

Single-nucleotide polymorphisms of ABCG2 increase the efficacy of tyrosine kinase inhibitors in the K562 chronic myeloid leukemia cell line

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Title Page

Title: Single nucleotide polymorphisms of *ABCG2* increase the efficacy of tyrosine kinase inhibitors in the K562 chronic myeloid leukemia cell line

Running head: *ABCG2* SNPs increase the efficacy of TKIs

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Abstract

Objective: The tyrosine kinase inhibitors (TKIs) used in the treatment of chronic myeloid leukemia are substrates for the efflux transport protein ABCG2. Variations in ABCG2 activity might influence pharmacokinetics and therapeutic outcome of TKIs. The role of *ABCG2* single nucleotide polymorphisms (SNPs) in TKI treatment is not clear and functional *in vitro* studies are lacking. The aim of this study was to investigate the consequences of *ABCG2* SNPs for transport and efficacy of TKIs (imatinib, N-desmethyl imatinib (CGP74588), dasatinib, nilotinib and bosutinib).

Methods: *ABCG2* SNPs 34G>A, 421C>A, 623T>C, 886G>C, 1574T>G and 1582G>A were constructed from *ABCG2* wild type cDNA and transduced to K562 cells by retroviral gene transfer. Variant ABCG2 expression in cell membranes was evaluated and the effects of *ABCG2* SNPs on transport and efficacy of TKIs were measured as the ability of ABCG2 variants to protect against TKI cytotoxicity.

Results: Wild type ABCG2 had a protective effect against the cytotoxicity of all investigated compounds except bosutinib. It was found that ABCG2 expression provided a better protection against CGP74588 than its parent compound, imatinib. *ABCG2* 421C>A, 623T>C, 886G>C and 1574T>G reduced cell membrane expression of ABCG2 and the protective effect of ABCG2 against imatinib, CGP74588, dasatinib and nilotinib cytotoxicity.

Conclusion: These findings show that the *ABCG2* SNPs 421C>A, 623T>C, 886G>C and 1574T>G increase the efficacy of investigated TKIs, indicating a reduced transport function that might influence TKI pharmacokinetics *in vivo*. Furthermore, the active imatinib metabolite CGP74588 is to a greater extent than the parent compound influenced by ABCG2 expression.

Keywords: imatinib, CGP74588, N-desmethyl imatinib, ABCG2, chronic myeloid leukemia, pharmacogenetics, single nucleotide polymorphism

Introduction

Imatinib remains the standard first-line therapy of chronic myeloid leukemia (CML) even though the second generation tyrosine kinase inhibitors (TKIs) dasatinib and nilotinib have been approved for first-line treatment. Bosutinib, the most recently TKI approved for CML treatment, can be used after failure on imatinib, dasatinib and nilotinib. The primary aim of CML therapy is to prevent the progression to advanced disease phases. In recent years it has been shown that it might also be possible to achieve curative treatment with TKIs in cases where patients with deep and durable responses to imatinib can remain in remission after cessation of therapy [1]. Despite the fact that most CML patients respond well to imatinib, a significant fraction still have to discontinue imatinib therapy due to adverse events or suboptimal response [2, 3]. In order to further improve the response rates in CML, a better understanding of the underlying mechanisms of response and resistance to the individual TKIs is needed. It has been observed that imatinib plasma concentrations have a large inter-individual variation and that higher concentrations are associated with a better response to therapy [4, 5]. These findings indicate a variability in pharmacokinetic parameters that might be of clinical significance for imatinib treatment and perhaps also for the second generation TKIs.

Imatinib, dasatinib and nilotinib are all substrates for the efflux transport protein ATP-binding cassette sub-family G member 2 (ABCG2), while bosutinib does not seem to be transported by ABCG2 [6-8]. ABCG2 is expressed in tissues involved in the absorption and elimination of drugs, including intestinal epithelium and the bile canalicular membrane of hepatocytes [9, 10]. ABCG2 transport activity might therefore affect the systemically circulating amount of TKIs that can reach target cells. Moreover, ABCG2 is expressed in normal hematopoietic stem cells and more abundantly in primitive CML progenitor cells [11, 12]. Potentially, ABCG2 expression may regulate the exposure of CML stem cells to TKIs, which could be important for the prospects of successful CML therapy cessation.

The *ABCG2* gene is polymorphic and harbors single nucleotide polymorphisms (SNPs) that might affect the *ABCG2* transport capacity. The most frequent *ABCG2* SNPs, 421C>A and 34G>A, have been studied in terms of their influence on the pharmacokinetics and therapeutic efficacy of imatinib *in vivo* [13-18]. However, the results are not consistent and the clinical significance of *ABCG2* SNPs in CML therapy is still being debated. Functional *in vitro* studies of *ABCG2* SNPs and their consequences for the transport and efficacy of imatinib and second generation TKIs might aid the interpretation of *in vivo* association studies but are currently lacking.

The aim of this study was to investigate the consequences of *ABCG2* SNPs for transport and efficacy of TKIs in K562 cells stably transduced with wild type (wt) and variant *ABCG2*. The ability of *ABCG2* variant cell lines to express *ABCG2* protein and protect against imatinib, N-desmethyl imatinib (CGP74588), dasatinib, nilotinib and bosutinib cytotoxicity were investigated. Non-synonymous SNPs with a relevant minor allele frequency of >2% were selected for the study.

Methods

Drugs and chemicals

Imatinib and its metabolite CGP74588 were provided by Novartis Pharma AG (Basel, Switzerland). Dasatinib, nilotinib and bosutinib were purchased from Selleck Chemicals (Houston, TX, USA). Stock solutions of 10mM were prepared for all drugs, stored in -20°C, aliquoted to avoid repeated freeze-thawing and were used within one year of preparation.

Imatinib and CGP74588 stock solutions were prepared in water while nilotinib, dasatinib and bosutinib were prepared in DMSO. Unless stated otherwise, all other chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The CML cell line K562 (ATCC-LGC Standards, Teddington, UK) as well as transduced K562/ABCG2 cells were kept at 37°C, 5% CO₂ in RPMI 1640 medium, supplemented with penicillin, streptomycin and 10% fetal bovine serum. Human embryonic kidney 293T cells (ATCC-LGC Standards) were grown in DMEM medium supplemented with penicillin, streptomycin and 10% heat-inactivated fetal bovine serum. All cell lines tested negative for mycoplasma infection. Cell culture reagents were obtained from Life Technologies (Paisley, UK).

***ABCG2* single nucleotide polymorphisms**

Non-synonymous single base-pair exchanges in *ABCG2* with a minor allele frequency (MAF) of >2% in any human population were selected for this study. SNPs resulting in stop codons were excluded. SNP frequencies were obtained from the NCBI dbSNP database. The selected SNPs were 34G>A, 421C>A, 623T>C, 886G>C, 1574T>G and 1582G>A. The 1574T>G MAF was retrospectively corrected to a lower frequency (1.4%) than the initial inclusion limit of >2%.

Characteristics and MAFs of the individual SNPs are summarized in Table 1. *ABCG2* wt transcript cDNA (Genbank ID: NM_004827.2) was purchased in the pCMV6-XL5 vector (OriGene, Rockville, MD, USA) and variant nucleotides of the selected SNPs were incorporated

in *ABCG2* cDNA using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA). The *ABCG2* wt cDNA sequence and also incorporation of variant nucleotides were confirmed by automated Sanger sequencing using the services of GATC Biotech AG (Konstanz, Germany).

Generation of K562 cells with expression of wild type or variant ABCG2

K562 cells were chosen for *ABCG2* transductions based on their chemosensitivity to TKIs and the fact that K562 do not have a natural expression of *ABCG2* or *ABCB1*. The pCMV6-XL5 *ABCG2* vector was cut by NotI restriction enzyme (New England Biolabs, Ipswich, MA, USA) and *ABCG2* was ligated into NotI cleaved MSCV-IRES-enhanced yellow fluorescent protein (EYFP) retroviral vector (MIY) [19] using the Rapid DNA Dephos & Ligation Kit (Roche, Basel, Switzerland). Correct orientation of the gene insert was confirmed by Sanger sequencing (GATC Biotech AG). 4 µg of MIY-*ABCG2* and 2 µg/each of the helper vectors VSVG and POL-GAG were mixed with a final concentration of 125 mM CaCl₂, followed by calcium phosphate transfection of 1x10⁶ 293T cells. Viral 293T supernatants were collected over 48 h and filtered through a 0.45 µm sterile cellulose acetate filter (Whatman GmbH, Dassel, Germany). 0.5x10⁶ K562 cells were transduced with viral supernatants using spin-infection (1.5 h in 1 200 x g, 22°C) in the presence of 4 µg/mL polybrene. Empty MIY vector was transduced to generate the vector control cell line referred to as K562/ve. EYFP⁺ cells were sorted for pure EYFP expressing populations and equal EYFP median fluorescence intensity (MFI) on a FACS Aria (BD Biosciences, Franklin Lakes, NJ, USA). Transduction efficiency measured as fraction of EYFP⁺ cells varied between experiments (4-72 %). However, since cells were sorted for EYFP MFI, any variations due to transduction efficiency between experiments were eliminated.

Cell membrane and total ABCG2 protein expression

Cell membrane expression of *ABCG2* was quantified using the PerCP-Cy5.5 conjugated mouse anti-human *ABCG2*, clone 5D3 (Biolegend, San Diego, CA, USA). While the 5D3 antibody binds to the extracellular domain of the *ABCG2* protein, the exact epitope has not been

characterized [20, 21]. All ABCG2 variants investigated in the present study are localized to the intracellular or trans membrane domains of ABCG2 [22] and consequently, do not directly alter the extracellular binding site of the 5D3 antibody.

1×10^6 cells were labeled with anti-ABCG2, washed in phosphate buffered saline and analyzed with a Gallios flow cytometer (Beckman Coulter, Bromma, Sweden) for simultaneous detection of ABCG2 and EYFP protein expression. In order to study the relation of ABCG2 protein expressed in the cell membrane as compared to the total (membrane and intracellular) amount of ABCG2 protein in cells transduced with variant ABCG2, cells were labeled according to the procedure described above, followed by fixation and permeabilization using BD Cytofix/Cytoperm (BD Biosciences) prior to a second round of anti-ABCG2 labeling. Each experiment was repeated three times.

Efficacy of TKIs in wild type and variant ABCG2 cells

The cytotoxic effect of TKI drugs on K562 cells expressing wt or variant ABCG2 was determined using the MTT assay [23]. Cells were seeded at 20 000/well in a final volume of 100 μ L in 96-well plates. Dilution series of imatinib, CGP74588, dasatinib, nilotinib and bosutinib were added and plates were incubated for 72 h at 37°C before adding 10 μ L 5mg/mL MTT. The reaction was stopped after 4 h of incubation by adding 100 μ L of 10% sodium dodecyl sulfate in 10 mM HCl and incubated overnight. Absorbance was recorded in a plate reader at 580 nm (SynergyHT, BioTek, Winooski, VT, USA). Each experiment was performed with a dilution series in triplicate and each experiment was repeated at least three times. The relative cell survival compared to cells treated with control vehicle was determined and a dose- response regression with variable slope was fitted to all replicates. IC₅₀ values and the 95% confidence intervals of the same were calculated using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA).

Quantification of intracellular drug accumulation

To confirm that ABCG2 drug efflux was the cause of observed TKI resistance, parental K562 cells as well as K562/ve and K562/ABCG2 wt were incubated with TKIs, followed by

quantification of intracellular drug concentrations using the UPLC tandem mass spectrometry method modified from a previously described method [24]. Two methods were developed, one for the simultaneous quantification of imatinib, CGP74588 and bosutinib using dasatinib (800 ng/mL) as the internal standard and another for dasatinib and nilotinib where imatinib (80ng/mL) served as the internal standard.

Cells were seeded 400 000/mL in 5 mL of growth medium and incubated for 0 up to 240 min in the presence of TKIs at IC₅₀ concentrations obtained in parental K562 cells using the MTT assay (imatinib = 0.45 μM; CGP74588 = 2.2 μM; dasatinib = 1.6 nM; nilotinib = 35 nM; bosutinib = 1.7 μM). Cells were separated from the medium by centrifugation (4 000 x g, 5 min at 22°C) on 1.5 mL silicone oil. Cell pellets were disrupted by adding 200 μL of 4% formic acid in water (v/v) containing the internal standard, except for pellets incubated with dasatinib or nilotinib, which were disrupted using 100 μL of 4% formic acid. Lysates were centrifuged at 10 000 x g for 10 min at 4°C and supernatants were collected and diluted 1:10 in water before analysis, except for extracts from dasatinib and nilotinib incubations, which were analyzed as concentrates.

The chromatographic system was an Acquity UPLC System (Waters, Milford, MA, USA) coupled to the tandem quadrupole mass spectrometer Xevo TQ MS (Waters). 5 μL of samples were injected and separated on an Acquity UPLC BEH C18 (2.1 x 50 mm, 1.7 μm) column (Waters), using a gradient mobile phase of 0.1% formic acid in water (A) and 0.1% formic acid (v/v) in acetonitrile (B). The following gradient was delivered at 0.6 mL/min: 0.0–0.4 min: 80% A, 0.4–3.0 min: linear gradient to 20% A, 3.0–3.5 min: 20% A, followed by re-equilibration with 80% A to 4.0 min. Multiple reaction monitoring was applied for highly selective and sensitive detection of TKIs monitored at transitions m/z 494 > 394 for imatinib, 480 > 394 for CGP74588, 488 > 232 and 488 > 401 for dasatinib, 530 > 289 for nilotinib and 530 > 141 for bosutinib.

Calibrators were prepared in blank lysates and extracted according to the same procedure as for the samples. Calibration curve ranges were 10 to 3 000 ng/mL for imatinib, CGP7488 and bosutinib; 1 to 500 ng/mL for dasatinib and 25 to 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 sample concentration was calculated from the respective calibration curves and normalized to the internal standard. Assay imprecision for all compounds at the investigated concentrations in quality control samples was found to be <10% with an accuracy ranging from 85 to 113% (N=5).

Statistical analysis

The difference in ABCG2 protein expression levels, as well as intracellular drug accumulation, was analyzed using Student's independent *t*-tests in IBM SPSS Statistics 19 (IBM, Armonk, NY, USA). *P*-values <0.05 were considered significant.

Results

ABCG2 variants influence the expression of *ABCG2* protein in cell membranes

ABCG2 cDNA was inserted immediately upstream of the reporter gene for *EYFP* in the MIY retroviral vector used for transductions. Since transcription of both genes was driven by the same promoter, an equal expression of the two genes was obtained. The vector also contained an internal ribosome entry site (IRES) between the *ABCG2* cDNA and *EYFP*, which ensured the translation of *ABCG2* and *EYFP* into separate proteins in transduced cells. Flow cytometry analysis showed that all *ABCG2* transduced cell lines had similar levels of vector expression visualized as overlapping *EYFP* fluorescence profiles, although variations in MFI between cell lines were observed (Fig. 1). *EYFP* MFI ranged from 14.7 for K562/*ABCG2* 421 to 46.5 for K562/*ABCG2* wt. K562/*ve* expressed extremely high amounts of *EYFP* (MFI = 1015.4), possibly due to less efficient translation of the longer transcript in vectors containing *ABCG2* cDNA.

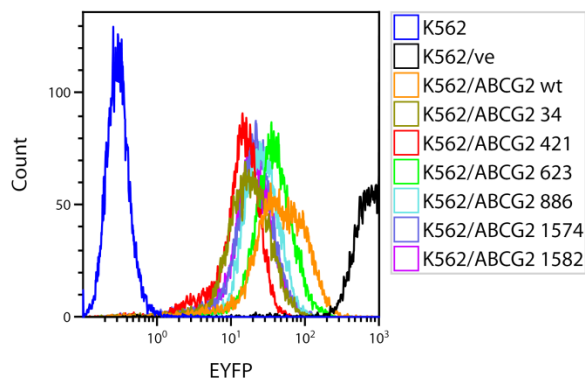


Figure 1: Expression of enhanced yellow fluorescence protein (*EYFP*) in parental K562 (K562), K562 transduced with an empty vector (K562/*ve*), and K562 transduced with wild type (wt) and variant *ABCG2*.

Surface detection of the *ABCG2* protein in the cell membrane was normalized to the *EYFP* expression to correct for differences in basal vector transcription. It was found that K562/*ABCG2* 623 had undetectable levels of *ABCG2* in the cell membrane. The *ABCG2* variants 421, 886 and 1574 had an intermediate reduction of membrane *ABCG2* protein while

ABCG2 variants in K562/ABCG2 34 and K562/ABCG2 1582 did not affect the expression of ABCG2 compared to K562/ABCG2 wt (Fig. 2A). However, uncorrected ABCG2 expression showed that K562/ABCG2 34 and K562/ABCG2 1582 had lower surface expression of ABCG2 than K562/ABCG2 wt cells, which was due to a lower transcriptional activity rather than an impact of the specific polymorphisms in these vectors (Fig. 2B). An analysis of the fraction of membrane ABCG2 protein expression out of the total ABCG2 protein level revealed that K562/ABCG2 623 had a significantly lower ratio than K562/ABCG2 wt (Figure 3). However, the absolute level of ABCG2 in the particular variant of K562/ABCG2 623 was extremely low, and the significance of an altered ratio is probably small since there is very little ABCG2 protein present at all in this cell line. All other ABCG2 variant cell lines had the same ratio (membrane: total ABCG2) as K562/ABCG2 wt.

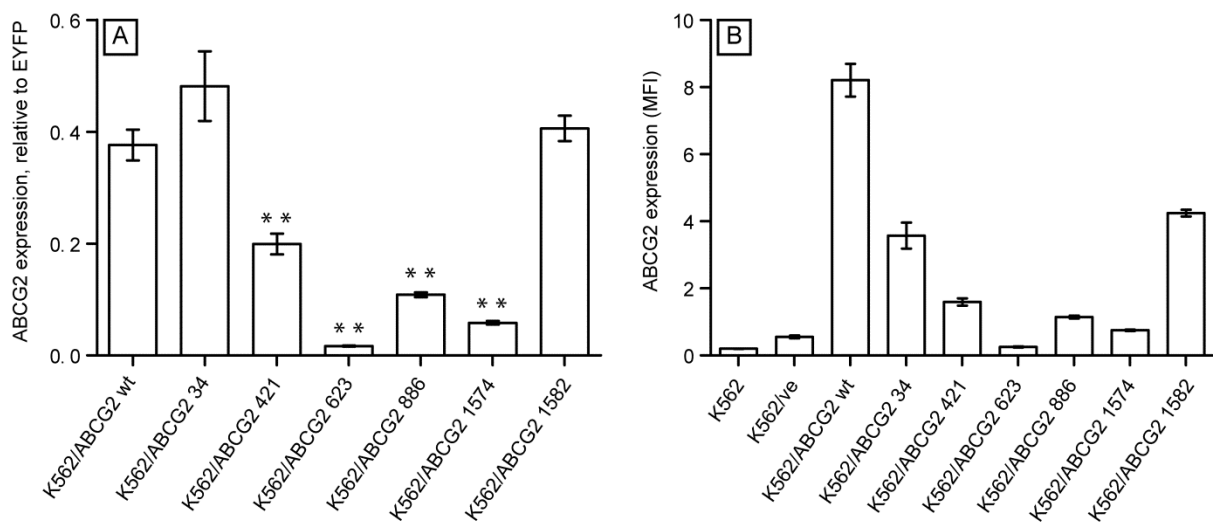


Figure 2: Expression of ABCG2 in cell membranes. Parental cells (K562), K562 transduced with empty vector (K562/ve), and K562/ABCG2 wild type (wt) and variants were analyzed in parallel for expression of ABCG2 and the reporter protein, enhanced yellow fluorescent protein (EYFP). **A)** ABCG2 expression corrected for differences in basal vector expression by normalizing ABCG2 median fluorescence intensity (MFI) to EYFP MFI. **B)** ABCG2 MFI without EYFP normalization. Bars represent the mean \pm s.e.m. of three replicates. Differences in ABCG2 expression were analyzed in **A)**, using Student's independent *t*-tests comparing all cell lines to K562/ABCG2 wt. * $P < 0.05$; ** $P < 0.01$.

Wild type ABCG2 expression has a greater influence on CGP74588 than imatinib efficacy

The expression of ABCG2 wt resulted in a significant 2–3-fold increase in resistance to imatinib, dasatinib and nilotinib in K562/ABCG2 wt cells compared to K562/ve (Fig. 4, Table 2).

However, a greater impact of ABCG2 wt expression was seen on the resistance to the imatinib metabolite CGP74588, which induced a 6.6-fold higher IC_{50} in K562/ABCG2 wt than in K562/ve. No significant difference in cell survival was found for any drug on comparing parental K562 cells and K562/ve, except in the case of bosutinib treatment. On comparing K562/ABCG2 wt and the parental K562 cells in terms of bosutinib cytotoxicity, a small (1.4-fold) increase in resistance to bosutinib was detected in K562/ABCG2 wt (K562 IC_{50} = 1.73 μ M, K562/ABCG2 wt IC_{50} = 2.49 μ M). This effect might be associated with the transduction procedure since K562/ve was more resistant than parental K562 and was also considered too small to be further investigated in cells transduced with variant ABCG2.

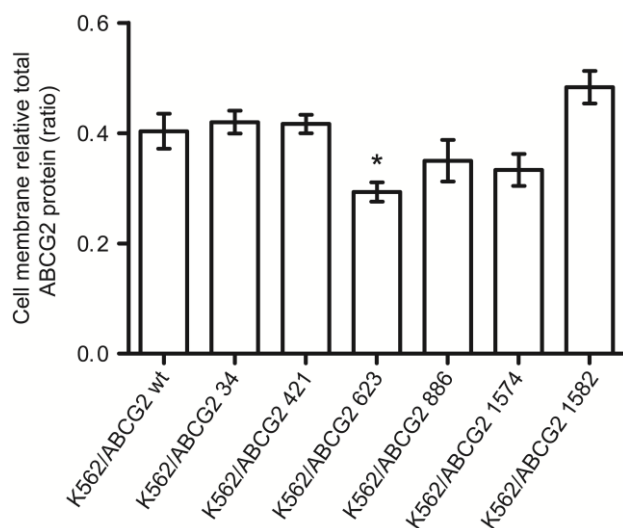


Figure 3: Cell membrane ABCG2 expression in relation to total ABCG2 protein level. Cells expressing wild type (wt) or variant ABCG2 were labeled with fluorescent anti-ABCG2 for detection of either surface or total ABCG2 protein using flow cytometry. Bars represent the mean ratio of EYFP-normalized ABCG2 membrane expression out of EYFP-normalized total ABCG2 protein level (n=3). Error bars represent standard error of the mean. Differences in ratios between cell lines were analyzed using Student's independent *t*-test comparing all cell lines to K562/ABCG2 wt. * $P < 0.05$.

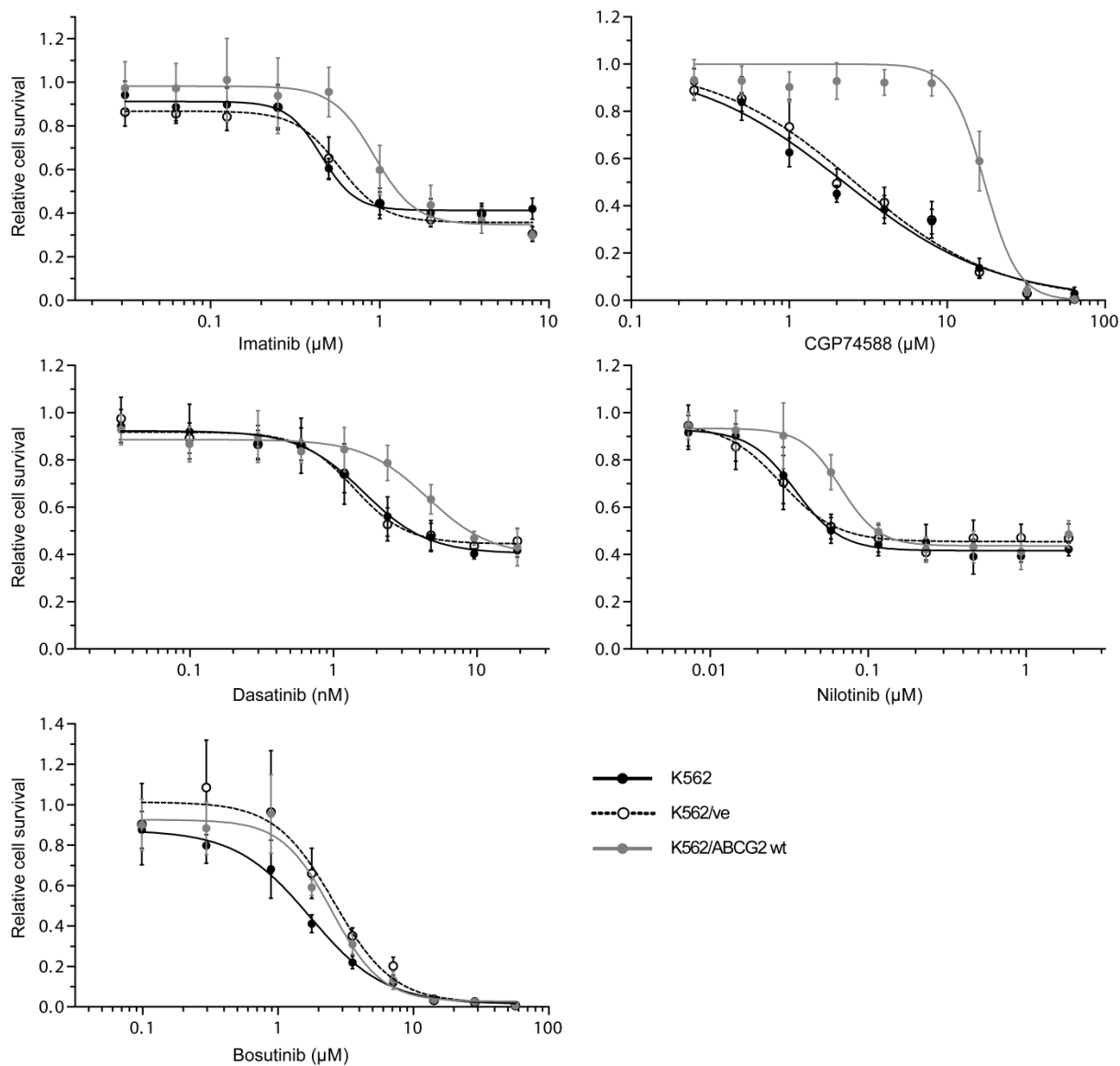


Figure 4: Influence of ABCG2 wild type (wt) expression on the cytotoxicity of tyrosine kinase inhibitors (TKIs).

Parental K562, K562 transduced with empty vector (K562/ve) and K562/ABCG2 wt were exposed to serial dilutions of TKIs for 72 h and cell survival was analyzed. The relative number of living cells compared to cells treated with control vehicle is displayed in the graphs and each sample point represents the mean \pm SD of at least nine replicates.

Variant ABCG2 influences TKI efficacy

Variant ABCG2 was shown to influence the efficacy of imatinib, CGP74588, dasatinib and nilotinib, as evaluated by the MTT assay (Fig. 5, Table 2). K562/ABCG2 34, 421, 623, 886 and

1574 showed significantly reduced resistance to all TKIs compared to K562/ABCG2 wt.

K562/ABCG2 1582 cells had reduced protection to all TKI treatments except for nilotinib. The particular nucleotide variants in K562/ABCG2 623 and 886 cells were shown to completely abolish the effect of ABCG2 expression and were not significantly different from K562/ve with respect to drug sensitivity, irrespective of drug treatment.

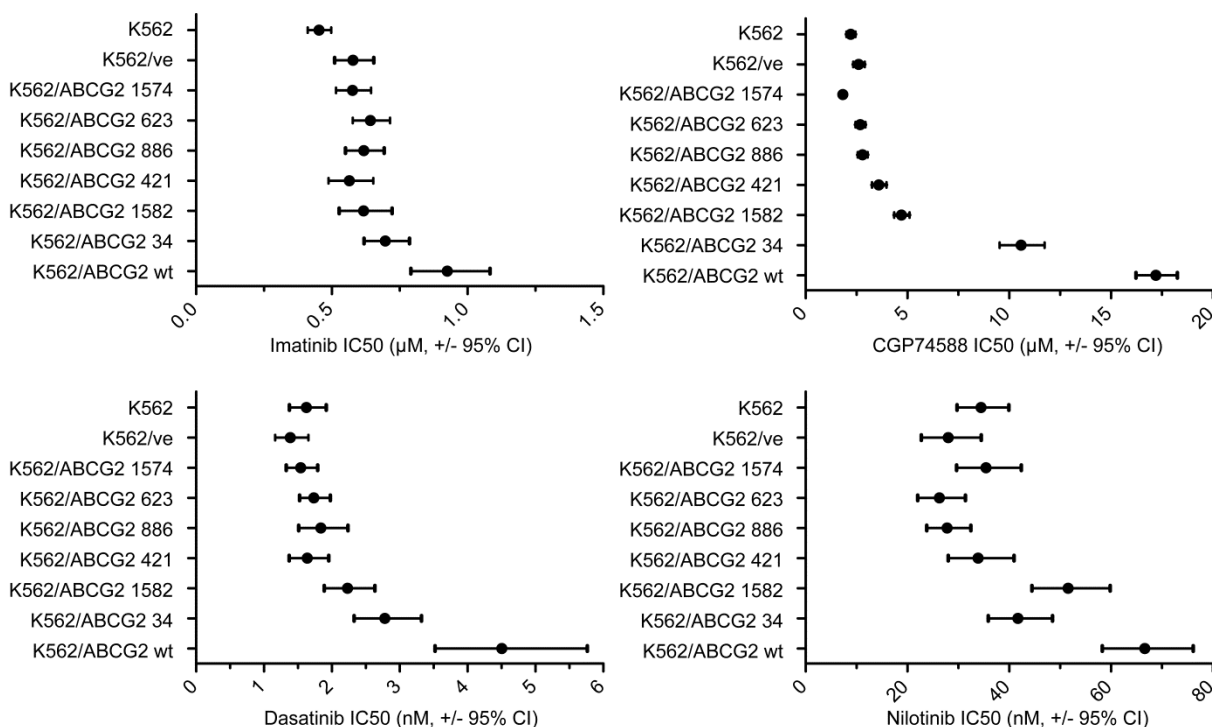


Figure 5: Influence of ABCG2 wild type (wt) and variant expression on the cytotoxicity of tyrosine kinase inhibitors (TKIs). Parental K562, K562 transduced with empty vector (K562/ve), K562/ABCG2 wt, as well as K562/ABCG2 variant cells, were exposed to serial dilutions of TKIs for 72 h and cell survival was analyzed. IC₅₀ values extracted from a dose-response regression from each drug and cell line are plotted in the figure (•) together with error bars representing \pm 95% confidence intervals (CIs), n=9.

Reduced intracellular accumulation of TKIs in cells expressing wild type ABCG2

Parental K562 cells and K562/ABCG2 wt were incubated with TKIs for 0, 30, 60, 120, 180 and 240 min, followed by quantification of intracellular accumulation of the drug. Influx-efflux

equilibrium was reached at 120 min for imatinib, CGP74588 and bosutinib, while 180 min was required for dasatinib and nilotinib (data not shown). Incubation of K562, K562/ve and K562/ABCG2 wt with TKI drugs for the defined equilibrium time points revealed that K562/ABCG2 wt cells accumulated significantly lower amounts of imatinib, CGP74588 and dasatinib than K562/ve. The same trend was observed for K562/ABCG2 wt incubated with nilotinib or bosutinib, but this was not statistically different from K562/ve. Furthermore, parental K562 accumulated significantly larger quantities of dasatinib and nilotinib than K562/ve (Fig. 6).

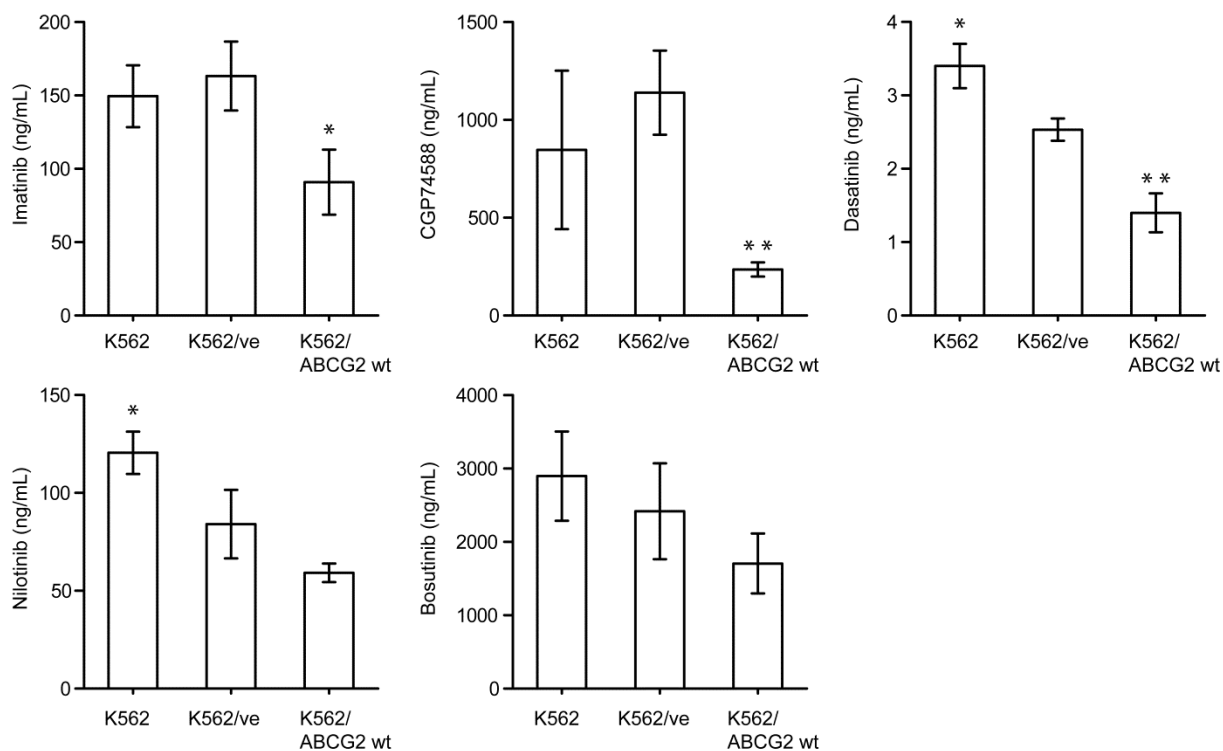


Figure 6: Influence of ABCG2 wild type (wt) expression on the intracellular accumulation of tyrosine kinase inhibitors (TKIs). Parental K562, K562 transduced with empty vector (K562/ve) and K562/ABCG2 wt were incubated with TKIs at parental K562 IC_{50} concentrations (as determined by MTT assay) and intracellular drug accumulation was quantified. Bars represent the mean concentration \pm SD of three replicates. Differences in drug accumulation were analyzed using Student's independent *t*-tests comparing K562 and K562/ABCG2 wt to K562/ve. * $P < 0.05$; ** $P < 0.01$.

Discussion

ABCG2 wt protein expression has previously been shown to affect the transport of imatinib, dasatinib and nilotinib *in vitro*, and in this study we add the finding of *ABCG2* SNPs that increase the cellular sensitivity to TKI drugs. To the best of our knowledge, this is also the first report in which it has been shown that *ABCG2* expression has a significant impact on the efficacy of the pharmacologically active imatinib metabolite CGP74588.

The main CYP3A metabolic product of imatinib, CGP74588, is known to be pharmacologically active although less potent than the parent drug *in vitro* [25]. Since this metabolite, as well as imatinib, has a large inter-individual variation in plasma concentrations [4, 26], we wanted to investigate the influence of *ABCG2* expression on CGP74588 transport and efficacy. One of the main physiological functions of ABC transporters is the protective excretion of xenobiotics. Therefore, it is likely that the hepatic metabolism of drugs might result in metabolites with a better affinity for excretion transporters such as *ABCB1* and *ABCG2*. We and others have previously demonstrated that CGP74588 is extensively transported by *ABCB1* [25, 27]. In the present study, we demonstrate that *ABCG2* wt expression reduces intracellular accumulation as well as decreases the efficacy of CGP74588. It was also noted that the efficacy of CGP74588 was influenced by *ABCG2* wt expression to a much larger extent than that of imatinib, indicating that reduced *ABCG2* transport function might influence metabolite excretion to a larger extent than excretion of the parent compound *in vivo*. Even though CGP74588 is less potent than imatinib, we speculate that individuals with a combination of low *ABCB1* and/or *ABCG2* transport capacity and high CYP3A activity might experience a clinical benefit from CGP74588.

ABCG2 wt conferred increased cell survival compared to the non-*ABCG2* expressing control cells K562/ve when treated with imatinib, dasatinib or nilotinib. In addition, drug accumulation assays showed an efflux of imatinib and dasatinib and the same trend for nilotinib in *ABCG2* wt expressing cells, supporting our and other investigators' conclusion that these drugs are substrates for *ABCG2*. The relative resistance of K562/*ABCG2* wt, as compared to K562/ve treated with

imatinib, dasatinib and nilotinib, was similar to previously reported findings [6], but somewhat lower than those found in other studies [7], which might be explained by differences in ABCG2 expression level depending on method used for ABCG2 gene transfer. No protective effect of ABCG2 against bosutinib cytotoxicity was observed when K562/ABCG2 wt and the K562/ve control cells were compared. This finding was abrogated by a significant difference in the efficacy of bosutinib on parental K562 cells compared to K562/ve. However, bosutinib was not accumulated to a lesser degree in K562/ve than in parental cells. In the light of these data, a firm conclusion cannot be drawn, but the influence of ABCG2 expression on bosutinib transport and efficacy is likely to be small, which conclusion is supported by other investigators [7].

Consequently, we did not investigate the influence of ABCG2 SNPs on bosutinib efficacy.

K562/ABCG2 623 cells had undetectable levels of ABCG2 protein expression and MTT assays revealed that, irrespective of drug treatment, this variant cell line was equally sensitive to treatment as K562/ve, completely abolishing the protective effect of ABCG2 expression.

Although the 623T>C SNP has to our knowledge not previously been investigated *in vivo*, our data are supported by the previous finding that 623C markedly reduces ABCG2 expression in insect Sf9 as well as human cell membranes [28, 29], probably due to increased ABCG2 susceptibility to proteasomal degradation [29].

The ABCG2 421C>A variant resulted in reduced cellular resistance to all investigated drugs compared to ABCG2 wt. K562/ABCG2 421 showed reduced ABCG2 expression in the cell membrane, which is consistent with previous findings where it was shown that ABCG2 421C>A has impaired functionality and reduced membrane incorporation due to increased susceptibility to proteasomal degradation [30-32]. It was also shown that this particular ABCG2 variant has reduced capacity for imatinib efflux when expressed in human embryonic kidney cell lines [15]. The 421C allele was associated with lower chances of achieving cytogenetic and molecular responses to imatinib in a large-scale study on CML patients [16]. This is in line with our results of a reduced transport activity of the 421A allele that might lead to increased drug accumulation and potentially better response rates *in vivo* compared to the 421C allele.

The 34G>A and 1582G>A SNPs resulted in reduced protective effects to TKI cytotoxicity but did not affect the levels of ABCG2 detected in cell membranes. The impact of these SNPs on TKI efficacy might have been due to the lower transcriptional activity seen in K562/ABCG2 34 and 1582 cells rather than the specific SNPs. In a previous study, *ABCG2* 34G>A induced higher resistance to the camptothecin analog SN-38 compared to wild type ABCG2 expressed in human cell lines [33]. We could not identify any increased resistance to TKIs in K562/ABCG2 34 cells. Furthermore, the 34G allele has been associated to less frequent achievement of complete cytogenetic responses to imatinib treatment. However, the 34G>A SNP was found to be in linkage disequilibrium with the 421C>A SNP [16]. Based on our findings, the association of 34G>A with outcome of imatinib treatment might be due to the effects of the 421C>A SNP.

The rare alleles of *ABCG2* 886G>C and 1574T>G generated low ABCG2 expression on the cell surface and significantly reduced protective effects against TKI treatment compared to cells expressing ABCG2 wt. To our knowledge, neither 886G>C nor 1574T>G has been functionally investigated previously. 886G>C results in a substitution of Asp296His in one of the intracellular loops of the ABCG2 protein structure which does not seem to be involved in any particularly important functions of the protein such as dimerization, substrate binding or membrane incorporation [22]. Nonetheless, the substitution from a negative to positive charge of the amino acid side chain, as well as steric conformation changes, might lead to the impaired functionality of *ABCG2* 886G>C detected here. The 1574T>G SNP results in the substitution of the hydrophobic leucine with the positively charged arginine on position 525 in the trans-membrane domain of ABCG2, which leads to speculation on reduced capability of successful incorporation in the non-polar cell membrane for this ABCG2 variant.

The activity of other transport proteins such as the organic cation transporter-1 (OCT-1), responsible for the cellular uptake of imatinib, have previously been shown to influence the outcome of CML therapy [34, 35]. These findings indicate that variable transport protein activity might be important for the prediction of TKI pharmacokinetics and therapeutic outcome of these drugs. So far, no consensus has been reached with respect to *ABCG2* genotype from *in vivo*

association studies and the present work provides basic knowledge of the effects of *ABCG2* SNPs applied specifically to the situation of TKIs for use in CML treatment. However, the SNPs with reduced function (623T>C, 886G>C, 1574T>G and 1582G>A) observed here are rare with minor allele frequencies of <4%. Nonetheless, their effects on transport function were substantial and should be considered in future studies of large patient cohorts. Perhaps most importantly, the 623T>C and 886G>C SNPs should be considered in the context of *ABCG2* transport since they proved to have a detrimental effect on *ABCG2* functionality. However, the overall significance of *ABCG2* transport in TKI treatment of CML will have to be confirmed in future clinical studies.

In conclusion, *ABCG2* wt expression provided protective effects against imatinib, CGP74588, dasatinib and nilotinib cytotoxicity. *ABCG2* also provided a better protection against CGP74588 cytotoxicity than against imatinib itself. The *ABCG2* SNPs 421, 623, 886 and 1574 reduced the protective effects of *ABCG2* against all investigated TKIs, indicating a reduced *ABCG2* transport function for these drugs. Future investigations of *ABCG2* genotype and its influence on TKI pharmacokinetics and therapeutic outcome should be pursued.

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Tables

Table 1: ABCG2 single nucleotide polymorphisms

rs#	Nucleotide substitution	Amino acid substitution	Minor allele frequency ^a
rs2231137	34G>A	Val12Met	0.29 (AS) ^b
rs2231142	421C>A	Gln141Lys	0.34 (AS) ^c
rs1061018	623T>C	Phe208Ser	0.04 (AS) ^d
rs41282401	886G>C	Asp296His	0.02 (CA) ^e
rs58818712	1574T>G	Leu525Arg	0.014 (mixed) ^f
rs45605536	1582G>A	Ala528Thr	0.02 (CA) ^g

AS, asian population; CA, Caucasian population

^aMinor allele frequencies were obtained from NCBI dbSNP with submission identification numbers (ss#): ^b48428447, ^c76894509, ^d12675225, ^e70352856, ^f86245712, ^g70352893; accessed 02 Feb 2013

Table 2: Influence of ABCG2 wild type (wt) and variant expression on the cytotoxicity of tyrosine kinase inhibitors.

Cell line	Imatinib		CGP74588		Dasatinib		Nilotinib		Bosutinib	
	IC ₅₀ , μ M (95% CI)	FC	IC ₅₀ , μ M (95% CI)	FC	IC ₅₀ , nM (95% CI)	FC	IC ₅₀ , nM (95% CI)	FC	IC ₅₀ , μ M (95% CI)	FC
K562	0.45 (0.41-0.50)	0.8	2.22 (2.01-2.45)	0.9	1.62 (1.38-1.92)	1.2	34.5 (29.7-40.0)	1.2	1.73 (1.51-1.97)	0.7
K562/ve	0.58 (0.51-0.66)	-	2.60 (2.34-2.90)	-	1.39 (1.17-1.66)	-	28.0 (22.7-34.5)	-	2.60 (2.11-3.20)	-
K562/ABCG2 wt	0.93 (0.79-1.08)	1.6	17.2 (16.2-18.3)	6.6	4.51 (3.52-5.77)	3.3	66.6 (58.3-76.2)	2.4	2.49 (2.19-2.83)	1.0
K562/ABCG2 34	0.70 (0.62-0.79)	1.2	10.6 (9.53-11.7)	4.1	2.78 (2.33-3.32)	2.0	41.7 (35.9-48.5)	1.5	n.d.	
K562/ABCG2 421	0.56 (0.49-0.65)	1.0	3.60 (3.26-3.97)	1.4	1.64 (1.37-1.95)	1.2	33.9 (28.0-41.0)	1.2	n.d.	
K562/ABCG2 623	0.64 (0.58-0.72)	1.1	2.68 (2.45-2.94)	1.0	1.74 (1.53-1.98)	1.3	26.3 (22.0-31.4)	0.9	n.d.	
K562/ABCG2 886	0.62 (0.55-0.69)	1.1	2.80 (2.56-3.05)	1.1	1.84 (1.51-2.24)	1.3	27.8 (23.8-32.5)	1.0	n.d.	
K562/ABCG2 1574	0.58 (0.52-0.65)	1.0	1.83 (1.67-1.99)	0.7	1.54 (1.33-1.79)	1.1	35.5 (29.7-42.4)	1.3	n.d.	
K562/ABCG2 1582	0.62 (0.53-0.62)	1.1	4.71 (4.35-5.11)	1.8	2.23 (1.89-2.64)	1.6	51.6 (44.5-59.9)	1.8	n.d.	

FC, IC₅₀ fold change compared to IC₅₀ of K562/ve; n.d., not determined; CI, confidence interval