Influence of CYP3A enzymes and ABC transporters on the activity of tyrosine kinase inhibitors in chronic myeloid leukemia

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Anybody who has been seriously engaged in scientific work of any kind realizes that over the entrance to the gates of the temple of science are written the words: 'Ye must have faith.'

Max Planck

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PAPERS IN THE THESIS

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I. **Skoglund K.**, Boiso Moreno S., Baytar M., Jönsson J.I. and Gréen H. ABCB1 haplotypes do not influence transport or efficacy of tyrosine kinase inhibitors in vitro. *Pharmgenomics Pers. Med.* 6: 63-72 (2013).

II. **Skoglund K.**, Boiso Moreno S., Jönsson J.I., Vikingsson S., Carlsson B. and Gréen H. Influence of variant ABCG2 on tyrosine kinase inhibitor transport and efficacy in the K562 chronic myeloid leukemia cell line. *Submitted manuscript.*

III. Gréen H., **Skoglund K.**, Rommel F., Mirghani R.A. and Lotfi K. CYP3A activity influences imatinib response in patients with chronic myeloid leukemia: a pilot study on in vivo CYP3A activity. *Eur. J. Clin. Pharmacol.* 66: 383-86 (2010).

IV. **Skoglund K.**, Richter J., Olsson-Strömberg U., Bergquist J, Aluthgedara W., Ubhayasekera K., Vikingsson S., Svedberg A., Söderlund S, Sandstedt A., Johnsson A., Aagesen J., Alsenhed J., Hägg S., Peterson C., Lotfi K. and Gréen H. In vivo CYP3A activity and pharmacokinetics of imatinib in relation to therapeutic outcome in chronic myeloid leukemia. *Manuscript.*

ABBREVIATIONS

3S-Q: 3S-3-hydroxy quinine

ABCB1: ATP-binding cassette family B member 1

ABCG2: ATP-binding cassette family G member 2

BCR-ABL1: breakpoint cluster region-c-abl oncogene 1

CML: chronic myeloid leukemia

CYP: cytochrome P450

ELN: European LeukemiaNet

EYFP: enhanced yellow fluorescence protein

IC_{50}: half maximal inhibitory concentration

IRES: internal ribosome entry site

MFI: median fluorescence intensity

MIY: MSCV-IRES-EYFP

MSCV: murine stem cell virus

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

OCT1: organic cation transporter 1

Ph chromosome: Philadelphia chromosome

SNP: single nucleotide polymorphism

TKI: tyrosine kinase inhibitor

QC: quality control

ABSTRACT

The introduction of imatinib, a tyrosine kinase inhibitor (TKI), in the treatment of chronic myeloid leukemia (CML) was a major break-through and the first drug that was successfully designed to target the specific mechanism of a malignant disease. Imatinib still remains as the standard treatment of newly diagnosed CML patients although a second generation of TKIs has also been approved for first-line CML treatment.

Most patients achieve a good therapeutic effect with imatinib, but some patients are resistant to the drug and are at greater risk of disease progression. In order to further improve CML treatment, a better understanding of the underlying reasons for variable responses to imatinib and the second generation TKIs is important.

A number of potential determinants of imatinib response have been suggested, including inter-individual variability in pharmacokinetics. Variations in drug metabolism and cellular transport might contribute to the large variations observed in imatinib plasma concentrations and might, therefore, affect the amount of drug that reaches target CML cells. Imatinib is primarily metabolized by the CYP3A hepatic enzymes that are known to be highly variable in activity between different individuals. Imatinib is also a substrate of the ABCB1 and ABCG2 efflux pumps

that potentially regulate the elimination of imatinib from the plasma. The *ABCB1* and *ABCG2* genes are polymorphic and contain single nucleotide polymorphisms (SNPs) that might influence the transport capacity of these proteins. The primary aim of the present thesis was to investigate the influence of CYP3A metabolic activity and cellular transport mediated by genetic variants of *ABCB1* and *ABCG2* on the response to imatinib and the second generation TKIs used for CML therapy.

In vivo CYP3A activity and plasma concentrations of imatinib and its pharmacologically active metabolite CGP74588 were analyzed in CML patients treated with imatinib. CYP3A phenotypes were correlated to plasma concentrations and imatinib outcome 12 months after initiation of treatment. The influence of ABC transport on TKI efficacy was evaluated *in vitro* by the transduction of genetic variants of *ABCB1* and *ABCG2* into the CML cell line K562. Functionality of the transport proteins was evaluated by measuring protein expression levels on the cell surface, the intracellular accumulation of TKIs, and the ability of ABCB1 and ABCG2 variants to protect cells from TKI cytotoxicity.

We found that CYP3A metabolic activity does not influence the drug plasma concentrations or the therapeutic outcome of imatinib in CML patients. These findings indicate that even though imatinib is primarily metabolized by CYP3A this metabolic activity is not the rate-limiting step in imatinib elimination. CYP3A activity, therefore, is not a suitable predictive marker of imatinib outcome. The *in vitro* studies revealed that the ABCB1 variants investigated here do not alter the transport of imatinib, CGP74588, dasatinib, or nilotinib. In contrast, the *ABCG2* SNPs 421C>A, 623T>C, 886G>C, and 1574T>G significantly impaired the cellular efflux of imatinib, CGP74588, dasatinib, and nilotinib and could possibly influence transport of these TKIs *in vivo*. It was also found that CGP74588 is by far a better substrate than imatinib for both ABCB1 and ABCG2, and this might have implications in patients with high levels of CYP3A activity. In conclusion, our studies show that *ABCG2* SNPs might be important for prediction of imatinib outcome *in vivo*. On the other hand, CYP3A activity and the *ABCB1* SNPs investigated in this study are not likely to be useful as predictors of imatinib outcome.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Kronisk myeloisk leukemi (KML) är en form av blodcancer som drabbar cirka 100 personer per år i Sverige. Idag finns det tre olika läkemedel som är aktuella vid behandling av nyupptäckt KML: imatinib, dasatinib och nilotinib. Alla tre tillhör läkemedelsgruppen tyrosinkinas-inhibitorer (TKI'er) och imatinib är idag första-handsvalet vid KML-behandling. Merparten av alla KML-patienter får en tillfredsställande effekt av imatinib men det är fortfarande en betydande andel som blir resistenta, vilket innebär en ökad risk för utveckling av mer avancerade sjukdomsstadier som kan vara svåra att behandla. För att förbättra behandlingen av KML behövs en ökad förståelse för vilka faktorer som påverkar behandlingseffekt och resistensutveckling vid behandling med imatinib och övriga TKI'er.

Liksom det finns stora variationer i människors ögonfärg och kroppslängd så finns det lika stora, om inte större, variationer i mindre synliga egenskaper så som förmågan att omsätta och fördela läkemedel i kroppen. CYP3A-enzymerna som finns i levern ansvarar för nedbrytningen av imatinib och dessa enzymer varierar kraftigt i aktivitet mellan individer. Denna variation kan resultera i att läkemedlet förbrukas olika snabbt hos olika individer och kan då ge upphov till en allt för hög behandlingseffekt (patienten får biverkningar) eller en alltför låg behandlingseffekt (patienten blir resistent mot behandlingen). Imatinib och dess restprodukter

transporteras ut ur kroppen via levern till gallan och därefter ut i tarmen. Denna process kräver dock att transport-proteinerna ABCB1 och ABCG2 i levern pumpar ut läkemedlen till gallan. Även transportkapaciteten för ABCB1 och ABCG2 varierar mellan individer och verkar till viss del bero på den enskilda individens genetiska uppsättning. Målet med denna avhandling har varit att studera variationer i aktiviteten av CYP3A, ABCB1 och ABCG2 och huruvida dessa skulle kunna bidra till att förutsäga behandlingseffekten av imatinib och de övriga TKI-läkemedlen vid KML.

För att undersöka effekten av genetiska variationer i ABCB1 och ABCG2 studerades celler med olika genetiska varianter av transportproteinerna och hur väl dessa transporterade TKI'er ut från cellen. Resultaten visade att genetiska varianter i ABCG2 påverkar utflödet av läkemedlen från cellen och skulle därmed även kunna påverka den mängd läkemedel som finns kvar i kroppen efter intag av TKI-läkemedel. Ingen av de studerade genetiska varianterna av ABCB1 hade någon betydelse för transporten av TKI'er. CYP3A-enzymernas aktivitet mättes i KML-patienter som behandlades med imatinib. Därefter jämfördes CYP3A-aktivitet med vilken mäng läkemedel som fanns i blodet samt vilken behandlingseffekt patienterna fick av imatinib. Resultaten från dessa undersökningar visade att CYP3A-enzymernas aktivitet troligen inte påverkar mängden av imatinib som finns i cirkulationen och inte heller vilken effekt patienterna får av behandlingen.

Sammanfattningsvis har vi visat att genetiska variationer i transportproteinet ABCG2 har betydelse för utflödet av TKI'er från celler, vilket kan vara betydelsefullt för att förutsäga behandlingseffekt av dessa läkemedel. Vi har dessutom funnit indikationer som tyder på att aktiviteten av CYP3A-enzymerna samt utvalda genetiska variationer i ABCB1 troligen inte kommer att kunna användas för att förutsäga effekten av TKI-behandling vid KML.

INTRODUCTION

Chronic myeloid leukemia

Chronic myeloid leukemia (CML) is a type of blood cancer that arises in hematopoietic stem cells (*Huntly et al., 2004, Jamieson et al., 2004*) and is characterized by an excessive formation of myeloid blood cells. Every year, 1–2 persons per 100,000 individuals are diagnosed with CML (*Howlader et al., 2013*), and most diagnoses are made when the individual is in the initial, chronic phase of the disease. In contrast to cells in acute myeloid leukemia, chronic phase CML cells are well differentiated and CML is often asymptomatic at diagnosis. However, if left untreated CML is inexorably fatal due to the fact that within 3–5 years the chronic phase progresses into an accelerated phase leading to a blast crisis (*Faderl et al., 1999*). In the accelerated phase and during blast crisis, proper myeloid cell differentiation is gradually arrested and immature blast cells accumulate in the circulation (*Sawyers, 1999*). The leukemic clone eventually completely represses normal hematopoiesis, and vital functions in the body cannot be maintained.

CML can be successfully treated in most cases due to important scientific discoveries made over the last 50 years. In the 1960s, it was found that blood cells

from CML patients consistently had one abnormally short chromosome (*Nowell et al., 1960*). This shorter chromosome was called the Philadelphia (Ph) chromosome, referring to the city of discovery, and was not identified in other types of leukemia. The genetic explanation behind the Ph chromosome was later identified as a reciprocal translocation between the long arms of chromosomes 9 and 22 (*Rowley, 1973*). As gene technology improved, it was found that the re-arrangement of genes during the translocation results in a shorter chromosome 22 in which the *breakpoint cluster region* (*BCR*) gene from chromosome 9 is positioned next to the *c-abl oncogene 1* (*ABL1*) gene on chromosome 22 (Figure 1). *BCR* and *ABL1* are jointly transcribed and translated into the fusion protein BCR-ABL1, and this fusion protein drives leukemogenesis in CML (*Druker, 2008*).
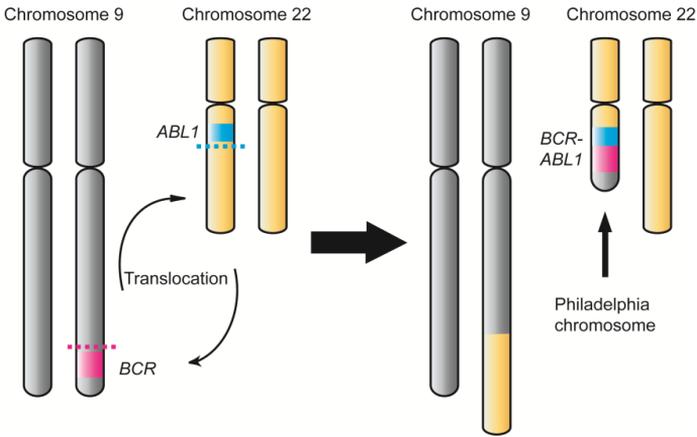
Figure 1: A translocation between chromosomes 9 and 22 causes the formation of the Philadelphia chromosome. The genetic rearrangement results in the positioning of the two genes *BCR* and *ABL1* next to each other. The fused transcript of the two genes translates into the BCR-ABL1 fusion protein, and this fusion protein is essential for CML leukemogenesis.

For many years it was uncertain whether BCR-ABL1 was a causative agent or simply an associated result of leukemogenesis in CML. The final evidence came in 1990 when it was shown that *BCR-ABL1* as a single oncogenic trait caused leukemia in animal models (*Daley et al., 1990, Heisterkamp et al., 1990*). The leukemogenic properties of BCR-ABL1 originate from the constitutive tyrosine kinase activity of the ABL1-encoded part of the protein in combination with a region in the BCR moiety that facilitates dimerization of BCR-ABL1. Dimerized BCR-ABL1 undergoes autophosphorylation at tyrosine residues that promote the

recruitment and activation of the intracellular signaling protein complex of growth factor receptor-bound protein 2 (GRB2), GRB2-associated binding protein 2 (GAB2), and son-of-sevenless (SOS). The activated GRB2/GAB2/SOS complex in turn activates several downstream signaling cascades, including the RAS/mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription 5 (STAT5), and phosphatidylinositol 3-kinase (PI3K)/AKT pathways (*O'Hare et al., 2011, Quintas-Cardama et al., 2009a*). Collectively, these pathways influence genetic transcription so that uncontrolled cell survival and proliferation is promoted in the CML cell clone.

CML treatment

The tyrosine kinase inhibitor revolution

Until the late 1990s, the most efficacious CML therapy consisted of interferon-α in combination with cytarabine. This therapeutic strategy delayed progression into blast crisis and provided a 5-year survival for 68% of CML patients (*Baccarani et al., 2002*). However, this treatment was associated with severe toxicities and less than 30% of patients achieved sufficient targeting of CML cells in the bone marrow that is predictive of long-term survival (*Lindauer et al., 2001*). Allogeneic hematopoietic stem cell transplantation was a therapeutic alternative for CML and still is the only known curative treatment. However, post-transplantation morbidity and mortality is high and the successful outcome depends on a number of risk factors including the patient's age and the availability of an HLA-matching donor (*Gratwohl et al., 1998*).

The identification of BCR-ABL1 as the cause of CML enabled the development of novel therapeutic approaches aiming at the inhibition of the tyrosine kinase activity of BCR-ABL1. Because the BCR-ABL1 fusion is not expressed in normal cells, it was predicted to be an ideal drug target because normal cells would be untouched by a selective BCR-ABL1 inhibitor. In 1992, tyrphostin compounds were shown to have inhibitory effects on BCR-ABL1 *in vitro* implying that it might be possible to eventually identify a compound with the right properties for use as a drug against CML (*Anafi et al., 1992, Levitzki, 1992*). The tyrphostins were not developed for clinical use, but further screening of chemical libraries and optimization of molecular structures lead to the discovery of a 2-phenyl-aminopyrimidine

compound that was called CGP57148 or STI571, currently known as imatinib (Glivec®, Gleevec®) (*Buchdunger et al., 1996, Druker et al., 1996*). Imatinib binds to and blocks the ATP-binding pocket of the BCR-ABL1 protein thereby preventing access of ATP that is essential for tyrosine kinase activity (*Manley et al., 2002, Schindler et al., 2000*). Consequently, imatinib shuts down all downstream signaling from BCR-ABL1 and specifically inhibits the survival of CML cells. Imatinib was approved by the regulatory agencies in the United States and Europe for treatment of CML in 2001, and it was the first example of a molecule that was successfully engineered to target a specific mechanism of a malignant disease. Imatinib significantly improved the treatment of CML by providing a means for suppressing the specific cause of the disease and has resulted in an 83%–89% 5-year overall survival rate (*de Lavallade et al., 2008, Druker et al., 2006*).

Monitoring of response

In contrast to many other malignant diseases, the minimal residual disease in CML can be easily monitored due to the fact that all malignant cells carry the tumor-specific marker of the Ph chromosome and express *BCR-ABL1* mRNA.

The evaluation of cytogenetic response to therapy is based on traditional karyotyping using chromosome-banding techniques on bone marrow aspirates. At least 20 mononuclear cells in metaphase are analyzed for the presence of the Ph chromosome, and the number and fraction of Ph$^+$ metaphases out of the 20 analyzed are determined (*Baccarani et al., 2013*). The cytogenetic response level has been standardized by the European LeukemiaNet (ELN), and 1%–35% Ph$^+$ metaphases defines a partial cytogenetic response. The best achievable response level is the absence of Ph$^+$ cells, and this corresponds to a complete cytogenetic response (*Baccarani et al., 2009*).

Molecular response is based on the quantification of *BCR-ABL1* transcripts by a *BCR-ABL1*-specific real-time polymerase chain reaction (PCR) on RNA isolated from whole blood. The molecular response is expressed as the percentage of *BCR-ABL1* transcripts compared to transcripts of a housekeeping gene. The basis for the interpretation of molecular response was provided in the International Randomized Study of Interferon vs. STI571 (IRIS). In the IRIS study, molecular response was defined as the reduction of *BCR-ABL1* transcripts on a logarithmic

scale compared to a standardized level that represented the median level of *BCR-ABL1* transcripts in untreated patients at the time of diagnosis (*Hughes et al., 2003*). The baseline used in the IRIS study was set to 100%, and a 3-log reduction was defined as a major molecular response and this has subsequently been assigned the level of *BCR-ABL1* <0.1% on an International Scale (*Muller et al., 2009*). The International Scale for molecular response is currently being implementing around the globe in order to harmonize test results from different laboratory facilities.

Some patients achieve deep levels of molecular response where *BCR-ABL1* transcripts are no longer detectable. This response level was previously defined as a complete molecular response (*Baccarani et al., 2006, Baccarani et al., 2009*). The absence of transcripts, however, is a matter of assay sensitivity, and the term complete molecular response was recently replaced by terms defining the log-reduction on the International Scale. For example, molecular responses (MRs) of *BCR-ABL1* <0.01% and *BCR-ABL1* <0.001% correspond to $MR^{4.0}$ and $MR^{5.0}$, respectively (*Baccarani et al., 2013, Cross et al., 2012*). The sensitivity of the PCR-based technology for the monitoring of molecular response by far exceeds the karyotyping method used for evaluation of cytogenetic response. Due to the higher methodological sensitivity, *BCR-ABL1* transcript levels continue to drop even after a complete cytogenetic response has been established (*Hughes et al., 2003*).

The achievement of specific levels of cytogenetic and molecular responses at specific time points after the start of treatment correlate with progression-free and overall survival of CML patients. Based on the most recent findings, an optimal response after 12 months of treatment is defined as a major molecular response (*Baccarani et al., 2013*), which is a sharpening of the previous guidelines defining an optimal response as only a complete cytogenetic response (*Baccarani et al., 2009*). Conversely, a failure after 12 months of treatment corresponds to *BCR-ABL1* >1% and/or less than a complete cytogenetic response (*Baccarani et al., 2013*). In general, it can be concluded that the faster and deeper cytogenetic and molecular responses the better the prognosis for the patient.

CML treatment today

Although imatinib is a superior therapeutic alternative compared to all previously investigated options, there is still room for improvements and the goals are set

higher for the CML therapy of today. There are two major challenges in the current treatment of CML. The first is to keep all patients stable in the chronic phase and to prevent progression into blast crisis. Despite the good prognosis for most CML patients on first-line imatinib treatment, 7%–9% progress into accelerated phase or blast crisis (*de Lavallade et al., 2008, Hochhaus et al., 2009*) and in total 16% stop imatinib due to unsatisfactory therapeutic effects (*Deininger et al., 2009*). An additional 10% of patients discontinue imatinib therapy due to adverse events (*Kantarjian et al., 2011*).

The second challenge is to obtain a cure for CML using tyrosine kinase inhibitor (TKI) treatment. Imatinib was initially predicted to be a life-long treatment in order to maintain the suppression of CML cell proliferation. However, in recent years it has been shown that a fraction of patients who achieve a sustained complete molecular response on imatinib can remain in remission after cessation of therapy (*Mahon et al., 2010*). Cessation of therapy is currently only an option for a few patients because only approximately 10% of patients on imatinib achieve a complete molecular response defined as $MR^{5.0}$ (*Cross et al., 2012*), which is the level suggested for successful discontinuation of therapy (*Mahon et al., 2010*).

In order to expand the therapeutic tools available to treat patients with imatinib intolerance or resistance, a second generation of TKIs has been developed, including the drugs dasatinib (Sprycel®), nilotinib (Tasigna®), and bosutinib (Bosulif®). The second generation TKIs are structurally related to imatinib (Figure 2) and have similar mechanisms of action as imatinib through their binding and blocking of the ATP-binding site of BCR-ABL1 (*Levinson et al., 2012, Vajpai et al., 2008*). Dasatinib and nilotinib have earned approval for first-line treatment because they induce more rapid and deeper response levels than imatinib while maintaining an adequate safety profile (*Kantarjian et al., 2010, Kantarjian et al., 2011*). However, imatinib still remains the standard choice for first-line treatment due to the longer follow-up studies on this TKI. The comparative clinical trial with first-line bosutinib versus imatinib showed an unfavorable safety profile for bosutinib with higher rates of adverse events while inducing similar rates of complete cytogenetic response as imatinib after 12 months of treatment (*Cortes et al., 2012b*). Currently, bosutinib is only approved for second-line treatment after failure on imatinib, dasatinib, and nilotinib.
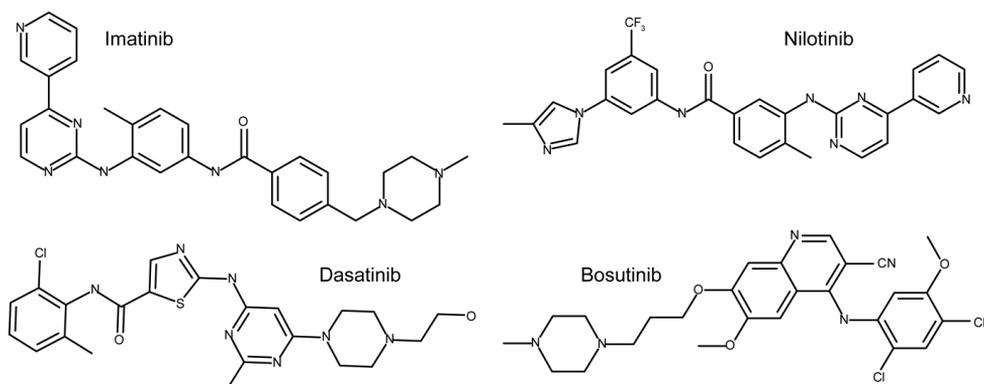
Figure 2: The chemical structures of imatinib and the second generation tyrosine kinase inhibitors dasatinib, nilotinib, and bosutinib.

Mechanisms of imatinib resistance

The underlying reasons for imatinib resistance are only partially known. Mutations sometimes arise in the *BCR-ABL1* gene that alter the conformation of the ATP-binding site in the protein and prevent the effective binding of imatinib. Most of the *BCR-ABL1* mutations can be targeted by the second generation TKIs, although drugs targeting the T315I amino acid substitution have been lacking (*Quintas-Cardama et al., 2009a*). However, the novel drug candidate ponatinib has shown therapeutic effects in patients carrying this BCR-ABL1 variant (*Cortes et al., 2012a*).

Intrinsic factors related to the patient or the disease also play a role in imatinib outcome, and patients can be categorized according to risk scores. The Sokal risk score divides patients into low, intermediate, and high risk based on age, spleen size, percent blast cells in circulation, and platelet count at diagnosis (*Sokal et al., 1984*). Although the Sokal score was developed for CML patients on conventional chemotherapy, it also predicts cytogenetic and molecular response levels as well as overall survival in patients treated with imatinib (*Hochhaus et al., 2009, Hughes et al., 2003*). However, the simpler imatinib-specific EUTOS score – based only on spleen size and percent basophils at diagnosis – has recently been shown to have better discriminatory power between high- and low-risk patients for the

achievement of a complete cytogenetic response within 18 months of therapy (*Hasford et al., 2011*).

Perhaps surprisingly in the context of treating a life-threatening disease, patient compliance has turned out to be a significant contributor to variable imatinib outcome. In a study of 87 CML patients treated with imatinib for more than two years, poor adherence was significantly associated with less frequent achievement of major and complete molecular responses (*Marin et al., 2010*).

In addition, plasma concentrations of imatinib are highly variable between patients (*Larson et al., 2008*) and are associated with variations in imatinib response (*Picard et al., 2007*). The reasons for variable imatinib pharmacokinetics have not yet been fully elucidated but might be due to the fact that the pharmacokinetic processes of absorption, distribution, metabolism, and elimination are known to vary among different individuals.

Imatinib pharmacokinetics

Imatinib is given in a standard oral dose of 400 mg/day. Variations in body size correlate weakly with imatinib plasma concentrations and clearance and are considered too small compared to other sources of variation to be accounted for in terms of body size-adjusted dose regimens (*Cohen et al., 2002, Larson et al., 2008*). Imatinib has a high bioavailability of 98% (*Peng et al., 2004*) indicating that it is not subjected to significant metabolism at the absorption site or to hepatic first-pass metabolism. Maximum plasma concentration is usually reached within 2–4 hours after administration and the elimination half-life ($t_{1/2}$) is approximately 18 hours (*Cohen et al., 2002, Peng et al., 2004*). The $t_{1/2}$ of 18 hours results in steady-state levels of imatinib in circulation within four days. Only 3%–5% of circulating imatinib corresponds to the free fraction of the drug due to the high affinity of imatinib for plasma proteins such as albumin and α_1-acid glycoprotein (*Gandia et al., 2012, Streit et al., 2011*). The level of α_1-acid glycoprotein varies between patients, and this can potentially affect the fraction of unbound drug and might have implications for the amount of drug available to exert therapeutic effects (*Gambacorti-Passerini et al., 2003, Widmer et al., 2006*).

Imatinib plasma concentration varies considerably between patients. In a study of 351 CML patients treated with an average daily dose of 393 mg imatinib (± 29 mg),

a 25-fold variation in plasma trough concentrations at steady-state was observed (*Larson et al., 2008*) and a trough concentration >1000 ng/mL was associated with better response rates (*Larson et al., 2008, Picard et al., 2007*). Imatinib is mainly excreted through the hepato-biliary pathway, and an average of 67% of a single oral dose of imatinib is recovered in feces either as parent drug or metabolites over a collection period of seven days and 13% is found in urine (*Gschwind et al., 2005*). Imatinib is subjected to extensive hepatic metabolism, and only 25% of the dose is excreted as unchanged compound while the rest is excreted as metabolites after a single dose (*Novartis, 2001*).

Metabolism

Hepatic metabolism of imatinib results in the formation of around 30 metabolites (*Marull et al., 2006, Rochat et al., 2008*). Most metabolites have not received much attention due to low plasma concentrations that do not appear to be clinically relevant. The chemical structure has been identified for a few metabolites, including CGP74588 (N-desmethyl imatinib), which is a de-methylation product of imatinib and is the metabolite found at the highest concentrations (*Gschwind et al., 2005*) (Figure 3).

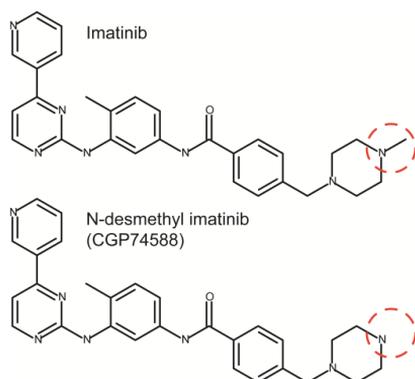Figure 3: The chemical structure of imatinib and its major metabolite, N-desmethyl imatinib (CGP74588). Hatched circles in red identify the de-methylation site.

CGP74588 was initially described as being equally potent as the parent compound (*Cohen et al., 2002*), but recent investigations have shown that it has a 3-fold reduction in *in vitro* potency compared to imatinib (*Mlejnek et al., 2011*). The mean

trough plasma concentration of CGP74588 reaches 27% of the parent drug 29 days after the start of imatinib treatment (*Larson et al., 2008*). The $t_{1/2}$ of CGP74588 has been estimated to be an average of 90 hours, which is considerably longer compared to imatinib (*le Coutre et al., 2004*), and this metabolite does not reach steady-state until after approximately three weeks of treatment. Experiments on pooled human liver microsomes as well as with recombinant expression of specific metabolic enzymes suggested that the cytochrome P450 (CYP) 3A isoenzymes CYP3A4 and CYP3A5 are the major contributors to imatinib metabolism. CYP1A2, CYP2C8, CYP2C9, CYP2C19, and CYP2D6 are also capable of imatinib metabolism but only to a minor extent (*Novartis, 2001, Peng et al., 2005*). The CGP74588 metabolite is primarily produced by CYP3A4 with contributions from CYP3A5 and CYP2D6 (*Novartis, 2001*).

The CYP3A enzymes are the most abundant form of CYP enzymes in the liver and consist of the four isoforms CYP3A4, CYP3A5, CYP3A7, and CYP3A43. CYP3A4 and CYP3A5 account for the majority of CYP3A activity in adults and metabolize a wide range of drugs. However, CYP3A5 is frequently inactivated in Caucasian populations due to the non-functional CYP3A5*3 genotype (*Mirghani et al., 2006, van Schaik et al., 2002*). CYP3A7 is mainly active during fetal development until approximately six months after birth (*Stevens et al., 2003*). Little is known about the CYP3A43 isoform with respect to drug metabolism, but no major contribution is expected because CYP3A43 is expressed at only 0.1%-0.2% of the level detected for CYP3A4 in human liver samples (*Gellner et al., 2001, Westlind et al., 2001*).

Due to the large number of drugs metabolized by CYP3A enzymes, drug-drug metabolic interactions are common. Some drugs are especially potent CYP3A inducers or inhibitors and can be predicted to affect the metabolism of co-administered drugs that have the same metabolic pathway. The concomitant treatment of imatinib with either of the potent CYP3A inducers rifampicin or St. John's wort resulted in reduced imatinib maximum plasma concentration (C_{max}) and area under the plasma concentration-time curve (AUC) (*Bolton et al., 2004, Frye et al., 2004, Smith et al., 2004*). In line with these findings, rifampicin increased the C_{max} and AUC of the CGP74588 metabolite (*Bolton et al., 2004, Frye et al., 2004*). Conversely, administration of the CYP3A4 inhibitor ketoconazole together with imatinib resulted in increased imatinib C_{max} and AUC (*Dutreix et al., 2004*).

CYP3A activity also has a large intrinsic inter-individual variation (5- to 20-fold) in the human population (*Wilkinson, 1996*). This variability primarily reflects variations in CYP3A4 activity because this enzyme represents the majority of CYP3A activity in the human liver (*Ingelman-Sundberg et al., 2007, Westlind-Johnsson et al., 2003*). In contrast to CYP3A5, there is currently no clear genetic explanation for the variability in CYP3A4 activity and the variations are probably due to a combination of genetic and environmental factors (*Ingelman-Sundberg et al., 2007, Lamba et al., 2002*).

Cellular transport

The passage of imatinib across cellular membranes is facilitated by the active uptake and efflux of transport proteins (Figure 4). It has previously been shown that the human organic ion transporters OCT1, OCTN2, OATP1A2, and OATP1B3 mediate the cellular uptake of imatinib *in vitro* (*Hu et al., 2008*). Of these transporters, OCT1 is the best studied. OCT1 is expressed in the basolateral membrane of hepatocytes (*Koepsell et al., 2007*) and potentially regulates the availability of imatinib for metabolism and excretion. OCT1 is also expressed in cell membranes of peripheral blood leukocytes in CML patients (*Thomas et al., 2004*) and regulates the amount of imatinib that reaches the intracellular target BCR-ABL1. OCT1 expression level and activity varies between patients, and transcript levels of OCT1 in circulating blood cells are predictive of imatinib response (*Wang et al., 2008*). Patients with low OCT1 activity benefit from imatinib dose escalation (*White et al., 2012*).

OCTN2, OATP1A2, and OATP1B3 have also been suggested to play a role in imatinib absorption and elimination due to their expression on the luminal side of enterocytes and/or in the basolateral membrane of hepatocytes (*Eechoute et al., 2011b, Glaeser et al., 2007, Koepsell et al., 2007*). However, much less is known about these transporters and the only clinical data that is available showed that inhibition of OATP1A2 *in vivo* did not seem to influence imatinib pharmacokinetics *(Eechoute et al., 2011a).*

Imatinib is also a substrate for the ATP-binding cassette (ABC) transporters ABCB1 (also known as P-glycoprotein and MDR1) and ABCG2 (also known as BCRP and MXR) that mediate cellular imatinib efflux *in vitro* (*Dohse et al., 2010,*

Shukla et al., 2008). The main physical role of ABCB1 and ABCG2 is protection against foreign substances by the cellular extrusion of such substrates and the mediation of their final excretion from the body through the liver and kidneys. Consequently, ABCB1 and/or ABCG2 are abundantly expressed in protective tissues of sensitive organs such as the blood-brain-barrier and the placenta as well as in locations important for uptake and elimination of drugs such as the intestine, kidneys, and liver (*Cooray et al., 2002, Fetsch et al., 2006, Thiebaut et al., 1987, 1989*).
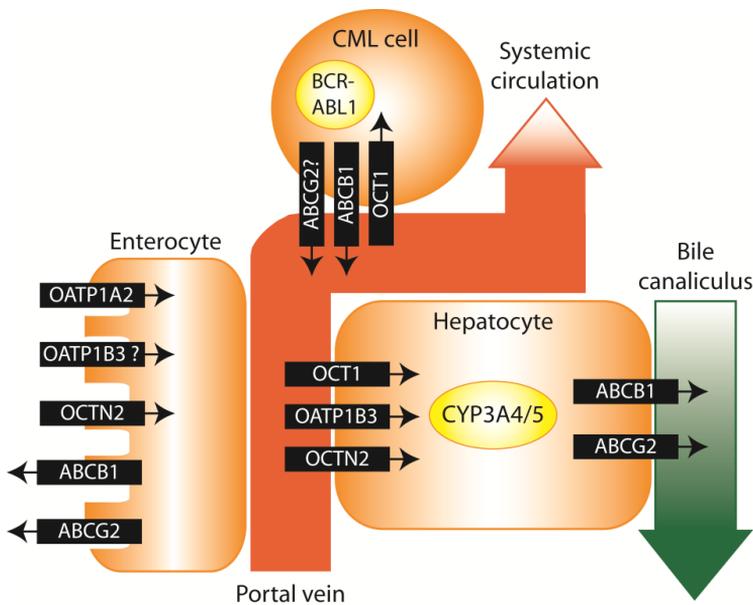
Figure 4: Trans-membrane transport of imatinib. Imatinib is a substrate for the organic ion transporters OCT1, OCTN2, OATP1A2, and OATP1B3 that facilitate its cellular uptake. OCTN2, OATP1A2, and to some extent also OATP1B3 are expressed on the luminal side of enterocytes and this makes them likely candidates for facilitation of imatinib absorption. OCT1 together with OATP1B3 and OCTN2 are expressed in basolateral membranes of hepatocytes and possibly regulate the availability of imatinib for hepatic CYP3A4 and CYP3A5 metabolism. OCT1 is also expressed on CML cells and plays an important role in the accumulation of imatinib in target cells. Imatinib is also a substrate for the efflux transporters ABCB1 and ABCG2 that work in the opposite direction to mediate imatinib efflux from enterocytes into the intestinal lumen as well as from hepatocytes into the bile for excretion. ABCB1 is expressed to varying degrees in CML progenitor cells as well as in differentiated mononuclear and polymorphonuclear cells. ABCG2 is expressed in primitive CML progenitors but it is not clear whether this transporter is also expressed in other stages of myeloid cell maturity.

ABCB1 and ABCG2 are expressed on the luminal side of enterocytes and at the bile canalicular membrane in hepatocytes and can potentially influence the excretion of imatinib from systemic circulation. It has been shown in mice that knocking out either of the ABCB1 or ABCG2 genes (*Mdr1a/1b* or *Bcrp1*) significantly decreases imatinib systemic clearance after an i.v. infusion (*Breedveld et al., 2005*).

Furthermore, ABCB1 and ABCG2 expression has been identified in isolated populations of primitive CML progenitor cells (*Jiang et al., 2007, Jordanides et al., 2006*). ABCB1 is also expressed in normal peripheral mononuclear as well as in polymorphonuclear leukocytes (*Racil et al., 2011*) potentially restricting the availability of imatinib for inhibition of BCR-ABL1.

ABCB1 and ABCG2 have overlapping substrate specificities and transport a wide range of substrates including the second generation TKIs dasatinib and nilotinib (*Dohse et al., 2010, Hegedus et al., 2009, Hiwase et al., 2008*). Bosutinib has not been well studied in terms of ABC transport, but a single report indicates that bosutinib is not transported by ABCB1 or ABCG2 *in vitro* (*Hegedus et al., 2009*). Recently, it was also reported that the imatinib metabolite CGP74588 is efficiently extruded from a multi-drug resistant cell line expressing ABCB1 (*Mlejnek et al., 2011*).

Single nucleotide polymorphisms in *ABCB1* and *ABCG2*

The *ABCB1* and *ABCG2* genes are highly polymorphic and harbor numerous single nucleotide polymorphisms (SNPs) that could potentially influence the functionality of the transporters and thus the pharmacokinetics and therapeutic effects of imatinib and the second generation TKIs.

About 100 SNPs have been identified in the coding regions of *ABCB1*. However, only a limited number have been investigated in terms of population frequencies and their functional consequences (*Cascorbi, 2011*). The synonymous *ABCB1* 3435T>C SNP frequently occurs in Caucasian populations and was the first variant found to be correlated with *in vivo* pharmacokinetics. It was found that the 3435T allele was associated with low intestinal ABCB1 expression and high digoxin plasma concentrations (*Hoffmeyer et al., 2000*) that were later suggested to be caused by reduced mRNA stability (*Wang et al., 2005*). The 3435T>C SNP was found to be in linkage disequilibrium with the synonymous 1236C>T and the non-

synonymous 2677G>T/A (Ala893Ser/Thr) SNPs (*Kim et al., 2001, Kroetz et al., 2003*). The three linked SNPs have been extensively investigated, either separately or as haplotypes, in association with the outcome of several drug classes (*Cascorbi, 2011, Leschziner et al., 2007*), including imatinib.

The first report on imatinib pharmacokinetics in relation to an *ABCB1* genotype showed that the 3435T>C SNP did not explain variations in imatinib oral clearance (*Gardner et al., 2006*). The year after, however, a significantly reduced rate of imatinib clearance was found in patients homozygous for a T at nucleotide positions 1236, 2677, and 3435 in the *ABCB1* gene (*Gurney et al., 2007*). Subsequent studies have been inconclusive with four reports indicating that the individual SNPs or the complete haplotype influence imatinib pharmacokinetics or therapeutic outcome (*Angelini et al., 2013, Deenik et al., 2010, Dulucq et al., 2008, Ni et al., 2011*) and four reports failing to identify such an effect (*Kim et al., 2009, Marin et al., 2010, Seong et al., 2012, Takahashi et al., 2010*). Furthermore, positive conclusions were not always consistent because one study reported higher rates of major molecular responses in CML patients carrying the 1236TT genotype (*Dulucq et al., 2008*) in contrast to another study where the 1236CC genotype was significantly associated with higher rates of major molecular response (*Deenik et al., 2010*).

In addition to the most commonly studied 1236, 2677, and 3435 SNPs, the rarer 1199G>A SNP (Ser400Asn) has been shown to alter cellular sensitivity to chemotherapeutic agents *in vitro* (*Crouthamel et al., 2006*). This SNP was suggested to influence the outcome of treatment in acute myeloid leukemia (*Green et al., 2012*) but has not yet been investigated in relation to imatinib.

The investigations of *ABCG2* SNPs in relation to pharmacokinetics and drug response have mainly focused on 421C>A (Gln141Lys), which is most frequently found in Asian populations. The 421A allele reduced the protein expression and transport activity of ABCG2 *in vitro* (*Furukawa et al., 2009, Morisaki et al., 2005*) and was shown to alter the pharmacokinetics of substrate drugs such as diflomotecan (*Sparreboom et al., 2004*) and rosuvastatin (*Zhang et al., 2006*) while other known ABCG2 substrates such as doxorubicin were not affected (*Lal et al., 2008*). The 421A variant had reduced capacity for imatinib transport when expressed in human embryonic kidney cell lines, but no similar influence could be detected in patients. However, it should be noted that no homozygously variant patients were

studied (*Gardner et al., 2006*). The *ABCG2* 421C allele and the G allele of *ABCG2* 34G>A (Val12Met) have been associated with lower rates of major molecular response and complete cytogenetic response, respectively (*Kim et al., 2009*). It was also shown that CML patients with the 421CC genotype had lower imatinib plasma concentrations than those with CA or AA genotypes (*Takahashi et al., 2010*). However, a large study on 189 CML patients did not identify any association between *ABCG2* 421C>A or 34G>A and imatinib response parameters (*Angelini et al., 2013*).

Predicting the response to CML therapy

Currently, imatinib remains the standard therapeutic option in CML treatment at diagnosis despite the introduction of second generation TKIs. Although imatinib induces an adequate therapeutic effect in the majority of patients, there is still a significant proportion of patients who do not achieve a satisfactory response and who are at greater risk of disease progression. Furthermore, it is not yet known why some patients achieve a deep response to imatinib that enables successful cessation of therapy while others do not. Currently, the majority of patients are started on standard imatinib therapy and switch to other alternatives when one or more of the milestones in the monitoring guidelines are not achieved. This strategy might not be optimal because it has been shown that a fast response to treatment is predictive of better long-term outcome (*Quintas-Cardama et al., 2009b*). A more personalized treatment strategy for CML in which imatinib is prescribed only to patients in whom an adequate response can be predicted might be beneficial. This approach would aim at faster responses and perhaps also enable more patients to achieve deep response levels to imatinib therapy.

In order to predict the outcome of imatinib treatment at diagnosis or early in the treatment, a better understanding of the parameters influencing imatinib response is necessary. The only imatinib resistance mechanism that has been translated into clinical practice is the appearance of *BCR-ABL1* mutations that are routinely investigated when patients fail on therapy. The monitoring of OCT1 activity has proven useful in clinical studies (*White et al., 2012*) but has not yet been implemented in clinical practice. The results from studies on OCT1 activity indicate that pharmacokinetic parameters might influence imatinib outcome, and

further studies on additional parameters such as metabolism and efflux transport are warranted in order to understand the variable response to imatinib among CML patients.

AIMS OF THE THESIS

The overall aim of this thesis was to investigate the potential influence of drug metabolism and transport on the response to the TKIs used in CML therapy.

The specific aims were:

* To investigate the influence of genetic variants of *ABCB1* and *ABCG2* on the transport and efficacy of imatinib, CGP74588, and the second generation TKIs.

* To study the influence of CYP3A enzyme activity on plasma concentrations of imatinib and CGP74588 in CML patients.

* To study the role of CYP3A enzyme activity on the therapeutic outcome of imatinib in CML patients.

METHODS

In vitro studies of ABC transporters (Papers I and II)

K562 cells as a model system

In Papers I and II, we investigated the effects of *ABCB1* and *ABCG2* genetic variations on protein expression levels of the transporters and on their transport capacity for TKIs. In order to minimize the influence from external factors typically seen in *in vivo* experimental designs, the CML cell line K562 was used as a model system. The K562 cell line is derived from a 53-year-old female with CML in terminal blast crisis (*Lozzio et al., 1975*) and consists of highly undifferentiated granulocytic cells (*Lozzio et al., 1979*).

K562 cells are Ph$^+$ and are, therefore, sensitive to TKI inhibition. We have exploited this trait by using cell survival assays to analyze the effect of TKI treatment on cells carrying genetic variants of *ABCB1* or *ABCG2*. The natural expression of ABCB1 and ABCG2 in K562 cells was investigated using real-time PCR and immunofluorescence detection during flow cytometry. K562 cells had low detectable levels of *ABCB1* and *ABCG2* transcripts, and no corresponding

proteins were detected on the cell surfaces. The absence of intrinsic ABCB1 and ABCG2 protein expression in K562 cells enabled an adequate estimation of TKI transport activity by the experimentally transduced *ABCB1* and *ABCG2* genes because there was no interference from residual background expression of wild-type *ABCB1* and *ABCG2* in the cells.

Real-time PCR showed low but detectable transcript levels of *CYP3A4* in K562 cells, which was in accordance with a previous report (*Nagai et al., 2002*). The *CYP3A4* transcript level was 1.7% of that found in the hepatic HepG2 cell line, and no significant metabolism of imatinib in K562 cells would be expected. Also, the major CYP3A4 imatinib metabolite, CGP74588, could not be detected when measuring intracellular drug concentrations after incubating K562 cells for 2 hours with imatinib.

Selection and construction of variant *ABCB1* and *ABCG2*

The overall aim of SNP selection for the studies in Papers I and II was to study SNPs located in the coding regions of the genes with a minor allele frequency of >2% in any human ethnic population. SNPs resulting in stop codons and synonymous substitutions were excluded. However, some exceptions to the initial aim were made, for example, the *ABCG2* 1574T>G minor allele frequency was in retrospect corrected to a lower frequency (1.4%) than the initial inclusion limit of >2%. Furthermore, it has been suggested that synonymous *ABCB1* SNPs might influence translation efficacy of *ABCB1* transcripts (*Kimchi-Sarfaty et al., 2007*). To ensure that any potential differences between variant cell lines were not caused by synonymous SNPs, the 1236C>T and 3435T>C SNPs were also included in the study. These SNPs do not alter the amino acid sequence but are in high linkage disequilibrium with the non-synonymous 2677G>T/A SNP. The *ABCB1* 1795G>A SNP was included primarily based on the fact that this SNP was already in its variant form in the cDNA that was purchased for the construction of the SNPs. No population frequency data were available for this specific variant. The selected *ABCB1* and *ABCG2* SNPs, nucleotide and amino acid substitutions, and minor allele frequencies are detailed in Table 1.

Table 1: *ABCB1* and *ABCG2* single nucleotide polymorphisms

rs#	Nucleotide substitution[a]	Amino acid substitution	Minor allele frequency
ABCB1			
rs9282564	61A>G	Asn21Asp	0.11 (CA)[b]
rs2229109	1199G>A	Ser400Asn	0.06 (CA)[b,c]
rs1128503	1236C>T	synonymous	0.45 (CA)[d]
rs2235036	1795G>A	Ala599Thr	n.d.
rs2032582	2677G>T/A	Ala893Ser/Thr	T=0.42 (CA), A=0.02 (CA)[b]
rs1045642	3435T>C	synonymous	0.42 (CA)[d]
ABCG2			
rs2231137	34G>A	Val12Met	0.29 (AS)[e]
rs2231142	421C>A	Gln141Lys	0.34 (AS)[f]
rs1061018	623T>C	Phe208Ser	0.04 (AS)[g]
rs41282401	886G>C	Asp296His	0.02 (CA)[h]
rs58818712	1574T>G	Leu525Arg	0.014 (mixed)[i]
rs45605536	1582G>A	Ala528Thr	0.02 (CA)[j]

AS, Asian population; CA, Caucasian population; n.d., not determined

[a]position from ATG in Ensemble Genome Browser reference transcripts ENST00000265724 (*ABCB1*) and ENST00000237612 (*ABCG2*)

Frequencies obtained from [b](*Cascorbi et al., 2001*), [c](*Green et al., 2012*), [d]NCBI 1000 Genomes Browser v.2.2.2, or NCBI submission identification numbers [e]48428447, [f]76894509, [g]12675225,[h]70352856,[i]86245712, and [j]70352893

ABCB1 SNPs were constructed in haplotypes corresponding to those previously identified as [1236T; 2677T; 3435T] or [1236C; 2677G; 3435C] and referred to here as the TTT or CGC haplotypes. In approximately 2% of Caucasian populations, 2677G>T is instead a G>A transition (*Cascorbi et al., 2001*) giving rise to the CAC haplotype [1236C; 2677A; 3435C] that was also constructed here. The 61A>G and 1199G>A SNPs were constructed together with the TTT and CGC

haplotypes, respectively, because these are the haplotypes with which they are most frequently associated. The *ABCB1* 1795G>A SNP has not previously been associated with a haplotype, and this was constructed with the CGC haplotype because this was the haplotype of the transcript that was purchased to construct this SNP. An overview of the *ABCB1* haplotypes of the generated cell lines is provided in Table 2.

Table 2: *ABCB1* haplotypes of the constructed cell lines

Cell line	cDNA position						Haplotype frequency[a]
	61	1199	1795	1236	2677	3435	
K562/ABCB1 TTT	A	G	G	**T**	**T**	**T**	0.32
K562/ABCB1 61	**G**	G	G	T	T	T	0.08
K562/ABCB1 CGC	A	G	G	**C**	**G**	**C**	0.15
K562/ABCB1 1199	A	**A**	G	C	G	C	0.01
K562/ABCB1 1795	A	G	**A**	C	G	C	n.d.
K562/ABCB1 CAC	A	G	G	**C**	**A**	**C**	0.02

Variant nucleotides are shown in red. Brackets indicate the three distinct haplotypes [1236C>T; 2677G>T/A; 3435T>C].
[a]haplotype frequency from (*Kroetz et al., 2003*); n.d., not determined

The *ABCG2* SNPs were constructed as single substitutions in the *ABCG2* gene. The resulting K562 variant cell lines were referred to with the nucleotide position of the specific substitution in each cell line.

Vectors carrying human wild-type *ABCB1* or *ABCG2* cDNA together with an ampicillin resistance gene were purchased and amplified in *Escherichia coli* under ampicillin selection. Selected SNPs were introduced in the corresponding wild-type gene using site-directed mutagenesis. Primers containing the substituted nucleotide of the corresponding SNP were used in a PCR to introduce the mutation and for amplification of the mutated gene. Due to DNA methylation by DNA adenine methyltransferase in *E. coli* during vector amplification, unmutated parental vector DNA could then be digested using the methylation-specific endonuclease *DpnI* (*Buryanov et al., 2005*). The mutated genes were transferred into K562 cells using retroviral gene transductions.

Retroviral gene transfer

Retroviral vectors can be used to transfer genomic material for insertion and stable expression in mammalian cells. The gene of interest is transferred into a viral vector that, together with helper vectors, is transfected into a packaging cell line that produces live viruses. Viruses carrying the gene of interest can then be used to infect and transduce the genetic material into the final host cell line.

In the present project, variant *ABCB1* and *ABCG2* genes were inserted into an *MIY* vector. This vector was constructed from the murine stem cell virus (<u>M</u>SCV) retroviral expression vector and modified to contain an internal ribosome entry site (<u>I</u>RES) between the gene insert and the enhanced yellow fluorescence protein (*E<u>Y</u>FP*) reporter gene (*DeKoter et al., 2007*). The *MIY-ABCB1* and *MIY-ABCG2* vectors were transfected into the human embryonic kidney 293T packaging cell line together with the helper vectors *VSV-G* and *POL-GAG*. Inside the 293T cells, the helper vectors are transcribed and translated into proteins that are essential for the production of infectious virus particles carrying the genetic material of the *MIY* vector. *VSV-G* encodes an envelope protein that facilitates infection of target cells and *POL* and *GAG* provide proteins for viral core and capsid structures as well as reverse transcriptase and integrase for the conversion and integration of viral RNA into host DNA (*Burns et al., 1993, Vogt, 1997*). Viral 293T supernatants were harvested over 72 hours and used for transduction of the *ABCB1* and *ABCG2* variant genes into K562 cells. The *MIY* vector, carried by the virus, randomly integrates into the genome of K562 cells, and the host transcriptional machinery is used for expression of the transferred genes. In the *MIY* vector, *ABCB1* or *ABCG2* were inserted downstream of a 5'-long terminal repeat (5'-LTR) that enhances transcriptional activation of the insert. The IRES site was located downstream of the gene insert followed by the *EYFP* gene. The 5'-LTR ensured constitutive transcription of the gene of interest (*ABCB1* or *ABCG2*), IRES, and *EYFP* in a single mRNA transcript. Although transcription of *ABCB1* or *ABCG2* and *EYFP* are driven by the same promoter, the IRES site is capable of recruiting its own set of ribosomal units (*Balvay et al., 2009*) and this leads to the separate translation of ABCB1 or ABCG2 and the EYFP proteins (Figure 5). The co-expression of *ABCB1* or *ABCG2* and *EYFP* ensures equal levels of transcription and translation that enables the analysis of EYFP as a reporter of *ABCB1* or *ABCG2* expression from the transduced vector. EYFP$^+$ cells were sorted by flow cytometry for equal median EYFP fluorescence intensity in order to ensure a

similar transcriptional activity of the transduced vectors for comparisons of cell lines expressing different variants of *ABCB1* and *ABCG2*. K562 cells transduced with an empty *MIY* vector served as controls in all experiments and were referred to as K562/ve.
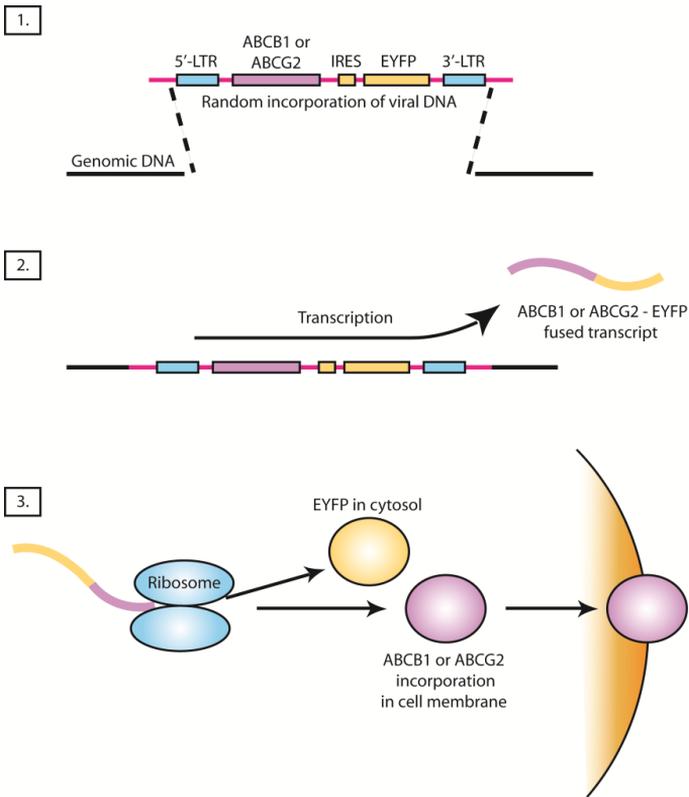

Figure 5: Overview of retroviral transduction of *ABCB1* or *ABCG2* in K562 cells. 1) The genetic material in the *MIY* retroviral vector randomly incorporates into the K562 genome. 2) The host cell machinery is used for transcription and is facilitated by the retroviral 5'-LTR promoter. *ABCB1* or *ABCG2* is co-transcribed with the reporter gene *EYFP* as a single transcript. 3) The IRES site between *ABCB1* or *ABCG2* and the *EYFP* gene ensures the translation of separate proteins from the two genes. *EYFP* is translated into a cytosolic protein while *ABCB1* or *ABCG2* is expressed in the cell membrane. Because *EYFP* is co-transcribed with the genes of interest, it serves as a suitable reporter protein of vector activity in transduced cells.

Analyzing EYFP, ABCB1, and ABCG2 with flow cytometry

In order to evaluate the influence of genetic variants on transporter expression in cell membranes, ABCB1 and ABCG2 were fluorescently labeled using antibodies and analyzed by flow cytometry. All transduced cell lines were simultaneously analyzed, and EYFP fluorescence was used for normalization when comparing the transporter expression levels between cell lines transduced with different genetic variants.
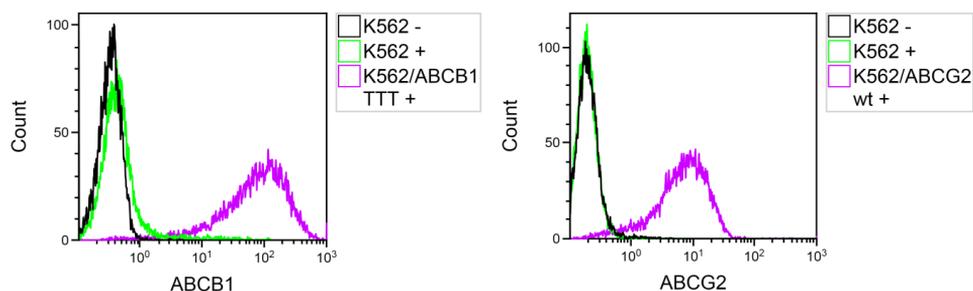

Figure 6: Detection of ABCB1 and ABCG2 cell membrane expression by flow cytometry. Parental K562 cells, labeled with the respective ABCB1 or ABCG2 antibody (K562 +), had similar fluorescence profiles as unlabeled K562 cells (K562 -) proving the absence of unspecific binding of antibodies and confirming that K562 cells do not have a natural expression of these transporters. The fluorescent profiles of labeled K562 cells transduced with ABCB1 or ABCG2 are adequately separated from their parental counterparts.

Antibodies for flow cytometry were selected based on specificity for the extracellular moiety of the target proteins and a conjugated fluorochrome with minimum overlap with the EYFP emission spectrum. For ABCG2, the mouse anti-human ABCG2 clone 5D3 was used with a conjugation of PerCP-Cy5.5. The 5D3 antibody binds to the extracellular domain of the ABCG2 protein, but the exact epitope has not been characterized (*Ozvegy-Laczka et al., 2008, Zhou et al., 2001*). All of the constructed *ABCG2* SNPs result in amino acid substitutions that are located in the intracellular or trans-membrane domain of the ABCG2 protein. This excludes the possibility of direct structural alterations of the extracellular antibody epitope, but indirect conformational effects cannot be excluded. Several antibodies for detection of ABCB1 were investigated during assay development, but none fulfilled the requirements of having a low nonspecific binding and a conjugated fluorochrome with a fluorescent emission that did not interfere with that of EYFP. Consequently, an indirect assay was developed with a primary

mouse anti-human ABCB1, clone 17F9, and a secondary anti-mouse antibody conjugated with allophycocyanin/Cy7. The final assays resulted in an adequate separation between positive and negative cells. Furthermore, the assays had low nonspecific binding and showed that parental K562 cells did not express ABCB1 or ABCG2 because they had similar fluorescent profiles as unlabeled cells (Figure 6).

Cell survival assay

The functionalities of the ABCB1 and ABCG2 variants were indirectly measured by their ability to efflux TKI drugs and to protect cells from TKI inhibition of survival and proliferation. Cells were seeded in 96-well plates and exposed to nine serially diluted concentrations of TKIs for 72 hours. The MTT assay was used to determine the relative number of living cells in each well compared to cells treated with control vehicle. The MTT assay is a colorimetric assay that is based on the conversion of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into a dark blue formazan product by dehydrogenase enzymes in the active mitochondria of living cells. The quantity of formazan can be measured using a spectrophotometric plate reader and reflects the number of living cells (*Mosmann, 1983*). In the present investigations, the relative cell survival was plotted against the TKI concentration and the concentration that inhibits 50% of the cell survival (IC_{50}) was calculated and used to compare the different *ABCB1* or *ABCG2* genotypes (Figure 7). Because the MTT assay measures the number of living rather than dead cells, it does not distinguish between inhibited cell proliferation and cell death after 72 hours. However, the studies of TKI mechanisms for inhibition of K562 cell survival and proliferation was not our aim, and the MTT assay was used to determine variations in the ability of different ABCB1 and ABCG2 variants to protect the cells from TKI activity by pumping the drugs out of the cells.
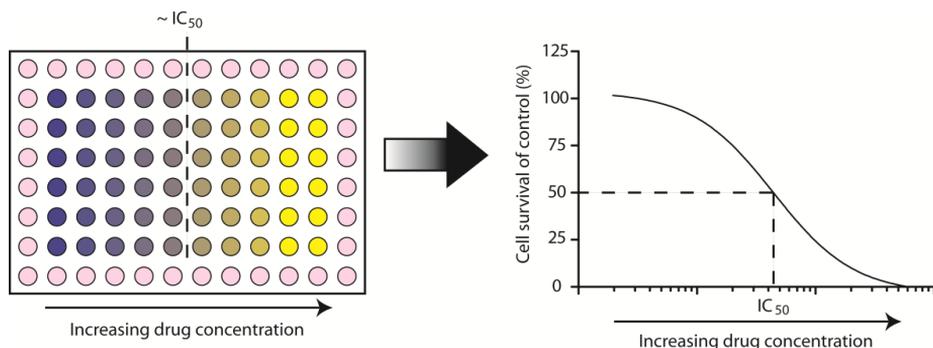
Figure 7: MTT experimental layout. Cells were seeded in 96-well plates together with dilution series of TKIs. After 72 hours of incubation, the yellow MTT salt was added to all wells. Living cells metabolize the salt into a dark blue product that is quantified in an absorbance plate reader. The relative number of living cells was plotted against drug concentration and a dose-response regression was fitted to the sample points enabling the extraction of IC_{50} concentrations.

Quantification of TKIs in cell lysates

Because the MTT assay only indirectly measures the transport activity of ABCB1 and ABCG2 through their ability to protect cells from TKI cytotoxicity, an additional method using LC-MS/MS was used to determine the intracellular accumulation of imatinib, CGP74588, dasatinib, nilotinib, and bosutinib in ABCB1- and ABCG2-transduced cell lines. Cells were incubated with TKIs for 0–240 minutes at the approximate IC_{50} concentrations in parental K562 cells to analyze the time point for influx-efflux equilibrium. To reduce the loss of accumulated TKIs during repeated washing steps, cells in incubation medium were layered on silicone oil and centrifuged for rapid separation of cells from drug-containing medium. Cell pellets were disrupted using formic acid in water containing the internal standard. Imatinib was used as the internal standard for the dasatinib and nilotinib assays, and dasatinib was the internal standard for the imatinib, CGP74588, and bosutinib assays. Cell lysates were injected on a UPLC C18 column and separated using a mobile phase gradient of 20%–80% acetonitrile and 0.1% formic acid in water. The chromatographic system was coupled to a tandem quadropole mass spectrometer monitoring m/z transitions of 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 in the ranges of 10–3,000 ng/mL for imatinib, CGP74588, and bosutinib; 1–500 ng/mL for dasatinib; and 25–500

ng/mL for nilotinib. Calibration curves for imatinib, CGP74588, nilotinib, and bosutinib had a 2nd order regression while the dasatinib curve had a linear fit. All curve fits had coefficients of correlation >0.99. Quality control (QC) samples, prepared in two concentrations for each compound, were analyzed for intra-assay performance. All samples were analyzed in a single batch eliminating the need for inter-assay validation. Assay imprecision in QC samples was found to be <10% with an accuracy of 85%–113% for all compounds (Table 3).

Influx-efflux equilibration occurred at 120 minutes for imatinib, CGP74588, and bosutinib incubations and at 180 minutes for dasatinib and nilotinib. These fixed times were applied to the experiments in which parental K562, control cells transduced with an empty vector (K562/ve), and K562/ABCB1 TTT and ABCG2 wt (wild type) were incubated with TKIs to determine the effect of transporter expression on intracellular drug accumulation.

Table 3: Performance of LC-MS assay for quantification of TKIs in K562 cell lysates.

QC (ng/mL)	Mean conc. (ng/mL)	Imprecision (%)	Accuracy (%)	QC (ng/mL)	Mean conc. (ng/mL)	Imprecision (%)	Accuracy (%)
Imatinib (n = 5)				**CGP74588** (n = 5)			
100	108.0	2.4	108	100	100.4	2.9	100
2,500	2,514	1.1	101	2,500	2,451	3.1	98.1
Dasatinib (n = 5)				**Nilotinib** (n = 5)			
8	6.8	9.5	85.0	30	28.2	5.1	94.0
200	189.0	2.6	94.5	200	225.8	3.3	113
Bosutinib (n = 5)							
100	101.1	2.6	101				
2,500	2,650	1.1	106				

In vivo studies on CML patients

CML patients were studied with the primary aim of determining if *in vivo* CYP3A activity influences the outcome of imatinib therapy. Because this subject had not been previously investigated, a pilot study on a small number of patients was performed to evaluate the magnitude and relevance of variable CYP3A activity for therapeutic response to imatinib. A larger follow-up study was designed to confirm

the findings of the pilot study. The studies were approved by the regional ethical review board in Linköping, Sweden, and written informed consent was obtained from all participants before enrollment in the studies.

Pilot study design (Paper III)

Irrespective of previous treatment, fourteen patients treated with imatinib were recruited from Linköping University Hospital between the years 2002 and 2004. All patients were phenotyped for CYP3A metabolic activity using quinine as a probe drug, and patient outcome on imatinib therapy was collected from medical records. CYP3A activity was correlated to the level of molecular response achieved after 12 months of imatinib treatment.

Follow-up study design (Paper IV)

The difference and mean standard deviation of CYP3A activity in responders and non-responders from the pilot study were used for a power analysis preceding the follow-up study. A minimum number of seven patients in the smallest group was predicted for reproduction of the observed difference in means between the groups in the pilot study with a power of 80%. In order to achieve a sufficient number of non-responding patients, we aimed at an inclusion of 50 patients in the follow-up study. Patients were recruited from eight Swedish hospitals between the years 2009 and 2012. The inclusion criteria were chronic phase CML at diagnosis with first-line treatment of imatinib at 400 mg/day. Imatinib dose adjustments as well as pre-treatment with interferon, hydroxyurea or allopurinol were allowed for inclusion. Exclusion criteria were significant treatments such as transplantation or the use of drugs other than the ones mentioned above before the start of imatinib. Patients with contraindications for quinine (concomitant treatment or other illnesses) were not eligible for inclusion.

Patients were recruited both prospectively before the start of imatinib and retrospectively after initiation of imatinib. The primary endpoint of the study was the achievement of cytogenetic and molecular responses 12 months after start of imatinib. In retrospectively included patients, CYP3A phenotyping with quinine as a probe drug and sampling for quantification of trough imatinib and CGP74588 concentrations were performed at the time of inclusion. One additional sampling

was performed at the next routine visit to the clinic in order to analyze plasma concentrations at a time when quinine was not co-administered. In the prospective set of patients, CYP3A activity was analyzed before start of treatment and three months afterwards in order to determine any potential change in enzyme activity caused by imatinib treatment. Trough samples for plasma concentrations were taken at one, three, and six months after the start of therapy. The prospective and retrospective outlines of the study are depicted in Figure 8.
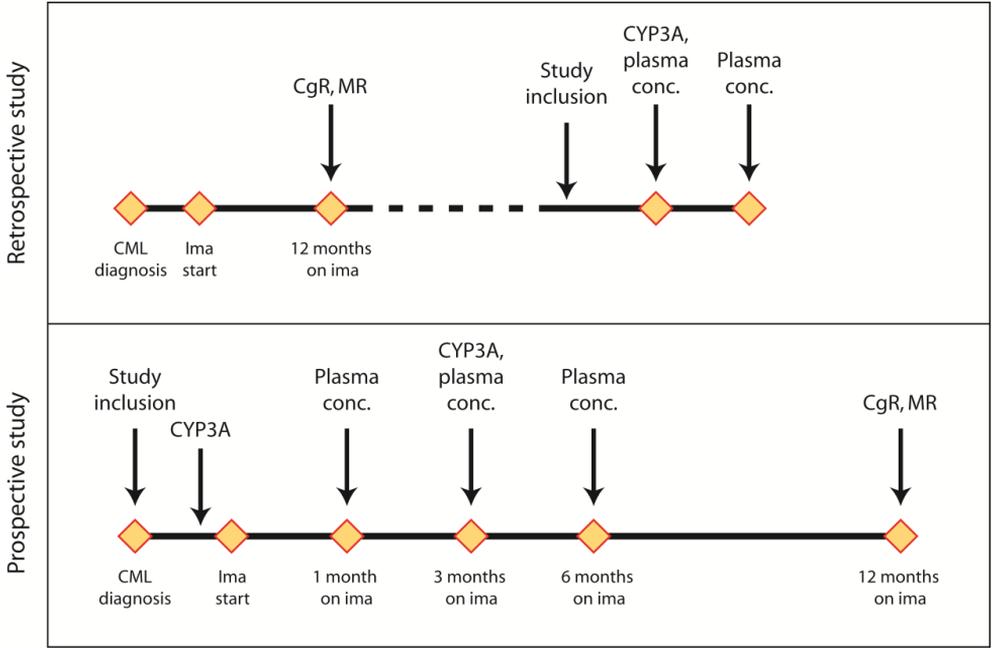

Figure 8: Retrospective and prospective inclusion of CML patients. In the retrospective arm, CYP3A activity and plasma concentrations of imatinib (ima) and CGP74588 were analyzed at the time of inclusion that occurred at any time point after start of imatinib therapy. Plasma concentrations were analyzed at one additional occasion where quinine was not co-administered. Patient cytogenetic response (CgR) and molecular response (MR) after the 12 first months of treatment were collected retrospectively from patient medical records. In the prospective arm, CYP3A activity was analyzed before and three months after start of imatinib. Plasma concentrations of imatinib and CGP74588 were determined after one, three, and six months. As in the retrospective setting, documented information on patient CgR and MR after 12 months on imatinib was used as the measure of therapeutic outcome.

CYP3A phenotyping

One of the most frequently used strategies to determine metabolic activity *in vivo* is to administer a probe drug to the test subject. The probe drug is enzymatically converted into one or more metabolites that can be quantified together with the parent drug in plasma. The ratio of parent drug and its metabolite concentration can then be used to determine the metabolic activity of the enzyme. In Papers III and IV, the *in vivo* activities of the CYP3A4 and CYP3A5 (CYP3A) isoenzymes were determined using quinine as a probe drug. A 250 mg dose of quinine was orally administered to patients followed by a blood sampling 16 hours later. Quinine and its CYP3A metabolite, 3S-3-hydroxy quinine (3S-Q), were quantified in plasma and the ratio of the two was used as a measure of CYP3A activity.

All probe drugs have different pharmacokinetic properties that should be carefully considered with respect to the aim and design of the study. In the case of CYP3A activity and its influence on imatinib metabolism, it was important to consider that imatinib has a high bioavailability and is relatively insensitive to CYP3A metabolism at the intestinal absorption site. Furthermore, imatinib is metabolized by both CYP3A4 and CYP3A5. In order to assess the relevant enzyme activity involved in imatinib metabolism, the optimal probe drug should ideally be a substrate for both CYP3A isoforms and should not be metabolized by intestinal CYP3A. Quinine was considered a suitable probe drug because it has a high bioavailability of 76% (*Paintaud et al., 1993*) indicating a small metabolic contribution from intestinal CYP3A enzymes. This was further supported by the finding that grape fruit juice inhibition of intestinal CYP3A enzymes does not influence quinine pharmacokinetics (*Ho et al., 1999*). Furthermore, both *in vitro* and *in vivo* studies have provided evidence that quinine is metabolized by both CYP3A isoforms (*Allqvist et al., 2007, Mirghani et al., 2006*). Quinine has previously been validated as a CYP3A probe drug and correlated to the metabolic ratio of omeprazole, which is another known CYP3A substrate (*Mirghani et al., 2003*).

The quantification of quinine and its 3S-Q metabolite in Paper III was performed at the Karolinska Institute using the validated HPLC assay published previously (*Mirghani et al., 2001*). For Paper IV, this assay was adapted for in-house analysis. Plasma samples from patients were precipitated using methanol and injected onto a reversed-phase C18 column. A gradient mobile phase of 10%–26% acetonitrile in acetate buffer was used for separation. Quinine and 3S-Q were quantified using a

fluorescence detector at 350 nm excitation measuring 450 nm emissions. The chromatography resulted in elution of 3S-Q and quinine at 5.2 min and 9.6 min, respectively. The separation of 3S-Q from its diastereomer 3R-3-hydroxy quinine was verified (Figure 9).

Calibration curves were prepared in plasma within the range of 10–2,000 nM for 3S-Q and 100–15,000 nM for quinine. Calibration curves were found to be linear with correlation coefficients >0.99. Low, medium and high concentration QC samples were prepared in plasma at 30, 300, and 1,000 nM for 3S-Q and 300, 3,000, and 12,000 nM for quinine. Intra-assay and inter-assay imprecision of QC samples were <3.9% and the accuracy ranged from 99.5% to 109% for both compounds (Table 4). Patient sample concentrations ranged from 70.62 nM to 356.8 nM and from 1,284 nM to 5,132 nM for 3S-Q and quinine, respectively.
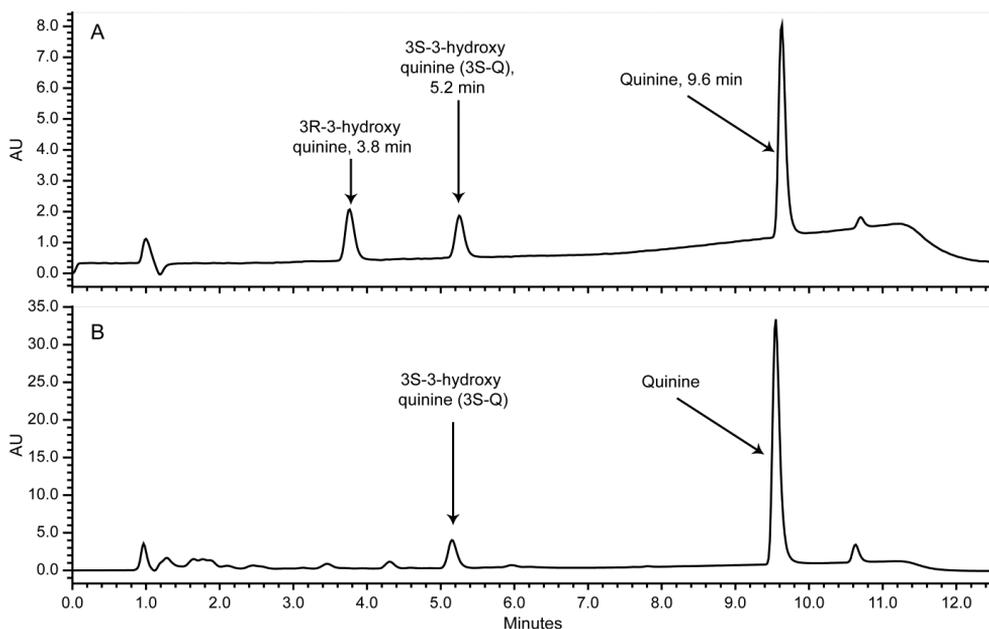

Figure 9: Chromatography of quinine and its metabolites. A) Plasma spiked with quinine, 3S-3-hydroxy quinine (3S-Q), and 3R-3-hydroxy quinine. B) Plasma from a CML patient 16 hours after administration of a single dose of 250 mg quinine. Quinine and 3S-Q were quantified as 354 nM and 3654 nM, respectively, in this sample.

Table 4: Performance of the HPLC assay for quantification of quinine and its metabolite, 3S-3-hydroxy quinine (3S-Q), in patient plasma.

Quinine				3S-Q			
QC (nM)	Mean conc. (nM)	Imprecision (%)	Accuracy (%)	QC (nM)	Mean conc. (nM)	Imprecision (%)	Accuracy (%)
Inter-assay (n = 5)				Inter-assay (n = 5)			
300	325.5	1.2	109	30	31.31	3.4	104
3,000	3,028	3.3	101	300	298.4	3.3	99.5
12,000	11,940	3.3	99.5	1,000	1,022	3.9	102
Intra-assay (n = 5)				Intra-assay (n = 5)			
300	328.3	0.9	109	30	31.50	0.9	105
3,000	3,046	1.1	102	300	299.9	1.6	100
12,000	12,004	1.0	100	1,000	1,031	1.6	103

Quantification of imatinib and CGP74588 in patient plasma

In Paper IV, trough plasma concentrations of imatinib and its main metabolite, CGP74588, were quantified using liquid chromatography coupled to a mass spectrometer. Patient plasma was mixed with the internal standard trazodone and precipitated with methanol. Samples were separated using reversed-phase liquid chromatography on a C18 column with a gradient elution profile of methanol and 0.1% formic acid in water. The detection of imatinib, CGP74588, and trazodone was made in a time-of-flight mass spectrometer using m/z ranges of 494.15–494.3, 480.2–480.3, and 372.1–372.2, respectively.

Calibration curves were prepared in the ranges of 30–7,000 ng/mL for imatinib and 30–3,000 ng/mL for CGP74588. The respective calibration curve regressions were linear with coefficients of correlation >0.99. QC samples were prepared in three concentrations for intra- and inter-assay validation of the method. Imprecision was found to be less than 8% with accuracy ranging from 93% to 110% for both compounds (Table 5).

Table 5: Performance of LC-MS assay for quantification of imatinib and its main metabolite, CGP74588, in patient plasma.

Imatinib				CGP74588			
QC (ng/mL)	Mean conc. (ng/mL)	Imprecision (%)	Accuracy (%)	QC (ng/mL)	Mean conc. (ng/mL)	Imprecision (%)	Accuracy (%)
Inter-assay (n = 3)				Inter-assay (n = 3)			
100	109	4.9	109	30	28	6.9	93.3
1000	944	4.2	94.4	100	105	4.7	105
2,000	2,151	7.7	108	500	517	5.9	103
Intra-assay (n = 3)				Intra-assay (n = 3)			
100	106	5.1	106	30	33	6.3	110
1,000	1,031	3.9	103	100	103	4.4	103
2,000	1,892	7.2	94.6	500	487	6.4	97.4

RESULTS AND DISCUSSION

In vitro studies of ABCB1 and ABCG2 (Papers I and II)

Despite the efforts of previous investigators to determine the potential association of *ABCB1* and *ABCG2* genotypes with the pharmacokinetics or therapeutic outcome of imatinib in CML treatment, no consensus has yet been reached. The studies included in this thesis were designed to establish the effect of *ABCB1* and *ABCG2* genetic variants on the transport and efficacy of imatinib, its major metabolite, and the second generation TKIs *in vitro*.

TKIs as substrates of ABCB1 and ABCG2

The first step toward the evaluation of the *ABCB1* and *ABCG2* genotypes and their influence on the transport of imatinib and the second generation TKIs was to investigate how efficiently the wild-type proteins transport the different drugs. Cells expressing ABCB1 TTT [1236T; 2677T; 3435T] or ABCG2 wt were exposed to TKIs, and the protective effect of ABCB1 and ABCG2 against TKI cytotoxicity was evaluated along with the intracellular TKI accumulation as a function of

transporter expression (Figure 10). CGP74588 was the compound most affected by expression of ABCB1 TTT and ABCG2 wt and exceeded the influence of these transporters on its parent compound imatinib. Compared to control cells, CGP74588 had a 10- and 6.6-fold increase in IC_{50} in ABCB1 TTT- and ABCG2 wt-expressing cells, respectively. This should be viewed in relation to the more modest 1.5- and 1.6-fold increase that was found after exposing the same cells to imatinib. ABCB1 and ABCG2 expression also resulted in a significant reduction in accumulation of intracellular CGP74588 compared to control cells.
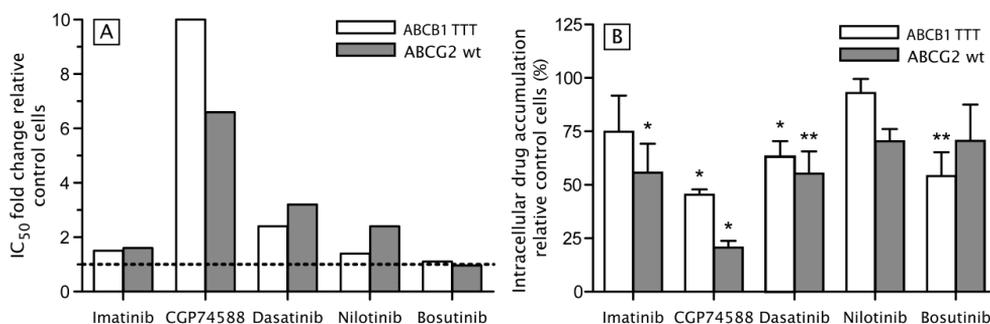

Figure 10: Influence of ABCB1 and ABCG2 expression on TKI efficacy and transport. A) Ratios of TKI mean IC_{50} in K562/ABCB1 TTT or K562/ABCG2 wt cells and control cells (K562/ve). A fold change of 1.0 is represented by the dotted line. B) Intracellular TKI accumulation in K562/ABCB1 TTT and K562/ABCG2 wt cells relative to control cells (K562/ve) after incubation with compounds at approximate IC_{50} concentrations obtained in parental K562 cells. Bars represent mean drug accumulation +SD as a percentage of the mean accumulation in K562/ve cells (n = 3). * = P <0.05; ** = P <0.01, Student's t-test.

Compared to CGP74588, the protective effect of ABCB1 TTT and ABCG2 wt expression against imatinib, dasatinib, and nilotinib cytotoxicity was small although a significant 1.4- to 3.3-fold increase in IC_{50} was seen in the ABCB1- and ABCG2-expressing cells, respectively. Of these compounds, dasatinib was the most strongly influenced. The protective effect was also reflected in a reduced intracellular accumulation of imatinib and dasatinib in K562/ABCG2 wt cells and of dasatinib in K562/ABCB1 TTT cells. No significantly reduced accumulation was observed for nilotinib in either cell type. The cellular resistance to bosutinib was not influenced by ABCB1 or ABCG2 expression when comparing transporter-expressing cell lines and control cells transduced with an empty vector. However, control cells appeared to be more resistant to bosutinib than parental K562 cells

(data shown in Papers I and II). Furthermore, ABCB1 expression conferred a significant reduction in intracellular accumulation of bosutinib.

Imatinib, dasatinib, and nilotinib have all previously been identified as substrates of the ABCB1 and ABCG2 transporters (*Dohse et al., 2010, Hegedus et al., 2009, Hiwase et al., 2008, Shukla et al., 2008*). However, most previous investigations were performed on the individual drugs and only a few studies have compared ABCB1 and ABCG2 transport of the different TKIs. One prior study has reported the comparison of imatinib, dasatinib, and nilotinib in terms of their transport by ABCB1 and ABCG2 *in vitro*. That study confirms our result of dasatinib being a superior substrate for ABCB1 compared to the other two. However, dasatinib and nilotinib seemed to be equally efficiently transported by ABCG2 (*Dohse et al., 2010*).

Because the protective effect of ABCB1 on imatinib, dasatinib, and nilotinib transport was fairly small, albeit statistically significant, we decided to compare the ABCB1 transport of TKIs to that of a well-known ABCB1 substrate. Vincristine is a vinca alkaloid commonly used in cancer chemotherapy and is susceptible to ABCB1-mediated multi-drug resistance (*Moore et al., 2009, Szakacs et al., 2006, Xia et al., 2012*). The exposure of K562/ABCB1 TTT cells to vincristine conferred a 17-fold increase in IC_{50} compared to control cells suggesting that imatinib, dasatinib, and nilotinib are all rather weak substrates of ABCB1. Only CGP74588 can be regarded as a relatively good substrate. These results suggest that because of the relatively inefficient ABCB1 transport, the influence of ABCB1 on the pharmacokinetics of imatinib, dasatinib, and nilotinib might not be as great as for traditional chemotherapeutic agents.

The pharmacokinetics of CGP74588 have not received much attention previously, and this is the first study to show that CGP74588 is transported by ABCG2 and is transported more efficiently than imatinib. In line with our findings, a recent study found that multi-drug resistant K562 cells expressing ABCB1 were resistant to and had reduced intracellular levels of CGP74588 compared to parental K562 cells (*Mlejnek et al., 2011*). The fact that ABCB1 and ABCG2 transport of CGP74588 is much more effective than that of imatinib suggests that variations in transport activity, e.g. by SNPs, might be more influential on CGP74588 than imatinib. This should be considered when associating *ABCB1* or *ABCG2* genotypes with the efficacy of imatinib treatment *in vivo*.

The evaluation of ABCB1 and ABCG2 transport of bosutinib encountered methodological issues. Even though ABCB1- and ABCG2-expressing cells were equally resistant to bosutinib as the control cells, the control cells showed protective effects to bosutinib cytotoxicity compared to parental K562 cells. However, there was no significant difference between control cells and parental cells in terms of bosutinib intracellular accumulation and, in contrast to the results from the cytotoxicity assay, ABCB1 expression resulted in a significantly lower accumulation of intracellular bosutinib. The reason for the effects of bosutinib but not of any of the other investigated TKIs on control cells could not be determined, and this precluded any firm conclusions being drawn from the investigations with bosutinib. Bosutinib has not been extensively investigated in terms of ABC transport before, but a single report showed that bosutinib is not a substrate of ABCB1 or ABCG2 transporters (*Hegedus et al., 2009*). Based on our data, however, we cannot rule out an ABCB1 influence on bosutinib transport.

In summary, we found that CGP74588 is extensively transported by ABCB1 and ABCG2 and that this transport by far exceeds that of imatinib. We could also confirm that imatinib, dasatinib, and nilotinib are transported by ABCB1 and ABCG2 even though in the case of ABCB1 neither of these drugs can be considered as very good substrates compared to vincristine. The effectiveness of both ABCB1 and ABCG2 transport was ordered as CGP74588 >> dasatinib > nilotinib = imatinib. Due to methodological issues, no firm conclusion could be reached with respect to ABCB1 and ABCG2 transport of bosutinib. It can be concluded that of the TKIs investigated here, CGP74588 and dasatinib are the compounds that should be most affected by variations in ABCB1 and ABCG2 transport activity *in vivo*.

ABCB1 haplotypes and their influence on TKI transport

Six variant haplotypes of the *ABCB1* gene were transduced into K562 cells, and the influence of these gene variants on ABCB1 protein expression in cell membranes as well as on the protective effects of ABCB1 against TKI cytotoxicity were investigated.

All *ABCB1* haplotypes conferred a lower level of membrane expression than what was observed in cells carrying the TTT haplotype (Figure 11A). However, after

correcting the ABCB1 expression for the level of expressed EYFP reporter protein only the CGC haplotype showed an increase in membrane expression (Figure 11B). These findings indicate that the reduced expression levels of the ABCB1 variants in cell membranes were not due to their different genetic backgrounds but to variations in vector expression levels. We concluded that except for the CGC haplotype none of the *ABCB1* variants influenced membrane expression.
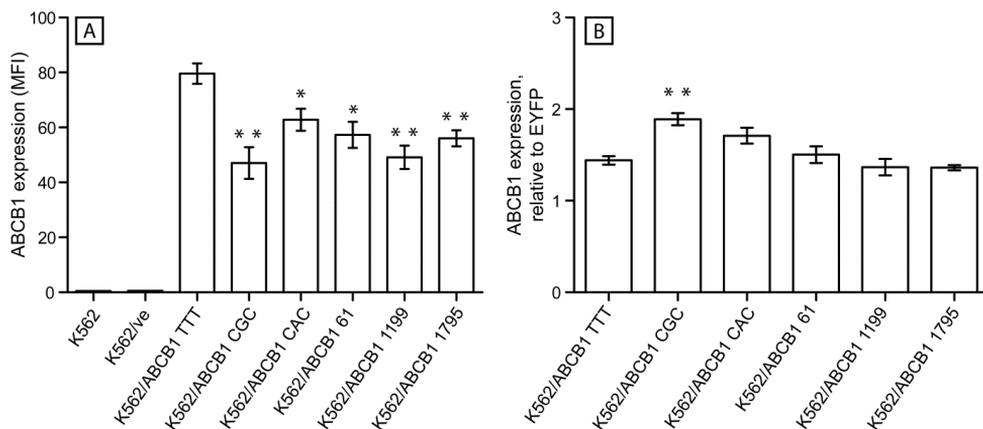

Figure 11: Influence of genotype on ABCB1 membrane expression. A) Uncorrected median fluorescence (MFI) of ABCB1 (n = 3). B) ABCB1 MFI normalized to enhanced yellow fluorescent protein (EYFP) MFI (n = 3). Bars represent the mean of three replicates ± s.e.m. * = *P* <0.05; ** = *P* <0.01, Student's *t*-test using K562/ABCB1 TTT as the reference cell line.

Analyzing the protective effect of *ABCB1* variants against TKI cytotoxicity revealed that, except in the case of the *ABCB1* CGC haplotype, none of the ABCB1 variants significantly influenced cell survival compared to the TTT haplotype (Figure 12). K562/ABCB1 CGC cells had reduced protection against imatinib, CGP74588, and dasatinib. However, these cells also had the lowest expression of uncorrected ABCB1 protein in the cell membrane indicating that the reduced protection against TKI cytotoxicity might have been due to reduced vector activity in this particular cell line rather than an effect of this specific haplotype.

In line with our findings, all possible combinations of different nucleotides of *ABCB1* 2677G>T/A in combination with those of 3435T>C have previously been expressed in porcine kidney epithelial cells. No difference in ABCB1 protein expression or transport function was detected in those experiments
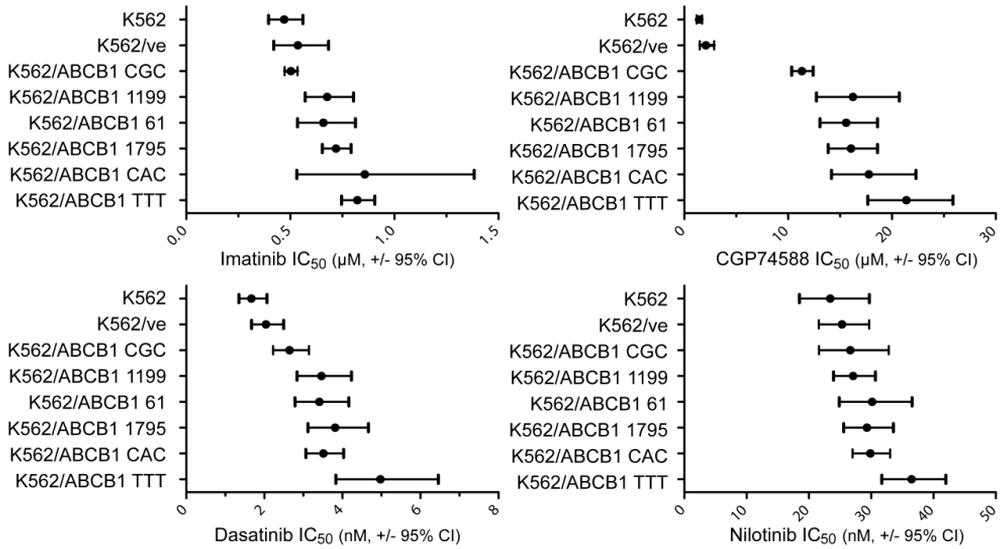
Figure 12: Influence of *ABCB1* haplotypes on TKI cytotoxicity. K562 cells transduced with *ABCB1* haplotypes were exposed to serial dilutions of TKIs followed by analysis of cell survival. TKI mean IC_{50} (•) ± 95% confidence intervals are presented for each cell line treated with the respective TKI.

(*Morita et al., 2003*). Moreover, Kimchi-Sarfaty *et al.* studied the *ABCB1* SNPs 61A>G, 307C>T, 1199G>A, 2677G>T, and 2995G>A expressed individually in HeLa cells but could not find any effects of the SNPs on the transport of substrates (*Kimchi-Sarfaty et al., 2002*).

More than a dozen studies have been published on the possible association of *ABCB1* genotype with imatinib pharmacokinetics or with therapeutic outcome (*Angelini et al., 2013, Deenik et al., 2010, Dulucq et al., 2008, Gardner et al., 2006, Gurney et al., 2007, Kim et al., 2009, Marin et al., 2010, Ni et al., 2011, Seong et al., 2012, Singh et al., 2012, Vivona et al., 2012*), and additional studies are still under way (*Dulucq et al., 2010*). Most of the studies were based on 50 to 100 CML patients and almost exclusively concern the 1236, 2677, and 3435 SNPs that constitute the TTT, CGC, and CAC haplotypes studied in this thesis. The conclusions from these studies deviate considerably. The two largest studies by Kim *et al.* and Angelini *et al.* included 229 and 189 CML patients, respectively (*Angelini et al., 2013, Kim et al., 2009*). Kim *et al.* investigated the *ABCB1* 1236, 2677, and 3435 SNPs and could not find any association with imatinib response parameters. Angelini *et al.* were the first to also study additional SNPs. In contrast to Kim *et al.*, a near significance was found between a promoter SNP, the 3435 SNP, and imatinib molecular response

rates. When stratifying the population based on ethnicity, the significance between the 3435 SNP and molecular response became clear. The discrepancy between these two studies might in part be due to population specific traits because Angelini *et al.* show an increased significance in a population specific analysis. Together, these studies might indicate that the influence of *ABCB1* genotype on imatinib treatment is small. Large and well defined patient populations are needed in order to clearly demonstrate any potential influence of *ABCB1* SNPs.

Like Kim *et al.*, we did not see an influence on TKI transport by the most common *ABCB1* SNPs at positions 1236, 2677, and 3435 in the gene. The significant association of *ABCB1* genotype and imatinib response found by Angelini *et al.* was suggested to be due to the synonymous SNP 3435T>C. In our study, only post-translational effects could be investigated because our method of choice was based on an artificial transcription. This means that our results do not necessarily contradict those of Angelini *et al.* but underscore the notion that other regulatory mechanisms or pre-translational effects of *ABCB1* SNP's might influence TKI transport.

In conclusion, the post-translational effects of the *ABCB1* haplotypes studied here did not alter the protective effects of ABCB1 against TKI cytotoxicity. This finding indicates that if there is a significant influence of *ABCB1* genotype on TKI pharmacokinetics and clinical outcome it should be sought in other regulatory mechanisms or pre-translational effects of SNPs.

ABCG2 SNPs and their influence on TKI transport

The investigations of *ABCG2* SNPs and their influence on TKI transport followed a similar experimental design as the investigations of *ABCB1* SNPs in which the effects of SNPs were evaluated in terms of cell membrane expression and protection against TKI cytotoxicity in cell survival assays. In contrast to *ABCB1*, several *ABCG2* SNPs were found to have a significant impact on TKI transport. Analyzing the membrane expression of variant ABCG2 proteins showed that all variant cell lines had lower levels of membrane expression than those carrying the gene for wild-type *ABCG2* (Figure 13A). Correcting the ABCG2 expression level for expression of the EYFP reporter confirmed that the lower expression levels of the 421C>A, 623T>C, 886G>C, and 1574T>G SNPs were not associated with a
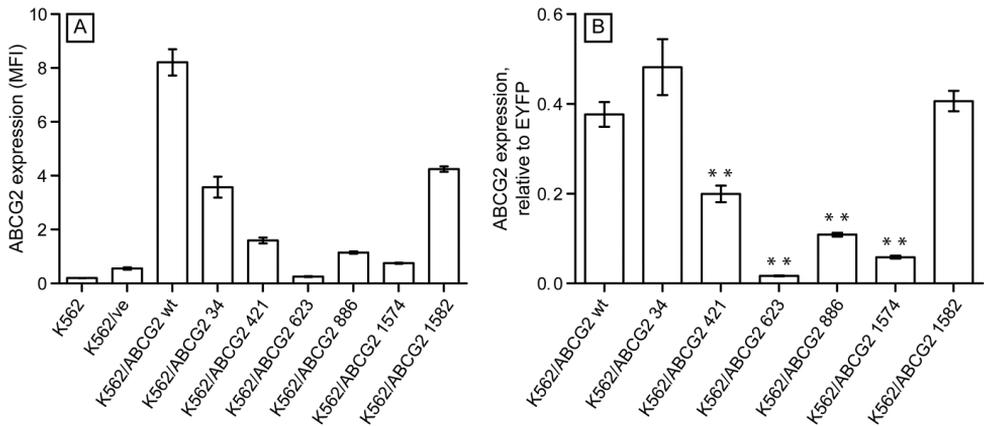
Figure 13: Influence of genotype on ABCG2 membrane expression. A) Uncorrected median fluorescence (MFI) of ABCG2 (n = 3). B) ABCG2 MFI normalized to enhanced yellow fluorescent protein (EYFP) MFI (n = 3). Bars represent the mean of three replicates ± s.e.m. ** = P <0.01, Student's t-test using K562/ABCG2 wt as the reference cell line.

lower degree of vector activity but were a direct result of the genetic variants (Figure 13B). However, the 34G>A and 1582G>A variants did not affect ABCG2 membrane expression.

These findings were reflected in the protective effects of the ABCG2 variants against TKI cytotoxicity (Figure 14). The most prominent effects were seen for the 623T>C SNP that did not show any detectable membrane ABCG2 expression and thus completely abolished the protective effect of ABCG2 irrespective of the TKI type applied. Also, K562/ABCG2 34, 421, 886, and 1574 were significantly less resistant to all TKIs compared to cells with wild-type ABCG2 expression, and K562/ABCG2 1582 had reduced resistance against all compounds except nilotinib. Based on the results of the membrane expression assay, the reduced resistance in K562/ABCG2 34 and 1582 could have been due to the lower expression of these vectors.

Although the 623T>C SNP has to our knowledge not previously been investigated *in vivo*, the pronounced effects of 623T>C on ABCG2 functionality found here are supported by the marked reduction of ABCG2 expression in insect Sf9 as well as human cell membranes expressing the 623C allele (*Nakagawa et al., 2008, Tamura et al., 2006*). The reduced expression is probably due to increased ABCG2 623C susceptibility to proteasomal degradation as compared to the wild type protein (*Nakagawa et al., 2008*).
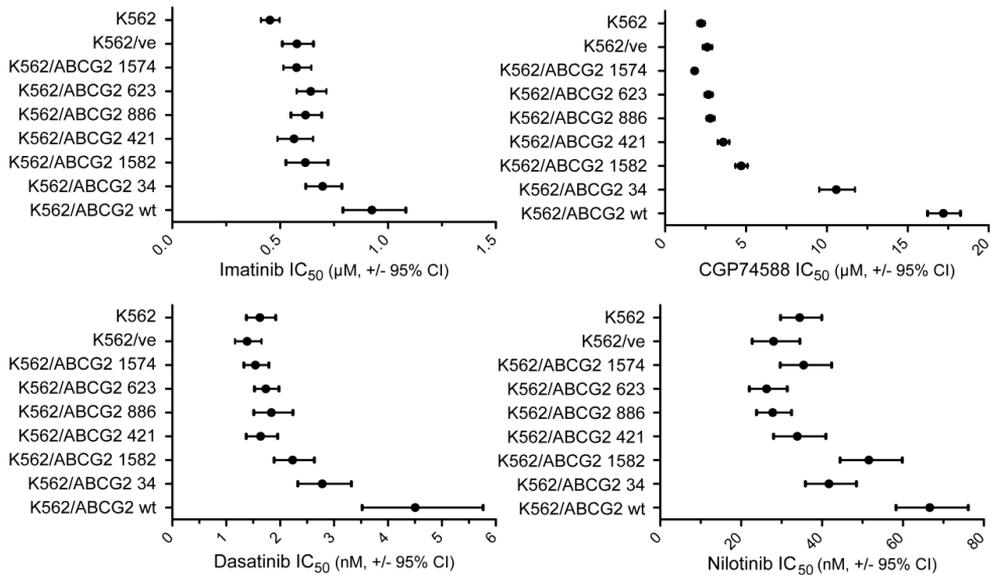
Figure 14: Influence of *ABCG2* SNPs on TKI cytotoxicity. K562 cells transduced with *ABCG2* SNPs were exposed to serial dilutions of TKIs followed by analysis of cell survival. TKI mean IC_{50} (•) ± 95% confidence intervals are presented for each cell line treated with the respective TKI.

The 421C>A SNP has previously shown a reduced level of membrane incorporation due to increased susceptibility to proteasomal degradation (*Furukawa et al., 2009, Kondo et al., 2004, Morisaki et al., 2005*). It has also been shown that the 421A allele results in reduced capacity for cellular efflux of imatinib (*Gardner et al., 2006*), and a number of smaller *in vivo* studies have identified a significant influence of this SNP on the pharmacokinetic parameters or the outcome of imatinib (*Petain et al., 2008, Seong et al., 2012, Takahashi et al., 2010*). Our findings also support the results from a large-scale study on CML patients in which the 421C allele was associated with reduced probability of achieving cytogenetic and molecular responses to imatinib (*Kim et al., 2009*). This finding is indirectly in line with our results of 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 rare *ABCG2* 886G>C and 1574T>G alleles have not been functionally investigated before, and our results showed a reduced expression and transport activity of these variants. The basis for their influence on ABCG2 functionality can only be speculated on at this time. The 886G>C SNP results in a substitution (Asp296His) in one of the intracellular loops of the ABCG2 protein structure that

does not seem to be involved in any particularly important functions of the protein such as dimerization, substrate binding, or membrane incorporation (*Polgar et al., 2008*). Nonetheless, the substitution from a negatively to positively charged 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 at position 525 in the trans-membrane domain of ABCG2. This suggests that the effect on ABCG2 function by this SNP might be the result of reduced incorporation within the non-polar cell membrane.

The 34G>A and 1582G>A SNPs resulted in reduced protection against 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 SNPs themselves. In a previous study, *ABCG2* 34G>A resulted in higher resistance to the camptothecin analog SN-38 compared to wild-type ABCG2 expressed in human cell lines (*Tamura et al., 2007*), but we found no increase in resistance to TKIs in K562/ABCG2 34 cells. Furthermore, Kim *et al.* found that the 34G allele was associated with a reduced likelihood of achieving complete cytogenetic responses to imatinib treatment. However, they also found that the 34G>A SNP was in linkage disequilibrium with the 421C>A SNP (*Kim et al., 2009*). 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.

In summary, the *ABCG2* 421C>A, 623T>C, 886G>C, and 1574T>G SNPs decrease the level of ABCG2 protein expression in the cell membrane. Our data support previous *in vivo* findings that the 421C>A SNP could be a determinant of imatinib pharmacokinetics and outcome. The 623T>C, 886G>C, and 1574T>G SNPs all had a reduced level of detectable ABCG2 in the cell membrane and low transport capacity of TKIs, and this effect was most prominent in the 623T>C variant cell line. Although these SNPs are rare, their effects on transport function were substantial and should be considered in future studies of large patient cohorts to validate their significance *in vivo*.

In vivo studies of CYP3A activity (Papers III and IV)

CML patients were investigated in terms of CYP3A activity and its significance for imatinib pharmacokinetics and therapeutic outcome in two separate studies. The characteristics of the patient populations in the respective studies are shown in Table 6.

Table 6: Characteristics of CML patients studied in Papers III and IV

	Patients, n	M/F, %	Median age, years (range)	Sokal high risk, % (evaluable patients)	Treatment prior to imatinib	Primary endpoint[a]
Pilot study (Paper III)	14	57/43	51 (20–79)	25 (n = 8)	All allowed	CMR
Follow-up (Paper IV)	55	64/36	60 (18–87)	40 (n = 43)	allopurinol, hydroxyurea (interferon, n = 1)	CCgR or *BCR-ABL1*<1%

M: male; F: female; CMR: complete molecular response; CCgR: complete cytogenetic response

[a]Evaluated 12 months after start of imatinib treatment

CYP3A significance for imatinib outcome in the pilot study

The outcome of imatinib treatment in the 14 CML patients included in the pilot study was evaluated based on achievement of a complete molecular response 12 months after start of imatinib treatment. The patients were grouped according to response level, and six patients had achieved a complete molecular response while eight patients had not. The assessment of *in vivo* CYP3A activity revealed a 5-fold variation in the whole set of patients with quinine metabolic ratios ranging from 6.2 to 33.3. Comparing the CYP3A activity between patients that achieved a complete molecular response and those who did not showed that complete molecular response patients had significantly higher levels of CYP3A activity (lower quinine metabolic ratio) (Figure 15). The association of high CYP3A activity to better outcome indicated not only a clinical significance of CYP3A activity for imatinib outcome but also a potential role of imatinib CYP3A metabolites. This pilot study lacked plasma samplings for quantification of imatinib metabolites and the conclusion was based only on a small number of patients. Therefore, we decided to design a follow-up study for validation of these preliminary findings.
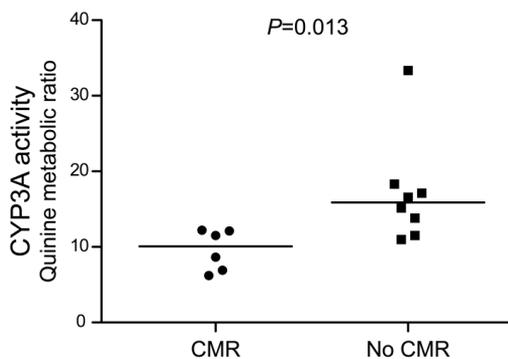
Figure 15: CYP3A activity in CML patients based on level of molecular response to imatinib after 12 months of treatment. Complete molecular responders (CMR) had significantly lower quinine metabolic ratios (higher CYP3A activities) than those who did not achieve this level of response. Horizontal lines represent the median CYP3A activity (n = 14).

Influence of CYP3A on imatinib pharmacokinetics and outcome

Fifty-five CML patients were included in the follow-up study of which eight patients where prospectively included. Forty-three patients were evaluable for imatinib outcome, and this was analyzed 12 months after the start of imatinib treatment. Not all patients had an evaluable complete cytogenetic response, which, according to ELN guidelines, reflects the optimal response at the 12 month time point (*Baccarani et al., 2009*). However, other investigators have shown that a complete cytogenetic response corresponds to a molecular response level of *BCR-ABL1* <1% (*Hanfstein et al., 2012, Hehlmann et al., 2011*). In order to optimize for as large a sample size as possible, optimal response was defined here as either the achievement of complete cytogenetic response or *BCR-ABL1* <1%. The traditional response parameters of complete cytogenetic response and major molecular response were analyzed separately as well.

Similar to the pilot study, the CYP3A activity showed a 5-fold variation in the total set of patients. However, in contrast to the findings of the pilot study, no influence of CYP3A activity was found on the achievement of any of the investigated response parameters (Figure 16).
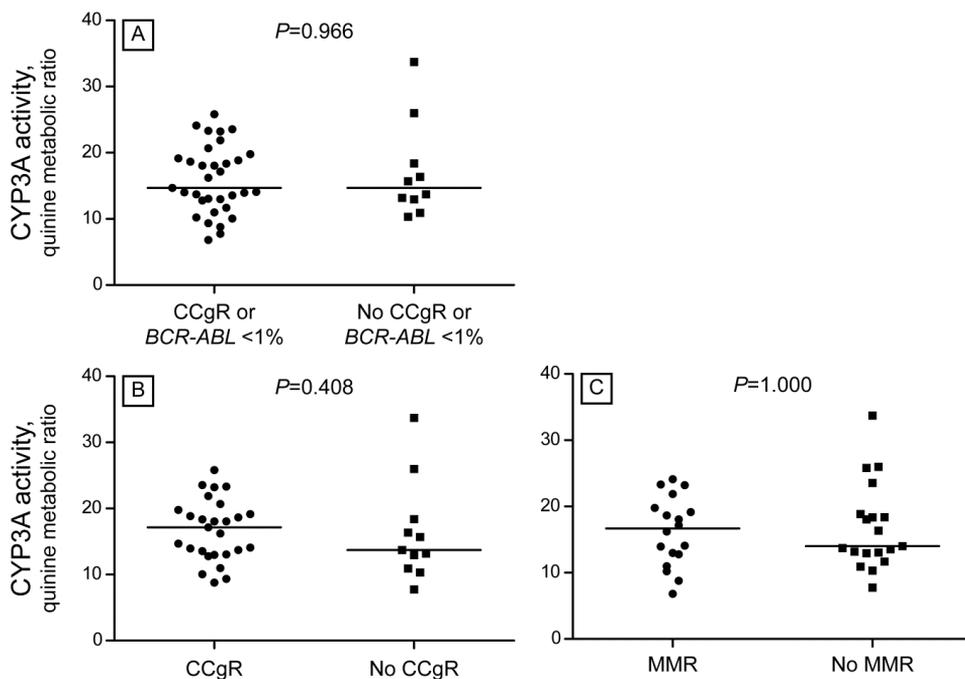
Figure 16: CYP3A activity in CML patients based on the level of response to imatinib after 12 months of treatment. In A), patients who achieved a complete cytogenetic response (CCgR) or a *BCR-ABL* <1% were compared to those who did not achieve either of these response levels (n = 43). In B) and C), CCgR and major molecular response (MMR) were evaluated as separate response parameters (B, n = 38; C, n = 37). Horizontal lines represent the median CYP3A activity in the respective group.

The reason for this discrepancy is most likely the limited number of patients in the pilot study, and this confirms the importance of validating significant outcomes from small patient cohorts. In addition, the fact that not all patients in the pilot study received imatinib as a first-line treatment might have included biases that affected the outcome of the study.

Out of the 55 CML patients that were studied in terms of CYP3A activity, 34 patients were successfully sampled for steady-state trough plasma concentrations of imatinib and 31 patients were successfully sampled for steady-state trough plasma concentrations of CGP74588. Imatinib and CGP74588 showed large inter-individual variations with concentrations ranging from 509 ng/mL to 3485 ng/mL for imatinib and 65 ng/mL to 829 ng/mL for CGP74588. However, the CYP3A metabolic activity did not explain the variations in imatinib plasma concentration. There was also no association between CYP3A activity and the CGP74588/imatinib plasma concentration ratio (Figure 17).
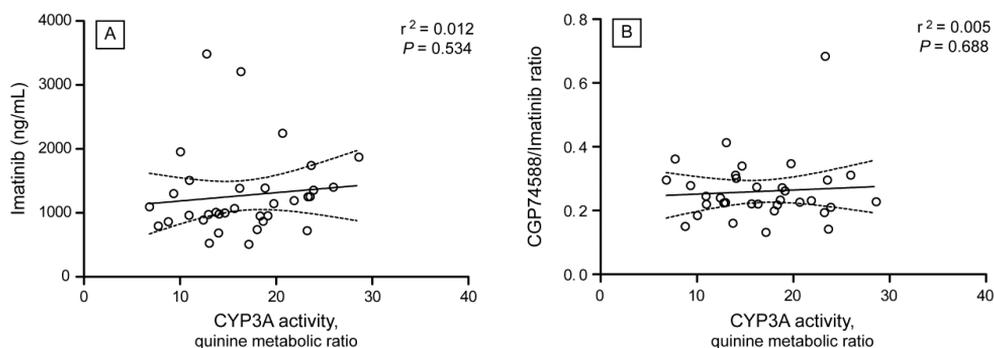
Figure 17: Correlation of plasma levels of imatinib (A) and CGP74588/imatinib ratios (B) with CYP3A metabolic activity (n = 34). The linear regression (solid lines) is plotted in the figures together with the ±95% confidence interval (dotted lines) of the same.

The follow-up study with a more homogenous patient population and a larger sample size provided a more reliable data set from which it could be concluded that variable CYP3A activity in the range observed in our studies does not influence the plasma concentration or outcome of imatinib. However, drug plasma concentrations are the result of all pharmacokinetic events including absorption, distribution, metabolism, and elimination. These findings indicate that despite the fact that imatinib is metabolized by CYP3A, metabolic activity is not the rate-limiting step in imatinib elimination and that there should be other, more influential, pharmacokinetic parameters that could explain variable imatinib levels in plasma.

The finding that CYP3A activity did not correlate with the CGP74588/imatinib ratio might indicate altered pharmacokinetics of the metabolite compared to the parent compound. One possible explanation for this might be the fact that CGP74588 was shown to be a far better substrate for ABCB1 and ABCG2 transport than the parent compound in Papers I and II. A non-uniform excretion of imatinib versus its metabolite by efflux pumps might influence the plasma concentration ratio of the two and overpower the influence of CYP3A metabolic activity on the accumulation of CGP74588 in plasma.

It has previously been shown that imatinib pharmacokinetics are altered when it is co-administered with potent CYP3A modulators such as ketoconazole or rifampicin (*Bolton et al., 2004, Dutreix et al., 2004, Frye et al., 2004, Smith et al., 2004*)

indicating that under certain circumstances imatinib is influenced by CYP3A activity. However, the magnitude of CYP3A variation might play a role here. Other studies have reported that a 20-fold variation in intrinsic CYP3A activity should be expected (*Wilkinson, 1996*), and we cannot rule out that extreme intrinsic CYP3A activities might influence imatinib elimination and outcome.

The results from measuring *in vivo* CYP3A activity using different probe drugs do not always correlate with each other (*Kinirons et al., 1993, Krivoruk et al., 1994, Stein et al., 1996*). In light of these data, it is possible that quinine does not properly reflect imatinib pharmacokinetics. The lack of correlation in previous studies was suggested to be due to differential pharmacokinetic properties of the probes such as extra-hepatic metabolism or the route of administration. In our study, quinine was believed to be the most optimal choice of probe because it has the same route of administration and a similar pharmacokinetic profile as imatinib. There is still a possibility though, that differences in pharmacokinetics might influence the correlation between quinine metabolic ratios and imatinib plasma concentrations.

CONCLUSIONS

★ The variant *ABCB1* haplotypes studied here do not alter the ABCB1 transport activity of imatinib, CGP74588, dasatinib, or nilotinib *in vitro*.

★ The *ABCG2* SNPs 421C>A, 623T>C, 886G>C, and 1574T>G impair the *in vitro* transport of imatinib, CGP74588, dasatinib, and nilotinib and could possibly influence transport of these TKIs *in vivo*.

★ The pharmacologically active imatinib metabolite, CGP74588, is a better substrate for both ABCB1 and ABCG2 than imatinib. This finding might have implications in patients with high CYP3A activity in combination with reduced ABCB1 and ABCG2 transport.

★ The 5-fold variability of CYP3A metabolic activity identified using quinine as a probe drug does not influence plasma concentrations or therapeutic outcome of imatinib in CML patients. Although imatinib is primarily metabolized by CYP3A, the enzyme activity is not the rate-limiting step in imatinib elimination.

FUTURE ASPECTS

To fully understand the role of CYP3A activity in imatinib pharmacokinetics, it might be of interest to investigate patients with a larger range of CYP3A activities than what could be observed in the present work. Such studies would elucidate the potential influence of CYP3A activity on imatinib pharmacokinetics also in extreme metabolizers. Furthermore, our conclusion with respect to CYP3A could be made firmer if validation was performed using additional CYP3A probe drugs.

In patients with an average CYP3A phenotype, the source of variability in imatinib plasma concentrations is most likely found in other pharmacokinetic parameters than metabolic activity. For example, we found that the *ABCG2* 421C>A, 623T>C, 886G>C and 1574T>G SNPs clearly impair ABCG2 transport function and are interesting candidates for future studies. Although the majority of these SNPs are rare, their significance *in vivo* should be determined since large-scale genotyping techniques are likely to become more accessible in the future, facilitating the analysis also of rare traits that do not seem efficient to investigate at present. Moreover, the *ABCG2* SNPs with reduced function should be further investigated in order to determine the functional mechanism of altered transport functions which might not only prove important for the TKIs but perhaps also for other ABCG2 substrate drugs.

No prominent effects on TKI transport and activity were found of the *ABCB1* haplotypes studied here, indicating that future TKI studies of ABCB1 should be focused on pre-translational mechanisms. However, imatinib and the second generation TKIs proved to be rather poor substrates of ABCB1 compared to vincristine, indicating that ABCB1 transport might be more interesting to study in relation to other chemotherapeutic drugs than TKIs.

Expanded knowledge of the interplay between imatinib metabolism and transport might be useful. So far, little is known about the role of the active imatinib metabolite CGP74588 in CML treatment. This CYP3A metabolite was shown to be more efficiently transported by ABCB1 and ABCG2 than its parent compound, and therefore, it might also be more sensitive to variations in transporter activity. It would be interesting to study CGP74588 accumulation in patients with a combination of high metabolism and reduced transport function. This strategy might provide explanations to the fact that some patients have a sufficient response to therapy despite a low imatinib plasma concentration. However, CML is a rare disease and this makes it difficult to study rare traits with sufficient power. The main challenge for future investigations is to achieve sufficiently large and well characterized study populations that will enable stratification of high and low enzyme activity in combination with transporter genotypes.

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