 1236C>T, 2677G>T/A, and 3435T>C SNPs. These SNPs have been evaluated for their influence on imatinib plasma concentrations and therapeutic efficacy in CML patients. However, the reports are inconclusive: some show influence of the individual SNPs or the complete haplotype on plasma concentrations and/or therapeutic outcome of imatinib, while others do not.

In addition, there are other nonsynonymous ABCB1 SNPs that have been associated with the therapeutic outcome of ABCB1 substrate drugs, but that have not yet been studied with regard to TKI transport. In light of contradictory results and the ongoing debate about ABCB1 SNPs and their significance in CML treatment, a functional study of ABCB1 SNPs in relation to their influence on TKI transport is needed. Therefore, the aim of this study was to investigate the influence of ABCB1 variant haplotypes on TKI transport and efficacy. For that purpose, the ABCB1 SNPs 61A>G, 1199G>A, 1236C>T, 1795G>A, 2677G>T/A, and 3435T>C were constructed in combinations to result in variant haplotypes that were transduced to a CML cell line. The impact of ABCB1 variant haplotypes on transport and efficacy of imatinib, CGP74588, dasatinib, nilotinib, and bosutinib was investigated.

Materials and methods

Drugs and chemicals

Imatinib and CGP74588 were provided by Novartis Pharma (Basel, Switzerland). Dasatinib, nilotinib, and bosutinib were purchased from Selleck Chemicals (Houston, TX, USA). Stock solutions of 10 mM were prepared for all drugs, stored at −20°C, aliquoted to avoid repeated freeze–thawing, and were used within 1 year of preparation. Imatinib and CGP74588 stock solutions were prepared in water, while nilotinib, dasatinib, and bosutinib were prepared in dimethyl sulfoxide. Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich (St Louis, MO, USA).

Cells and culturing conditions

The CML cell line K562 (LGC Standards, Teddington, UK) was used for ABCB1 transduction and parental as well as transduced cell lines were kept in Roswell Park Memorial Institute 1640 medium supplemented with penicillin, streptomycin, and 10% fetal bovine serum (FBS). Human embryonic kidney 293T cells (LG Standards) were cultured in Dulbecco’s Modified Eagle’s Medium, supplemented with penicillin, streptomycin, and 10% heat-inactivated FBS. Cell-culture reagents were purchased from Life Technologies, Paisley, UK. All cell lines were verified to be mycoplasma-free.

ABCB1 single-nucleotide polymorphisms and haplotypes

The ABCB1 SNPs studied here were selected based on their single-nucleotide substitution, location in the coding region of the ABCB1 gene, and a minor allele frequency of >2% in the Caucasian population. SNP and haplotype frequencies were obtained from previously published population studies or from the National Center for Biotechnology Information dbSNP database. The ABCB1 SNPs included in this study were 61A>G (rs9282564), 1199G>A (rs2229109), 1236C>T (rs1128503), 1795G>A (rs2235036), 2677G>T/A (rs2032582), and 3435T>C (rs1045642). The 1236C>T, 2677G>T/A, and 3435T>C SNPs are in linkage disequilibrium and are commonly inherited together as one of the two haplotypes (1236T, 2677T, 3435T) or (1236C, 2677G, 3435C), referred to here as the TTT or CGC haplotype. In approximately 2% of the Caucasian population, 2677G>T is substituted by an A, giving rise to the CAC haplotype (1236C, 2677A, 3435C). The 1236 and 3435 SNPs are silent substitutions; the method of retroviral gene transfer with artificial transcriptional regulation, our main focus was to study the posttranslational effects of ABCB1 variants. However, the complete haplotypes of...
1236, 2677, and 3435 SNPs were constructed to ensure that any differences between the variant cell lines had not been caused by altered efficacy of translation due to linked silent SNPs in the ABCB1 transcript. Consequently, the three haplotypes were constructed in separate vectors for transduction into K562 cells. The 61A>G and 1199G>A SNPs were constructed together with TTT and CGC, respectively, the haplotypes with which they are most frequently associated.\(^17\) The 1795G>A SNP was included in the study because it was already in its variant form in the transcript used for vector constructs. To the best of our knowledge, this SNP has not been associated with a specific haplotype, thus it was kept together with the CGC haplotype, since this was the haplotype of the transcript used for vector constructs. An overview of the genotypes of generated cell lines and frequencies of minor alleles and haplotypes appears in Table 1.

\(ABCB1\) complementary DNA (cDNA; GenBank ID NM_000927.3) in a pCMV6-XL4 vector (OriGene, Rockville, MD, USA) was used for incorporation of variant nucleotides corresponding to each specific SNP, using the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). Incorporation of variants and \(ABCB1\) reference sequence was confirmed by automated Sanger sequencing, using the services of GATC Biotech (Konstanz, Germany).

**Generation of K562 cells with variant \(ABCB1\) expression**

\(ABCB1\) cDNA was ligated into a murine stem cell virus-IRES-enhanced yellow fluorescent protein (EYFP) retroviral vector (MIY),\(^29\) using the Rapid DNA Dephos and Ligation Kit (Roche, Basel, Switzerland). The correct orientation of the gene insert was confirmed by Sanger sequencing (GATC Biotech). The MIY-\(ABCB1\) vector (11 kb long) exceeded the optimal size of insert cDNA to the retroviral transfer system and had to be shortened for efficient transductions. The restriction enzyme Pmel (New England Biolabs, Ipswich, MA, USA) was used to cleave off a 750-base-long fragment in the vector–gene complex from position +199 in the \(ABCB1\) 3′-untranslated region into nonsignificant parts of the vector. MIY-\(ABCB1\) (4 μg) and 2 μg each of the helper vectors vesicular stomatitis virus glycoprotein G and Pol-Gag were mixed with a final concentration of 125 mM CaCl\(_2\), followed by calcium phosphate transfection of \(1 \times 10^6\) 293T cells. Viral 293T supernatants were collected over 48 hours and filtered through a 0.45 μm sterile cellulose acetate filter (Whatman, Dassel, Germany). K562 cells (0.5 \times 10^6) were transduced with viral supernatants using spin infection (1.5 hours in 1200 g, 22°C) in the presence of 4 μg/mL polybrene. EYFP/\(ABCB1\) cells were sorted using flow cytometry. Sorting aimed at ensuring equal median fluorescence intensities (MFIs) of EYFP between cell lines. An empty MIY vector was transduced to K562 cells to obtain the control cell line, referred to here as K562/ve.

**Cell-membrane expression of \(ABCB1\) protein**

The expression level of \(ABCB1\) protein in the cell membrane after retroviral gene transfer was evaluated using flow cytometry. Cells (\(1 \times 10^6\)) were labeled with 0.25 μg of primary mouse antihuman \(ABCB1\), clone 17F9 (BD Biosciences, Rockville, MD, USA) and 0.25 μg of goat antimouse secondary antibody conjugated to fluorescein isothiocyanate (BD Biosciences). Cell membranes were permeabilized using 0.1% saponin solution in 1× PBS. Cells were stained for 15 minutes at room temperature and sorted using FACS ARIA (BD Biosciences). The expression level of \(ABCB1\) protein in the cell membrane was determined by sorting gates setting threshold on both the forward and side scatter intensities. The expression level was calculated as a percentage of the mean fluorescence intensity of the cellular population, normalized to the mean fluorescence intensity of the control cell line.

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**Table 1 ABCB1 genotype of transduced cell lines**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Complementary DNA position</th>
<th>1236</th>
<th>2677</th>
<th>3435</th>
<th>Haplotype frequency(^{17})</th>
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<tr>
<td>K562/ABCB1 TTT</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td></td>
<td>T</td>
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<tr>
<td></td>
<td>(0.45)(^{24})</td>
<td></td>
<td></td>
<td></td>
<td>(0.42)(^{28})</td>
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<tr>
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<td>G</td>
<td>G</td>
<td>G</td>
<td></td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>(0.11)(^{28})</td>
<td>T</td>
<td>T</td>
<td></td>
<td>(0.58)(^{24})</td>
</tr>
<tr>
<td>K562/ABCB1 CGC</td>
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<td>G</td>
<td>G</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>(0.55)(^{44})</td>
<td></td>
<td></td>
<td>C</td>
<td>(0.42)(^{44})</td>
</tr>
<tr>
<td>K562/ABCB1 1795</td>
<td>A</td>
<td>A</td>
<td>G</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>(0.06)(^{28,26})</td>
<td></td>
<td></td>
<td>G</td>
<td>C</td>
</tr>
<tr>
<td>K562/ABCB1 CAC</td>
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<td>G</td>
<td>A</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>(nd)</td>
<td></td>
<td></td>
<td></td>
<td>G</td>
</tr>
<tr>
<td>K562/ABCB1 CAC</td>
<td>A</td>
<td>G</td>
<td>G</td>
<td></td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>(0.02)(^{28})</td>
<td></td>
<td></td>
<td></td>
<td>A</td>
</tr>
</tbody>
</table>

San Jose, CA, USA), washed twice in phosphate-buffered saline supplemented with 2% FBS, followed by labeling with 0.5 μg of secondary goat anticonfused immunoglobulin G2b conjugated with allophycocyanin/Cy7 (Abcam, Cambridge, UK). Cells were washed once more in phosphate-buffered saline before simultaneous detection of ABCB1 and EYFP on a Gallios flow cytometer (Beckman Coulter, Bromma, Sweden).

**Efficacy of TKIs and vincristine in variant ABCB1 cells**

All cell lines were investigated in terms of resistance to TKIs, using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, performed as described in a previous report. In addition, the resistance of K562, K562/ve, and K562/ABCB1 TTT to vincristine was investigated in order to verify the functionality of transduced ABCB1. Vincristine is a vinca alkaloid that is commonly used in chemotherapy and is a known ABCB1 substrate. Experiments were performed in a total of nine replicates for TKIs and six replicates for vincristine. A dose-response regression with variable slope and a top plateau constrained to 100% was fitted to each of the replicates in GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Half-maximal inhibitory concentration (IC$_{50}$) values were extracted from each dose-response regression; the mean IC$_{50}$ and 95% confidence intervals of the same were calculated and used for comparisons of drug sensitivity between cell lines with different ABCB1 variant haplotypes. Of note, the MTT assay reflects the number of living cells rather than dead cells, and thus the obtained IC$_{50}$ values reflect the sum of cell death and inhibition of proliferation caused by TKIs.

**Intracellular accumulation of TKIs in ABCB1-expressing cells**

In order to confirm that ABCB1 drug efflux was involved in the TKI resistance observed using the MTT assay, parental K562 cells, as well as K562/ve and K562/ABCB1 TTT, were incubated with TKIs until influx–efflux equilibrium was attained. Intracellular accumulation of TKIs was quantified using an ultraperformance liquid chromatography (UPLC) tandem mass-spectrometry method (see Supplementary materials for details).

**Statistical analysis**

Differences in ABCB1 protein expression and intracellular TKI accumulation between cell lines were analyzed with Student’s independent t-tests. P-values <0.05 were considered significant.

**Results**

**Characterization of ABCB1 transduced cell lines**

In this study, we used the MIY retroviral vector containing an internal ribosome entry site between the ABCB1 cDNA and the reporter gene EYFP to ensure the same protein-translation level of both genes in transduced cells. K562 cells transduced with variants of ABCB1 were sorted based on equal EYFP protein expression. Flow-cytometry analysis showed that similar EYFP fluorescence profiles had been obtained in all ABCB1 transduced cell lines (Figure 1). The EYFP MFI ranged from 24.8 for K562/ABCB1 CGC to 55.3 for K562/ABCB1 TTT. K562/ve had an extremely high EYFP expression (MFI = 1,017), which may be related to more efficient translation of this shorter transcript compared to EYFP–ABCB1 transcripts.

ABCB1 MFI, normalized to EYFP MFI, revealed that none of the variant cell lines, with the exception of K562/ABCB1 CGC, had significantly different quantities of ABCB1 expressed in the membrane than K562/ABCB1 TTT (Figure 2A). However, upon analyzing the uncorrected ABCB1 MFI, all variant cell lines had significantly lower ABCB1 membrane expression than K562/ABCB1 TTT (Figure 2B). Parental K562 cells and K562/ve did not express ABCB1 in the cell membrane, as demonstrated by the fact that these cell lines had similar mean ABCB1 MFI (MFIs = 0.45 and 0.52) as unlabeled K562 (MFI = 0.35) (Figure 3).

Exposing cells to the ABCB1 substrate vincristine and assessing cell survival using the MTT assay evaluated functionality of transduced ABCB1. It was shown that K562/ABCB1 TTT treated with vincristine had a 17-fold increased IC$_{50}$ value compared to K562/ve (Figure 4 and Table 2), indicating a functional ABCB1-transport protein.

![Figure 1](https://www.dovepress.com/figure1-expression-of-enhanced-yellow-fluorescent-protein-eyfp-all-abcb1-transduced-cell-lines-had-similar-expression-of-eyfp-as-shown-by-the-overlapping.jpg)

**Figure 1** Expression of enhanced yellow fluorescent protein (EYFP). All ABCB1 transduced cell lines had similar expression of EYFP, as shown by the overlapping fluorescent profiles. Cells transduced with empty vector (K562/ve) express high EYFP fluorescence.
ABCBI expression mainly influences the efficacy of CGP74588 and dasatinib

The cells that expressed the ABCBI TTT variant haplotype had the highest IC\textsubscript{50} value of all cell lines when treated with any of the TKIs, with the exception of imatinib, where the K562/ABCBI CAC had a slightly higher IC\textsubscript{50} but with a wide confidence interval. The fold change in IC\textsubscript{50} of ABCBI TTT, compared to the IC\textsubscript{50} of K562/ve control cells, was used for the comparative evaluation of ABCBI protection against cytotoxicity of the investigated TKIs. ABCBI expression seemed to have the largest impact on CGP74588: the IC\textsubscript{50} value was ten times higher in K562/ABCBI TTT than K562/ve. A 2.4-fold increase in IC\textsubscript{50} was seen after treating K562/ABCBI TTT with dasatinib. Compared to K562/ve, K562/ABCBI TTT had a 1.5-fold increase in IC\textsubscript{50} when treated with imatinib and a 1.4-fold increase when treated with nilotinib. Bosutinib efficacy was not affected by ABCBI expression when comparing the IC\textsubscript{50} obtained in ABCBI-expressing cells to that of K562/ve (Figure 4 and Table 2). However, the K562/ve cells appeared to be more resistant to bosutinib than parental K562, although the 95% confidence intervals of these IC\textsubscript{50} values overlapped (Figure 4).

ABCBI variants do not influence the efficacy of TKIs

No prominent effects of ABCBI variants on TKI resistance were detected in this study. The IC\textsubscript{50} values obtained when treating cells with the two drugs (dasatinib and CGP74588) most affected by ABCBI expression showed that only K562/ABCBI CGC had reduced resistance to the drugs compared to cells that expressed ABCBI TTT (Figure 4). However, the K562/ABCBI CGC cells also had the lowest EYFP and ABCBI expression compared to other cell lines (Figures 1 and 2B). All other ABCBI variant cell lines had similar resistance to dasatinib and CGP74588 as K562/ABCBI TTT. The protective effect of ABCBI expression was small using imatinib and nilotinib and nonsignificant for bosutinib. Similar to CGP74588 and dasatinib, a reduced protective effect of the ABCBI CGC variant was observed when treating the cells with imatinib. No influence of other genetic variants was detected on the efficacy of these drugs.
ABCB1 expression leads to low intracellular accumulation of dasatinib and CGP74588

As expected from the results of the MTT assays, K562/ABCB1 TTT accumulated significantly lower quantities of CGP74588 and dasatinib than did K562/ve (Figure 5). No significant influence of ABCB1 expression on accumulation of imatinib or nilotinib was detected, although K562 accumulated a smaller quantity of imatinib than K562/ve. Surprisingly, there was also a significantly lower accumulation of bosutinib in K562/ABCB1 TTT than K562/ve.

Discussion

The therapeutic efficacy of drugs, including TKIs used in the treatment of CML, that rely on the long-term effect of steady-state plasma concentrations might be particularly sensitive to alterations in such pharmacokinetic parameters as drug-transport function. But despite substantial efforts, it has proven difficult to establish an association of common ABCB1 SNPs with imatinib pharmacokinetics and outcome in vivo. We report that none of the ABCB1 haplotypes investigated in this study had any major influence on the efficacy of TKIs in K562 cells.

ABCB1 expression provided substantial protection against the cytotoxic effects of CGP74588 and dasatinib. These findings were also supported by reduced intracellular accumulation of CGP74588 and dasatinib in ABCB1-expressing cells compared to the K562/ve control cells. The influence of ABCB1 on imatinib and nilotinib efficacy was minor, albeit significant. These results were in agreement with previous findings showing that ABCB1 expression provides better protection against dasatinib than imatinib or nilotinib cytotoxicity.\(^3,4\) The observed resistance of ABCB1-expressing cells to CGP74588 is also in agreement with previous findings showing that CGP74588 is extensively transported in a multidrug-resistant cell line that expresses ABCB1.\(^6\) K562/ve cells were equally as sensitive to bosutinib cytotoxicity as ABCB1-expressing cells, but appeared to be more resistant than the parental K562 cells. However, ABCB1 expression significantly reduced the intracellular quantity of bosutinib. Based on these data, an influence of ABCB1 expression on bosutinib transport cannot be ruled out, even though this was not reported by previous investigators.\(^4\)

Plasma concentrations of TKIs measured in patients at standard dosing reach approximately 1.7 \(\mu\)M of imatinib,\(^13\) 0.4 \(\mu\)M of CGP74588,\(^13\) 2.1 \(\mu\)M of dasatinib,\(^12\) 4.2 \(\mu\)M of...
nilotinib,\textsuperscript{33} and 0.4 µM of bosutinib.\textsuperscript{34} The drug concentrations studied here were well within clinically attainable plasma concentrations of imatinib, dasatinib, and nilotinib. CGP74588 and bosutinib concentrations were higher than the mean observations in vivo. However, considering the range of individual variation in plasma concentrations of CGP74588 and bosutinib in vivo,\textsuperscript{13,34} the concentrations used in the present study can be considered as clinically attainable.

A comparison of the efficacies of TKIs and the previously validated ABCB1 substrate vincristine in K562/ABCB1 TTT revealed that imatinib, dasatinib, and nilotinib should all be regarded as rather poor ABCB1 substrates. The K562/ABCB1 TTT had only 1.4- to 2.4-fold better protection against the toxicity of these TKIs, compared to the 17-fold protection observed when using vincristine. Only CGP74588 may be regarded as a relatively good substrate of ABCB1, given its tenfold-higher IC\textsubscript{50} in K562/ABCB1 TTT compared to K562/ve. These data suggest that CGP74588 should be affected by ABCB1-transport activity to a much larger extent than imatinib in vivo. This could be important to keep in mind when interpreting association studies of ABCB1 function and therapeutic efficacy of imatinib, considering that although this metabolite is less potent than imatinib, it might accumulate in patients with low ABCB1-transport activity. We previously found an inverse association of CYP3A metabolic activity and imatinib therapeutic outcome, indicating the possible significance of imatinib metabolites in CML therapy.\textsuperscript{8}

Table 2 Influence of ABCB1 variant haplotypes on drug efficacy

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Imatinib IC\textsubscript{50} µM</th>
<th>FC</th>
<th>CGP74588 IC\textsubscript{50} nM</th>
<th>FC</th>
<th>Dasatinib IC\textsubscript{50} µM</th>
<th>FC</th>
<th>nilotinib IC\textsubscript{50} nM</th>
<th>FC</th>
<th>Bosutinib IC\textsubscript{50} µM</th>
<th>FC</th>
<th>Vincristine IC\textsubscript{50} µM</th>
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<tbody>
<tr>
<td>K562</td>
<td>0.47</td>
<td>0.9</td>
<td>1.43</td>
<td>0.7</td>
<td>1.67</td>
<td>0.8</td>
<td>23.4</td>
<td>0.9</td>
<td>1.26</td>
<td>0.6</td>
<td>18.1</td>
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<td>25.3</td>
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</tbody>
</table>

Abbreviations: IC\textsubscript{50}, half-maximal inhibitory concentration; FC, fold change compared to K562/ve cells.

Figure 5 The influence of ABCB1 expression on intracellular accumulation of tyrosine kinase inhibitors (TKIs). Parental K562, K562 transduced with empty vector (K562/ve), and ABCB1 TTT haplotype (K562/ABCB1 TTT) were incubated with TKIs, and intracellular drug accumulation was quantified. The bars represent the mean concentrations of triplicate incubations, with error bars corresponding to ± standard deviation. Differences in drug accumulation were analyzed using Student’s independent t-tests, comparing K562 and K562/ABCB1 TTT to K562/ve.

Notes: *P<0.05; **P<0.01.

Figure 6 No influence of ABCB1 haplotypes on TKI transport.
the CGC haplotype significantly reduced cellular resistance to these drugs compared to the ABCB1 TTT haplotype. However, K562/ABCB1 CGC had the lowest EYFP expression among the transduced cell lines, indicating less transcriptional activity of the vector that slightly reduced ABCB1 expression compared to other cell lines. Therefore, the reduced resistance of K562/ABCB1 CGC might be an effect of lower transcriptional activity in this particular cell line rather than of the variant haplotype. None of the other investigated ABCB1 variant haplotypes had any significant influence on either ABCB1 membrane expression or TKI efficacy. It was concluded that neither of the investigated ABCB1 haplotypes were likely to influence TKI transport. Our findings were in agreement with other in vitro studies that did not find any association of ABCB1 genotypes and transport functions. Considering the chosen method of retroviral gene transfer, it was not possible to investigate the influence of ABCB1 SNPs on pretranslational mechanisms such as messenger RNA stability. It has previously been suggested that the 3435T>C SNP affects ABCB1 messenger RNA levels, which our data could neither confirm nor refute.

We have shown that investigated ABCB1 haplotypes do not influence the efficacy of TKIs in vitro, at least on a posttranslational level; this is supported by the negative explorations in several association studies of the ABCB1 genotype and outcome of imatinib in vivo. Given our results that TKIs currently used in CML therapy (imatinib, dasatinib, and nilotinib) appear to be rather poor ABCB1 substrates compared to traditional chemotherapeutic agents, a large impact on TKI efficacy from varying ABCB1 activity is perhaps not to be expected. Our results indicate that the most common ABCB1 SNPs are not likely to predict response and resistance to TKI therapy in vivo. However, there is still a possibility that other ABCB1 SNPs or haplotypes not yet investigated have a greater impact on ABCB1-transport function than those found here. According to our findings, dasatinib and the major metabolite of imatinib – CGP74588 – would be among the compounds most likely to be affected.

All TKIs studied here are subjected to additional sources of pharmacokinetic variability. It is known that ABCB1 and CYP3A4 to some degree have overlapping substrate specificities and tissue distributions. Indeed, all TKIs used in the present study were CYP3A4 substrates and might be influenced by variations in CYP3A4 metabolic activity, but also by other sources of variation, such as degree of plasma protein binding and the variable uptake and efflux by other transport proteins. To some extent, the outcome of imatinib can be predicted by the activity of the OCT-1 uptake transporter, while dasatinib and nilotinib do not seem to be OCT-1 substrates. In addition, all TKIs except bosutinib are transported by ABCG2, which is coexpressed with ABCB1 in the liver and in the CML stem cells, potentially influencing the distribution and elimination of TKIs. Further studies on imatinib as well as the second-generation TKIs are needed in order to understand fully the rate-limiting steps of TKI pharmacokinetics, any potential additive effects of the different pharmacokinetic parameters, and their significance as predictors of response and resistance to the TKIs used in CML therapy.

**Conclusion**

ABCB1 expression substantially influenced the transport and efficacy of dasatinib and the imatinib metabolite CGP74588, which is a far better substrate for ABCB1 than the parent compound. None of the investigated ABCB1 variant haplotypes influenced the efficacy of TKIs used in CML therapy. This result indicates that any influence of ABCB1-transport activity on TKI efficacy in vivo is not limited to non-synonymous SNPs, but might involve other regulatory elements of ABCB1 activity in addition to SNPs or haplotypes not yet investigated.

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**Disclosure**

The authors report no conflicts of interest in this work.

**References**


Supplementary materials

Method for quantification of intracellular TKI accumulation

K562, K562/ve, and K562/ABCB1 TTT were incubated with TKIs, followed by quantification of intracellular drug concentrations using a method modified from a previous report. Two methods were developed: one for quantification of imatinib, CGP74588, and bosutinib where dasatinib (800 ng/mL) served as the internal standard, and another for dasatinib and nilotinib, using imatinib (80 ng/mL) as the internal standard.

Cells were seeded 400,000/mL in 5 mL of growth medium and incubated for 120 minutes (imatinib, CGP74588, or bosutinib) or 180 minutes (dasatinib or nilotinib), depending on the time to attain influx–efflux equilibrium. TKIs were used in concentrations in the same range as mean IC50 concentrations found in parental K562 cells using the MTT assay (imatinib 0.5 μM, CGP74588 2.0 μM, dasatinib 1.5 nM, nilotinib 20 nM, bosutinib 2.0 μM). Cells were separated from the medium by centrifugation (4,000 g, 5 minutes at 22°C) on 1.5 mL silicone oil. Cell pellets were disrupted by adding 200 μL of internal standard in 4% formic acid (FA) in water (v/v), with the exception of pellets incubated with dasatinib or nilotinib, which were disrupted using 100 μL of 4% FA. Lysates were centrifuged at 10,000 g for 10 minutes at 4°C, and supernatants were collected and diluted 1:10 in water before analysis, with the exception of extracts from dasatinib and nilotinib incubations, which were analyzed as concentrates.

Samples were analyzed on a chromatographic system (Acquity UPLC System; Waters, Milford, MA, USA) coupled to the tandem-quadrupole mass spectrometer Xevo TQ MS (Waters). Five microliters of samples were separated on an Acquity UPLC BEH C18 (2.1 × 50 mm, 1.7 μm) column (Waters), using a gradient mobile phase of 0.1% FA (v/v) in water (A) and 0.1% FA (v/v) in acetonitrile (B). A gradient was delivered at 0.6 mL/minute – 0.0–0.4 minutes, 80% A; 0.4–3.0 minutes, linear gradient to 20% A; 3.0–3.5 minutes, 20% A – followed by reequilibration with 80% A to 4.0 minutes. Multiple-reaction monitoring was applied, and TKIs were monitored at transitions m/z 494 > 394 for imatinib, 480 > 394 for CGP74588, 488 > 322 and 488 > 401 for dasatinib, 530 > 289 for nilotinib, and 530 > 141 for bosutinib.

Calibrators were prepared in blank lysates and extracted in accordance with the same procedure as for the samples. The calibration curve ranges were 10–3,000 ng/mL for imatinib, CGP74588 and bosutinib; 1–500 ng/mL for dasatinib; and 25–500 ng/mL for nilotinib. Quality-control samples were prepared in two concentrations in blank lysates for each calibration curve: imatinib, CGP74588, and bosutinib were analyzed at 100 ng/mL and 2,500 ng/mL; dasatinib at 8 ng/mL and 200 ng/mL; and nilotinib at 30 ng/mL and 200 ng/mL.

The calibration curves were used for calculation of the TKI concentration in samples and normalized to the internal standard. All compounds had assay imprecision <10%, with accuracy ranging from 85% to 113% at the investigated quality-control concentrations (n = 5).

Reference