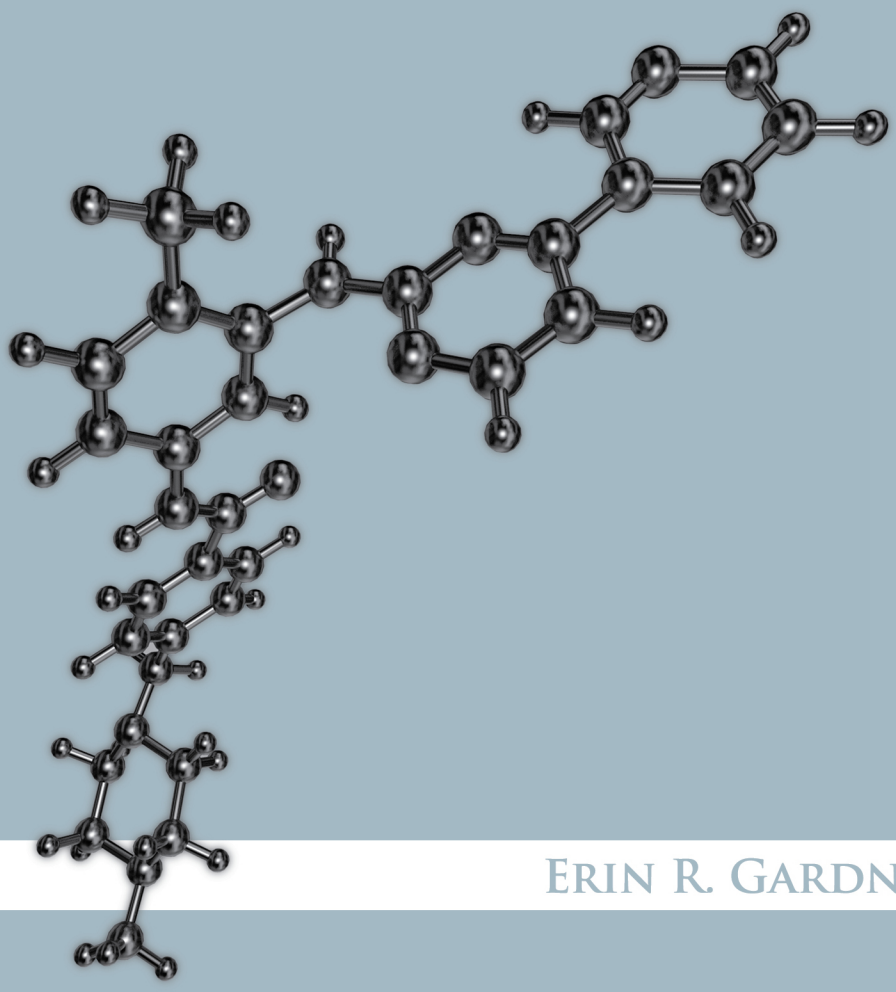
Factors Affecting Pharmacokinetic Variability of imatinib Mesylate



Erin R. Gardner

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Factors Affecting Pharmacokinetic Variability of Imatinib Mesylate (Gleevec, STI-571)

Factoren van invloed op de farmacokinetische variabiliteit van imatinib mesylate (Glivec, STI-571)

Thesis

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Chapter 1

Introduction to the Thesis

Imatinib mesylate (Gleevec, Glivec) is a rationally designed tyrosine kinase inhibitor, originally created to target Bcr-Abl in chronic myelogenous leukemia (CML). This synthetic small molecule was subsequently found to have activity in gastrointestinal stromal tumors (GIST) against c-kit and is now approved for both indications worldwide. Clinical studies are currently underway to evaluate imatinib in a range of additional malignancies.

Initial clinical response to imatinib was extremely promising. In addition to impressive results in chronic-phase CML (one study reported complete hematological and cytogenic responses in 95% and 74% of patients, respectively), imatinib was shown to significantly improve the previously poor prognosis of individuals with unresectable or metastatic GIST [1,2]. Unfortunately, along with primary resistance noted in patients with advanced CML, acquired resistance to imatinib developed in a number of individuals. Numerous mechanisms of resistance have been identified, many of which involve genetic changes in Bcr-Abl or c-kit. However, it has also been suggested that decreased plasma concentrations of imatinib, leading to sub-optimal tumor cell exposure, may also be contributing [3].

Imatinib is typically administered once daily as an oral tablet and exhibits very high bioavailability (98%) [4]. Though the pharmacokinetics of this agent are considered to be linear over a wide dose range, substantial interindividual variability has been observed in many studies, yet remains largely unexplained.

The aim of the work described in this thesis was to not only improve understanding of the factors affecting the pharmacokinetic variability of imatinib in patients with cancer, but to determine whether these factors might be contributing to pharmacokinetic resistance and assess strategies for overcoming this.

This research was initiated following the identification of imatinib as a substrate for both ABCB1 (P-glycoprotein) and ABCG2 (BRCP) [5,6]. The role of these polymorphic transporters as mechanisms of resistance to anticancer drugs is reviewed in Chapter 2.

Imatinib is highly metabolized by Cytochrome P450 isozymes 3A4 and 3A5, both of which are polymorphic [4]. Midazolam, a sedative, can be used as a phenotyping probe to assess CYP3A4 and CYP3A5 activity *in vivo* [9]. Chapter 3 describes the method development and validation of an analytical assay to measure midazolam concentrations in human plasma. Chapter 4 evaluates the impact of polymorphisms in *CYP3A4* and *CYP3A5* on the activity of the encoded enzymes in patients with cancer, by determining the correlation between midazolam pharmacokinetics and genotype.

Combining anticancer agents with inhibitors of transporter proteins has been tested clinically for substrates of ABCB1 and remains of interest for modulation of tumor exposure to substrate drugs. We hypothesized that addition of an ABCG2 inhibitor to an imatinib treatment regimen may increase *in vivo* drug exposure (through decreased drug efflux in the gut and liver) and potentially modulate brain penetration, due to the expression of ABCG2 on endothelial cells in the brain. Chapter 5 evaluates the pharmacokinetic effect of administering tariquidar, an inhibitor of both ABCB1 and ABCG2, prior to oral treatment with imatinib in mice.

Judson *et al.* reported on the clinical observation that drug clearance appeared to increase with chronic drug treatment, suggesting that imatinib may be inducing its own efflux or metabolism [10]. Burger *et al.* subsequently demonstrated an upregulation of both ABCB1 and ABCG2 in Caco-2 cells that were treated chronically, leading to increased drug efflux [11]. In Chapter 6, the effect of chronic imatinib treatment on plasma pharmacokinetics and the expression of these transporters was evaluated *in vivo*.

A letter by Miyazwa *et al.* appeared in the International Journal of Hematology, questioning whether 400 mg daily imatinib is an optimal starting dose of imatinib in Japanese patients, after observing much higher rates of thrombocytopenia than had previously been seen in a large multi-center study performed in Europe and the US [7]. In contrast, doses of up to 800 mg per day have been relatively well tolerated in a number of studies performed in the US [8]. These observations further supported the hypothesis that genetic variation could be

contributing to the pharmacokinetic variability. In Chapter 7, correlations between the pharmacokinetics of imatinib and polymorphisms in a range of transporters and enzymes, including ABCB1, ABCG2, CYP3A4 and CYP3A5, were explored in a large population of patients with cancer, to determine whether these mutations might contribute to the large interindividual variability in imatinib pharmacokinetics.

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Chapter 2

Mechanisms of Resistance to Anticancer Drugs: the Role of Polymorphic ABC Transporters ABCB1 and ABCG2

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Summary

ATP-binding cassette (ABC) genes play a role in the resistance of malignant cells to anticancer agents. The ABC gene products, including ABCB1 (P-glycoprotein) and ABCG2 (BCRP, MXR, ABCP), are also known to influence oral absorption and disposition of a wide variety of drugs. As a result, the expression levels of these proteins in humans have important consequences for an individual's susceptibility to certain drug-induced side effects, interactions, and treatment efficacy. Naturally occurring variants in ABC transporter genes have been identified that might affect the function and expression of the protein. This review focuses on recent advances in the pharmacogenetics of the ABC transporters ABCB1 and ABCG2, and discusses potential implications of genetic variants for the chemotherapeutic treatment of cancer.

Introduction

Cells exposed to toxic compounds can develop resistance by a number of mechanisms including decreased uptake, increased detoxification, alteration of target proteins, or increased excretion [1]. Several of these pathways can lead to multidrug resistance (MDR) in which the cell is resistant to several commonly used drugs in addition to the initial compound to which it was exposed. This is a particular limitation to cancer chemotherapy, and cells with an MDR phenotype often display other properties, such as genome instability and loss of checkpoint control, that complicate further therapy [1]. The ATP-binding cassette (ABC) genes play an important role in MDR, and various genes have been identified that are associated with altered anticancer drug transport. These genes belong to a large family of transmembrane proteins that bind and subsequently hydrolyze ATP, using the energy to drive the transport of various molecules across cell membranes [2-4].

It is now well established that ABC gene products may also play a major role in host detoxification and protection against xenobiotic substances [5]. Indeed, genetic knock-outs of murine ABC transporter genes have shown altered blood-brain barrier function [6,7], intestinal drug absorption [8,9], fetal drug exposure [10], and drug-induced damage to testicular tubules [11], choroid plexus epithelium and oropharyngeal mucosa [12]. Furthermore, recent re-sequencing of various human ABC transporters has revealed a number of naturally-occurring allelic variants that appear to affect the functional activity of the encoded protein *in vivo* [13-15]. This genetic variation may potentially modulate transporter phenotypes in humans and thereby affect their predisposition to toxicity and response to drug treatment. This review provides an update on the pharmacogenetics of the two best characterized ABC transporters, ABCB1 (P-glycoprotein) and ABCG2 (BCRP, MXR, ABCP), and discusses the potential implications of genetic variation of the proteins on anticancer drug response in patients.

Structural organization of the ABCB1 and ABCG2 genes

Proteins are classified as ABC transporters based on the sequence and organization of their ATP-binding domains, also known as nucleotide-binding folds (NBFs). The NBFs contain characteristic motifs (viz. the Walker A and B motifs), separated by approximately 90–120 amino acids, found in all ATP-binding proteins. ABC genes also contain an additional element, the signature (C) motif, located upstream of the Walker B site [16]. The functional protein typically contains two NBFs and two transmembrane domains. These domains contain 6–11 membrane-spanning α-helices which can form pores for allowing molecules through the membrane and therefore dictate substrate specificity. The NBFs are located in the cytoplasm and transfer the energy to transport the substrate across the membrane. ABC transporters are mostly unidirectional. In eukaryotes, most ABC genes move compounds from the cytoplasm to the outside of the cell or into an intracellular compartment (e.g., the endoplasmic reticulum, mitochondria, or peroxisome). Most of the known functions of eukaryotic ABC transporters involve the shuttling of hydrophobic compounds either within the cell as part of a metabolic process or outside the cell for transport to other organs or for excretion from the body.

The eukaryotic ABC genes are organized either as full transporters containing two transmembrane domains and two NBFs, or as half transporters. The latter must form either homodimers or heterodimers to form a functional transporter. ABC genes are widely dispersed in eukaryotic genomes and are highly conserved between species, including yeast, indicating that most of these genes have existed since the beginning of eukaryotic evolution [17]. The genes can be divided into subfamilies based on similarity in gene structure (half *versus* full transporters), order of the domains, and on sequence homology in the NBF and TM domains. There are seven mammalian ABC gene subfamilies, and all have standard nomenclature, developed by the Human Genome Organization (see: http://www.humanabc.org) [18].

Physiological and pharmacological function

Two ABC genes appear to account for nearly all of the MDR phenotypes in both humans and rodent models, which are selected with natural product cytotoxic drugs in vitro and show decreased drug accumulation: *ABCB1*[1] and *ABCG2* encoding ABCB1 (P-glycoprotein) and ABCG2 [formerly breast cancer resistance protein (BCRP), mitoxantrone resistance protein (MXR), or ABC transporter in placenta (ABCP)], respectively. Overexpression of these genes confers resistance to a wide variety of hydrophobic natural product drugs [19].

Inhibitors of the major ABC genes contributing to MDR have been developed, and extensive preclinical and clinical research has been carried out aimed at blocking the development of drug resistance during chemotherapy. Moreover, activity of the ABC transporter proteins can be blocked with doses of the inhibitor that do not have adverse side effects or significantly affect the pharmacokinetic profile of the anticancer drug regimen [20,21]. However, early ABCB1 inhibitors have not been very successful at reversing MDR in clinical trials, for a variety of reasons, including poor pharmacokinetic profiles and the inability to accurately evaluate the effect on ABCB1[22].

ABCB1

The *ABCB1* gene maps to chromosome 7q21.1 and is the best characterized ABC drug transporter. It consists of 28 exons and 27 introns. Formerly known as *MDR1* or *PGY1*, *ABCB1* was the first human ABC transporter gene cloned and characterized through its ability to confer an MDR phenotype to cancer cells that had developed resistance to certain chemotherapy drugs [23]. The gene product ABCB1 (P-glycoprotein) is a 170 kDa, 1280 amino acid protein: two 610 amino acid half-transporters, each comprised of one TM and one NBF, joined by a sixty amino acid flexible linker. It has been shown to be a promiscuous transporter of a large number of hydrophobic substrates from diverse therapeutic classes [15], including several anticancer drugs [24], and its

[1] Italicized symbols designate genes, alleles or loci to distinguish them from phenotype. All Human Gene Mapping designations are capitalized; for genes from other species, the nomenclature committee recommendation is given.

function can be inhibited by a large variety of chemical compounds (Table 1). The gene is expressed in multiple healthy organs, and is thought to play an important role in removing toxic substances or metabolites from cells. For example, the protein is highly expressed in cells at the blood–brain barrier and presumably plays a role in transport of toxic compounds out of the brain, effectively preventing uptake [25].

The ABCB1 protein is also expressed in many excretory cell types such as kidney, liver, intestine, and adrenal gland, where the normal physiological function is thought to involve the secretion of toxic xenobiotics and their metabolites, in addition to steroids [26-28]. Mice have two closely related homologues of *ABCB1*, *Abcb1a* and *Abcb1b*. Mice homozygous for disrupted *Abcb1* genes are phenotypically normal but are extremely sensitive to certain neurotoxins such as ivermectin [6,7]. These studies led to the characterization of an important role of ABCB1 in transport of several agents across the blood-brain barrier, since lack of gene expression led to an accumulation within the brain. ABCB1 is also highly expressed in hematopoietic stem cells, where it may serve to protect these cells from toxic substrates [29,30] and has been shown to play a role in the migration of dendritic cells [31].

The expression of ABCB1 on the apical surface of epithelial cell of the lower gastrointestinal tract (jejunum, ileum, and colon) has been shown to influence intestinal drug absorption and limit oral bioavailability of many drugs, including important anticancer agents derived from natural sources [28]. This finding has been the starting point for various proof-of-concept studies in cancer patients by administering these drugs orally in combination with inhibitors of ABCB1 to enhance intestinal drug uptake and increase the oral bioavailability [32-34].

ABCG2

The *ABCG2* (formerly *BCRP/MXR/ABCP*) gene maps to chromosome 4q22 and consists of 16 exons and 15 introns [35]. It encodes a 655 amino acid half transporter with a NBF-TM orientation [36-38]. Analysis of cell lines resistant to mitoxantrone that did not overexpress *ABCB1* or *ABCC1* led several laboratories in the late 1990s to almost simultaneously identify *ABCG2* as a gene encoding a drug transporter [39-41]. *ABCG2*

Table 1. Selected Substrates and Inhibitors of ABCB1 and ABCG2

Substrates	Inhibitors
ABCB1	
Actinomycin D [149]	Biricodar (VX-710) [150]
Daunorubicin [151]	Cyclosporin A [152]
Docetaxel [153]	Dexniguldipine [154]
Doxorubicin [155]	GF120918 [156]
Etoposide [157]	JTV-519 [158]
Imatinib [159]	LY335979 [160]
Irinotecan [161]	R101933 [162]
Mitoxantrone [163]	Ritonavir [164]
Paclitaxel [165]	Tariquidar (XR9576) [166]
SN-38 [161]	Valspodar (PSC 833) [167]
Teniposide [168]	Verapamil [152]
Topotecan [169]	
Vinblastine [155]	
Vincristine [170]	
ABCG2	
9-Aminocamptothecin [171,172]	17-B-estradiol [64]
Ciprofloxacin [173]	Biricodar (VX-710) [174]
Daunorubicin [175]	CI1033 [176]
Diflomotecan (BN-80915) [177]	Circumin [178]
Dipyridamole [179]	Cyclosporin A [180]
DX-8951f [181]	Diethylstilbestrol [182]
Epirubicin [183]	Elacridar [184]
Etoposide [100,185]	Fumitremorgin C [62]
Flavopiridol [186]	GF120918 [60]
Gefitinib [187]	Gefitinib [188]
Heterocyclic amines [55,189]	Imatinib [188]
Homocamptothecin [177]	Ko143 [54]
Imatinib [190]	Nelfinavir [191]
J-107088 [192]	Nicardapine [179]
Methotrexate [100,185]	Nimodipine [179]
Mitoxantrone [175]	Nitrendipine [179]
NB-506 [192]	Novobiocin [193]
Norfloxacin [173]	Ortataxel [174]
Ofloxacin [173]	Pantoprazole [184]
SN-38 [194]	Reserpine [195]
Teniposide [196]	Ritonavir [197]
Topotecan [198]	Rosuvastatin [199]
UCN-01 [98]	Saquinavir [191]
	Sirolimus [180]
	Tacrolimus [180]
	Tariquidar (XR9576) [98]
	Tryprostatin A [200]

confers resistance to various topoisomerase I and II inhibitors and is amplified or involved in chromosomal translocations in cell lines selected with topotecan, mitoxantrone, or doxorubicin treatment [42]. It might be expected that ABCG2 functions as a homodimer [43], although more recently it was shown that human ABCG2 probably exists as a homotetramer with possibly a higher form of oligomerization (Figure 1) [44].

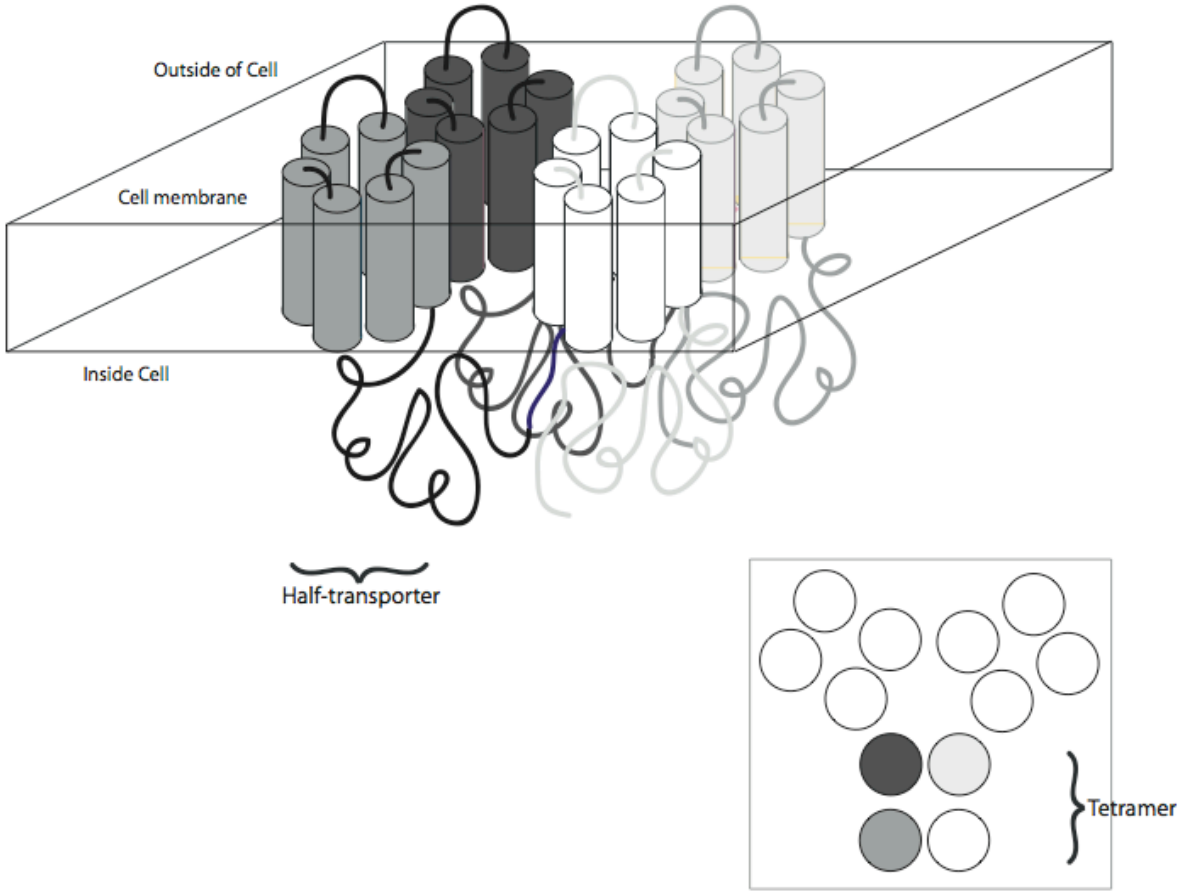

Figure 1. **Upper panel,** Diagram of generic ABC transporter, showing half-transporter (blue), full transporter (blue and green), and the formation of a tetramer. Tubes represent helices through cell membrane. **Lower panel,** Schematic representation of higher order organization of ABCG2, with three tetramers grouping together.

ABCG2 can also transport several dyes, such as rhodamine 123 and Hoechst 33342, and the coding gene is highly expressed in a subpopulation of hematopoietic stem cells (side population) that stain poorly for these dyes [45-49]. However, the normal function of the gene in these cells is not completely understood. ABCG2 is also highly expressed in the trophoblast cells of the placenta [39,50]. This suggests that the protein is responsible either for transporting compounds into the fetal blood supply or removing toxic compounds [8,51]. The gene is also highly expressed in the intestine [52,53], and restricts the exposure to ingested carcinogens and potentially toxic xenobiotics by decreasing uptake from the gut lumen [51,52,54-56]. Indeed, a food carcinogen present in overcooked meat, which has been linked to breast cancer, has been shown to be a substrate for ABCG2, providing further evidence of an evolutionary protective function [55]. Like with ABCB1, inhibitors of the ABCG2 transporter proteins have been shown to be useful in making anticancer substrate drugs orally available [57,58].

In cells from mice deficient in the *Abcb1a*, *Abcb1b*, and *Abcc1* genes, exposure to mitoxantrone, topotecan, or doxorubicin results in amplification of the *Abcg2* gene [19,59]. This strongly suggests that *ABCG2* is one of three major transporter genes involved in drug resistance in mammalian cells. Inhibitors of ABC drug transporters represent a potential strategy for preventing the development of drug-resistant tumors [21]. Effective inhibitors of ABCG2, including GF120918 and (analogues of) fumitremorgin C have been described [42,60-62] (Table 1), and several of these are currently being tested clinically in an attempt to reverse resistance to anticancer substrate drugs. Recently, ABCG2 expression has been shown to be upregulated by hypoxia, through activation of the hypoxia-inducible transcription factor (HIF-1) signaling pathway [49]. This might have great implications for cancer, due to the hypoxic conditions that exist within tumors.

ABCG2 also plays a role in the transport of sterols [63]; 17-β-Estradiol is a competitive substrate for ABCG2, though it was originally believed to be an inhibitor [64], and it also stimulates transcription of *ABCG2* [65]. An estrogen response element has recently been identified in the 5′ flanking region of the *ABCG2* gene. In addition,

Table 2. Summary of common genetic variants in the *ABCB1* gene

cDNA position[a]	Region	Wild-type allele	Variant allele	Amino Acid	Change
-274	Intron -1	G	A		
-223	Intron –1	C	T		
-146	Intron –1	T	C		
-60	Intron –1	A	T		
-41	Intron -1	A	G		noncoding
-241	Exon 1	G	A		noncoding
-145	Exon 1	C	G		noncoding
-129	Exon 1	T	C		noncoding
-43	Exon 1	A	G		noncoding
+140	Intron 1	C	A		
+237	Intron 1	G	A		
-4	Exon 2	C	T		Noncoding
-1	Exon 2	G	A		Noncoding
61	Exon 2	A	G	21	Asn to Asp (N21D)
-8	Intron 3	C	G		
266	Exon 4	T	C	89	Met to Thr (M89T)
-25	Intron 4	G	T		
307	Exon 5	T	C	103	Phe to Leu (F103L)
+139	Intron 6	C	T		
+145	Intron 6	C	T		
548	Exon 7	A	G	183	Asn to Ser (N183S)
729	Exon 8	A	G	243	Syn
781	Exon 8	A	G	261	Ile to Val (I261V)
-44	Intron 9	A	G		
-41	Intron 10	T	G		
1199	Exon 11	G	A	400	Ser to Asn (S400N)
-4	Intron 11	G	A		
1236[c]	Exon 12	C	T	412	Syn
1308	Exon 12	A	G	436	Syn
+17	Intron 12	G	A		
+44	Intron 12	C	T		
1474	Exon 13	C	T	492	Arg to Cys (R492C)
+24	Intron 13	C	T		
1617	Exon 14	C	T	539	Syn
+38	Intron 14	A	G		

+38	Intron 15	G	A		
1985	Exon 16	T	G	662	Leu to Arg (L662R)
2005	Exon 16	C	T	669	Arg to Cys (R669C)
-27	Intron 17	A	G		
+8	Intron 20	C	G		
+24	Intron 20	G	A		
+40	Intron 20	C	T		
2547	Exon 21	A	G	849	Ile to Met (I849M)
2650	Exon 21	C	T	884	Syn
2677	Exon 21	G	T	893	Ala to Ser (A893S)
2677[d]	Exon 21	G	A	893	Ala to Thr (A893T)
+31	Intron 22	G	A		
2956	Exon 24	A	G	986	Met to Val (M986V)
2995	Exon 24	G	A	999	Ala to Thr (A999T)
3151	Exon 25	C	G	1051	Pro to Ala (P1051A)
3320	Exon 26	A	C	1107	Gln to Pro (Q1107P)
3322	Exon 26	T	C	1108	Trp to Arg (W1108R)
3396	Exon 26	C	T	1132	Syn
3421	Exon 26	T	A	1141	Ser to Thr (S1141T)
3435[e]	Exon 26	C	T	1145	Syn
3751	Exon 28	G	A	1251	Val to Ile (V1251I)
3767	Exon 28	C	A	1256	Thr to Lys (T1256K)
4030	Exon 28	G	C		Noncoding
4036	Exon 28	A	G		Noncoding
+21	Intron 28	T	C		

[1] cDNA numbers are relative to the ATG site and based on the cDNA sequence from GenBank accession number M14758 with an A as the reference at position 43.
[b] The location of the variants in the DNA sequence is indicated as intronic or non-coding; intron -1 is the intronic sequence immediately preceding exon 1. The amino acid position is indicated for those variants in the coding exonic sequence. The addition Syn indicates a synonymous nucleotide change, so the resulting protein is unchanged.
[c] Also referred to as *ABCB1*8* [76]; [d] Also referred to as *ABCB1*7* [76]; [e] Also referred to as *ABCB1*6* [76]

testosterone and tamoxifen have all been shown to stimulate ATPase activity, similar to estradiol [66].

Genetic variants of the ABCB1 and ABCG2 genes

ABCB1 variants

With respect to the ABC transporters, genetic variations of the human *ABCB1* gene have been most extensively studied. Several recombinant variants have been generated either by *in vivo* drug selection or by site-directed mutagenesis techniques, which show altered substrate specificity or impaired function of a properly assembled protein [24,67]. Hoffmeyer et al. were the first to report a systematic screen of the *ABCB1* gene for the presence of single-nucleotide polymorphisms (SNP) [68,69]. The original *ABCB1* sequence was defined as wild-type and all 28 exons, including the core promoter region and exon-intron boundaries, were sequenced. Sequencing the *ABCB1* genes from 188 Caucasian volunteers revealed 15 polymorphisms (in 8 exons and 7 introns), of which 3 had been described previously. A summary of the various commonly observed SNPs and insertion/deletion (indel) polymorphisms in the *ABCB1* gene is provided in Table 2 [70-73].

A detailed analysis of the potential functional consequences of different *ABCB1* variants has not yet been performed, except for five most common non-synonymous coding SNPs (i.e., Asn21Asp, Phe103Leu, Ser400Asn, Ala893Ser/Thr, and Ala998Thr) as assessed by a vaccinia virus-based transient expression system [74]. Transport studies of several tested substrates indicated that the substrate specificity of the protein was not substantially affected by any of the SNPs, whereas cell surface expression and function of even double mutants showed no difference from the wild-type protein. This suggests that these SNPs result in mutant proteins with a cellular distribution and function similar to the wild-type protein. One of the tested SNPs, the *ABCB1* 2677G>T/A [75] contains a tri-allelic polymorphism (with G at nucleotide 2677 found in the wild-type sequence, and with A or T at that position being the two possible variants), which results in an amino acid change in exon 21 (Ala893Ser/Thr). However, work by others has shown that the

Ser893 substitution is associated with an altered MDR pattern in AdrR MCF-7 cells as well as approximately 2-fold enhanced efflux transporting ability in stably transduced NIH3T3 GP+E86 cells [76]. These disparities between the studies might reflect differences in the substrates used in these assays, in intrinsic transporter function in the various expression systems, or in the *ABCB1* haplotypes that were heterologously expressed [73]. It is of note that most of these studies have not addressed the possibility that changes in expression of the encoded protein can have a greater influence on function than changes in transport activity itself. Clearly, further work is required to evaluate these contradictory findings regarding the *ABCB1* 2677G>T/A polymorphism with stratification according to common haplotypes.

The most extensively studied *ABCB1* variant to date is a common synonymous mutation at the transition C to T at position 3435 (3435C>T) at a wobble position in exon 26. Although this transition does not change its encoded Ile amino acid (i.e., a silent mutation), it is the only *ABCB1* variant identified thus far that may be associated with altered protein expression in different human tissues (Table 3). Greiner et al. first evaluated the expression of ABCB1 in duodenal biopsies of 21 subjects, and Hoffmeyer et al. subsequently identified a 2-fold decrease in protein level ($P = 0.056$) for individuals with the homozygous T allele (TT genotype) as compared to those with the wild-type alleles (CC genotype) [68,77]. In addition, it has been shown that rhodamine 123 fluorescence in $CD56^+$ natural killer cells was significantly lower (i.e. greater efflux) in individuals with the CC genotype (51.1 ± 11.4%; n = 10) compared to those with the TT genotype (67.5 ± 9.5%; n = 11) [78]. The TT genotype also led to a decrease in ABCB1 mRNA level in this study, as compared to the CC genotype. Despite the silent nature of this SNP, it is also possible that the T allele produces an Ile codon that is infrequently utilized in the human genome, leading to reduced translation efficiency. Alternatively, changes in ABCB1 expression and function in individuals homozygous for the TT genotype by other, yet unknown causes might have been incorrectly attributed to the synonymous change at this position. Differences in protein expression and function might also result from other non-coding and intronic nucleotide changes that are in phase with the *ABCB1* 3435C>T polymorphism. Nonetheless, the association of the 3435C>T polymorphism with ABCB1

protein expression and function remains controversial. In fact, various investigators have reported that the T allele is associated with *increased* protein expression or has no clearly discernible effect (Table 3). This degree of variability observed in the various studies might also reflect the ethnic haplotype diversity in the population samples [73]. However, many of these studies have used different methodology for determination of protein expression, which may be a contributing factor. Additionally, expression of ABCB1 is known to be regulated by the pregnane X receptor, a ligand-activated, nuclear receptor, which may further obscure any effects of the polymorphism [79,80]. More recently, Takane et al. have identified novel variants in the promoter region of the *ABCB1* gene with increased transcriptional activity and mRNA expression in the placenta [81]. However, because the frequency of these variants in the Japanese and Caucasian populations is very low, further studies are required to establish their impact on drug absorption and disposition as well as response to treatment.

ABCG2 variants

In contrast to the extensive investigations into the pharmacological significance of *ABCB1* SNPs, surprisingly little has still been reported regarding naturally-occurring variants in other ABC transporter genes [82]. To date, *ABCG2* has been systematically screened for genetic variations in 13 different ethnic groups [83-90], and various genetic variants have been identified (Figure 2 and Table 4). The common *ABCG2* 421C>A allele in exon 5, in which the C to A transversion results in an amino acid change of Gln to Lys at codon 141 has been associated with low ABCG2 expression levels [50,85,91-93] as well as increased sensitivity to several anticancer drugs *in vitro* as compared to the wild-type protein, due to decreased efflux from the cells [85]. This allele is carried by 46% of a normal Japanese population [85]. Interestingly, this SNP has been found in African-Americans and Africans from North or South of the Sahara at only very low frequencies or not at all, as illustrated in Table 5 [86,87]. In addition to the possible decrease in expression levels, ATPase activity in the *ABCG2* 421C>A mutant has been shown to be significantly lower than with wild-type ABCG2 [89,94].

Table 3. Effect of *ABCB1* polymorphism 3435C>T on protein expression and function

Tissue	Population (genotype, CC/CT/TT)	Effect on expression	Reference
Hematopoietic cells			
Bone marrow	AML patients (total 136)	significantly lower with CC alleles[e]	[128]
Bone marrow	AML patients (total 23)	no difference	[201]
$CD56^+$ natural killer cells	volunteers (10/0/10)	lower in TT than CC[a]	[202]
$CD56^+$ natural killer cells	volunteers (10/10/11)	significantly lower with T allele[a]	[78]
Leukocytes	volunteers (10/10/11)	no difference[c]	[78]
Lymphocytes	volunteers (total 18)	no difference[f]	[203]
Lymphocytes	male volunteers (17/14/15)	no difference[g]	[204]
Lymphocytes	volunteers (13/15/17)	no difference[g]	[205]
Peripheral blood mononuclear cells	HIV-1-infected patients (total 59)	significantly lower with T allele	[142]
Peripheral blood mononuclear cells	volunteers (10/0/10)	no difference[b]	[202]
Intestine			
Duodenum	volunteers and patients (6/10/5)	significantly lower with T allele	[68]
Duodenum	volunteers (9/18/10)	no difference	[206]
Duodenum	volunteers (5/4/4)	significantly higher with T allele	[207,208]
Duodenum	volunteers (13 total)	significantly higher with T allele	[209]
Duodenum/rifampin induction	volunteers (3/4/1)	significantly lower with T allele	[68]
Small intestine	LT recipients (21/35/13)	no difference[d]	[105]
Miscellaneous			
Heart	CABGS subjects (12/25/14)	no difference	[210]
Placenta	pregnant subjects (31/39/19)	no difference	[211]
Placenta	pregnant subjects (73 total)	significantly lower in TT than CC	[212]
Kidney cortex and RCC	nephrectomized patients (24 total)	no differences[g]	[213]
Renal parenchyma	healthy volunteers (85)	significantly lower in TT than CC	[131]

Abbreviations: CC, wild-type; CT, heterozygous variant; TT, homozygous variant; LT, liver transplant; AML, acute myeloid leukemia; CABGS, coronary-artery bypass graft surgery; RCC, renal cell carcinoma. [a] Higher rhodamine fluorescence; [b] 42% lower in TT than in CC; [c] The expression levels were lowest in TT; [d] Genotype affected the expression level of *CYP3A4* mRNA; [e] Highest in the CT genotype; Significant increase in the TT group following rifampin induction; [g] Haplotype analysis performed for the polymorphisms 1236C>T, 2677G>A/T, and 3435.

Table 4. Summary of genetic variants in the *ABCG2* gene, occurring in humans

Position in gene[a]	Nucleotide[b]	Region	Wild-type allele	Variant allele	Amino Acid	Change
-39141		5′Flanking region	CTCA	-		CTCA deletion
-20445		5′ Flanking region	C	T		
-20296		5′ Flanking region	A	G		
-19781		5′ Flanking region	A	G		
-19202		5′ UTR	G	C		
-18845		5′ UTR	T	C		
-18604		5′ UTR	A	-		deletion
-18482	-113	Exon 1	C	T		noncoding
-18398	-29	Exon 1	A	G		noncoding
34	34	Exon 2	G	A	12	Val to Met (V12M)
114	114	Exon 2	T	C	38	synonymous
239		Intron 2	A	G		
7268		Intron 2	T	C		
7491		Intron 3	-	T		insertion
8007		Intron 3	G	A		
8184	369	Exon 4	C	T	123	synonymous - (wobble)
8191	376	Exon 4	C	T	126	Gln to Term (Q126Stop)
8253		Intron 4	G	C		
8825	421	Exon 5	C	A	141	Gln to Lys (Q141K)
8878	474	Exon 5	C	T	158	synonymous
8883	479	Exon 5	G	A	R160Q	
18186		Intron 5	A	G		
18286	616	Exon 6	A	C	206	Ile to Leu (I206L)
21540		Intron 6	C	T		
21903		Intron 7	A	G		
26297	1098	Exon 9	G	A	366	synonymous
32367		Intron 9	A	T		
32604		Intron 10	T	A		
38369		Intron 10	A	G		
38398	1291	Exon 11	T	C	431	Phe to Leu (F431L)
38429	1322	Exon 11	G	A	441	Ser to Asn (S441N)
38485		Intron 11	A	G		
40121		Intron 11	G	A		
40303	1425	Exon 12	A	G	475	synonymous
40343	1465	Exon 12	T	C	489	Phe to Leu (F489L)

40408		Intron 12	G	A		
40417		Intron 12	A	G		
40419		Intron 12	G	T/C		
42127	1515	Exon 13	C	-	506, 507, 508, 509	Frame shift: F506S, F507L, V508L, M509stop
42289		Intron 13	C	T		
42314		Intron 13	T	G		
44061		Intron 13	A	T		
44072		Intron 13	C	T		
44168	1723	Exon 14	C	T	575	Arg to Term (R575Stop)
44997		Intron 14	A	G		
45073	1768	Exon 15	A	T	590	Asn to Tyr (N590Y)
47355	1858	Exon 16	G	A	620	Asp to Asn (D620N)
	2332	3′ UTR	A	TA		
	2364	3′ UTR	A	C		
	2512	3′ UTR	C	T		

a Position with respect to translation start of ABCG2 gene. (GenBank accession number, AC084732)
b From mRNA, (GenBank accession number, XM_032424)
c Syn denotes a synonymous nucleotide change, so the encoded protein remains unchanged.

An additional potentially functional polymorphism has been identified in the *ABCG2* gene at nucleotide position 34 resulting in a V12M amino acid change. All Mexican-Indians screened possessed at least one variant allele, while the frequency in Caucasians was only 4.7% [86]. It is believed that this mutation results in poor localization of the ABCG2 protein and a subsequent decrease in efflux from the cell [85].

A mutation at the arginine on amino acid position 482 in human cancer cells expressing ABCG2 or mouse cells expressing Abcg2 has been shown to have a crucial role in protein function and in altering the MDR phenotype by changing substrate specificity [95-98]. Interestingly, this mutation has never been seen in individuals [83,95-97,99]. Cell lines carrying this variant have been shown to have altered substrate specificity; e.g. mitoxantrone is transported by both wild-type and variant ABCG2. However, cells expressing either the R482G or R482T mutant both demonstrated greater resistance to mitoxantrone than the wild-type, suggesting that the mutant is a better

Table 5. Ethnic variability in the most common *ABCG2* mutations

Population	34 G>A (Exon 2) Variant Allele frequency	239 A>G (Intron 2) Variant Allele frequency	369 C>T (Exon 4) Variant Allele frequency	421 C>A (Exon 5) Variant Allele frequency	Reference
	q	*q*	*q*	*q*	
White American	0.02	0.03	0.00	0.12-0.14	[86,87]
White European (includes Swedish and Dutch populations)	0.02 -0.06	0.06	-	0.10 – 0.12	[87,88,214]
African-American	0.04 -0.06	0.05	0.07	0.00 – 0.05	[86,87,93]
African (sub-Sahara)	-	-	-	0.01	[87]
Chinese	0.20	0.20	-	0.34 – 0.35	[86,87]
Japanese	0.15-0.19	0.18-0.15	-	0.27 -0.26	[85,87,93]
S.E. Asians (not Japanese or Chinese)	0.45	0.45	-	0.15	[86]
Pacific Islanders	0.64	0.14	-	0.14	[86]
Mexican Indians	0.90	0.90	-	0.10	[86]
Mexicans	0.10	0.10	-	0.05	[86]
Middle Eastern	0.05	0.05	-	0.13	[86]
Ashkenazi Jewish	0.10	0.10	-	0.10	[86]
African (North of the Sahara)	0.14	0.14	-	0.07	[86]

transporter for this agent [97]. In contrast, the anticancer drug methotrexate is transported by wild-type ABCG2, but not by either mutant [99,100], whereas daunorubicin is transported only by the mutant ABCG2 protein.

Phenotype-genotype relationships

Variation in the pharmacokinetic behavior of an anticancer drug in a patient population is the net result of complex interactions between genetic, physiological, and environmental factors. It is reasonable to assume that genetic variations in ABC transporter genes could alter drug disposition and might have clinical consequences. If the function or expression level of interstitial ABC transporters is altered due to genetic changes, intestinal secretion of substrate drug into the gut lumen may change. Such information may be valuable in predicting a change in bioavailability of orally administered substrate drugs in individual patients.

ABCB1 genotype in relation to drug absorption and disposition

Phenotypical consequences of variants in ABC genes have so far been most extensively described for *ABCB1*, and have mainly focused on the 3435C>T and 2677G>T/A SNPs. These data are summarized in Table 6 and Table 7, respectively. In line with their initial observation of lower duodenal expression of ABCB1 in individuals homozygous for the T-allele of the 3435C>T SNP, Hoffmeyer et al. found a higher area under the concentration-time curve (AUC) and maximum concentration (C_{max}) for the substrate drug digoxin in 22 individuals with the TT genotype after oral drug administration [68]. However, because plasma concentrations of oral digoxin are dependent on both absorption and elimination pathways [77], it cannot be concluded that the higher drug concentration observed in the TT genotype group was simply due to decreased ABCB1 function at the intestinal level. Indeed, possible effects of the 3435C>T polymorphism on the pharmacokinetics of substrate drugs remain highly controversial. For example, Kurata et al. reported that the AUC of oral digoxin was significantly higher in

Table 6. Effect of *ABCB1* polymorphism 3435C>T on substrate drug effects

Drug	*Population (CC/CT/TT)*	*Parameter*	*Effects*	*Ref*
Anticancer				
Diflomotecan	cancer patients (5/9/7)	AUC, F	no significant differences	[147]
Docetaxel	cancer patients (5/16/7)	clearance	no significant difference	[119]
Docetaxel	cancer patients (21 total)	clearance	no significant difference	[215]
Docetaxel	cancer patients (62 total)	survival	no significant difference	[216]
Etoposide	children with ALL (35/41/26)	clearance	significantly lower with T allele	[217]
Irinotecan	cancer patients (16/35/8)	AUC, clearance	no significant differences	[120]
Irinotecan	cancer patients (7/17/6)	AUC, clearance	no significant differences	[218]
Irinotecan	cancer patients (49 total)	renal clearance	no significant difference	[118]
Tipifarnib	cancer patients (29 total)	AUC	no significant difference	[121]
Vincristine	cancer patients (52 total)	AUC, clearance	no significant differences	[219]
Antihypertensives/antiarrhythmics				
Digoxin	volunteers (3/4/1)	AUC (rifampin induction)	significantly higher with T allele	[68]
Digoxin	volunteers (7/0/7)	C_{max}	significantly higher in TT	[68]
Digoxin	volunteers (5/4/3)	AUC (0-4 and 0-24 h)	no significant differences	[220]
Digoxin	volunteers (5/4/4)	AUC (0-4 hours)	significantly lower with T allele	[221]
Digoxin	volunteers (50 total)	AUC, C_{max}	no significant differences	[222]
Digoxin	volunteers (8/8/8)	AUC (0-4 hours), C_{max}	significantly higher in TT[c]	[114]
Digoxin	volunteers (5/5/5)[a]	F	significantly higher in TT	[101]
Digoxin	volunteers (117 total)	AUC, T_{max}	significantly lower in TT	[223]
Digoxin	volunteers (14/8/10)	AUC (0-4 and 0-24 h)	significantly higher in TT[c]	[224]
Digoxin	volunteers (7 total)	AUC, C_{max}	no significant differences	[225]
Digoxin	volunteers	absorption rate	significantly lower in TT	[226]
Talinolol	volunteers (13/29/13)	AUC, F, C_{max}	no significant differences	[206]
Immunosuppressants				
Cyclosporine	RT recipients (31/52/41)	trough levels	no significant differences	[227]
Cyclosporine	volunteers (7/6/1)	AUC, C_{max}	no significant differences[b]	[228]
Cyclosporine	HT recipients (14 total)	AUC, C_{max}	no significant differences[c]	[115]
Cyclosporine	RT recipients (108 total)	trough levels	no significant differences	[229]
Cyclosporine	RT recipients (98 total)	trough levels	no significant differences[c]	[230]

Drug	*Population (CC/CT/TT)*	*Parameter*	*Effects*	*Ref*
Cyclosporine	RT recipients (10 total)	oral clearance	significantly higher with T allele	[231]
Cyclosporine	RT recipients (15/18/17)	trough levels	no significant differences[c]	[232]
Cyclosporine	RT recipients (28/43/35)	AUC, C_{max}	no significant differences[c]	[233]
Cyclosporine	LT recipients (44 total)	dose requirement	significantly lower in TT	[234]
Tacrolimus	LT recipients (15/22/9)	concentration/ dose ratio	no significant difference	[105]
Tacrolimus	RT recipients (48/70/62)	plasma concentrations	no significant differences	[235]
Tacrolimus	LT recipients (4/10/3)	neurotoxicity	no significant difference	[236]
Tacrolimus	HT recipients	concentration/dose	no significant difference	[237]
Tacrolimus	RT recipients (62 total)	trough concentrations	no significant differences	[229]
Tacrolimus	RT recipients (29/34/18)	dose requirement	no significant difference[c]	[238]
Tacrolimus	RT recipients (19/24/7)	trough levels	no significant differences	[232]
Tacrolimus	LT recipients	trough levels	no significant differences[c]	[239]
Tacrolimus	RT recipients (73 total)	trough levels	no significant differences[c]	[240]
Tacrolimus	RT recipients (30 total)	trough levels	no significant differences	[241]
Miscellaneous				
Dicloxacillin	volunteers (17 total)	clearance, C_{max}	no significant differences	[242]
Fexofenadine	volunteers (9/16/12)	AUC (0-4 hours)	significantly lower in TT	[76]
Fexofenadine	volunteers (10/0/10)	AUC, clearance	no significant differences	[243]
Fexofenadine	volunteers (15/12/6)	AUC, C_{max}	no significant differences[c]	[116]
Loperamide	volunteers (20 total)	AUC, C_{max}	no significant differences[c]	[117]
Loperamide	volunteers (8/0/8)	AUC, C_{max}	no significant differences[c]	[244]
Midazolam	cancer patients (5/16/7)	clearance	no significant difference	[119]
Midazolam	volunteers (9/5/7)	metabolic ratio	no significant difference[c]	[112]
Nelfinavir	HIV-1 patients (10/30/14)	plasma concentrations	significantly lower with T allele	[142]
Nortriptyline	depressed patients (78 total)	postural hypotension	risk increased in TT	[245]
Phenytoin	volunteers (28/45/23)	plasma concentrations	lower in CC	[246]
Prednisone	HT recipients (69 total)	steroid dose/weaning	more CC remain on steroids at 1 year	[247]
Warfarin	AT patients (31/100/70)	dose requirement	no significant difference[c]	[248]

Abbreviations: CC, wild-type; CT, heterozygous variant; TT, homozygous variant; AUC, area under the time-concentration curve; RT, renal transplant; HT, heart transplant; LT, liver transplant; C_{max}, maximum concentration; F, oral bioavailability; ALL, acute lymphoblastic leukemia; AT, atrial fibillation.

[a] Linked to the polymorphism 2677G>A/T; [b] AUC and C_{max} in the CT and TT genotypes were higher than those in CC group; [c] Haplotype analysis performed for the polymorphisms 1236C>T, 2677G>A/T, and 3435C>T.

Table 7. Effect of *ABCB1* polymorphism 2677G>T/A on substrate drug effects

Drug	Population (GG/GT/TT/GA/AA)	Parameter	Effects	Ref
Anticancer drugs				
Diflomotecan	cancer patients (8/8/3/1/0)	AUC, F	no significant differences	[147]
Docetaxel	cancer patients (21)	clearance	no significant difference	[215]
Irinotecan	cancer patients (12/23/13/4/1)	AUC, clearance	no significant differences	[120]
Irinotecan	cancer patients (6/16/6/0/0)	AUC, clearance	no significant differences	[218]
Irinotecan	cancer patients (49)	renal clearance	significantly lower in TT[a]	[118]
Tipifarnib	cancer patients (29)	AUC	no significant difference	[121]
Antihypertensives/antiarrhythmics				
Digoxin	volunteers (50)	AUC, C_{max}	no significant differences	[222]
Digoxin	volunteers (15)	F	significantly higher in TT	[101]
Digoxin	volunteers (117)	AUC, T_{max}	significantly lower in TT	[223]
Digoxin	volunteers (12/11/7/1/1)	AUC (0-4 and 0-24 hours)	no significant differences	[224]
Talinolol	volunteers (67 total)	AUC, F, C_{max}	no significant differences	[206]
Immunosuppressants				
Cyclosporine	HT recipients (14 total)	AUC, C_{max}	significantly higher in TT	[115]
Cyclosporine	RT recipients (98 total)	trough levels	no significant differences	[230]
Cyclosporine	RT recipients (20/21/9/0/0)	trough levels	no significant differences	[232]
Cyclosporine	RT recipients (32/51/15/3/0)	AUC, C_{max}	no significant differences	[233]
Tacrolimus	LT recipients (69 total)	concentration/dose ratio	no significant correlation	[105]
Tacrolimus	LT recipients (17 total)	neurotoxicity	no significant difference	[236]
Tacrolimus	HT recipients	concentration/dose ratio	no significant difference	[237]
Tacrolimus	RT recipients (26/38/12/3/0)	dose requirement	no significant difference	[238]
Tacrolimus	RT recipients (23/16/8/3/0)	trough levels	no significant differences	[232]
Tacrolimus	LT recipients	trough levels	no significant differences[a]	[239]
Tacrolimus	RT recipients (73 total)	trough levels	no significant differences[a]	[240]
Tacrolimus	RT recipients (30 total)	trough levels	no significant differences	[241]

Drug	Population (GG/GT/TT/GA/AA)	Parameter	Effects	Ref
Miscellaneous				
Fexofenadine	volunteers (60 total)	AUC (0-4 hours)	significantly lower in TT	[76]
Fexofenadine	volunteers (33 total)	AUC, C_{max}	no significant differences[a]	[116]
Loperamide	volunteers (20 total)	AUC, C_{max}	no significant differences	[117]
Loperamide	volunteers (16 total)	AUC, C_{max}	no significant differences	[244]
Midazolam	volunteers (10/7/4/0/0)	metabolic ratio	no significant difference	[112]
Prednisone	HT recipients (69 total)	steroid dose/weaning	more CC remain on steroids at 1 year	[247]
Warfarin	AF patients (57/91/40/9/1)	dose requirement	no significant difference	[248]

Abbreviations: GG, wild-type; GT/GA, heterozygous variant; TT/AA, homozygous variant; AUC, area under the time-concentration curve; RT, renal transplant; HT, heart transplant; LT, liver transplant; C_{max}, maximum concentration; F, oral bioavailability; AT, atrial fibrillation.
[a] When analyzed as haplotype for the polymorphisms 1236C>T, 2677G>A/T, and 3435C>T.

subjects with the TT genotype, but there was no difference in AUC after intravenous administration of digoxin [101].

To explain the observed inconsistencies between the various substrate drugs tested (as shown in Tables 6 and 7), a number of potentially confounding factors can be put forward. Numerous environmental factors affecting the phenotypical activity of ABCB1 must be considered, which may include exogenous chemicals, food constituents, herbal preparations, and/or therapeutic drug use that may induce or inhibit the function or expression of the protein (e.g., rifampin [102] and St. John's wort [103]). This is particularly problematic for cancer patients, who are known to take a wide variety of medications concomitantly with their chemotherapeutic regimen [104]. Also, the physiological status of the patient may alter ABCB1 function [71]; Goto et al. have reported that the 3435C>T polymorphism was associated with neither the expression level of *ABCB1* mRNA nor systemic concentrations of the immunosuppressive drug tacrolimus during the first seven postoperative days in liver transplant recipients [105]. It is likely that the expression and phenotypical activity of ABCB1 in these recipients is strongly influenced by other medications taken prior to the transplantation and/or by the function of the transplanted organ. Thus, these non-genetic factors might mask the potential genetic

effects. Therefore, though studies of this type with a heavily pretreated patient population may be of limited value in determining the absolute effects of transporter polymorphisms, they do provide an indication of the clinical relevance.

Another explanation for the discrepancies is related to route of drug administration and drug-specific differences in metabolism and excretion for various substrates of ABCB1 [106,107]. For example, cyclosporine is a substrate of the cytochrome P450 3A4 (CYP3A4) isoform and is transported by ABCB1, whereas digoxin is also a substrate of ABCB1 but is not metabolized by CYP3A4. In the case of cyclosporine, reduced ABCB1 function by possible genetic effects might be compensated for by (inducible) CYP3A4 activity. Despite the inconsistent results, the significant geographic, ethnic and racial differences in the allele frequency distribution of several ABC transporter genes, including the *ABCB1* 3435C>T variant, interethnic variabilities could also have an impact on drug disposition profiles and thus may provide a mechanistic basis for some of the observed discrepancies among different populations [73,76,108-111]. Finally, as mentioned earlier, distribution of other (unidentified) variation in the same gene and/or other genes relevant to drug disposition that may be linked to the 3435C>T polymorphism might be different among the different human populations studied. In this context, it is noteworthy that a reduced expression of intestinal CYP3A4 mRNA was observed in subjects carrying the TT genotype of the 3435C>T polymorphism in a Japanese population [105], although this finding could not be confirmed by others [112]. It has also been suggested that the interindividual variation of protein expression levels in the human intestine might be associated with SNPs in the 5'-regulatory region of the *ABCB1* gene (i.e., at nucleotides -692T>C and -2352G>A) that are possibly in linkage with the 3435C>T polymorphism [113].

Altogether, the seemingly contradictory findings may indicate that genetic variation of *ABCB1* 3435C>T is not the causal modulator of any of the observed functional differences. In addition, the 3435C>T SNP is a silent mutation that does not result in an amino acid change. Therefore, it is very likely that this SNP is in linkage disequilibrium with other (unidentified) functional polymorphism(s), including the *ABCB1* 2677G>T/A polymorphism. This suggests that functional effects of genetic variants in the *ABCB1* gene

should be considered as haplotypes rather than independent SNPs. For example, Kim et al. demonstrated close linkage of the polymorphisms 1236C>T, 2677G>T, and 3435C>T, which makes a variant allele referred to by the investigators as *ABCB1*2*, whereas an allele with the original published sequence is referred to as *ABCB1*1* [76]. According to their pharmacogenetic study, individuals who are homozygous for *ABCB1*1* allele had approximately a 40% higher AUC value of fexofenadine, a probe of the ABCB1 activity, compared to those who are homozygous for *ABCB1*2*. Recent work also indicates that the use of *ABCB1* haplotypes is superior to unphased SNP analysis to predict the pharmacokinetics of digoxin [114], cyclosporine [115], and fexofenadine [116], whereas Goto et al. suggested a correlation between the haplotype of these three SNPs and intestinal expression of *ABCB1* mRNA [105]. Assessing haplotypes in the *ABCB1* gene and consideration of their interethnic differences in future investigations will likely provide greater power to detect associations with functional differences [73,111,115,117,118].

Although no standard nomenclature for the *ABCB1* SNPs and haplotypes is currently available, a comprehensive sequence diversity analysis has recently found 33 haplotypes in at least three chromosomes in a collection of 247 ethnically diverse DNA samples [73]. These authors noted two very common haplotypes in the *ABCB1* gene, referred to as *ABCB1*1* for the reference allele and *ABCB1*13*, which contains three common codon polymorphisms (i.e., 1236C>T, 2677G>T, and 3435C>T) as well as three intronic variants (10.1, 13.1, and 14.2; see Table 2) [73]. In line with data presented by Kimchi-Sarfaty et al. [74], no functional difference was found in the transport of ABCB1 probe drugs in cells transfected with the *ABCB1*1* or *ABCB1*13* constructs, either in the presence or absence of the ABCB1 inhibitor, GF120918 [73]. As far as anticancer drugs are concerned, studies investigating clinical consequences of the *ABCB1* polymorphisms in terms of their ability to modulate the pharmacokinetic profile of substrates in adults are very scarce (Tables 5 and 6). Goh et al. examined the 3435C>T polymorphism in 28 Asian patients with cancer who were treated with docetaxel [119]. The genotyping results showed no statistically significant difference in docetaxel clearance among the three genotypes. Another study explored the relationships in 59 Caucasian patients between irinotecan disposition and multiple SNPs in several ABC transporter genes, including the

ABCB1 3435C>T polymorphism [120], and no statistical associations were observed. Likewise, the *ABCB1* 3435C>T or 2677G>T/A genotypes did not predict the systemic exposure to the farnesyltransferase inhibitor tipifarnib (R115777; Zarnestra) administered orally [121]. It should be noted that the lack of relationships between some of the known *ABCB1* polymorphisms and the pharmacokinetics of these agents is consistent with preclinical observations in *Abcb1*-deficient mice as well as clinical data that metabolism rather than transport is the prominent elimination pathway for docetaxel [122,123], irinotecan [124,125], and tipifarnib [126].

ABCB1 genotype in relation to disease risk

In contrast to the negative data obtained with studies evaluating *ABCB1* genotype in relation to substrate drug pharmacokinetics, recent studies suggest that common allelic variants in the *ABCB1* gene may influence the susceptibility to specific diseases and therapy outcome (recently reviewed by Marzolini et al. [15] and Jamroziak and Robak [127]). These include associations between the *ABCB1* 3435C>T polymorphism and survival of patients with acute myeloid leukemia [128] or acute lymphoblastic leukemia[129], response to preoperative chemotherapy for breast cancer [130], the risk for developing non-clear cell renal-cell carcinoma [131], response to antiepileptic treatment [132], susceptibility to ulcerative colitis [133-137] or Parkinson's disease [138,139], and the incidence of osteonecrosis of the femoral head [140] or chronic renal dysfunction [141]. The *ABCB1* 3435C>T polymorphism has also been recently found to be associated with the CD4 response to treatment of HIV infections [142,143], although in subsequent studies this effect was not demonstrated to translate into changes in phase I viral decay [144], disease progression before treatment [145], or the response to antiretroviral therapy in drug-naive HIV-positive patients [146]. A recent study has also found significant associations between *ABCB1* haplotypes and both refractory Crohn's disease and ulcerative colitis[136]. Nonetheless, these intriguing findings require confirmation in larger patient populations as well as other ethnic groups with known differences in allele and genotype frequencies.

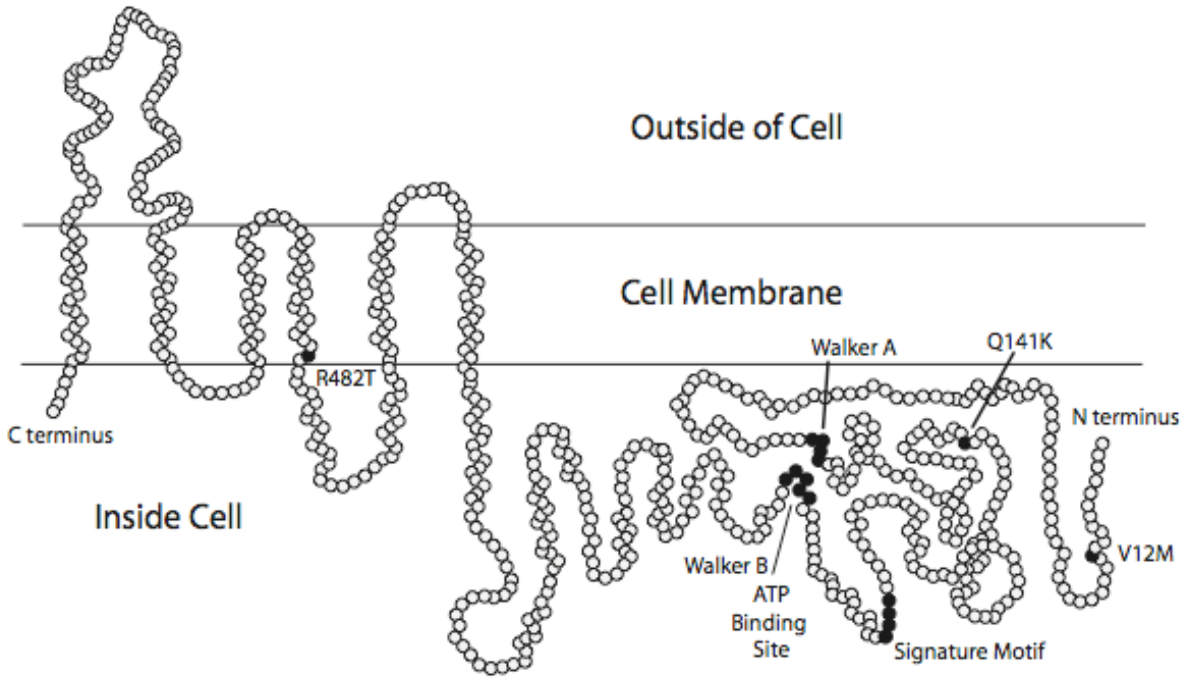

Figure 2. Schematic structure of ABCG2 and its variants.

ABCG2 genotype in relation to drug absorption and disposition

In contrast to ABCB1, there have been only few studies reporting links between genotype and phenotype for ABCG2 (Table 8). However, those that have been carried out exhibit promising and fairly consistent results. The focus of these studies has been primarily on the *ABCG2* 421C>A SNP. Initial experiments evaluated the effects of this polymorphism along with the 34G>A SNP, in comparison with wild-type ABCG2 transfected PA317 cells [85]. The drug concentrations causing 50% inhibition of cell growth (IC_{50}) values in *in vitro* assays were determined for the anticancer substrate drugs SN-38, mitoxantrone, and topotecan. ABCG2 was shown to mediate the efflux of all three drugs, with significantly higher IC50 values for cells with ABCG2 (transfected) as compared to those without ABCG2 (non-transfected). There was no significant difference between the G34A mutant and wild-type ABCG2. However, the variant 421A cells

demonstrated a significant decrease in IC_{50} for all three drugs as compared to wild-type, reducing, but not eliminating, drug efflux. For example, the IC_{50} for the irinotecan metabolite SN-38 was 2.5, 98, and 30 ng/ml in untransfected PA317 cells, cells transfected with wild-type ABCG2, and homozygous transfected C421A mutant cells, respectively. Similar experiments were carried out with stably transfected HEK-293 cells in which the homozygous 421A variant cells demonstrated a significantly lower mitoxantrone efflux and an IC_{50} value that was 6-fold lower than that for the cells transfected with the wild-type protein [94].

Table 8. Effect of *ABCG2* polymorphism 421C>A on anticancer drug effects

Drug	Test system (CC/CA/AA)	Parameter	Effect	Ref
Diflomotecan	cancer patients (15/5/0)	AUC (i.v.)	significantly higher with A allele	[147]
Irinotecan	cancer patients (68/14/2)	AUC of SN-38	no significant difference	[87]
Mitoxantrone	PA317 cells (CC vs AA)	IC_{50}	significantly decreased in AA	[85]
Mitoxantrone	HEK-293 cells (CC vs AA)	drug efflux	significantly decreased in AA	[94]
Mitoxantrone	LLC-PK1 cells (CC vs AA)	IC_{50}	significantly decreased in AA	[89]
SN-38	PA317 cells (CC vs AA)	IC_{50}	significantly decreased in AA	[85]
Topotecan	cancer patients (10/2/0)	AUC (oral), F	significantly higher with A allele	[91]
Topotecan	PA317 cells (CC vs AA)	IC_{50}	significantly decreased in AA	[85]
Topotecan	LLC-PK1 cells (CC vs AA)	IC_{50}	significantly decreased in AA	[89]

Abbreviations: CC, wild-type; CA, heterozygous variant; AA, homozygous variant; AUC, area under the time-concentration curve; i.v., intravenous; IC_{50}, drug concentration causing 50% inhibition of cell growth; F, oral bioavailability.

The first study linking the *ABCG2* 421C>A polymorphism with altered drug exposure *in vivo* has recently been completed. Diflomotecan, a topoisomerase I inhibitor and substrate for ABCG2, was administered to 22 patients both intravenously and orally [147]. After intravenous administration, the AUC in patients heterozygous for the variant allele (5 individuals) showed an area under the curve (AUC) that was 299% of that for those patients homozygous for the wild-type allele. After oral dosing, the bioavailability was significantly lower in the heterozygous variant group as compared to the homozygous

mutant (31.5% compared to 73.7%), suggesting a more prominent role for ABCG2 in elimination than absorption for this particular agent. Indeed, the direct effect of *ABCG2* genotype on the pharmacokinetics of diflomotecan after oral administration was much less pronounced that that seen after intravenous administration. This suggests that there may be other factors affecting intestinal transport of this drug. However, this study provides a starting point for further, larger-scale studies on the effect of this polymorphism on the pharmacokinetics of diflomotecan and other ABCG2 substrates.

A pilot study of the *ABCG2* 421C>A polymorphism in cancer patients undergoing treatment with the structurally-related agent topotecan, a combined substrate for ABCB1 and ABCG2, produced similar results [91]. The heterozygous CA allele was associated with a 1.34-fold increased oral bioavailability of topotecan (31.4% (wild-type) *versus* 42.0% (heterozygous); P = 0.037). Interestingly, the lowest combined mRNA expression levels of ABCG2 and ABCB1 in small intestinal biopsies was observed in one of the patients that was heterozygous variant for *ABCG2* 421C>A [91].

In contrast to the observations with diflomotecan and topotecan described above, circulating concentrations of the ABCG2 substrates SN-38 and SN-38-glucuronide (SN-38G) following the administration of irinotecan to 84 Caucasian cancer patients were not statistically significantly dependent on *ABCG2* 421C>A genotype [87]. The frequency of the variant allele in this retrospective study was 10.7%, with 14 heterozygotes and 2 homozygous variants within the group. Overall, the results from this investigation suggest that other processes involved in irinotecan metabolism and elimination that exhibit great interindividual variation might be over-shadowing any effect of this *ABCG2* polymorphism. It is noteworthy, however, that one of the two patients homozygous for *ABCG2* 421C>A exhibited very extensive accumulation of SN-38 and SN-38G, developed very severe side effects including grade 4 leukocytopenia, neutropenia, and diarrhea, and died within 9 days after drug administration. Hence, it cannot be ruled out that patients with the rare genotype of two variant alleles for *ABCG2* 421C>A may have severely impaired ability to eliminate SN-38 or other ABCG2 substrate drugs.

Expert opinion

The ABC transporters ABCB1 (P-glycoprotein) and ABCG2 (BCRP, MXR, ABCP) have an established role in the pharmacokinetic behavior of many substrate drugs, including anticancer agents, as well as in the occurrence of multidrug resistance in malignant cells. Several polymorphic variants of ABC genes have been described recently, of which some, including those of the *ABCB1* and *ABCG2* genes, may alter protein expression and/or function in humans. The effects of genetic variants in ABC transporter genes in relation to its phenotypical consequences are still debatable, as contradictory and unexplainable results have been reported, especially for the common naturally-occurring *ABCB1* 3435C>T variant. Most studies published to date clearly suffer from small sample sizes evaluated in relation to the often low allele frequency of the studied variant, as well as from a host of other potentially confounding factors that are likely to influence their outcome. Most important among these are environmental and physiological factors that may affect expression of the transporters, and links to other (unknown) genes or variants of putative relevance for drug absorption and disposition pathways. The use of haplotype profiles as opposed to testing unphased SNPs to predict certain phenotypes has been proposed [73], and it cannot be excluded that this may have clinical importance, but this remains to be clarified for most drugs. In addition, more detailed investigations into the influence of ethnicity on ABC transporter function and expression is urgently needed. Nevertheless, in view of the substantial degree of inter-individual variability in systemic exposure to most anticancer drugs (up to 40-fold; [148]), it can be predicted that variability in ABC transporter expression as a result of a certain genotype (up to 2-fold; [68,86]) is presumably relatively unimportant. This hypothesis is consistent with literature findings indicating that *ABCB1* and *ABCG2* genotype has little, if any, effect on the pharmacokinetics of substrate drugs unless transport is the crucial process in the agent's oral absorption or disposition pathways (e.g., as in the case of *ABCG2* 421C>A in relation to the pharmacokinetics of diflomotecan [147] and topotecan [91]). In the field of drug resistance, the pharmacogenetic aspects of ABC transporters are unfortunately still largely

unexplored, but this may eventually contribute to individualizing treatment of patients with cancer on the basis of tumor genotyping.

Outlook

Substantial progress has been made in recent years toward an understanding of the functional significance of polymorphic ABC transporter proteins, although various aspects in this area require more work before it becomes more useful clinically as a prospective tool to predict treatment outcome. Over the next decade, the importance of ABC transporter pharmacogenetics with classical anticancer drugs will be defined; however, it will also be of importance for the rational development of new agents designed to exploit advances in molecular oncology and those acting on oncogenes, tumor suppressor genes and related signal transduction pathways, including apoptosis, as well as agents used to inhibit invasion, angiogenesis, and metastasis. The ongoing development of improved genomic methods, for example those based on DNA chip technology, will also be a major factor in the growing influence of ABC transporter pharmacogenetics as a factor contributing to anticancer drug pharmacology. Similar to the discoveries of functional genetic variations in drug efflux transporters of the ABC family, SNPs will be identified in transporters that facilitate cellular uptake of anticancer drugs, and this will likely have a profound impact on attempts to further optimize cancer chemotherapeutic treatment.

Highlights

- ATP-binding cassette (ABC) genes play a significant role in the resistance of malignant cells to a variety of anticancer agents, including taxanes, anthracyclines, epipodophyllotoxins, and *Vinca* alkaloids.
- The ABC gene products, including ABCB1 (P-glycoprotein) and ABCG2 (BCRP, MXR, ABCP) are also known to influence oral absorption and disposition of a wide variety of drugs.

- The expression levels of the ABC transporter proteins in humans have important consequences for an individual's susceptibility to certain drug-induced side effects, interactions, and anticancer treatment efficacy.
- Naturally occurring variants in the *ABCB1* and *ABCG2* genes have been identified that might affect the function and expression of the encoded protein.
- The frequency of many variant alleles in the *ABCB1* and *ABCG2* genes are significantly ethnically/racially dependent.
- Whereas the functional significance of variants in *ABCB1* is inconclusive and controversial, there is increasing evidence that certain single-nucleotide polymorphisms (SNPs) in *ABCG2* are promising as a predictor of exposure to anticancer substrate drugs.
- The usefulness of haplotypes as opposed to testing unphased SNPs to predict certain phenotypes has been documented.
- Additional clinical studies are needed to evaluate the clinical usefulness of ABC transporter genotyping as a prospective tool to predict the outcome of anticancer treatment.

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Chapter 3

Determination of midazolam in human plasma by liquid chromatography with mass-spectrometric detection

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Abstract

A liquid chromatographic assay with mass-spectrometric detection was developed for the quantitative determination of the cytochrome P450 3A phenotyping probe midazolam in human plasma. Sample pretreatment involved a one-step extraction of 600 µl aliquots with ethyl acetate. Midazolam and the internal standard, lorazepam, were separated on a column (150 × 4.6 mm, I.D.) packed with 5-µm Zorbax Eclipse XDB-C8 material, using a mobile phase composed of methanol and 10 mM aqueous ammonium acetate (60:40, vol/vol). Column eluants were analyzed using mass-spectrometry with an atmospheric pressure chemical ionization interface. Calibration curves were linear in the concentration range of 1.00 to 200 ng/ml. The accuracy and precision ranged from 92.8% to 112% and from 0.056% to 13.4%, respectively, for 4 different concentrations of quality control samples analyzed in triplicate on 8 separate occasions. The developed method was subsequently applied to study the pharmacokinetics of midazolam in a group of 35 human subjects at a single dose of 25 µg/kg.

Introduction

Midazolam is an imidiazobenzodiazepine that is used clinically for sedative purposes prior to minor medical procedures and surgery [1]. Midazolam is almost completely metabolized to 1'-hydroxy-midazolam and, to a lesser extent, to 4'-hydroxy-midazolam via two enzymes in the cytochrome P450 (CYP) CYP3A subfamiliy, CYP3A4 and CYP3A5 [2]. Currently, medications are dosed based upon the assumption that the general population metabolizes drugs at approximately the same rate. However, it has been shown that hepatic CYP3A microsomal activity may vary as much as 40 fold *in vitro*, and 5-fold differences have been observed *in vivo* [3]. Such a wide range of metabolic activity present in the population may lead to unwanted therapeutic outcomes such as sub-therapeutic or toxic drug levels. Due to this wide variability in CYP3A expression, the need for the use of an *in vivo* probe such as midazolam to predict the CYP3A metabolic phenotype of patients treated with narrow therapeutic window agents is evident [3,4]. For the purpose of CYP3A phenotyping, doses of midazolam administered to humans are typically very low, which poses a significant challenge on analytical methods. In contrast to gas chromatographic methods with electron capture or mass spectrometric detection [5-9], most of the currently available validated liquid chromatographic methods used to determine midazolam in human plasma are hampered by a lack in sensitivity to allow for accurate estimation of complete pharmacokinetic profiles, necessitate the use of large sample volumes for extraction (≥ 1 mL) [10,11], and/or require triple-quadrupole mass spectrometry [12], which is not available in most laboratories (Table 1). Here, we describe a novel, sensitive analytical method for the determination of midazolam concentrations in human plasma based on liquid chromatography coupled with single-quadrupole mass-spectrometric detection.

Experimental

Chemicals

Midazolam (lot # 081-122-1; purity, 99.5%) was obtained from UFC Ltd. (Manchester, United Kingdom). The internal standard lorazepam originated from Sigma (St.

Table 1. Published liquid chromatographic methods for the analysis of midazolam in human plasma

Sample pretreatment	Internal standard	Detection	LLOQ[a]	Reference
LLE (cyclohexane)	Flurazepam	200 nm	10 ng/ml	Portier et al. [16]
LLE (diethyl ether)	Diazepam	254 nm	7 ng/ml	Carrillo et al. [17]
LLE (diethyl ether)	Flurazepam	215 nm	10 ng/ml	Blackett et al. [18]
LLE (diethyl ether)	Prazepam	APCI-MS	6.5 ng/ml	Shiran et al. [15]
LLE (diethyl ether)	Flurazepam	220/254 nm	15 ng/ml	Vasiliades et al. [19]
LLE (diethylether)	None	215 nm	30 ng/ml	Vree et al. [20]
LLE (diethylether)	Ro 05-6669	245 nm	5 ng/ml	Ha et al. [21]
LLE (diethyl ether – cyclohexane)	Diazepam	240/300 nm	1 ng/ml	Eeckhoudt et al. [11]
LLE (diethyl ether – methylene chloride)	None	254 nm	50 ng/ml	Puglisi et al. [22]
LLE (diethyl ether – methylene chloride)	Climazolam	220 nm	30 ng/ml	Vletter et al. [23]
LLE (diethyl ether – methylene chloride)	Flurazepam	220 nm	30 ng/ml	Chan et al. [24]
LLE (heptane – isoamylalcohol)	Desmethylclomipramine	250 nm	23.4 ng/ml	ter Horst et al. [25]
LLE (methylene chloride)	Clonazepam	232 nm	25 ng/ml	Hayball et al. [26]
LLE (methylene chloride)	Medazepam	240 nm	7.5 ng/ml	Odou et al. [27]
LLE (n-butyl chloride)	Methylclonazepam	220 nm	10 ng/ml	Manjoub et al. [28]
LLE (isopropanol – methylene chloride)	Climazolam	220 nm	12.5 ng/ml	Lee et al. [29]
LLE (t-butylmethyl ether)	Diazepam	240 nm	7 ng/ml	Johnson et al. [30]
LLE (toluene)	Flurazepam	207 nm	100 ng/ml	Van Brandt et al. [31]
SPE (C18)	Clonazepam	254 nm	15 ng/ml	Mastey et al. [32]
SPE (C18)	Climazolam	254 nm	50 ng/ml	Sautou et al. [33]
SPE (C18)	Alprazolam	nano ESI-MS-MS	1.5 ng/ml	Kapron et al. [34]
SPE (C18)	Alprazolam	ESI-MS-MS	0.25 ng/ml	Kashuba et al. [12]
Column switching	Flurazepam	230 nm	10 ng/ml	Lauber et al. [35]

[a]*Abbreviations:* LLOQ, lower limit of quantitation; LLE, liquid-liquid extraction; SPE, solid phase extraction; APCI, atmospheric pressure chemical ionization; MS, mass spectrometry; ESI, electrospray ionization

Louis, MO, USA). All other chemicals and solvents were of analytical grade or better. Deionized water was generated with a Hydro-Reverse Osmosis system (Durham, NC, USA) connected to a Milli-Q UV Plus purifying system (Malbourough, MA, USA). Drug-free heparinized human plasma was obtained from the National Institutes of Health Clinical Center Blood Bank (Bethesda, MD, USA).

Preparation of Stock Solutions and Standards

Stock solutions of midazolam were prepared independently in triplicate by dissolving the appropriate amount of drug, corrected for impurity, in HPLC-grade methanol at a concentration of 0.500 mg/ml, and were then stored in glass at –20ºC for up to 4 weeks. The difference in drug concentration in each of the triplicate stock solutions, estimated from the mean peak area following repeat analysis of a dilution of the stock, was determined to be within 5%. Out of one of the midazolam stock solutions, a working solution containing 100 μg/ml was prepared in methanol, which was further used for the construction of calibration samples and quality control (QC) samples. An internal standard working solution of 4 μg/ml was prepared by dilution with HPLC-grade methanol, and was stored for later use at –20ºC.

Sample pretreatment

Samples were prepared by spiking 600 μl blank human plasma in 15 ml polypropylene tubes with midazolam at the indicated concentrations (below), and lorazepam at a final concentration of 100 ng/ml (25 μl of the methanolic working solution). Blank samples and samples containing only internal standard were corrected for methanol content, so all samples had a final methanol content of 7.7% . After adding 600 μl of 0.1 M ammonium acetate, the samples were vortex-mixed and 4 ml of water saturated ethyl acetate was added to each tube. The samples were vortex mixed for 1 min and then centrifuged at 2000 *g* for 5 min (4˚C). The organic phase was transferred to a clean glass tube using a disposable plastic pipette, and evaporated to dryness under a continuous stream of air at 40˚C. The extracts were reconstituted with 125 μl of a mixture

of methanol and 0.01 M ammonium acetate (60:40, vol/vol), and 75-µl was injected for chromatographic analysis.

Equipment

Chromatography was performed on a HP1100 system (Agilent Technology, Palo Alto, CA, USA), which included a binary pump, a refrigerated autosampler, a degasser, a photodiode-array detector, and a single-quadrupole mass-spectrometric (MS) detector (Agilent 1100 MSD) equipped with an atmospheric pressure chemical ionization source. The autosampler was maintained at 4 °C and the column was at ambient temperature. The analytes were separated on a stainless steel column (150 × 4.6 mm I.D.) packed with 5-µm particle size Zorbax Eclipse XDB-C8 material (Agilent), preceeded by a Zorbax phenyl (4.6 × 12.5 mm I.D., 5 µm particle size) guard colum (Agilent). Samples were eluted isocratically using a mobile phase composed of methanol – 0.01 M ammonium acetate (60:40, vol/vol) at a flow rate of 1.0 ml/min. The MS conditions were as follows: fragmentor, 90 V; gain, 1; drying gas flow, 5 liter/min; nebulizing gas pressure, 55 psi; drying gas temperature, 330˚C; and capillary voltage, 2200 V. Selected-ion monitoring was accomplished at *m/z* 326 for midazolam, and *m/z* 322 for the internal standard.

The chromatographic data were collected and analyzed using the software package Chemstation (Agilent). Calibration graphs were calculated by least-squares linear regression analysis of the peak area ratio of midazolam and the internal standard *versus* the drug concentration of the nominal standard. The zero concentration sample (blank) was used to visually verify the purity of the reagents and the lack of other potentially interfering (endogenous) substances, but was not considered for the regression analysis of standards. The goodness-of-fit of various calibration models was evaluated by visual inspection, the correlation coefficient and an ANOVA lack-of-fit test.

Validation procedures

Method validation with respect to accuracy and precision was performed according to procedures described in detail elsewhere [13]. Calibration standards in drug-free human heparinized plasma were prepared freshly by serial dilution at midazolam concentrations

of 1.00, 2.00, 5.00, 10.0, 30.0, 100, and 200 ng/ml, such that the total amount of methanol added was identical in each sample. Pools of quality-control (QC) samples of midazolam in plasma were prepared similarly at concentrations of 1.00, 3.00, 85.0, and 170 ng/ml, and stored in batch at –20ºC for the duration of the validation procedure. Validation runs included a calibration curve and QC samples analyzed in triplicate and were performed on 8 separate occasions. The lower limit of quantitation (LLOQ) of the assay was assessed by determining the concentration of midazolam at which the values for precision and accuracy were less than 20%, excluding outliers.

The accuracy and precision of the assay were assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within-run and between-run precision, respectively. The accuracy for each tested concentration was calculated as:

$$DEV = 100 \times \{([\text{analyte}]_{\text{mean}} - [\text{analyte}]_{\text{nominal}})/[\text{analyte}]_{\text{nominal}}\}$$

Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square (MS_{bet}), the within-groups mean square (MS_{wit}), and the grand mean (GM) of the observed concentrations across runs were calculated using the software package NCSS 2001 (J. Hintze, Number Cruncher Statistical Systems, Kaysville, UT, USA). The between-run precision (BRP), expressed as a percentage relative standard deviation, was defined as:

$$BRP = 100 \times (\sqrt{((MS_{\text{bet}} - MS_{\text{wit}})/n)}/GM)$$

where n represents the number of replicate observations within each run. For each concentration, the estimate of the within-run precision (WRP) was calculated as:

$$WRP = 100 \times (\sqrt{(MS_{wit})}/GM)$$

The extraction efficiency of the assay, expressed as a percentage, was measured by comparison of extracted plasma samples and aqueous samples injected without extraction in replicates of five at midazolam concentrations of 1.00, 3.00, 85.0, and 170 ng/ml.

Possible matrix effects were investigated by infusing a 15 μM solution of midazolam into the MS, post column using a syringe pump via a tee. After a constant response was established, five blank plasma samples that had been extracted and reconstituted, as

detailed above, were injected. A slight matrix effect was noted at a retention time of 2 minutes. No further matrix effects were noted, showing that there is no interference with midazolam and lorezepam, both of which have significantly longer retention times.

Pharmacokinetic analysis

Midazolam (Roche Laboratories, Nutley, NJ, USA) was injected intravenously over a 30 to 60-second period at a dose of 25 μg/kg to 35 human subjects. Blood samples (7-ml each) were collected prior to drug administration, and at 5 and 30 min, and 1, 2, 4, 5, and 6 hours post-infusion. The samples were centrifuged at 2000 *g* for 10 min (4 °C) immediately after collection, and were stored at –20 °C at the day of collection and at –80 °C afterwards, until the day of analysis. The current experiment was approved by the Institutional Review Board, and the patients signed informed consent before study entry for the blood sampling procedure. Plasma concentration-time data of midazolam were analyzed by a non-compartmental model using the software package WinNonlin v4.0 (Pharsight Corporation, Mountain View, CA, USA).

Results and discussion

Specificity

Figure 1 displays typical chromatograms of an extract of a blank human plasma sample (**A**), an extract of a plasma sample spiked with midazolam at a concentration of 1 ng/ml (**B**), and an extract of a plasma sample taken at 5 hours after the administration of midazolam (dose, 25 μg/kg) (**C**). The chromatographic peaks showed symmetrical resolution, with no interfering peaks present for all compounds in drug-free specimens, obtained from 6 individuals. Midazolam (t_R = 11.36 min) and the internal standard lorazepam (t_R = 6.79 min) were well separated, and the overall chromatographic run time was established at 15 minutes. The selectivity and resolution of the chromatographic system was also confirmed by co-injection of midazolam with one of its major metabolites, 1′-hydroxy-midazolam.

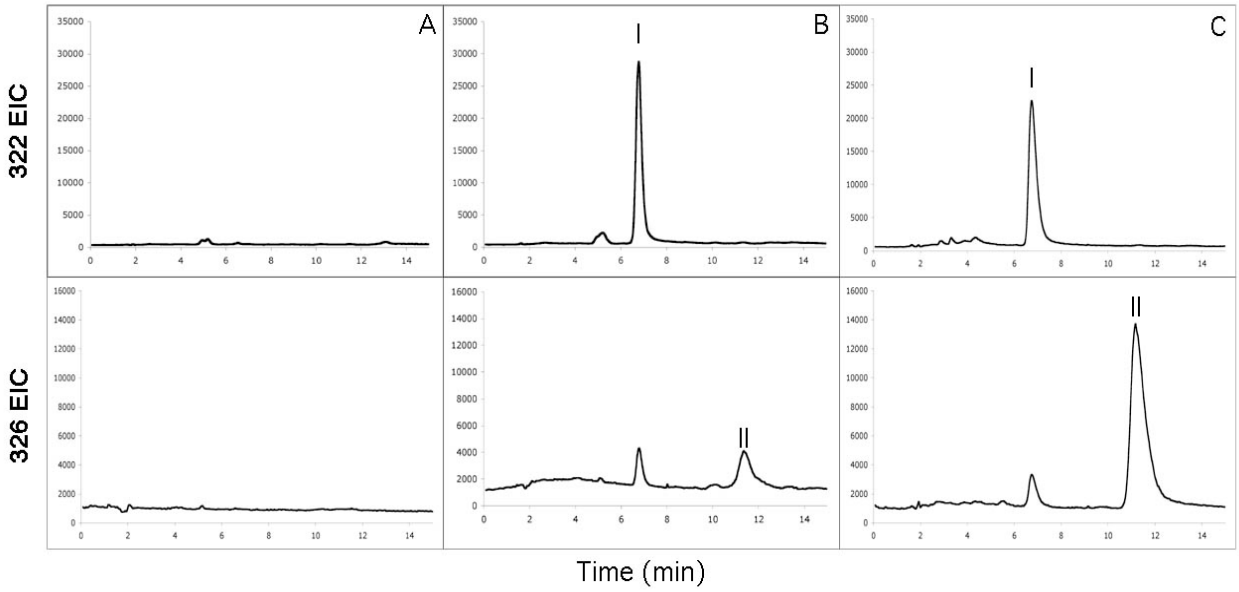

Fig. 1. Chromatograms from reversed-phase HPLC analysis of a blank human plasma sample (**A**), a human plasma sample spiked with midazolam at a concentration of 1 ng/ml (**B**), and a plasma sample obtained from a female patient with cancer at 5 hours after intravenous administration of midazolam (dose, 25 µg/kg) (**C**). The labeled chromatographic peaks indicate midazolam (**II**) and the internal standard lorazepam (**I**).

Validation characteristics

The measurement variance over the range of 1.00 to 200 ng/ml increased proportionally with the midazolam concentration, as detected by a one-sided *F*-test at an α-value of 5%. Therefore, a weighting factor was applied inversely proportional to the variance at the given concentration level (1/[nominal midazolam concentration]^2). Using least-squares linear-regression, a mean (± standard deviation) correlation coefficient of 0.9974 ± 0.00176 (range, 0.9946 to 0.9988) was obtained (Table 2). The statistical evaluation of the coefficients of the mean ordinary least-squares line indicated an acceptable degree of bias in the slope (coefficient of variation, 8.20%) and in the intercept (coefficient of variation, 40.7%), suggesting minor matrix and blank effects [14]. In blank human plasma spiked with midazolam at a concentration of 1.00 ng/ml, only 2 out of 24 samples were outside the acceptable ± 20% deviation limits for accuracy [13], while the remaining samples had a mean percentage deviation from the nominal concentration and within and between-run variability of ±7.23%, 0.056%, and 13.4%, respectively. Based on

Table 2. Back-calculated concentrations from calibration curves run in duplicate on four occasions

Nominal (ng/ml)	GM[a] (ng/ml)	SD (%)	DEV (%)	RSD	*n*
1	1.05	0.062	5.13	5.89	8
2	1.93	0.262	-3.68	13.6	6
5	4.64	0.227	-7.23	4.89	8
10	10.2	0.247	1.52	2.43	8
30	31.9	1.43	6.34	4.48	7
100	101	1.36	1.4	1.34	8
200	199	5.27	-0.388	2.65	8

[a] *Abbreviations*: GM, grand mean; SD, standard deviation; DEV, percent deviation from nominal value; RSD, relative standard deviation; *n*, total number of replicate observations within the validation runs.

these results, the lower limit of quantitation for midazolam was established at 1.00 ng/ml. This represents a 7 to 100-fold increase in sensitivity as compared to assays based on HPLC with UV detection (Table 1).

Validation data of the analytical method in terms of accuracy and precision are summarized in Table 3. Based upon analysis of QC samples analyzed on 8 different occasions, the final method was shown to be accurate, with an average accuracy at the 4 tested concentrations within 12%, and precise, with a within-run and between-run precision always within 15%. The mean overall extraction efficiency for midazolam was independent of the spiked concentration, and not significantly different from 100%.

Table 3. Assessment of accuracy and precision from quality-control samples*

Nominal (ng/ml)	GM[a] (ng/ml)	SD (%)	DEV (%)	BRP (%)	WRP	*n*
1	0.928	0.476	-7.23	13.4	0.056	22
3	3.13	0.312	4.48	12.1	0.112	22
85	94.9	8.42	11.6	2.58	0.865	24
170	160	8.89	-5.6	4	0.608	24

[a] *Abbreviations*: GM, grand mean; SD, standard deviation; DEV, percent deviation from nominal value; WRP, within-run precision; BRP, between-run precision; *n*, total number of replicate observations during the validation runs. * Three samples at each concentration were run on 8 different occasions.

Plasma concentration-time profile

The mean observed concentration-time profile of midazolam in a group of 35 human subjects (median dose, 1.8 mg; range, 1.2 – 2.7 mg) is shown in Figure 2. The mean (± standard deviation) observed peak concentration of midazolam in this group was 62.8 ± 22.0 ng/ml, with a mean area under curve extrapolated to infinity of 96.1 ± 42.7 ng·h/ml and a mean systemic clearance of 360 ± 132 ml/min (range, 118 – 704 ml/min), which is similar to previously obtained values [2]. In 28 of 35 subjects, the observed midazolam concentration had already dropped below 5 ng/ml at 4 hours after drug administration (overall mean concentration, 4.46 ± 2.50 ng/ml). This provides further evidence that for the purpose of CYP3A phenotyping with non-sedative doses of midazolam, an analytical method is required with a lower limit of quantitation at the low ng/ml level to avoid the generation of overestimated values for drug clearance.

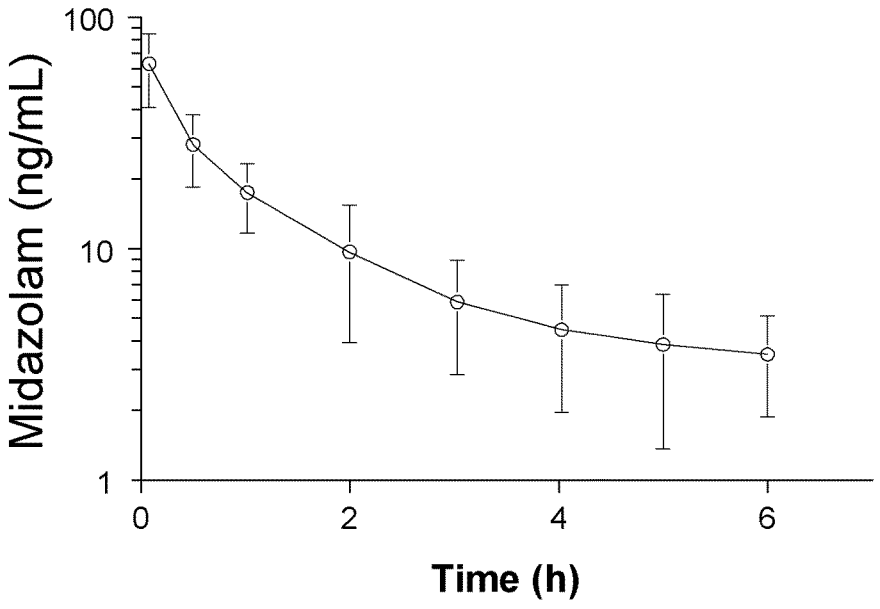

Fig. 2. Mean plasma concentration-time profile of midazolam following intravenous administration of midazolam at a dose of 25 μg/kg to a group of 35 human subjects (median total dose, 1.8 mg). Symbols represent mean values, and error bars represent standard deviations.

Conclusion

In conclusion, we have described a novel method for the quantitative determination of midazolam in human plasma, which is specific, accurate and precise, and can be easily implemented in routine practice. The sample pretreatment procedure is based on a simple and efficient solvent extraction, thereby eliminating the need of solid phase extraction, column switching procedures, and/or the use of large volumes of plasma for sample clean-up. Compared to a previously described assay for the determination of midazolam with single-quadrupole mass spectrometric detection [15], this new method provides superior sensitivity, at a lower limit of quantitation of 1.00 ng/ml, using only 600-µl sample aliquots. The method will be implemented in current and future clinical pharmacokinetic investigations in which midazolam is used as a CYP3A phenotyping probe.

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Chapter 4

Impact of Common *CYP3A4* and *CYP3A5* Variants on the Pharmacokinetics of the Cytochrome P450 3A Phenotyping Probe Midazolam in Cancer Patients

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Abstract

Purpose: To evaluate the impact of naturally-occurring variants in genes encoding the cytochrome P450 isoforms CYP3A4 and CYP3A5 in patients with cancer receiving midazolam as a phenotyping probe.

Experimental Design: Five variants in *CYP3A4* and *CYP3A5* were evaluated in 58 patients (21 women and 37 men) receiving a short i.v. bolus of midazolam (dose, 0.0145 or 0.025 mg/kg). Midazolam concentrations in plasma were determined using liquid chromatography-mass spectrometry, and pharmacokinetic parameters were calculated using non-compartmental analysis. Genomic DNA was characterized for the variants by PCR-restriction fragment length polymorphism, and all genotypes were confirmed by direct nucleotide sequencing.

Results: The mean clearance of midazolam was 24.4 ± 9.12 L/h, and phenotypic CYP3A activity varied about 4-fold in this population (range, 10.8 to 44.3 L/h). There were six carriers of the *CYP3A4*1B* allele (allele frequency, 0.061). No variant alleles for *CYP3A4*17*, *CYP3A4*18A*, or *CYP3A5*6* were identified. Forty-eight of the 58 patients were homozygous variant for *CYP3A5*3C*, eight were heterozygous, and two were homozygous wild-type (allele frequency, 0.897). No associations were noted between any of the studied genotypes and the phenotypic measures ($P \geq 0.16$). Likewise, a common variant in exon 26 in the gene encoding P-glycoprotein (i.e., *ABCB1* (*MDR1*) 3435C>T) that was previously reported to be linked to *CYP3A4* mRNA levels was unrelated to any of the studied phenotypic measures ($P \geq 0.49$).

Conclusions: The studied genetic variants in *CYP3A4* and *CYP3A5* are unlikely to have an important functional significance to phenotypic CYP3A activity in patients with cancer.

Introduction

Isoforms of the cytochrome P-450 (CYP) 3A subfamily are the most abundantly expressed CYP enzymes in the human liver [1]. In adults, CYP3A4 and CYP3A5 are predominant among the four known isoforms (CYP3A4, CYP3A5, CYP3A7, and CYP3A43) in the liver and intestines [1]. However, metabolically active CYP3A5 is expressed only by an estimated 10 to 30% of Caucasians and 50 to 60% of African Americans [2,3]. The genetic basis for polymorphically expressed CYP3A5 was recently reported to be associated with a single nucleotide polymorphism (SNP) in intron 3 of the *CYP3A5* gene (*CYP3A5*3C*) [4,5]. This SNP causes alternative splicing and truncation of CYP3A5 protein, and provides the basis for the absence of CYP3A5 protein in most individuals of Caucasian descent. In people with at least one *CYP3A5*1A* wild-type allele, CYP3A5 accounts for at least 50% of the total CYP3A content, and results in approximately 2- to 3-fold higher total CYP3A activity *in vitro* [4,6]. The most prevalent polymorphism in *CYP3A4* (*CYB3A4*1B*) occurs in the 5′ flanking region of the gene. The frequency of this allele ranges from 2 to 9.6% in Caucasians to 35 to 67% in African Americans [1].

Wide variation in CYP3A expression and activity exists in humans, which is reflected by extensive interindividual differences in the clearance of CYP3A substrate drugs and phenotyping probes like midazolam [7]. For many anticancer drugs used therapeutically, pharmacokinetic variability is a significant contributor to the variability of both the severity of side effects and therapeutic response [8]. Furthermore, the metabolism of 37% of all currently approved cytostatic and/or cytotoxic anticancer agents is known to be mediated, at least in part, by the CYP3A isoforms. Hence, identification of factors affecting the clearance of CYP3A substrates could aid in predicting or adapting appropriate, individualized doses of anticancer drugs. Here, we evaluated the impact of common naturally-occurring variants in genes encoding CYP3A4 and CYP3A5 in cancer patients receiving midazolam as a phenotyping probe.

Methods

Patient Selection

This study was conducted under two Institutional Review Board approved protocols and all patients signed an informed consent form prior to treatment. Patients with a histologically or cytologically confirmed diagnosis of malignant solid tumor were eligible for two clinical trials in which patients underwent CYP3A phenotyping with intravenously administered midazolam [9,10]. Eligibility criteria included: age >18 years; performance status ≤ 1; adequate hematopoietic (leukocytes, ≥ 4.0×10^9/L; neutrophils, ≥ 1.5×10^9/L; and platelets, ≥ 100×10^9/L), hepatic (bilirubin within normal limits; transaminases, ≤ 2 times the upper limit of normal) and renal function (creatinine clearance, ≥ 50 mL/min). All patients were required to have an estimated life expectancy of more than 12 weeks, and no previous chemotherapy was allowed for at least 4 weeks prior to enrollment. The patients were asked to abstain from substances known to affect the function and/or expression of CYP3A and/or ABCB1 for a period of 2 weeks before, during, and up to 3 weeks after the administration of midazolam.

Blood Sampling and Processing

Blood samples were collected in glass tubes containing heparin. Samples were drawn at 5, and 30 minutes and 1, 2, 3, 4, and 5 hours following bolus i.v. administration of midazolam at a dose of either 0.0145 or 0.025 mg/kg. In about half of the patients, additional samples were obtained at 15 minutes and 6 hours after midazolam administration. Immediately after collection, each sample was centrifuged for 10 minutes at 2000 *g* (4 °C), and plasma supernatants were stored at –80 °C until the day of analysis.

Drug Measurement and Pharmacokinetic Analysis

Quantitation of midazolam in plasma was performed by high-performance liquid chromatography with mass spectrometric detection, as described previously [11]. Pharmacokinetic profiles of midazolam were analyzed by non-compartmental methods

using the software package WinNonlin version 4.0 (Pharsight, Mountain View, CA). The parameters calculated included area under the plasma concentration-time curve extrapolated to infinity (AUC), clearance (CL; calculated as dose/AUC), volume of distribution at steady-state (V_{ss}), and half-life of the terminal phase (t1/2,z). The AUC was normalized to a dose of 0.025 mg/kg. As a comparison, the AUC was also calculated based on the limited sampling model described previously [12].

CYP3A4 and CYP3A5 Genotype Analysis

Plasma was used to isolate genomic DNA according to the manufacturer's instructions using the UltraSens Virus Kit (Qiagen, Valencia, CA). Variations in *CYP3A4* (*CYP3A4*1B*, *CYP3A4*17*, *CYP3A4*18A*) and *CYP3A5* (*CYP3A5*3C*, and *CYP3A5*6*) were analyzed using restriction fragment length polymorphism (RFLP) based techniques, as previously described [13,14]. Confirmation of all the variant genotype assignments (i.e., for *CYP3A4*1B*, *CYP3A5*3C*, and *CYP3A5*6*) was done using direct nucleotide sequencing.

In preparation for sequencing, an initial polymerase chain reaction (PCR) was performed with concentrated DNA utilizing 20 pmol of each primer. For *CYP3A4*1B*, the primers were designed and chosen as follows: 5′-CTGTGTGAGGAGTTTGGTGAG-3′ (*CYP3A4*1B* F3), and 5′-TGGAAGAGGCTTCTCCACCTTG-3′ (*CYP3A4*1B* R3). These primers were added to a reaction mixture of 1× PCR buffer (Perkin-Elmer), 2 mM of each of the four deoxynucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, and 2 units of Platinum Taq polymerase (Invitrogen, Carlsbad, CA) in a reaction volume of 20 μL. The temperature profile for the PCR reaction was one cycle at 94°C for 5 minutes, followed by 20 cycles of 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 30 seconds, with a final 7-minute cycle at 72°C. After initial amplification, 3 μL of amplified product was removed and an additional PCR amplification was performed using 40 pmol of the primers 5′-GCTCTGTCTGTCTGGGTTTGG-3′ (*CYP3A4*1B* F4) and 5′-CACACCACTCACTGACCTCCT-3′ (*CYP3A4*1B* R4) in a 50-μL reaction. PCR conditions were 94°C for 5 minutes, followed by 40 cycles of 94°C for 30 seconds, 64°C for 30

seconds, and 72ºC for 30 seconds, with a final 7-minute cycle at 72ºC. Sequencing was performed using the following primers: (*CYP3A4*1B* F5) 5′-AGGTGTGGCTTGTTGGGATG-3′ for the forward strand, and (*CYP3A4*1B* R5) 5′-TCAGAAACTCAAGTGGAGCC-3′ for the reverse strand.

For the *CYP3A5*3C* allele, primers were designed and chosen as follows: (5′-TCCTCAGAATCCACAGCGCTG-3′ (*CYP3A5*3C* F1) and 5′-TTTATGTGCTGGAGAAGGACG-3′ (*CYP3A5*3C* R1). These primers were used in a 20 μL reaction that contained 1× PCR buffer (Perkin-Elmer), 1.5 mM $MgCl_2$, 2 mM dNTPs, and 2 units of Platinum Taq polymerase (Invitrogen). The cycle conditions were: one cycle at 94ºC for 5 minutes, followed by 20 cycles of 94ºC for 30 seconds, 64ºC for 30 seconds, and 72ºC for 30 seconds, ending with a final 7-minute cycle at 72ºC. After initial amplification, 3 μL of amplified product was removed and an additional PCR amplification (as described above in a total of 50 μL final volume) was performed using 40 pmol of primers 5′-AGCACTTGATGATTTACCTGCC-3′ (*CYP3A5*3C* F2) and 5′-CCAGGAAGCCAGACTTTGATC-3′ (*CYP3A5*3C* R2). PCR conditions for this reaction were 94ºC for 5 minutes, followed by 40 cycles of 94ºC for 30 seconds, 62ºC for 30 seconds, and 72ºC for 30 seconds, with a final 7-minute cycle at 72ºC. Sequencing primers were as follows: (*CYP3A5*3C* F3) 5′-AGTGGCATAGGAGATACCCAC-3′ and (*CYP3A5*3C* R3) 5′-AGGTTCTAGTTCATTAGGGTG-3′.

For the *CYP3A5*6* allele, an initial PCR was performed using the sample DNA and 20 pmol of each primer, 5′-AGGGAGTAGATGGAAGATGATTC-3′ (*CYP3A5*6* F1) and 5′-AGTTGATTATTGGATGCTTAGGGC-3′ (*CYP3A5*6* R1). These primers were used in the initial reaction along with 1× PCR buffer (Perkin-Elmer), 1.5 mM $MgCl_2$, 2mM dNTPs, and 2 units of Platinum Taq polymerase (Invitrogen) in a reaction volume of 20 μL. The temperature profile for the PCR reaction was one cycle at 94ºC for 5 minutes, 40 cycles of 94ºC for 30 seconds, 64ºC for 30 seconds, and 72ºC for 30 seconds, followed by a final 7-minute cycle at 72ºC. After initial amplification, 3 μL of amplified product was removed and an additional PCR amplification using the above reaction ingredients was performed using 40 pmol of the designed primers 5′-TGCTGCATGTATAGTGGAAGGAC-3′

(*CYP3A5*6* F2) and 5′-TTTGTGGTGGGGTGTTGACAGC-3′ (*CYP3A5*6* R2). PCR conditions for this reaction were 94ºC for 5 minutes, followed by 40 cycles of 94ºC for 30 seconds, 66ºC for 30 seconds, and 72ºC for 30 seconds, with a final 7-minute cycle at 72ºC. The sequencing primers were 5′-TTGGGGCCTACAGCATGGATG-3′ (*CYP3A5*6* F3) and 5′-TGTGTGAGGGCTCTAGATTGAC-3′ (*CYP3A5*6* R3). The existence of each variant was determined by direct nucleotide sequencing using the dRhodamine Terminator Cycle Sequencing Ready Reaction kit on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, CA). Concordance in results between RFLP and direct sequence analysis was observed in all but one sample (for *CYP3A5*3C* only); results from sequence analysis were used for correlation with midazolam pharmacokinetic parameters.

ABCB1 (MDR1) Genotype Analysis

The following primers were designed for the *ABCB1* 3435C>T variant in exon 26: 5′-AGCAACCTTACATCTACTAC-3′ (*ABCB1* 3435C>T 6 F) and 5′-GGAGAGACAGTCATGCCTAC-3′ (*ABCB1* 3435C>T R), and nested primers 5′-TCACAGTAACTTGGCAGTTTCA 3′ (*ABCB1* 3435C>T F) and 5′-GGGACCAGCCCCTTATAAATC 3′ (*ABCB1* 3435C>T R) (Invitrogen). The outside primers and approximately 100 ng of genomic DNA were added to a 15-μL reaction mixture consisting of 1 mM dNTPs, 1× PCR buffer, 2.5 mM $MgCl_2$ and 0.6 units of Amplitaq Gold (Applied Biosystems). For the nested PCR reaction, 2 μL of the first PCR product was used as template for amplification. The thermocycler conditions were: an initial cycle at 95°C for 12 minutes, followed by 10 cycles 94°C for 45 seconds, 55°C for 1 minute and 72°C for 1 minute, 20 cycles of 89°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, and ending with a final 10-minute cycle at 72°C. The restriction enzyme MboI (New England Biolabs, Beverly, MA) was used for the determination of the variants. Enzyme (0.5 μL) and 2 μL of enzyme buffer were added to 20 μL of PCR product and incubated at 37°C for 1 hour, followed by an incubation at 65°C for 20 minutes. Blue juice (1 μL of 5× solution) was added to 10 μL restricted PCR product, and run on a 2.0% agarose gel with ethidium bromide as a fluorescence detector. A "T" mutation was determined by bands of

408, 263, 158, 103, 59 bp; a "C" was determined by bands of 263, 236, 172, 158, 59 bp; heterozygous samples contained all bands. The existence of the variant was also determined by direct nucleotide sequencing after a cleanup step with MinElute PCR purification kit (Qiagen) in a total volume of 12 μL, which included 6 μL of DNA at 3 ng per 100 bp of amplified products and 6 μL of either the forward or reverse primer. Sequencing in the forward and reverse direction was performed using an ABI 3730XL Sequencer (Applied Biosystems). Concordance in results between RFLP and direct sequence analysis was observed in all but four samples; results from sequence analysis were used for correlation with midazolam pharmacokinetic parameters.

Statistical Considerations

All data are presented as mean values ± standard deviation (SD), unless stated otherwise. Genotype-frequency analysis of Hardy-Weinberg equilibrium was carried out using Clump version 1.9. Linkage disequilibrium was calculated using EMLD (Qiqing Huang; see: http://linkage.rockefeller.edu/soft/). The linkage between each pair of SNPs was determined in terms of the classical statistic D′. The absolute value for D′ (|D′|) of 1 denotes complete linkage disequilibrium, while a value of 0 denotes complete linkage equilibrium.

To relate pharmacokinetic parameters with the variant genotypes, the Kruskal-Wallis test, a nonparametric one-way analysis of variance, was used for each polymorphism examined. (NCSS v2001; J. Hintze, Number Cruncher Statistical Systems, Kaysville, UT). Although this study was mainly exploratory in intent, a Bonferroni adjustment was used to evaluate the significance of the multiple comparisons. Two-sided *P*-values of less than 0.017 (0.05 divided by the three observed variant genotypes) were regarded as statistically significant, and those less than 0.05 were considered a trend. These levels were chosen to reduce the risk of finding purely coincidental associations in view of the number of variant genotypes analyzed concurrently.

Results

Patients

A total of 58 adult patients (21 women and 37 men) were evaluated during this study. Patients were of Caucasian (*n* = 55), African American (*n* = 2) or Hispanic (*n* = 1) descent, and were between 29 and 73 years old (median age, 57 years). Additional patient demographics are shown in Table 1.

Table 1. Patient Demographics

Gender	Female:	27		
	Male:	31		
Race	Caucasian	55		
	African-American	2		
	Hispanic	1		
	Mean	*Median*	*Min*	*Max*
Age (years)	56.7	58	29	73
Height (m)	1.73	1.74	1.55	1.91
Weight (kg)	80.27	77.85	48	137
BSA (m^2)	1.94	1.91	1.46	2.46
Bilirubin (μg/dL)	0.50	0.47	0.23	1.23

Midazolam Disposition

Complete midazolam pharmacokinetic data following i.v. administration were available in all 58 patients. The mean dose-normalized AUC was 90.2 ± 35.8 ng×h/mL, whereas the mean clearance was 24.4 ± 9.12 L/h, which is consistent with earlier findings obtained in cancer patients following i.v. administration of midazolam [15,16]. Table 2 provides a comparison of the clearance values determined in previously conducted trials in cancer patients and healthy volunteers, which involved genotyping for *CYP3A5*3C*.

Table 2. Summary of midazolam pharmacokinetics

Parameter	Mean	Median	SD	Min	Max	Sample Size	Population (route)
AUC (ng×h/mL)	90.2	80.6	35.8	39.0	204		
Cmax (ng/ml)	79.2	71.2	31.9	37.5	194		
Vss (L)	78.0	70.1	40.1	24.9	228		
T1/2, z (h)	3.40	2.8	2.37	0.62	17.5		
CL (L/h)							
This study	24.4	22.8	9.12	10.8	44.3	58	Cancer patients (IV)
Ref [15]	25.6	[b]	9.88	[b]	[b]	31	Cancer patients (IV)
Ref [28]	34.9 [a]	[b]	[b]	[b]	[b]	22	*CYP3A5*3C/3C* volunteers (PO)
	31.9 [a]	[b]	[b]	[b]	[b]	17	*CYP3A5*1A/*3C* volunteers (PO)
Ref [42]	25.4	[b]	9.3	[b]	[b]	27	Cancer patients (IV)
	24.4	[b]	8.1	[b]	[b]	24	Volunteers (IV)
Ref [25]	23.2	[b]	6.3	9	47.6	23	Volunteers (IV)
Ref [24]	112 [a]	123 [a]	[b]	55.6	214	21	Volunteers (PO)
Ref [16]	26.9	[b]	12.8	8.7	68.7	45	Cancer patients (IV)
	81.7	[b]	41.5	7.74	185	22	Cancer patients (PO)
Ref [29]	32.5	[b]	10.2	[b]	[b]	6	*CYP3A5*1A/*1A* volunteers (IV)
	32.1	[b]	14.5	[b]	[b]	6	*CYP3A5*1A/*3C* volunteers (IV)
	28.4	[b]	12.5	[b]	[b]	7	*CYP3A5*3C/*3C* volunteers (IV)

Abbreviations: AUC, dose-normalized area under the plasma concentration versus time curve extrapolated to infinity; Cmax, dose-normalized peak plasma concentration; V_{ss}, volume of distribution at steady-state; T1/2,z, half-life of the terminal phase; CL, clearance or apparent oral clearance (CL/F) in case of oral administration. [a] Calculated based on AUC and dose published. [b] Data not provided.

Variant Genotypes

Genotyping results were available for all patients except for *CYP3A4*1B*, which had available data for 57 of the 58 patients (Table 3). Of these 57 patients, there were six carriers of the *CYP3A4*1B* allele (five heterozygous *CYP3A4*1A/*1B*, and one homozygous *CYP3A4*1B/*1B*). Both of the subjects of African-American descent were wild-type for *CYP3A4*1B* (homozygous *CYP3A4*1A/*1A*). No patients were identified carrying variant alleles for *CYP3A4*17*, *CYP3A4*18A*, or *CYP3A5*6*. Forty-eight of the 58 patients were homozygous variants for *CYP3A5*3C* and there were eight heterozygotes (*CYP3A5*1A/*3C*). The *ABCB1* 3435C>T genotypes were distributed as follows: wild-type (C/C), 12; heterozygous (C/T), 35; and variant (T/T), 11.

ABCB1 3435C>T was in linkage equilibrium with both *CYP3A4*1B* and *CYP3A5*3C* (D′ = 0.183 and 0.0933, respectively). Linkage disequilibrium was apparent between *CYP3A4*1B* and *CYP3A5*3C*, (D′ = 0.656), as described previously [17].

Genotype-Phenotype Relations

There were no significant correlations between any of the variant genotypes and clearance, volume of distribution at steady-state, or AUC (Figure 1 and Table 4). Although the mean midazolam clearance was 1.2 times higher in patients with the *CYP3A5*1A/*1A* genotype compared with patients that were homozygous variant (*CYP3A5*3C/*3C*), this difference was not statistically significant ($P = 0.50$). Furthermore, there was no correlation between *CYP3A5*3C* genotype status and clearance, when patients with the homozygous variant genotype (*CYP3A5*3C/*3C*) were compared with patients that had at least one wild-type allele (*CYP3A5*1A/*1A* and *CYP3A5*1A/*3C*) ($P = 0.24$).

Comparison of Predicted and Observed AUC

The AUC of midazolam was also calculated with a limited-sampling model based on the 4-hour time point, as done by Wong et al. [16]. This model was designed originally using a different data set, as described elsewhere [12]. Figure 2 shows the correlation of the observed AUC with the calculated AUC. The correlation between the predicted AUC

Table 3. Genotype and allele frequencies for the studied variants

Polymorphism [c]	*Nomenclature*	*Effect* [d]	*Genotype frequencies* [a]			*Allele frequencies* [b]	
			Wt [e]	*Het*	*Var*	*p*	*q*
CYP3A4 -392A>G	*CYP3A4*1B*	Promoter	51 (89.5)	5 (8.77)	1 (1.75)	0.939	0.061
CYP3A4 15615T>C	*CYP3A4*17*	F189S	58 (100)	0 (0)	0 (0)	1.000	0.000
CYP3A4 20070T>C	*CYP3A4*18A*	L293P	58 (100)	0 (0)	0 (0)	1.000	0.000
CYP3A5 6986A>G	*CYP3A5*3C*	Splicing defect	2 (3.45)	8 (13.79)	48 (82.8)	0.103	0.897
CYP3A5 14690G>A	*CYP3A5*6*	Splicing defect	58 (100)	0 (0)	(0)	1.000	0.000
ABCB1 3435C>T	(not available)	I1145I	12 (20.69)	35 (60.34)	11 (18.97)	0.509	0.491

[a] Number represent number of patients with percentage in parenthesis; the difference in total number of patients for *CYP3A4*1B* is due to the fact that one sample did not shown PCR amplification. [b] Hardy-Weinberg notation for allele frequencies (p, frequency for wild-type allele and q, frequency for variant allele). [c] Number represents position in nucleotide sequence based on gene AF280107 (for *CYP3A4*1B*, *CYP3A4*17*, and *CYP3A4*18A*). There is still no genomic *CYP3A5* sequence available corresponding to the *CYP3A5*1A* allele initially described on the cDNA level and encoding a functional CYP3A5 enzyme. The reference sequence for the *CYP3A5*1A* allele has been obtained by using the *CYP3A5* sequence found in accession number NG_000004.2 (corresponding to the *CYP3A5*3A* allele) and replacing base 6986 with an A and base 31611 with a C (numbering based on translation start as +1). See http://www.imm.ki.se/CYPalleles/cyp3a4.htm and http://www.imm.ki.se/CYPalleles/cyp3a5.htm).
[d] Number represents amino acid codon. [e] Wt, Homozygous wild type patient; Het, Heterozygous variant patient; Var, Homozygous variant patient.

and the observed AUC, using all time points, showed an $R^2 = 0.749$. The mean (± standard error) bias and precision of the prediction made by the model, determined by calculation of the percentage mean prediction error and the relative root mean squared prediction error, were -18.3 ± 5.82% and 30.2 ± 3.73%, respectively.

Discussion

Interindividual variability in drug efficacy and toxicity, resulting in unpredictable patient responses, is commonly observed in all therapeutic areas [18]. However, these differences are particularly important in the field of cancer therapy since many anticancer agents have a narrow therapeutic index. It is now well recognized that, in addition to environmental and physiological factors, such interpatient variation is commonly associated with polymorphisms in genes encoding drug metabolizing enzymes, drug transporters, and/or drug targets. In this study, we tested the hypothesis that the disposition of the prototypical CYP3A substrate drug midazolam in cancer patients is dependent on genetic variability in the *CYP3A4* and *CYP3A5* genes.

It has been proposed that genotyping for *CYP3A4* and *CYP3A5* variants may be useful for prediction of total CYP3A activity because of the genetic diversity in the genes encoding these proteins [2,3]. Over 30 SNPs in *CYP3A4* have been published; however, most are unlikely to contribute substantially to the interindividual variability of CYP3A4 activity *in vivo*, based on their limited functional significance and/or low allele frequency [3,19,20]. Similar to earlier findings [21], the very low allele frequency of the functional *CYP3A4*17* and *CYP3A4*18A* variants evaluated in the present study suggests that these SNPs most likely have no broad relevance to CYP3A4 activity and function in predominantly Caucasian populations. The one additional SNP that was studied here, *CYP3A4*1B*, is a promoter variant in the so-called nifedipine-specific element. The allele frequency of this variant is known to depend on racial ancestry, being absent in Japanese and Chinese subjects, and present in 2 to 10% in Caucasians, and 35 to 67% in African Americans, respectively [3]. The currently observed frequency of 6.1% is consistent with previous estimates for predominantly Caucasian populations. Although the functional

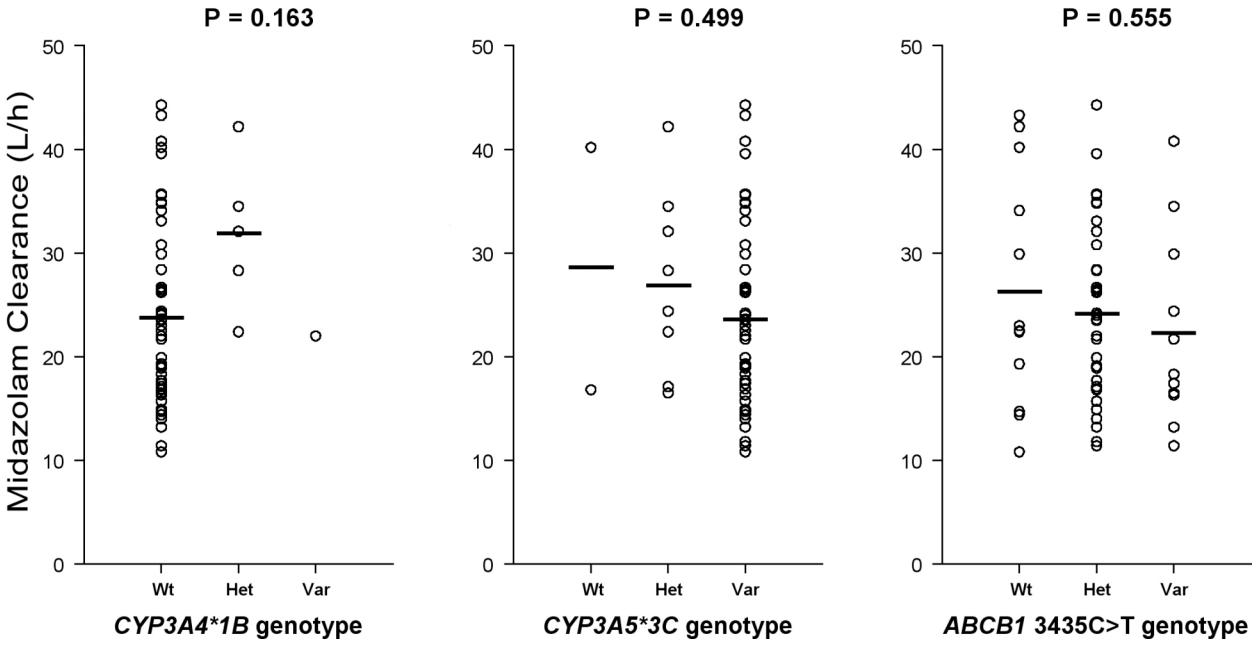

Fig. 1. Midazolam clearance as a function of the *CYP3A4*1B*, *CYP3A5*3C*, and *ABCB1* 3435C>T genotypes. Horizontal lines indicate mean values in each group. Wt, Homozygous wild-type patient; Het, Heterozygous patient; Var, Homozygous variant patient. Two-sided *P*-values are given above the panels

significance of this SNP presently remains unclear, the preponderance of the evidence suggests that *CYP3A4*1B* does not affect the metabolism and clearance of CYP3A4 substrate drugs [22-26]. Likewise, in the current study the pharmacokinetic profile of midazolam was not significantly different between patients with or without the *CYP3A4*1B* variant allele.

As mentioned previously, and in contrast to CYP3A4, the CYP3A5 protein isoform is known to be expressed in only a small percentage of Caucasian individuals, and this has been linked to a common transition in intron 3 of the *CYP3A5* gene (*CYP3A5*3C*), which introduces a frameshift during translation and results in a truncated, nonfunctional protein [4,5]. Approximately 70 to 90% of Caucasian subjects are homozygous variant for *CYP3A5*3C*, and thus are deficient in functionally active CYP3A5 [27], which is

consistent with the currently observed genotype frequency of 89.7%. In the present study, no change in midazolam pharmacokinetic parameters was noted between ten patients with at least one wild-type (*CYP3A5*1A*) allele and 48 patients carrying two variant (*CYP3A5*3C*) alleles. This is similar to findings obtained in healthy subjects using midazolam [24,25,28,29], or various other CYP3A phenotyping probes, including erythromycin [23,25], and nifedipine [30]. Interestingly, in a small cohort of Australian cancer patients, Wong et al. recently observed approximately 1.4-fold higher systemic clearance of midazolam in four patients with the *CYP3A5*1A/*3C* genotype compared to 39 patients with the *CYP3A5*3C/*3C* genotype ($P = 0.01$) [16]. In an effort to explain this discrepant finding, it is noteworthy that the interindividual variability in CYP3A phenotypic activity was substantially greater in the study reported by Wong et al. than that found in the various other studies [8-fold for systemic clearance and 24-fold for apparent oral clearance [16] *versus* 3.3-fold to 5.2-fold [24,25,28,29]], suggesting the possibility of altered expression of either CYP3A4, CYP3A5, or both, as a result of differences in advancement of the disease state of patients in the studied cohort [31].

Wong et al. estimated the systemic midazolam clearance from a previously developed limited-sampling model, based on the collection of one plasma sample obtained 4 hours after drug administration [16]. Although a retrospective validation of this model on our own data set indicated a good correlation between the predicted AUC and the observed AUC ($R^2 = 0.749$), the model had a statistically significant tendency for systematic negative error and demonstrated poor precision. To illustrate this problem, two different patients in our cohort that had very similar observed AUC values of 117.0 and 117.2 ng×h/mL, respectively, had a predicted AUC based on the limited-sampling model of 37.3 and 165 ng×h/mL, respectively. This raises the possibility that some of the proposed genotype-phenotype associations described previously that were based on parameter estimates from a limited-sampling model might be particularly prone to the risk of false-positive error due to inaccurate phenotypic assessments. In addition, some error may also be attributed to the smaller sample size used in the previous study [16]. Collectively, current evidence does not suggest that *CYP3A5*3C*-associated differences in

Table 4. **Genotype-phenotype relationships[a]**

	CL (L/h)				***V_{ss} (L)***				***AUC (ng×h/mL)***			
Polymorphism	*Wt[b]*	*Het*	*Var*	P	*Wt*	*Het*	*Var*	P	*Wt*	*Het*	*Var*	P
CYP3A4*1B	22.5 (10.8-44.3, 51)	32.1 (22.4-42.2, 5)	22.00 (1)	0.16	77.6 (24.9-228, 51)	49.9 (36.8-189, 5)	69.9 (1)	0.97	80.7 (39.0-204, 51)	69.0 (49.5-108, 5)	99.9 (1)	0.46
CYP3A5*3C	28.5 (16.8-40.2, 2)	26.4 (16.5-42.2, 8)	22.3 (10.8-44.3, 48)	0.50	81.0 (42.5-119, 2)	45.6 (24.9-189, 8)	73.8 (32.8-228, 48)	0.77	86.4 (60.0-113, 2)	73.2 (49.5-117, 8)	86.8 (39.0-204, 48)	0.56
***ABCB1* 3435C>T**	22.8 (10.8-43.3, 12)	23.6 (11.4-44.3, 35)	18.3 (11.4-40.8, 11)	0.56	88.8 (32.8-130, 12)	69.9 (31.9-228, 35)	69.7 (24.9-145, 11)	0.87	77.2 (47.8-204, 12)	89.0 (39.0-168, 35)	79.3 (53.4-166, 11)	0.85

Abbreviations: CL, clearance; V_{ss}, volume of distribution at steady-state; AUC, area under the plasma concentration-time curve normalized to dose.
[a] Numbers represent median (range, number of subjects with each genotype) or two-sided *P*-values. [b] Wt, Homozygous wild type patient; Het, Heterozygous variant type patient; Var, Homozygous variant type patient.

the extent of drug metabolism of CYP3A substrates are clinically important.

The extent to which the CYP3A isoforms metabolize midazolam is likely to be determined, at least in part, by intracellular drug concentrations in the liver and, to a lesser extent, the intestine. This process, in turn, is partially dependent on the ATP-binding cassette transporters like ABCB1 (P-glycoprotein) that are localized in the apical membrane of hepatocytes and enterocytes. Although it has been suggested that midazolam is not a substrate for ABCB1 [32], recent data have indicated that midazolam exhibits characteristics of a highly permeable ABCB1 substrate [33]. The clearance of midazolam has also been shown to be correlated ($R^2 = 0.6$; $P = 0.0005$) with the clearance of docetaxel [15], a known dual substrate of CYP3A and ABCB1 [34], suggesting that varying expression of ABCB1 may alter midazolam clearance. In recent years, various genetic variants in the *ABCB1* gene have been described that may impact transporter expression or function [35]. The most extensively studied *ABCB1* variant to date is a common synonymous C to T transition at nucleotide position 3435 at a wobble position in exon 26 [36]. Although this transition does not change its encoded amino acid, recent findings have indicated that this variant is associated with altered protein expression in different human tissues [37], and this may result in decreased hepatobiliary and/or intestinal secretion of substrate drugs. Furthermore, a reduced expression of intestinal *CYP3A4* mRNA has been observed in subjects carrying the homozygous variant genotype of the *ABCB1* 3435C>T polymorphism in a Japanese population [38], further pointing to the possibility of *ABCB1* genotype affecting midazolam clearance. However, in this study, midazolam pharmacokinetic parameters were not significantly influenced by the *ABCB1* 3435C>T genotype, which is consistent with prior observations in healthy volunteers [25,39]. It should be noted that the lack of relationships with this SNP is, as expected, consistent with preclinical observations in *Abcb1a*-deficient mice that metabolism rather than transport is the prominent elimination pathway for midazolam [40]. This further suggests that the involvement of ABCB1 in the disposition of midazolam may be relatively unimportant regardless of *ABCB1* genotype status.

The lack of statistically significant relations in this study does not necessarily mean that there are none, especially in light of the few individuals studied in our population.

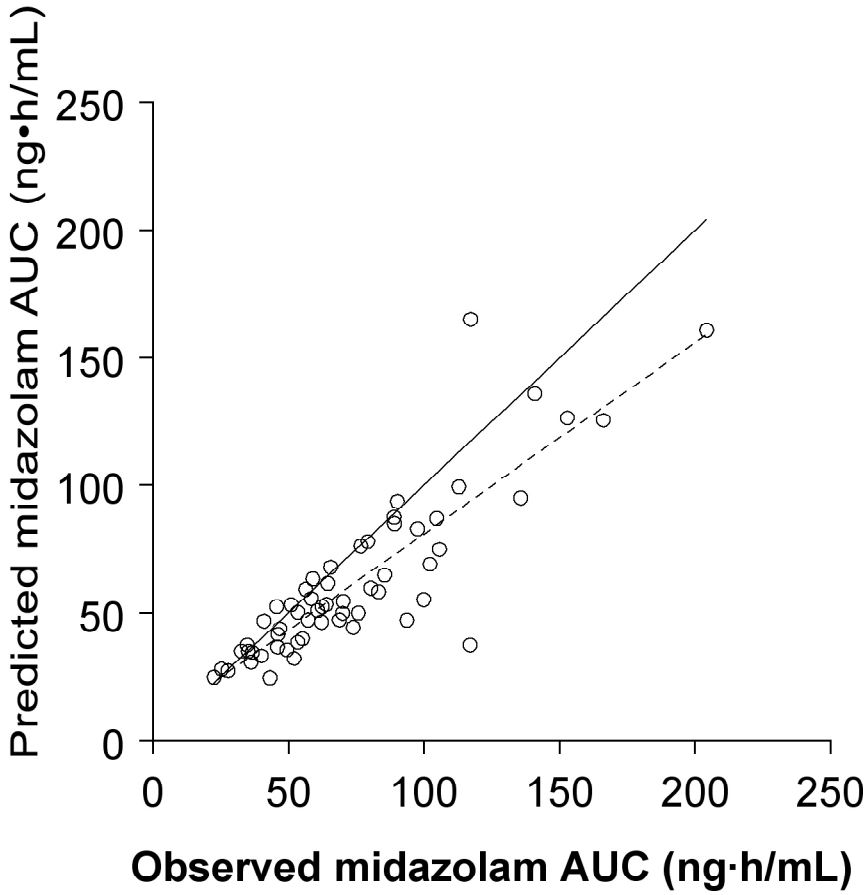

Fig 2. Scatter plot of the observed area under the curve (AUC) of midazolam versus the AUC of midazolam calculated based on the observed midazolam concentration at the 4-hour sampling time point (4-h conc.) using the limited sampling model equation: AUC = 9.91 + 12.2 × [4-h conc.], as described previously [12]. The dotted line indicates a linear regression fit, whereas the solid line represents the line of identity.

with a homozygous variant genotype. However, the variant genotype effects are minor and/or the genotype heterogeneity is sufficiently small to conclude that the presently studied variants in the *CYP3A4* and *CYP3A5* genes do not cause a substantial interindividual difference in midazolam clearance and, thus, are unlikely to have an important functional significance in cancer patients treated with CYP3A substrate drugs. For example, even in a population of this size, if CYP3A5 genotype accounted for the large degree of variability in midazolam pharmacokinetics, detection of this effect would have been expected.

There may be a number of other factors contributing to the variability in midazolam pharmacokinetics, since the tested genetic variants do not appear to explain

this finding. A recent study has shown an inverse correlation between liver dysfunction, particularly total bilirubin levels, and CYP3A activity [23]. Though all participants in the current study had total bilirubin levels within 1.5 times the upper limit of normal, there was still a considerable range represented (Table 1). Inflammatory response in patients with cancer, resulting in increased α-1 acid glycoprotein serum concentrations, has also been suggested to decrease clearance by the CYP3A isozymes [41]. Age and sex have also been postulated to influence CYP3A activity. Furthermore, many drugs and herbal medications are known to interact with CYP3A. Though patients were asked to abstain from any drugs known to affect the function and/or expression of either CYP3A or ABCB1, this possibility cannot be entirely excluded. Finally, it is possible that additional genes, genetic variants and/or haplotypes of importance to the pharmacokinetics of CYP3A substrate drugs like midazolam may yet be discovered and/or evaluated. Further investigation is needed to determine the relative role of genetic variation and environmental variables (e.g., concomitant drugs or herbs) on the pharmacokinetics of anticancer drugs that are substrates of CYP3A4 and CYP3A5.

Acknowledgements

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Chapter 5

Influence of the Dual ABCB1 and ABCG2 Inhibitor Tariquidar on the Disposition of Oral Imatinib in Mice

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Submitted

Abstract

Purpose: Imatinib, a tyrosine kinase inhibitor currently approved for treatment of several malignancies, has been shown to be a substrate for multiple efflux-transporter proteins, including ABCB1 (P-glycoprotein) and ABCG2 (BCRP). The effect of inhibiting these transporters on tissue exposure to imatinib remains unclear.

Methods: To assess the role of these transporters on drug disposition, 50 mg/kg imatinib was administered to Balb/C mice, 30 minutes after receiving tariquidar (10 mg/kg), an inhibitor of both ABCB1 and ABCG2, or vehicle, via oral gavage. Quantitative determination of imatinib in mouse plasma, liver and brain was performed using a newly-developed and validated liquid-chromatography-mass spectrometric method.

Results: Exposure to imatinib was 2.2-fold higher in plasma, liver and brain in mice that received tariquidar, as compared to those that received the vehicle ($P = 0.001$). The peak plasma concentration did not increase substantially, suggesting that tariquidar is affecting the distribution, metabolism and/or excretion of imatinib, rather than absorption. Though tariquidar increased the absolute exposure of imatinib, the brain-to-plasma ratio of imatinib was unaffected.

Conclusions: This study suggests that intentional inhibition of ABCB1 and ABCG2 function at the blood-brain barrier is unlikely to significantly improve clinical outcome of imatinib with currently used dosing regimens.

Introduction

Imatinib mesylate is an orally administered tyrosine kinase inhibitor, currently FDA approved for the treatment of Philadelphia chromosome-positive chronic myeloid leukemia (targeting Brc-Abl) and unresectable and/or metastatic malignant gastrointestinal stromal tumors (targeting c-KIT) [1]. This agent is also currently under intensive investigation in other tumor types, most notably as a single agent or in combination with hydroxyurea for the treatment of gliomas. However, there has been limited clinical success in brain tumors reported to date [2,3].

Imatinib was initially determined to be a substrate for ABCB1 (P-glycoprotein) *in vitro* [4]. Subsequently, it was demonstrated that the *in vivo* distribution of imatinib is limited by ABCB1-mediated efflux, resulting in limited brain penetration [5]. More recently, positron emission topography studies with [*N*-^{11}C-methyl]-imatinib have confirmed limited brain penetration in primates [6]. However, ABCB1 is not the sole transporter expressed in the blood-brain barrier that may limit the brain distribution of imatinib. In particular, imatinib is both an inhibitor [7] and substrate [8] of ABCG2 (BCRP). Experiments comparing the plasma and brain pharmacokinetics of imatinib following i.v. administration of radiolabeled drug to wild-type, *Abcb1* knockout and *Abcg2* knockout mice have confirmed a role of these transporter proteins in limiting brain exposure [9].

The potential influence of these efflux transporters is not limited to brain exposure. For example, ABCB1 and ABCG2 are also highly expressed in the small intestine, bile canaliculi of the liver and numerous other normal tissues [10,11]. In addition, expression of these proteins in human tumors has been associated with development of multidrug resistance [12]. Furthermore, *in vitro* studies have suggested that long-term treatment with imatinib leads to increased expression of both ABCB1 and ABCG2, resulting in decreased intracellular drug accumulation [13]. As such, it is of great interest to identify and characterize inhibitors of ABCB1 and ABCG2 *in vivo* that could potentially be used to intentionally alter the pharmacokinetics of and/or improve response to therapy with anticancer ABCB1 and ABCG2 substrates [11].

Several transporter inhibitors have previously been evaluated in preclinical models, including the ABCB1 inhibitors valspodar and zosuquidar, the ABCG2 inhibitor pantoprazol and the dual ABCB1/ABCG2 inhibitor elacridar [9,14]. Tariquidar, an orally available anthranilic acid derivative, has been shown to be an inhibitor of both ABCB1 and ABCG2 [15]. It is currently in clinical trials evaluating its utility as an inhibitor of ABCB1, in an effort to overcome resistance associated with anticancer chemotherapy [16]. Here, we evaluated the effect of tariquidar on the disposition of imatinib in mice, in order to provide a pharmacokinetic rationale for attempts to improve the agent's low brain penetration.

Materials and Methods

Chemicals and reagents

Imatinib mesylate was supplied by Novartis (East Hanover, NJ). Tariquidar was supplied by Dr. Susan Bates (NCI, Bethesda, MD). Glucose, harmine, absolute ethanol and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid (98%) was obtained from Fluka (through Sigma-Aldrich). Methanol (J.T. Baker, Phillipsburg, NJ) was of HPLC grade. Deionized water was generated with a Hydro-Reverse Osmosis system (Durham, NC) connected to a Milli-Q UV Plus purifying system (Billerica, MA). Blank mouse plasma was purchased from Innovative Research (Southfield, MI).

Sample preparation

Unknown and quality control (QC) plasma samples were thawed at room temperature, vortex mixed for 20 seconds, and 100 µL were transferred to a polypropylene centrifuge tube. For analysis of unknown tissue samples, approximately 100 mg of tissue were accurately weighed and water added (5 µL per mg). After vortex-mixing, samples were homogenized using a PowerGen 125, while kept on ice. One hundred µL of homogenate was transferred to a clean polypropylene centrifuge tube for further processing. To each tube, including calibrators (10, 25, 50, 100, 500 and 1000 ng/mL)

and QC samples (30, 450, 800 and 18,000 ng/mL), 250 µL of methanol (containing 25 ng/mL of internal standard, harmine) was added. All tubes were capped, vortex-mixed for 5 min and then centrifuged for 5 min at 18,000 x *g*. Following centrifugation, the supernatant was transferred to a vial for injection. Either 5 or 10 µL of the supernatant was injected for tissue or plasma samples, respectively. Calibration curves and QC samples were prepared in both brain and liver, for tissue sample analysis.The working ranges for liver and brain were 0.125-100 and 0.125-25 ng/mL, respectively.

Equipment

High performance liquid chromatography was carried out on an Agilent 1100 system (Agilent Technology, Palo Alto, CA), coupled with a single-quadrupole mass spectrometer, utilizing electrospray ionization in positive mode. Samples were cooled to 4 °C in a thermostated autosampler and the column compartment, containing a Waters SymmetryShield RP8 column (2.1 x 50 mm, 3.5 µm), was maintained at 35 °C. Samples were eluted using a gradient mobile phase, comprised of 10 mM ammonium acetate with 0.1% formic acid and methanol, running at a flow rate of 0.35 mL/min for 10 min, including re-equilibration. Mass spectrometric conditions were as follows: fragmentor, 150 V; gain, 2; drying gas flow, 10 L/min; drying gas temperature, 300 °C; nebulizer pressure, 40 psi; and capillary voltage, 1500 V. Selected-ion monitoring was accomplished at *m/z* 494.2 for imatinib and *m/z* 213.1 for the internal standard. The chromatographic data were acquired and analyzed using the Chemstation software package (Agilent).

Validation procedures

Calculation of accuracy and precision was carried out according to procedures reported in detail previously [17]. Calibration samples were prepared fresh each day in the relevant matrix and frozen QC samples were defrosted and analyzed. A $1/x^2$ weighting scheme was employed in the generation of standard curves to account for concentration dependent variance. Detector response for plasma was found to be linear

in the imatinib concentration range of 10-1000 ng/mL. Plasma accuracy and precision were evaluated with QC samples. Overall, the assay was found to be accurate (deviation of less than 10% for QCs) and precise (within run precision <10%, between run precision <12.6%) for plasma, liver, and brain.

Animals

All experiments were performed on six-week old, male, Balb/C mice obtained from Charles River Laboratories (Wilmington, MA). The mice weighed approximately 15 to 20 g at the time of study. All mice were allowed unlimited access to water and rodent chow prior to, and during the experiment. Blank mouse liver and brain samples were harvested from surplus mice following euthanasia. NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, DC). The study design and protocol were approved by the NCI Animal Care and Use Committee (Bethesda, MD).

Experimental design

Imatinib was dissolved in sterile water to make a 10 mg/mL dosing solution. Tariquidar was prepared as a 2 mg/mL solution in water with 5% glucose. Mice received either 10 mg/kg tariquidar or the vehicle (5 mL per kg weight) [15] 30 minutes prior to 50 mg/kg of imatinib [18]. All compounds were administered via oral gavage. At each time point, three mice in each treatment group were anesthetized with isoflurane, and bled via cardiac puncture into a tube containing sodium heparin as an anticoagulant. Blood samples were centrifuged at 18,000 x *g* for 5 minutes at 4 °C, the plasma layer transferred to a cryovial and frozen. Following euthanasia by cervical dislocation, brain and liver tissues were excised and snap-frozen. All samples were stored at -80 °C until the time of analysis.

Statistical and pharmacokinetic analysis

Concentration-time data were evaluated using a non-compartmental approach, with WinNonlin 5.0 (Pharsight, Mountain View, CA), using the mean concentration (n=3) at each time point. The peak plasma concentration (C_{max}) and the time to peak plasma concentration (T_{max}) are reported as observed values. The area under the curve (AUC) was calculated using the linear trapezoidal method from time zero to the time of the last sample with measurable drug concentration. To allow for direct comparison between the two groups and characterization of the terminal phase for the imatinib alone arm, the 24-hour plasma and liver samples, along with the 4-hour brain samples were estimated at LLQ/2, as drug was detectable, but measured concentrations were below the limit of quantitation. Bailer's method was employed to assess the variance, allowing for comparison of exposure between the two dose groups. The significance of the difference in AUC was evaluated by a Z-test. Brain concentrations were corrected for drug in the brain vascular space, by subtracting 1.4% of the plasma concentration from the measured brain concentration for each animal [5]. Brain-to-plasma concentration ratios were calculated for each animal at the 2-hour time point, and the groups compared using a t-test. All statistical tests were performed in Microsoft Excel 2004 (Redmond, WA). P-values <0.05 were considered significant.

Results

The administration of oral tariquidar 30 minutes prior to an oral dose of imatinib resulted in a significant increase in systemic exposure to imatinib (**Table 1**; **Figure 1**). Tariquidar increased the peak plasma concentration of imatinib by 19% (6,813 ± 1,548 vs 5,711 ± 1,472 ng/mL, P = ns), with no apparent change in the rate of absorption, as judged from the similar times to peak concentration (0.17 hours). In contrast, the AUC_{0-24} for imatinib was 2.2-fold higher in mice pretreated with tariquidar compared to the vehicle (26,725 vs 12,168 hr*ng/mL, P = 0.001). In liver tissue, tariquidar increased the peak concentration by 75% (46,139 vs 26,280 ng/g) and the AUC_{0-24} was also 2.2-fold higher (153,209 vs 68,331 hr*ng/mL, $P < 0.00001$). The maximal corrected concentration

Table 1. Pharmacokinetics of imatinib in Balb/C mice in the presence and absence of tariquidar

	Imatinib		Imatinib + Tariquidar		
Plasma	Mean	SD	Mean	SD	P-value
C_{max} (ng/mL)	5710.5	1472.3	6813.2	1547.9	-
T_{max} (hr)	0.17	-	0.17	-	-
AUC_{0-24} (hr*ng/mL)	12167.5	-	26724.6	-	0.001
Liver	Mean	SD	Mean	SD	P-value
C_{max} (ng/g)	26279.7	4560.2	46139.1	11000.6	-
T_{max} (hr)	0.25	-	0.17	-	-
AUC_{0-24} (hr*ng/g)	68330.8	-	153209.2	-	<0.00001
Brain	Mean	SD	Mean	SD	P-value
C_{max} (ng/g)	194.7	27.2	417.0	116.6	-
T_{max} (hr)	2	-	2	-	-
AUC_{0-4} (hr*ng/g)	574.23	-	1277.7	-	0.00001

of imatinib achieved in brain tissue was 114% higher in the imatinib plus tariquidar group (417 vs 195 ng/g), and the AUC_{0-4} was 2.2-fold higher (417 vs 195 hr*ng/mL, P = 0.00002). No imatinib was detectable in the brain within the first 5 minutes after administration in either group, and the maximal brain concentration was observed after two hours in both groups. The brain-to-plasma ratio of imatinib 2 hours after administration did not differ significantly between the two groups (P = 0.83), and similar brain-to-plasma AUC_{0-4} ratios were observed for each group (0.070 for imatinib plus vehicle *versus* 0.078 for imatinib plus tariquidar). In addition, the liver-to-plasma AUC_{0-24} ratios did not differ significantly between the two groups.

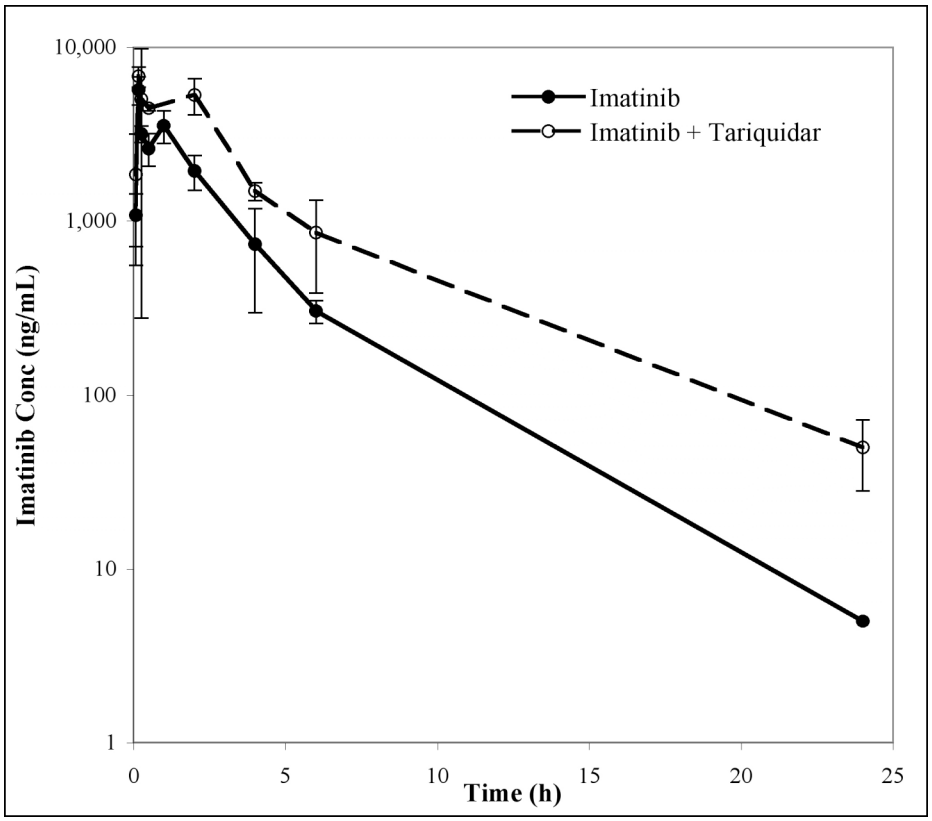
Conclusions

The current study indicates that administration of the dual ABCB1 and ABCG2 inhibitor tariquidar results in a statistically significantly increase in plasma, liver and brain exposure to imatinib. Since imatinib is known to have very high bioavailability (approximately 98%) [1], it is likely that the difference in plasma AUC is due to modified distribution and/or elimination of the drug, rather than a change in the extent of intestinal absorption. This hypothesis is supported by the fact that tariquidar increased the peak plasma concentration of imatinib by less than 20% and this change was not statistically significant. As expected, there was also no apparent change in the rate of absorption. Considering that imatinib is effluxed by both ABCB1 and ABCG2, the almost complete bioavailability may seem somewhat surprising. However, it is possible that the high concentrations of imatinib in the gut are actually leading to localized inhibition of these transporters, as has been suggested by inhibition data [7].

Inhibition of ABCB1 and ABCG2 by tariquidar may also alter the extent of imatinib metabolism. Bihorel *et al*. noted an increase in plasma concentrations of imatinib metabolites in both *Abcb1a/1b* knockout and *Abcg2* knockout mice; however, co-administration of elacridar, another dual ABCB1 and ABCG2 inhibitor, did not alter the concentrations of imatinib metabolites [14]. Therefore, it is unclear whether this observation may arise due to a compensatory mechanism in the knockout mice.

The brain-to-plasma concentration ratio of imatinib 2 hours after administration was not significantly affected by tariquidar. In addition, the AUC_{0-4} ratio for brain-to-plasma was similar in the presence or absence of tariquidar. This suggests that, rather than modifying the blood-brain barrier directly, tariquidar may simply be increasing plasma concentrations of the drug, leading to saturation of these efflux transporters at this site. The AUCs of imatinib in plasma and both of the tissues studied were 2.2-fold higher following pre-treatment with tariquidar. If modulation at the blood-brain barrier were occurring, independent of increased plasma concentrations of drug, it was hypothesized that the brain accumulation would be greater, not merely the same, as the increase in plasma.

A.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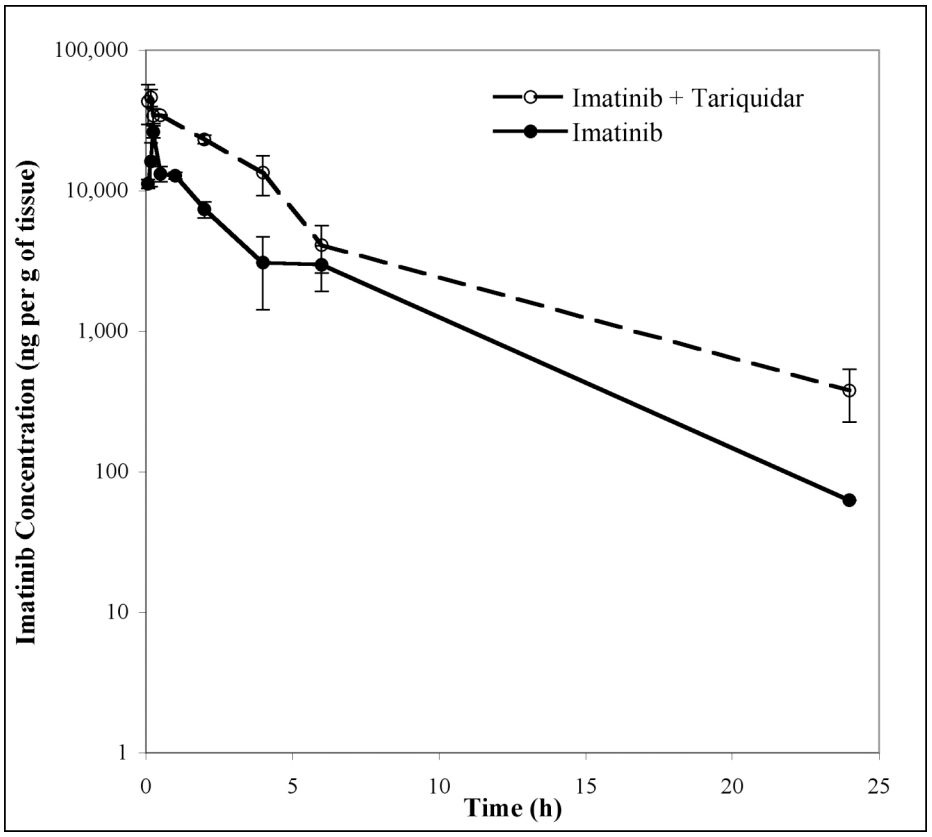
B.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Imatinib

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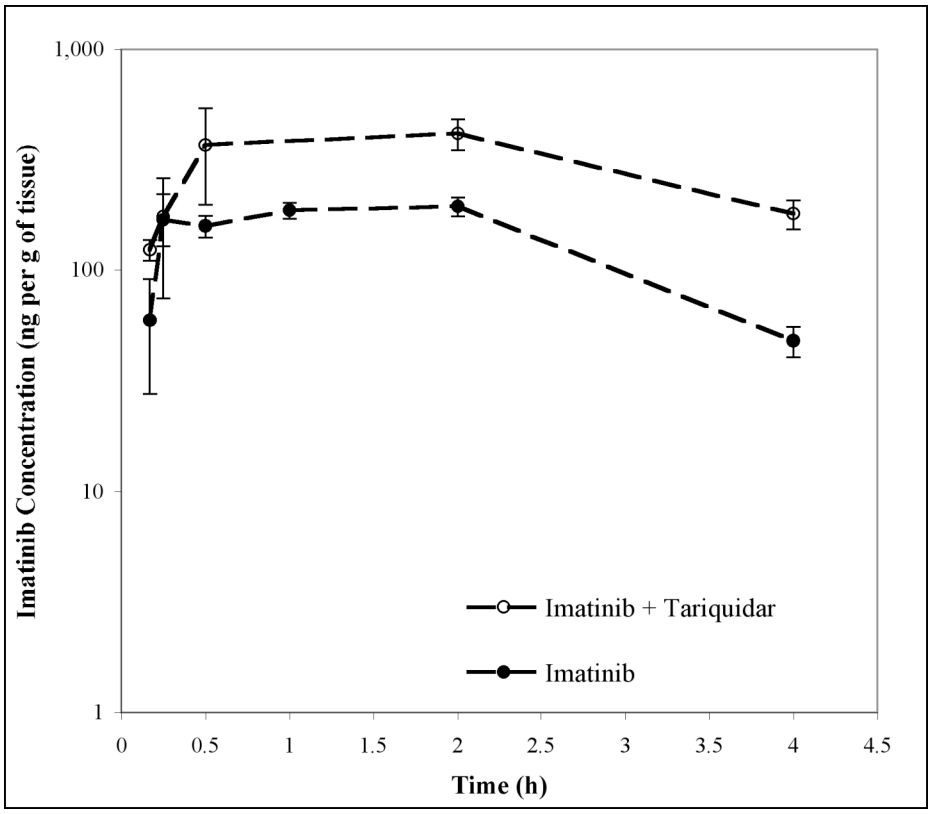

Figure 1. Concentration-time profiles of imatinib in A. plasma, B. liver and C. brain, for the imatinib plus vehicle group (solid line) and the imatinib plus tariquidar group (dashed line). Error bars for each timepoint represent the standard error.

Initial comparison of the inhibitory effects of tariquidar toward ABCB1 and ABCG2, as compared to elacridar, in the context of imatinib disposition, may suggest that tariquidar is less potent, in spite of previously published data that supports the opposite [19]. Specifically, elacridar has been shown to result in a 9.3-fold increase in the brain-to-plasma concentration ratio, as compared to administration of imatinib alone [14]. However, those experiments utilized significantly lower doses of imatinib as compared to the present study (12.5 *versus* 50 mg/kg), and the absolute concentrations of drug in brain were not stated. Hence, it is possible that the higher imatinib dose utilized in the current study results in higher plasma concentrations of drug and, therefore, saturation of drug efflux at the blood-brain barrier. In this context, it is particularly noteworthy that single dose plasma pharmacokinetics of imatinib in humans at the recommended oral dose of

400 mg per day results in overall drug exposure that is very similar to that found in the current study for mice (24.8 ± 7.4 *versus* 26.3 ± 4.6 h*μg/mL) [1].

Direct comparison between this study and prior experiments investigating the effect of ABC transporter inhibitors on imatinib pharmacokinetics are difficult due to a variety of reasons. The current study employed oral dosing at 50 mg/kg of imatinib, in an effort to closely mimic the clinical situation, whereas Breedveld et al. administered 12.5 mg/kg of imatinib intravenously (in combination with elacridar) [9]. These authors also examined the effect of oral pantoprazole on the pharmacokinetics of 100 mg/kg oral imatinib [9]. Though the increase in brain exposure to imatinib was reported to be higher with oral administration, as compared to i.v., this was only measured at 4 hours post-imatinib, and the analysis was based only on measurement of total radioactivity. As such, it is impossible to determine whether the higher radioactivity in the brain is due to the parent drug only or the parent drug plus metabolites.

Mistry et al. have demonstrated that the inhibitory effect of tariquidar on drug efflux *in vitro* persists for over two hours [15]. In healthy volunteers, a dose of 2 mg/kg i.v. or ≥ 200 mg orally, resulted in 100% inhibition of ABCB1 in CD56+ lymphocytes for over 24 hours. The maximal effect was observed between 2 and 6 hours after administration of tariquidar. In the current study, tariquidar was administered 30 minutes prior to imatinib administration in an effort to ensure sufficient distribution and inhibitory effects.
In conclusion, oral administration of tariquidar prior to oral imatinib resulted in increased imatinib exposure in plasma and tissues, including brain. The increase in brain exposure appears to be directly related to the increase in plasma concentrations of the drug, at a dose comparable to that used clinically. This further substantiates the possibility that ABC transporters localized in the blood brain barrier are more resistant to inhibition than at other tissue sites such as the intestine and liver [19]. In a clinical setting, the currently observed increase in plasma AUC would result in increased toxicity, as has been observed previously with the use of ABCB1 inhibitors [20]. One strategy that has been employed is dose reduction prior to combining the ABCB1 and ABCG2 substrate with the transporter inhibitor. However, based on the current findings, it should be anticipated that inhibition

of ABCB1 and ABCG2 function at the blood-brain barrier will not result in increased brain penetration or improved clinical outcome.

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*E. R. Gardner

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Chapter 6

Lack of ABC Transporter Autoinduction in Mice Following Long-term Exposure to Imatinib

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Abstract

Purpose: Chronic imatinib exposure has been shown to result in upregulation of the ABCB1 (P-glycoprotein) and ABCG2 (BCRP) transporters *in vitro*, for which imatinib is a substrate. This finding, along a trend towards increasing imatinib clearance over time in patients, has resulted in the suggestion that pharmacokinetic resistance may be contributing to eventual treatment failure. Here, we sought to determine the effects of long-term imatinib exposure on apparent oral clearance and transporter expression *in vivo*.

Methods: Male BALB/c mice were treated daily-times 5 with oral imatinib at a dose of 50 mg/kg for 4 consecutive weeks. Imatinib concentrations were measured in plasma and liver tissue prior to treatment and following each week of dosing. Western blotting of liver and intestinal tissue lysates was performed to assess expression of Abcb1 and Abcg2.

Results: Plasma and liver concentrations of imatinib did not change significantly ($P > 0.1$) over the course of treatment, suggesting that steady-state had been reached. There was no increase in Abcb1 or Abcg2 expression in liver samples, whereas expression of these transporters in intestinal samples was highly variable, and no increase was apparent.

Conclusions: Imatinib does not appear to cause upregulation of ABC transporters in the hepatic and intestinal compartments in mice. These data do not support the possibility that autoinduction contributes to the development of resistance to imatinib.

Introduction

Imatinib mesylate (Gleevec®, Glivec®) is a synthetic, small molecule tyrosine kinase inhibitor known to target Bcr-Abl, c-KIT and PDGFR α and β [1]. This agent is currently FDA approved for the treatment of Philadelphia chromosome-positive chronic myeloid leukemia (CML) and unresectable and/or metastatic malignant gastrointestinal stromal tumors (GIST). Though initial response rates to imatinib are high, clinical resistance develops in a large number of individuals [2]. For example, over 70% of patients with acute leukemia develop resistance within 3-6 months of starting treatment [2]. While mutations of the Bcr-Abl tyrosine kinase domain are directly responsible for many of these cases, there are also individuals in whom such mutations have not been detected [3]. As such, it has been postulated that pharmacokinetic resistance may be partly responsible for these cases, with drug exposure decreasing during chronic treatment. Judson et al. described a trend towards increasing apparent oral clearance over time in patients treated with imatinib, supporting this hypothesis [4]. In a group of patients with CML, significantly higher trough concentrations of imatinib in plasma were correlated with both cytogenic and molecular response [5]. Furthermore, multiple studies have demonstrated that intrapatient imatinib dose escalation can circumvent the resistance phenotype [6,7].

In adults, imatinib is administered daily as a tablet, at a recommended dose of 400 per day or higher for chronic myeloid leukemia and gastrointestinal stromal tumors. Pharmacokinetic studies have demonstrated that imatinib has high oral bioavailability (>98%), yet is extensively metabolized [1]. It has been hypothesized that chronic treatment with imatinib may induce expression of transporter proteins and metabolizing enzymes. This may result in decreased overall exposure to imatinib, by limiting drug absorption in the gastrointestinal tract and increasing elimination. The two ABC transporter proteins implicated in this process, namely ABCB1 (P-glycoprotein, MDR1) and ABCG2 (BCRP, MXR) [8-10], are particularly highly expressed in the small intestine, and the bile canaliculi of the liver [11,12].

A previous *in vitro* study, utilizing human Caco-2 cells as a model of intestinal drug transport, demonstrated that chronic exposure to imatinib was associated with maximal

induction of ABCB1 and ABCG2 after 39 days of treatment with 10 μM imatinib, showing 5-fold and 17-fold increases in mRNA, respectively [13]. At this point, intracellular imatinib accumulation was shown to be 2-fold higher in treatment naïve cells as compared to those having been subjected to chronic drug treatment. To determine whether this phenomenon is occurring *in vivo* and contributes to drug resistance, we assessed plasma concentrations of imatinib along with intestinal and liver expression of Abcb1 and Abcg2 during long-term treatment with imatinib in mice.

Materials and Methods

Chemicals and reagents

Imatinib mesylate was supplied by Novartis (East Hanover, NJ). Harmine, absolute ethanol, ammonium acetate, and sodium deoxycholate were each purchased from Sigma-Aldrich (St. Louis, MO). Formic acid (98%) was obtained from Fluka (through Sigma-Aldrich). Methanol (J.T. Baker, Phillipsburg, NJ) was of HPLC grade. Deionized water was generated with a Hydro-Reverse Osmosis system (Durham, NC) connected to a Milli-Q UV Plus purifying system (Billerica, MA). 10X TBS, 1M Tris-HCl, 5 M NaCl, and 0.5 M EDTA and 10% SDS solutions were purchased from KD Medical (Columbia, MD). Triton X-100 and Complete protease inhibitor were purchased from Roche Applied Science (Indianapolis, IN). Blank mouse plasma was purchased from Innovative Research (Southfield, MI).

Animals

All experiments were performed male BALB/c mice obtained from Charles River Laboratories (Wilmington, MA). The mice were weighed twice weekly. At the start of treatment, all mice were eight weeks old and weighed between 25 and 30 g. Blank mouse liver was harvested from surplus mice following euthanasia. NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research

Council; 1996; National Academy Press; Washington, DC). The study protocol was approved by the NCI Animal Care and Use Committee (Bethesda, MD).

Experimental design

Imatinib was dissolved in sodium citrate buffer (pH 3.0) to make a 10 mg/mL dosing solution. Three mice were sacrificed prior to the start of treatment. All remaining mice received 50 mg/kg of imatinib [14] once daily (5 µL of the dosing solution per g of body weight) via oral gavage, for five days per week for a total of 4 weeks. This dose was selected for its tolerability and drug exposure was expected to be comparable to that observed in humans [15]. Mice were weighed several times per week and dose adjusted for any changes in weight. Following each week of treatment, four hours after dosing, three mice were anesthetized with isoflurane and bled via cardiac puncture. Blood samples were centrifuged at 18,000 x *g* for 5 minutes at 4 °C, the plasma layer transferred to a cryovial and frozen. Following euthanasia by cervical dislocation, liver and intestines were removed and cleaned thoroughly with cold PBS (Gibco, Invitrogen, Carlsbad, CA), prior to snap freezing. All samples were stored at -80 °C until the time of analysis.

Blood and liver sample analysis

Plasma and liver samples were analyzed using a validated analytical assay reported elsewhere [15]. Briefly, 250 µL of methanol containing harmine (the internal standard) were added to 100 µL of the thawed plasma sample or liver homogenate (tissue homogenized with 5 µL/mg water using a PowerGen 125). After vortex-mixing for 5 min, the sample was centrifuged for 5 min at 18,000 x *g*. Following centrifugation, 10 µL of the supernatant were injected onto a SymmetryShield column (Waters Corp, Milford, MA), incorporated within an Agilent 1100 system (Agilent Technology, Palo Alto, CA) that was coupled with a single-quadrupole mass spectrometer utilizing positive mode electrospray ionization and selective ion monitoring. Samples were eluted using a gradient mobile phase, comprised of 10 mM ammonium acetate with 0.1% formic acid and methanol,

running at a flow rate of 0.35 mL/min. Drug concentrations were back-calculated from duplicate standard curves prepared in blank mouse plasma, analyzed within the same run.

Immunoblotting

Expression of the mouse Abcb1 and Abcg2 proteins was assessed by Western blot analysis. Protein lysates were prepared by adding cold modified RIPA buffer (50 mM Tris-HCL (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 5 mM EDTA and 1% deoxycholate) containing 1x Complete protease inhibitor to liver or intestinal tissue, while on ice, followed by homogenization, centrifugation and separation of the supernatant. Samples containing 40 μg of protein were subjected to electrophoresis on a NuPage 10% Bis-Tris gel with MOPS buffer (Invitrogen, Carlsbad, CA) under reducing conditions. Proteins were transferred electrophoretically to a PVDF membrane using the XCell II Blot Module (Invitrogen). Membranes were then blocked for 1 hr in 5% non-fat dried milk in TBST (0.1% Tween 20 in 1X TBS), followed by overnight incubation at 4° C with the diluted primary antibody in the milk solution. For detection of Abcg2, the rat monoclonal BXP-53 (Abcam, Cambridge, MA) was used as a 200-fold dilution for liver lysates and a 100-fold dilution for intestinal lysates. For detection of Abcb1, the mouse monoclonal C219 (Abcam) was used as a 2000-fold dilution for liver and intestinal lysates. Mouse IgG_1 anti-β-actin (BD Transduction Laboratories, San Jose, CA) was used to confirm equal amounts of protein in each lane, at a dilution of 1:5000. Following extensive washing with TBST, and 10 min of blocking, blots were incubated with anti-rat (Abcam) or anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) secondary antibodies, respectively, in milk solution at room temperature for 1 hr. After washing, results were visualized using the ECL Advanced Western Blotting Detection Kit (Amersham Biosciences, Piscataway, NJ).

Statistical analysis

Data are presented as mean ± standard deviation. Comparison between groups was performed using one-way ANOVA.

Results

The mean 4-hr imatinib concentration in plasma after four weeks of treatment was 445 ± 240 ng/mL, whereas the mean imatinib concentration in liver after four weeks of treatment was 3217 ± 1455 ng per g of tissue. There was no significant difference in plasma ($P = 0.21$) or liver ($P = 0.10$) concentrations between the weekly groups, suggesting that steady-state was reached by the end of treatment. (**Figure 1**). In the initial weeks, there was a trend towards increasing drug concentrations. Week 4 plasma concentrations were significantly higher than week 1. However, in contrast to the hypothesis that drug exposure would decrease over time with chronic treatment, no decline in plasma or tissue concentrations of imatinib was noted. Considering that the life span of a human is approximately 40 times longer than that of a BALB/c mouse, it was anticipated that the decline would occur rapidly during the four week experiment.

To assess potential changes in expression of Abcb1 and Abcg2 caused as a result of long-term exposure to imatinib, Western blotting of liver and intestinal tissue lysates was performed. There was no apparent increase in protein expression levels of either ABC transporter in mouse liver tissue lysates, over the four week course of treatment (**Figure 2**). In fact, there appears to be a decline in liver expression of Abcg2 following several weeks of treatment with imatinib. The intestinal expression of Abcb1 and Abcg2 was considerably lower than that observed in the liver and it was highly variable, with no apparent change in expression with chronic imatinib treatment (data not shown).

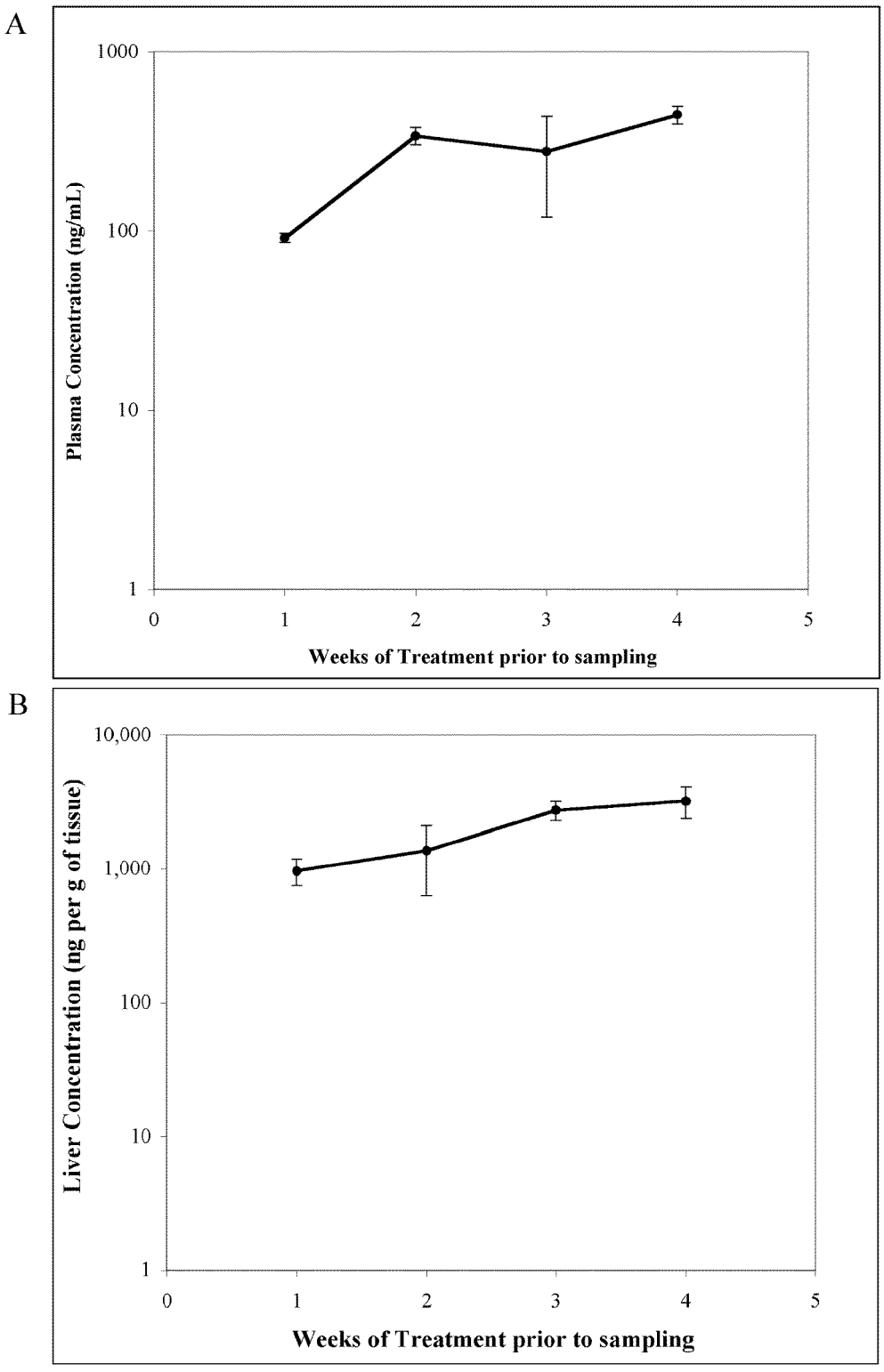

Figure 1. In vivo change in imatinib concentrations with long-term treatment Black dots and bars represent mean (n = 3) and standard error of imatinib concentration in A) plasma and B) liver sampled four hours after orally administering mice with 50 mg/kg, following 1, 2, 3 or 4 weeks of treatment.

Discussion

The current study demonstrates that, during long-term treatment in mice, imatinib is unlikely to induce its own transport or metabolism *in vivo*, as there was no decrease in plasma concentrations of the parent drug. In contrast to the original hypothesis, we found that Abcg2 expression actually appears to decline over the treatment period, though no significant corresponding increase in plasma or liver concentrations of imatinib was observed. The exact mechanism by which imatinib could potentially reduce expression of ABCG2 mRNA through activation of a pathway associated with lysosomal degradation [16,17]. This and other possible mechanisms are currently under investigation.

The discordance between the current findings and changes noted both *in vitro* and clinically can be attributed to many factors. It is possible that the upregulation of ABCB1 and ABCG2 in Caco-2 cells seen by Burger *et al.* is in fact overestimated, since the possibility that chronic imatinib treatment may be essentially selecting for a cell population that overexpresses these transporters cannot be entirely excluded. However, clinical observations of increased apparent oral clearance over time and reversal of resistance with dose escalation suggest that changes in drug metabolism or transport might be occurring with long-term treatment. The increased apparent oral clearance could result from changes in multiple processes, including decreased drug absorption, increased drug metabolism or increased drug excretion.

Drug disposition in the mouse can vary considerably from that in humans, due to physiological differences between the two species. For example, while two CYP3A isozymes (including CYP3A4, the major enzyme responsible for imatinib metabolism in humans), exist in human intestines and liver, the mouse expresses five different Cyp3a isoforms in the intestines, the most predominant of which is Cyp3a13 [18,19]. Based on our data, it is likely that similar interspecies differences are affecting long-term pharmacokinetics of imatinib and that the mouse may not be a suitable model for evaluating these changes *in vivo*.

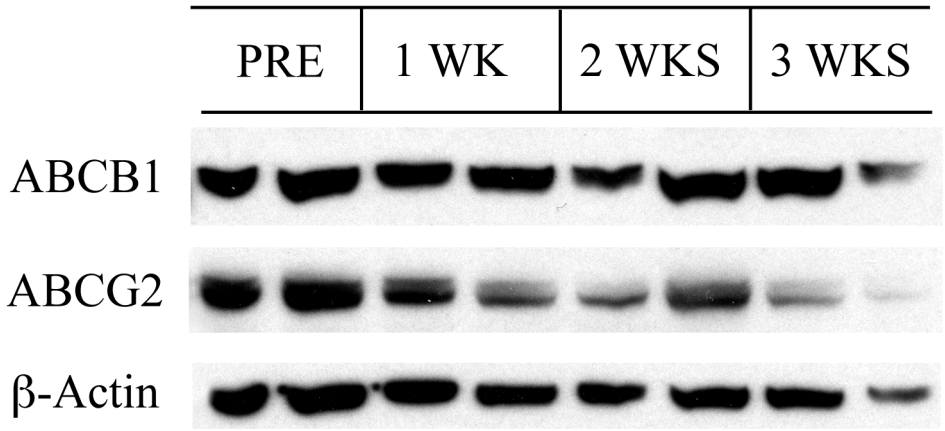

Figure 2. Change in expression of ABCB1 and ABCG2 during long-term treatment with imatinib. Mouse livers were harvested prior to, or following 1, 2 or 3 weeks of treatment with 50 mg/kg 5x per week, via oral gavage. Liver lysates from two different animals were assessed at each weekly time-point, by Western blot analysis.

Many studies have examined factors affecting the pharmacokinetic variability of imatinib. In humans, polymorphisms in the genes encoding metabolizing enzymes and transporter proteins can potentially affect the basal expression and/or functionality, thereby resulting in altered drug exposure. An initial study of 9 allelic variants in 82 patients found no correlation between polymorphisms in *ABCB1, ABCG2, CYP2C9, CYP2C19* or *CYP3A4* and imatinib clearance at steady-state.[20] However, a smaller subsequent study found a correlation between *ABCB1* polymorphisms and steady-state clearance of imatinib [21]. In direct contradiction to the findings of Judson *et al.*, in this study, an overall decrease in clearance was observed, from day 1 to steady-state. The most significant decreases in clearance were seen in individuals homozygous for the allele with the most transport activity (3435CC). Gurney et al. showed that there was no significant change in clearance for those individuals that already expressed variant ABCB1 associated with low activity, suggesting that imatinib may be actually decreasing the expression of ABCB1 or inhibiting its function [21].

α_1-acid glycoprotein (AAG) has also been purported to play several roles in imatinib treatment outcome. *In vitro* studies have demonstrated that imatinib is approximately 95% protein bound in plasma, primarily to AAG and albumin [1].

Clinically, AAG levels are higher in patients with leukemia than in healthy controls, and imatinib treatment does not appear to alter expression [22,23]. Le Coutre *et al*. have reported in two studies that patients with elevated AAG responded less rapidly to imatinib treatment, but there was no correlation with resistance [23]. In a subsequent clinical study, the same authors reported that relapsed patients had significantly elevated AAG as compared to responders [24]. While increased AAG may decrease the unbound fraction of imatinib, potentially altering both drug exposure and clearance, higher AAG has also been correlated with decreased CYP3A4 activity [25]. Indeed, Delbaldo et al. reported a correlation between increased AAG levels and decreased imatinib clearance [26].

In order to conclusively assess the possibility of pharmacokinetic resistance arising from modulation of drug disposition via changes in protein binding, metabolism, or transport, long-term pharmacokinetic studies must be embarked upon. Assessment of steady-state pharmacokinetics at monthly intervals would allow for determination of long-term changes in drug clearance. Additionally, measurement of the major metabolite of imatinib, CGP74588, which is formed by CYP3A4, possibly performed in conjunction with phenotyping experiments to determine CYP3A activity, could shed further light on potential alterations in metabolism. Furthermore, it would be of interest to measure the unbound fraction of imatinib in these serial samples. Direct measurement of unbound drug concentrations would allow for a confirmatory assessment of the role of AAG in imatinib pharmacokinetics, and would highlight any correlation between the pharmacologically active fraction unbound imatinib, that can penetrate tumor cells, and the development of resistance to treatment.

Collectively, the current study suggests that imatinib does not cause upregulation of ABC transporters in the hepatic and intestinal compartments in mice. These data do not support the possibility that autoinduction contributes to the development of resistance to imatinib.

Acknowledgments

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Chapter 7

Association of Enzyme and Transporter Genotypes with the Pharmacokinetics of Imatinib

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Abstract

Objective: To explore the relationships between imatinib pharmacokinetics and 9 allelic variants in 7 genes coding for ATP binding-cassette transporters (ABCB1, ABCG2) and enzymes (CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5) of putative relevance for imatinib.

Methods: Imatinib transport *in vitro* was studied using HEK293 cells transfected with wild-type ABCG2 and an ABCG2 Q141K clone. Steady-state pharmacokinetics of imatinib were obtained in 82 patients with gastrointestinal stromal tumors treated with oral imatinib at doses ranging from 100 to 1000 mg/day. Genotyping was carried out using direct sequencing or restriction fragment length polymorphism-based techniques.

Results: HEK293 cells transfected with ABCG2 Q141K exhibited greater drug accumulation in vitro than the cells expressing wild-type ABCG2 ($P = .028$). However, pharmacokinetic parameters of imatinib *in vivo* were not statistically significantly different in 16 patients that were heterozygous for *ABCG2* 421C>A compared to 66 patients carrying the wild-type sequence ($P = .479$). The apparent oral clearance of imatinib was reduced in individuals with at least one *CYP2D6*4* allele (median, 7.78 versus 10.6 L/h; $P = .0695$). Pharmacokinetic parameters were not related to any of the other multiple-variant genotypes ($P \geq .230$), possibly due to the low allele frequencies.

Conclusions: This study indicates that common genetic variants in the evaluated genes have only a limited impact on the pharmacokinetics of imatinib. Further investigation is required to quantitatively assess the clinical significance of homozygous variant *ABCG2* and *CYP2D6* genotypes in patients treated with imatinib.

Introduction

Imatinib mesylate (Gleevec™, Glivec®; hereafter referred to as imatinib), an orally administered selective Bcr-Abl and c-kit tyrosine kinase inhibitor, is currently approved for the treatment of Philadelphia chromosome-positive chronic myeloid leukemia and KIT positive, unresectable and/or metastatic malignant gastrointestinal stromal tumors. The degree of interindividual pharmacokinetic variability observed with imatinib is substantial, yet remains largely unexplained. For example, Judson et al. recently reported 73% variation in the apparent oral clearance of imatinib administered to 42 patients with gastrointestinal stromal tumor [1]. They also demonstrated a strong association between pharmacokinetic parameters of imatinib and the severity of hematological toxicity (i.e., neutropenia and thrombocytopenia) [1]. Similar variability was also seen following administration of imatinib to patients with chronic myeloid leukemia [2]. Hence, identification of factors affecting the pharmacokinetic profile of imatinib could aid in predicting or adapting appropriate, individualized doses of this drug.

We have recently shown that imatinib is a substrate for ABCG2 [formerly ABC transporter in placenta (ABCP), breast cancer resistance protein (BCRP), or mitoxantrone resistance protein (MXR)], a highly polymorphic transporter protein that influences the absorption and disposition of various substrates [3]. Like ABCB1 (P-glycoprotein), ABCG2 functions as an efflux transporter, possibly decreasing the amount of substrate drug absorbed after oral intake due to its localization on the apical surface of intestinal epithelial cells. Furthermore, ABCG2 may increase systemic drug elimination, as it is expressed in proximal renal tubular cells and on the biliary surface of hepatocytes [4]. Various naturally-occurring variants in *ABCG2* are known to affect the function and/or expression of its encoded protein. In particular, a functional single-nucleotide polymorphism (SNP) has been identified in exon 5 of the *ABCG2* gene, in which a C>A nucleotide transition at position 421 (*ABCG2* 421C>A) results in a non-synonymous variant protein with a glutamine to lysine amino acid substitution at codon 141 (Q141K) [5]. The purpose of the present study was to investigate the association between the *ABCG2* 421C>A polymorphism and the pharmacokinetics of imatinib in a cohort of

patients with gastrointestinal stromal tumors. Since i*n vitro* studies have shown that imatinib is transported by ABCB1[6] and extensively metabolized by the cytochrome P450 isoforms CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 [7], we also analyzed common genetic polymorphisms in the genes encoding these proteins, in order to provide a stronger scientific basis for optimizing imatinib therapy on the basis of each patient's genetic constitution.

Methods

In vitro cellular accumulation

Human embryonic kidney cells (HEK293) cells transfected with pcDNA3, wild-type ABCG2, or an ABCG2 Q141K clone were generated as described previously [8]. Cells were maintained in HEPES-buffered RPMI 1640 supplemented with 10% (v/v) fetal calf serum. ^{14}C-labeled imatinib was provided by Novartis (Basel, Switzerland).

Preliminary experiments indicated that the relative accumulation between ABCG2-overexpressing cells and the parental cells independent of the concentration in the range of 0.02 to 2.0 μM. In the experiments presented here, HEK293 cells (10^6/mL) were exposed to ^{14}C-labeled imatinib at a total drug concentration of 0.64 μM (corresponding to 0.03 μCi of radioactivity) for 2 hours at 37°C, and then washed three times with ice-cold phosphate-buffered saline. Accumulation of imatinib was calculated from the radioactivity measured in the cell pellet over that retained in the supernatant, as determined by liquid scintillation counting. The ABCG2 protein level was determined in the HEK293 cells by Western blot analysis using the monoclonal BXP-21 antibody (1:200 dilution), kindly provided by Dr. George L. Scheffer (Vrije Universiteit Medical Center, Amsterdam, The Netherlands). A mouse monoclonal anti-β-actin (1:5,000) was used to confirm equal loading, as described previously [9]. Since ABCG2 is a homo-oligomeric protein, total protein lysates were isolated in the presence of β-mercaptoethanol and sodium dodecyl sulfate, and boiled for 5 minutes prior to gel electrophoresis and detection of only the monomeric form of the ABCG2 protein.

Patient treatment

Patients with histological evidence of soft tissue sarcoma, including gastrointestinal stromal tumors (c-kit positivity before entry was required), were considered eligible for this study. Additional eligibility criteria for participation included: a) a measurable lesion with evidence of progression within 6 weeks prior to treatment (osseous lesions and pleural effusions are not considered measurable); b) no radiotherapy or embolization to the sole index lesion; c) age ≥15 years; d) World Health Organization performance status of < 2; e) adequate hematopoietic function (absolute neutrophil count, ≥ 1.5 × 10^9/1iter and platelet count, ≥ 100 × 10^9/L); and f) adequate renal function (serum creatinine, £ 120 mmol/L or calculated clearance (Cockroft method), > 65 mL/min) and hepatic function (bilirubin, £ 30 mmol/L; serum albumin, > 25 g/L). Specific exclusion criteria included: a) other severe medical illness, including psychosis and previous history of cardiovascular disease; b) concurrent treatment with warfarin; c) symptomatic or known central nervous system metastases; d) prior or concurrent second primary malignant tumors (except adequately treated in situ carcinoma of the cervix, or basal cell carcinoma); e) women of child-bearing potential unless in case of adequate contraceptive measures; and f) patients for whom regular follow-up attendance are impractical. None of the patients receive any medication aside from imatinib that could possibly influence the activity of ABCG2 or the pharmacokinetic profile of imatinib. The study protocol was approved by the Institutional Review Boards (Leuven, Belgium; and Rotterdam, the Netherlands), and before patient registration, written informed consent was obtained from each patient according to ICH/EU GCP and national/local regulations. Toxicity and efficacy data were not considered as pharmacodynamic endpoints in the current study due to the fact that different dosing schedules were used in the studied population.

Blood sampling

Blood sampling was performed on each patient after steady-state plasma concentrations had been achieved (at approximately 28 days after the start of drug administration). At this time, serial blood samples (8 mL each) were drawn immediately before drug administration and at 1, 2, 3, 4, 6, and 24 hours after drug intake, in order to

provide a full pharmacokinetic profile over one dosing interval. In 12 patients, additional samples were obtained at 30 minutes and at 5, 8, 10, and 18 hours after drug intake. All blood samples were collected in glass tubes containing heparin. Patency of the catheter between blood draws was maintained with a heparin lock (10 U/mL in normal saline) or slow normal saline drip (10 mL/h). Immediately after collection, blood samples were centrifuged at 4000 g for 15 minutes, and the plasma was separated and stored in polypropylene vials at –20°C until analysis.

Analytical measurements

Imatinib concentrations in plasma were determined by validated analytical methods using liquid chromatography with ultraviolet or tandem mass-spectrometric detection [10]. Briefly, imatinib and the internal standard [$^2H_8$]imatinib were extracted with a single protein precipitation step by the addition of acetonitrile to and plasma. The analytes of interest were then separated on a Waters Symmetry C18 column (50 × 2.1 mm internal diameter; 3.5 µm particle size) using a mobile phase composed of a mixture of methanol-0.05% ammonium acetate (72:28, v/v). Imatinib and the internal standard were detected by electrospray tandem mass spectrometry (Micromass Quattro Ultima triple quadrupole mass spectrometer; Micromass, Manchester, UK) in the positive mode, and monitored in the multiple reaction monitoring transitions 494>394 and 502>394, respectively. The linearity and reproducibility of the imatinib calibration curves in human plasma were satisfactory between 1 and 10,000 ng/mL, and the lower limit of quantitation was 1 ng/mL. The mean intraday accuracies at the lower limit of quantitation for imatinib were between 99.8 and 102%, with values for the relative standard deviation between 5.74% and 2.39%. Above the lower limit of quantitation, the mean intra-day accuracies ranged from 99.8 to 102%.

Pharmacokinetic analysis

Pharmacokinetic profiles were evaluated by non-compartmental analysis using the software package WinNonlin version 5.0 (Pharsight, Mountain View, Calif), and the parameters of interest included the area under the curve for one dosing interval (AUC_{0-24}),

the apparent oral clearance (CL/F), and the average steady-state plasma concentration (C_{ss}). Previously, it was shown that imatinib exposure is dose-proportional for the dose range of 25 to 1,000 mg [2], indicating linear pharmacokinetics. Therefore, AUC_{0-24} and C_{ss} estimates were dose-normalized to account for the different dosages administered.

Genotype analysis

Genomic deoxyribonucleic acid (DNA) was isolated from plasma using the UltraSens Virus Kit (Qiagen, Valencia, Calif). The *CYP2C9* 430C>T (*CYP2C9*2*), *CYP2C9* 1075A>C (*CYP2C9*3*), *CYP2C19* 681G>A (*CYP2C19*2*), *CYP2C19* 636G>A (*CYP2C19*3*), *CYP2D6* 1846G>A (*CYP2D6*4*), *CYP3A4* -392A>G (*CYP3A4*1B*), *CYP3A5* 6986A>G (*CYP3A5*3C*), *ABCB1* 3435C>T, and *ABCG2* 421C>A variants were analyzed by polymerase chain reaction (PCR)-restriction fragment length polymorphism-based techniques, as described previously [11-13]. The selection of these particular polymorphic variants in our predominantly Caucasian population was based on considerations provided elsewhere [14]. Confirmation of variant *ABCG2* genotype assignments was performed using direct nucleotide sequencing, following nested PCR. An initial PCR was performed with concentrated DNA utilizing 20 pmol of each primer. Primers were designed as follows: 5′-CTATTTGGGTGTACAGATAGGGG-3′ and 5′-CACGTTCATATTATGTAACAAGCC-3′. These primers were added to a reaction mixture of 1× PCR buffer, 2 mM of each of the four deoxynucleotide triphosphates (dNTPs), 1.5 mM $MgCl_2$, and 2 units of Platinum Taq polymerase (Invitrogen, Carlsbad, Calif) in a reaction volume of 20 μL. The temperature profile for the first reaction was: 5 minutes at 94°C, 20 cycles (30 seconds at 94 °C, 30 seconds at 63°C, 30 seconds at 72°C), then 7 minutes at 72°C. After initial amplification, 3 μL of amplified product was removed and an additional PCR amplification was performed using 40 pmol of the primers 5′-GCAGGTTCATCATTAGCTAGAAC-3′ and 5′-CCTACTTATGCTGATCATGAGC-3′ in a 50-μL reaction. Secondary PCR was carried out at the same temperatures, using 40 cycles. Direct nucleotide sequencing was performed using the following primers: 5′-CTTAAGGATGATGTTGTGATGGG-3′ for the forward strand, and 5′-

TCTTGAATGACCCTGTTAATCCG-3′ for the reverse strand with dRhodamine Terminator Cycle Sequencing Ready Reaction kit on an ABI 310 genetic analyzer (Applied Biosystems, Foster City, Calif). The genotype was called variant if it differed from the Refseq consensus sequence for the SNP position (http://www.ncbi.nlm.nih.gov/LocusLink/refseq.html). Genotype-frequency analysis of Hardy-Weinberg equilibrium was carried out using Clump version 1.9.

Statistical analysis

All data are presented as median values with 95% confidence intervals (95%CI), unless indicated otherwise. Differences in intracellular imatinib accumulation in the cell lines as well as the differences in pharmacokinetic parameters of imatinib as a function of drug dose, sex, and the different genotypes were evaluated using a non-parametric Kruskal-Wallis one-way analysis of variance on ranks. The relationship between patient age and the apparent oral clearance of imatinib was assessed using Spearman rank correlation analysis. In case of two dichotomous variables, a Fisher's exact test was performed. Because this study was mainly exploratory in intent, no adjustments were performed to evaluate the significance of the multiple comparisons. Two-tailed *P* values of less than .05 were considered to be statistically significant. All statistical calculations were performed in the software package NCSS version 2001 (Number Cruncher Statistical System; J. Hintze, Kaysville, Utah).

Results

In vitro transport of imatinib by ABCG2

In order to investigate the potential effect of the *ABCG2* 421C>A variant on the protein's ability to transport imatinib, human embryonic kidney cells (HEK293) cells transfected with pcDNA3, wild-type ABCG2, or an ABCG2 Q141K clone were exposed to ^{14}C-labeled imatinib for 2 hours and the intracellular concentration determined. The overexpression of wild-type ABCG2 resulted in significant decrease in drug accumulation compared to cells lacking ABCG2 ($P < .01$), confirming that imatinib is a substrate for the

wild-type ABCG2 protein (**Fig 1**). The cells homozygous for the studied variant exhibited significantly greater drug accumulation than the cells expressing wild-type ABCG2 (*P* = .028), suggesting impaired outward-directed transport of imatinib by the ABCG2 Q141K variant (**Fig 1**). Following this *in vitro* observation, we sought to determine whether patients carrying this genetic variant would have increased exposure to imatinib.

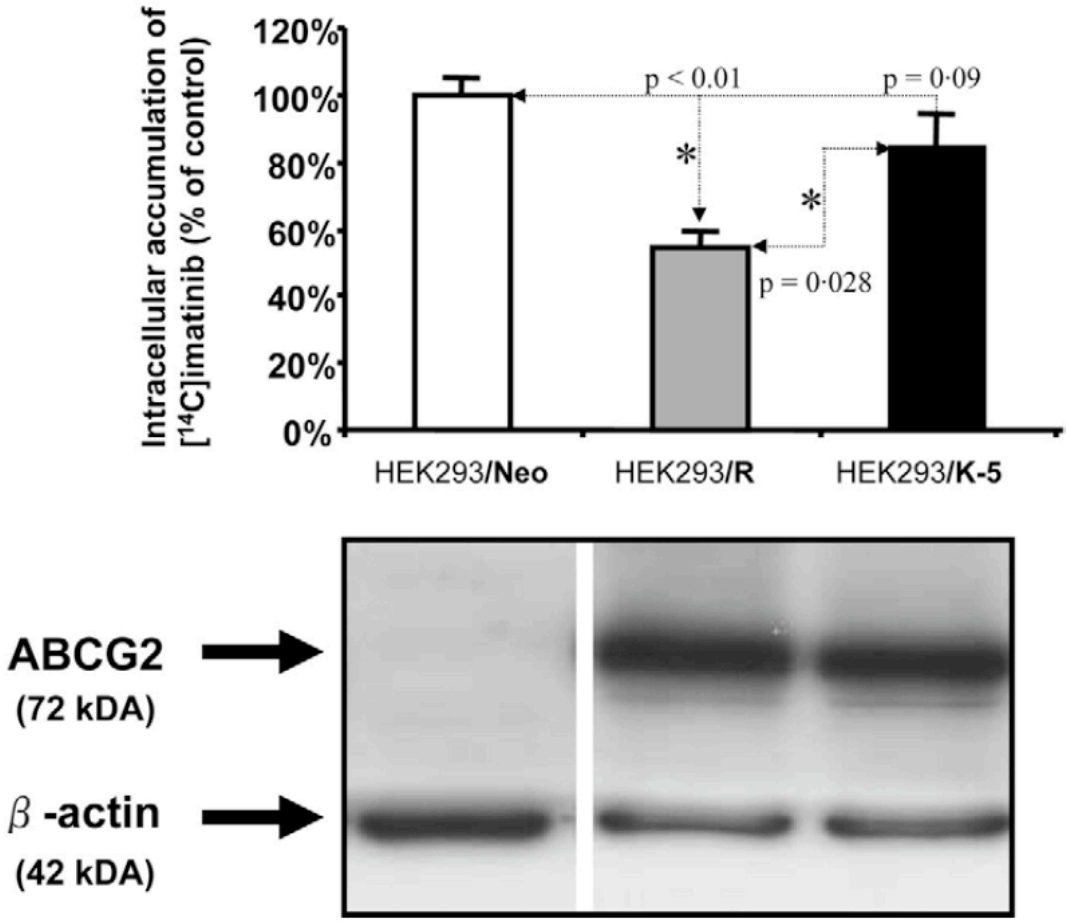

Fig 1. Comparative intracellular accumulation of ^{14}C-labeled imatinib ([^{14}C]imatinib) in human embryonic kidney cells (HEK293) transfected with wild-type ABCG2 (HEK293/R), and an ABCG2 Q141K clone (HEK293/K-5). Data are expressed as mean (bars) ± standard deviation (error bars) of two experiments each carried out in at least duplicate as the percent accumulation observed relative to that in HEK293 cells transfected with (empty vector) pcDNA3 (HEK293/**Neo**). The HEK293/**Neo**, HEK293/**R**, and HEK293/**K-5** cells showed normalized intracellular accumulation values of 100 ± 5%, 55 ± 4%, and 84 ± 9%, respectively. The star (*) indicates statistically significant differences ($P < .05$) in the intracellular accumulation of ^{14}C-labeled imatinib between the tested cell lines. The bottom panel shows a comparative Western blot analysis for ABCG2 in the 3 cell lines to demonstrate the similarity in protein expression levels between the HEK293/**R** and HEK293/**K-5** cells and the absence of ABCG2 expression in the HEK293/**Neo** cells [9].

Patients and imatinib pharmacokinetics

Eighty-two patients with cancer (80 patients with gastrointestinal stromal tumors and two patients with dermatofibrosarcoma protuberans), were treated with oral imatinib, given daily at a dose ranging from 100 to 1000 mg (median dose, 400 mg). The cohort consisted of 79 Caucasians, and 3 patients of Asian ancestry. In total, there were 53 males and 29 females, and the median age was 58 years (range, 25 to 80 years). The individual and average pharmacokinetic parameters of imatinib at steady-state are consistent with previous findings from patients on a similar regimen [7]. The apparent oral clearance was not statistically significantly dependent on drug dose (P = .299), consistent with a linear pharmacokinetic profile, as suggested previously [2]. Interindividual pharmacokinetic variability of imatinib was extensive, and the apparent oral clearance ranged more than 60-fold, from 0.86 to 62 L/h, in the studied population. The clearance histogram indicated a leptokurtic unimodal distribution with skewness to the right, with an overall median value of 9.14 L/h (95%CI, 7.78 to 10.7 L/h) and a coefficient of variation of 91.6% (**Fig 2**). To exclude the possibility of sex-related differences in the individuals evaluated in the present analysis, pharmacokinetic parameters were also compared between the groups. The apparent oral clearance was slightly higher in males (median, 8.47 L/h; 95% CI, 6.31 to 9.75 L/h) as compared to females (median, 7.80 L/h; 95% CI, 5.81 to 11.8 L/h), but this difference was not statistically significant (P = .163). Likewise, the apparent oral clearance of imatinib was unrelated to age (Spearman rank = -0.0420; P = .710).

Variant Genotypes

Of the 82 patients included in this analysis, 66 patients had two wild-type alleles (wild-type genotype) and 16 patients had inherited one copy of the variant *ABCG2* 421C>A allele (heterozygous variant genotype), resulting in an allele frequency of 0.0976, which is consistent with previous findings for a predominantly Caucasian population [15]. No patients homozygous for this variant allele were identified. Eight additional SNPs were analyzed in 6 genes of putative relevance for imatinib absorption and disposition. In one of the tested SNPs (i.e., *CYP2C19*3*), no variants were observed. In the other polymorphisms studied, frequencies of the rarest alleles ranged from 0.0429 to 0.494

(**Table I**). All genotype frequencies were found to be in Hardy-Weinberg equilibrium, and complete genetic linkage was not seen among polymorphisms from within the same gene. The genotype and allele frequencies are in excellent concordance with previously published values for a predominantly Caucasian population [14].

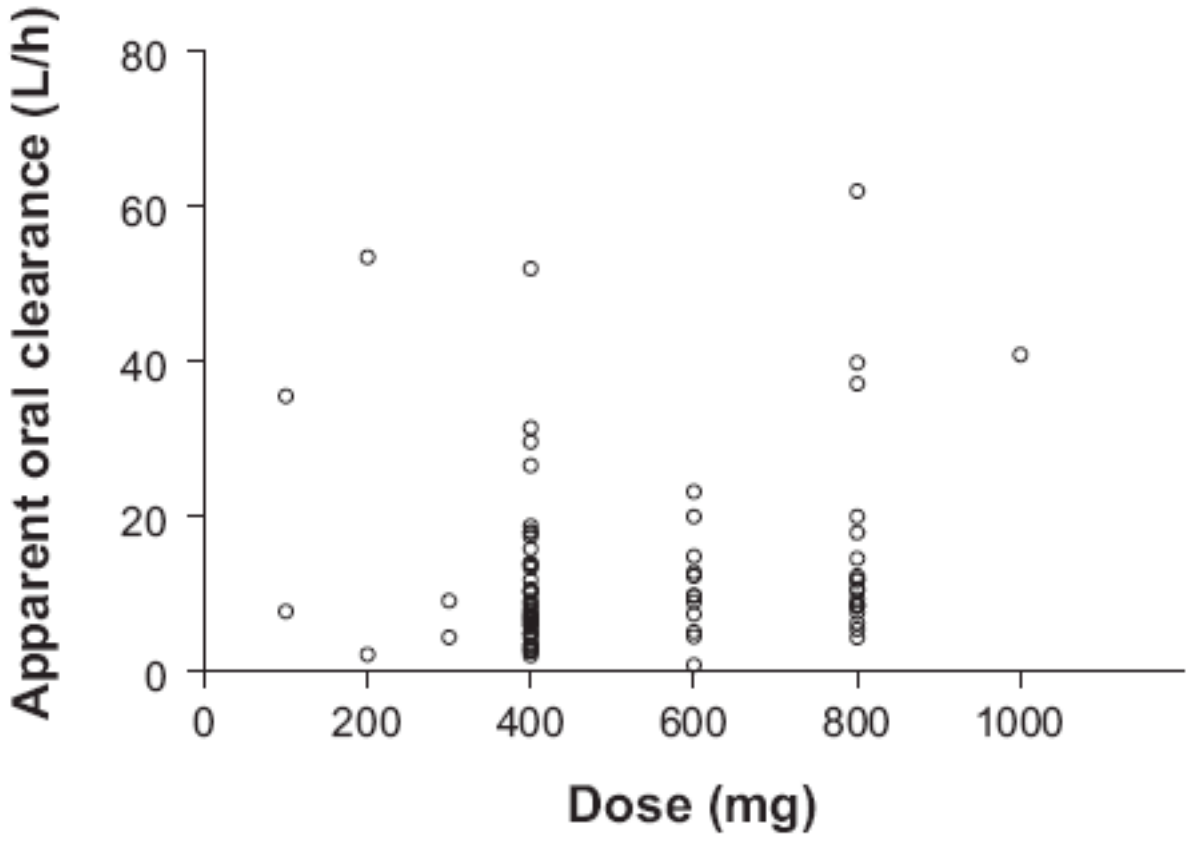

Fig 2. Histogram of the apparent oral clearance of imatinib (CL/F) determined at steady-state in a cohort of 82 patients with gastrointestinal stromal tumors.

Table 1. Genotype and allele frequencies for the studied variant genes

Variant[c]	Effect[d]	Activity[e]	Genotype frequencies[a]			Allele frequencies[b]	
			Wt	Het	Var	p	q
Enzyme genotypes							
*CYP2C9*2* (430C>T)	R144C (exon 3)	Decreased	60 (85.7)	9 (12.9)	1 (1.43)	0.921	0.0786
*CYP2C9*3* (1075A>C)	I359L (exon 7)	Decreased	60 (85.7)	10 (14.3)	0 (0)	0.929	0.0714
*CYP2C19*2* (681G>A)	Splice defect (exon 4)	None	40 (57.1)	27 (38.6)	3 (4.29)	0.764	0.236
*CYP2C19*3* (636G>A)	W212X (exon 5)	None	70 (100)	0 (0)	0 (0)	1	0
*CYP2D6*4* (1846G>A)	Splice defect (exon 4)	None	42 (62.7)	24 (35.8)	1 (1.49)	0.806	0.194
*CYP3A4*1B* (-392A>G)	Promoter	Normal (?)	64 (91.4)	6 (8.57)	0 (0)	0.957	0.0429
*CYP3A5*3C* (6986A>G)	Splice defect (intron 3)	Severely decr.	0 (0)	13 (18.6)	57 (81.4)	0.0929	0.907
Transporter genotypes							
ABCB1 3435C>T	I1145I (exon 26)	Reduced	21 (25.6)	41 (50.0)	20 (24.4)	0.506	0.494
ABCG2 421C>A	Q141K (exon 5)	Reduced	66 (80.5)	16 (19.5)	0 (0)	0.902	0.0976

[a] Number represents number of patients with percentage in parenthesis; the difference in the total number of patients is due to the fact that not all samples yielded PCR amplification.
[b] Hardy-Weinberg notation for allele frequencies (p, frequency for wild type allele and q, frequency for variant allele).
[c] Number represents position in nucleotide sequence.
[d] Number represents amino acid codon.
[e] Proposed functional activity of the variant protein *in vivo* relative to the wild-type protein. A comprehensive overview of published data has been reviewed recently by Lepper et al.[4] (transporters) and Van Schaik [14] (enzymes). *Abbreviations:* Wt, Homozygous wild type patient; Het, Heterozygous patient; Var, Homozygous variant patient; decr., decreased

Genotype-phenotype associations

Since the mechanistic basis for associations of the studied genetic variants would primarily be with (oral) clearance, we focused on presenting these results. For the 8 polymorphisms in 6 genes in which variant alleles were observed, none was associated with statistically significant differences in the oral clearance of imatinib (**Table II** and **Fig 3**). In contrast to what was predicted based on the *in vitro* data, the apparent oral clearance of imatinib was similar in 16 patients that were heterozygous for *ABCG2* 421C>A as compared to 66 patients with the wild-type sequence (P = .479). The apparent oral clearance of imatinib was reduced in carriers of at least one *CYP2D6*4* allele (median, 7.78 L/h; 95%CI, 6.17 to 9.75 L/h) compared to individuals carrying two wild-type alleles (median, 10.6 L/h; 95%CI, 8.57 to 14.9 L/h). This difference was not statistically significant (P = .0695), presumably as a result of the relatively small sample size and the presence of only one patient who carried two variant alleles. However, the proportion of individuals with an oral clearance of imatinib higher than the median value was significantly greater in the group with the reference *CYP2D6* sequence (15 of 42 patients; 35.7%) as compared to the group with at least one *CYP2D6*4* allele (3 of 25 patients; 12.0%; P = .0466). Differences in other pharmacokinetic parameters, including AUC, steady-state concentration, and peak concentration, were not statistically significantly different between the different genotype groups (data not shown).

Table 2. Apparent oral clearance of imatinib as a function of the studied genotypes.

	Genotype group						
	Wild-type		***Heterozygote***		***Variant***		
Variant	**Median (95%CI)[a]**	**n**	**Median (95% CI)[a]**	**n**	**Median (95%CI)[a]**	**n**	***P*-value[b]**
Enzyme genotypes							
*CYP2C9*2*	8.95 (7.23 – 10.7)	60	14.0 (4.54 – 39.9)	9	14.6	1	0.286[c]
*CYP2C9*3*	9.01 (7.23 – 10.7)	60	12.9 (6.31 – 35.5)	10	N/A	0	0.230
*CYP2C19*2*	10.5 (6.86 – 13.4)	40	8.65 (6.79 – 11.8)	27	5.14	3	0.850
*CYP2C19*3*	9.26 (7.67 – 11.8)	70	N/A	0	N/A	0	N/A
*CYP2D6*4*	10.6 (8.57 – 14.9)	42	7.88 (5.81 – 9.75)	25	6.24	1	0.0695[c]
*CYP3A4*1B*	9.08 (7.67 – 10.7)	64	13.4 (2.22 – 39.9)	6	N/A	0	0.706
*CYP3A5*3C*	N/A	0	8.47 (4.41 – 14.9)	13	9.75 (7.78 – 12.3)	66	0.450
Transporter genotypes							
ABCB1 3435C>T	7.23 (5.14 – 13.8)	21	10.4 (8.38 – 12.3)	41	8.90 (6.72 – 14.0)	20	0.271
ABCG2 421C>A	9.45 (7.43 – 11.8)	66	9.01 (6.72 – 10.7)	16	N/A	0	0.479

[a] Data represent apparent oral clearance of imatinib in units of L/h.
[b] *P*-values were obtained from a non-parametric Kruskal-Wallis test.
[c] Data were grouped from patients with one (heterozygote genotype) or two variant alleles (variant genotype).
Abbreviations: 95%CI, 95% confidence intervals; N, number of patients; N/A, not available

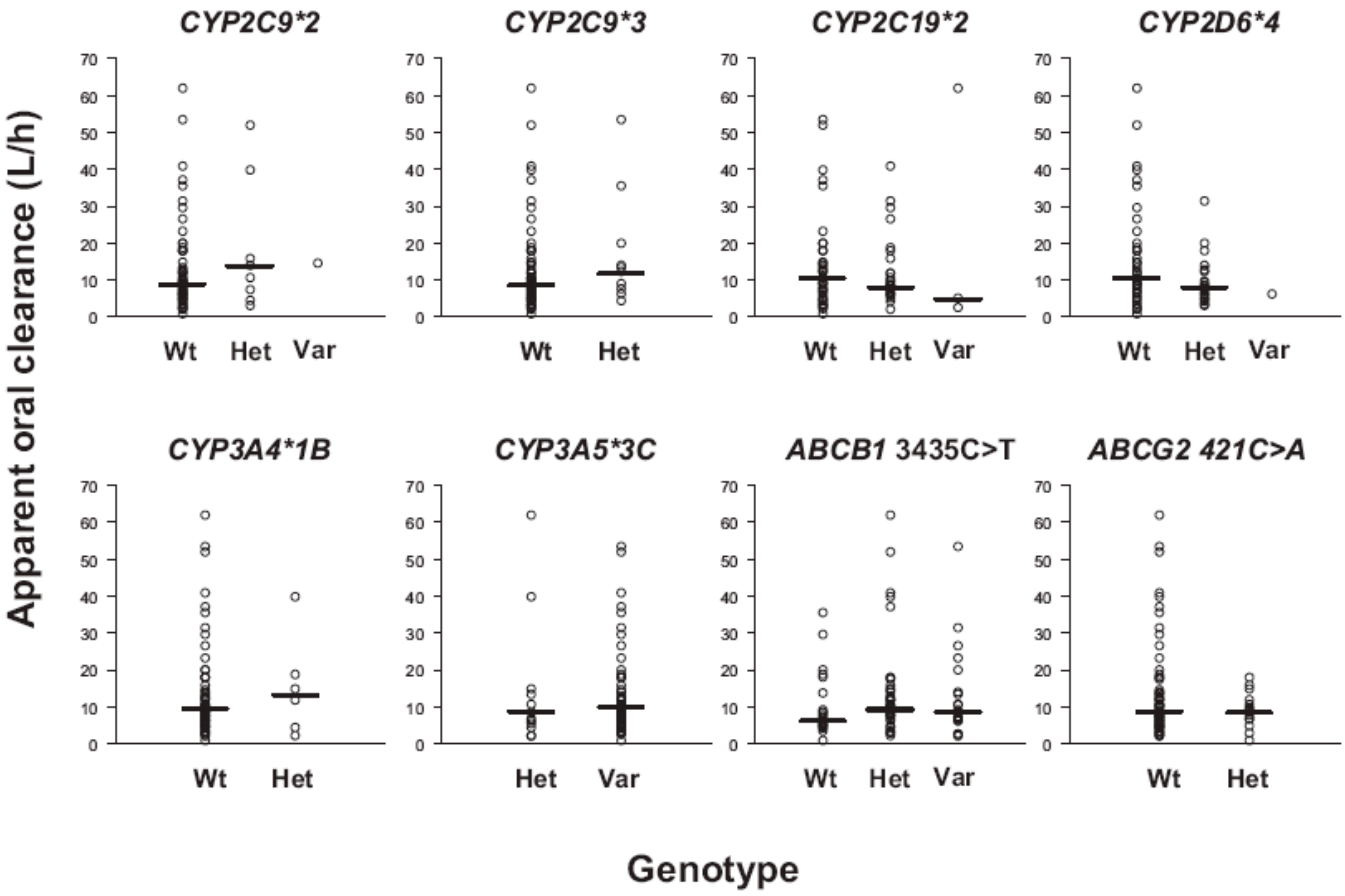

Fig 3. Individual apparent oral clearance values of imatinib as a function of variant genotypes. Wt, wild-type patient; Het, heterozygous variant type patient; Var, homozygous variant type patient. Each symbol represents an individual patient, and horizontal lines represent median values within each group.

Discussion

The present study suggests that patients carrying one defective *ABCG2* 421C>A allele do not have elevated plasma concentrations of the anticancer agent and ABCG2 substrate imatinib as compared to those patients with two wild-type alleles. The studied variant in *ABCG2* is a SNP causing a non-synonymous change in the protein sequence, in which a 421C to A transition in exon 5 leads to a glutamine to lysine amino acid substitution at codon 141 (Q141K). In contrast to the current clinical observations with imatinib, the *ABCG2* 421C>A allele has been shown previously to be associated with

altered pharmacokinetics of the topoisomerase I inhibitors diflomotecan [16], topotecan [9], and the irinotecan metabolite, SN-38 [17].

The underlying mechanistic reasons for the lack of a significant association between the *ABCG2* 421C>A polymorphism and the observed pharmacokinetic outcome in this study are not entirely clear. Although our own current *in vitro* transport studies in HEK293 cells transfected with the homozygous Q141K variant show an increased intracellular accumulation of imatinib, it is unclear whether this also holds true for the heterozygous situation as seen in the patients. It is also unknown whether ABCG2 is (equally) transcribed from both alleles. Furthermore, the functional ABCG2 transporter is a homo-oligomeric protein, and hence, in the heterozygous situation, various multimers with differing transport capacities may be formed. It is noteworthy that a recent preclinical investigation provided evidence that the mouse orthologue Abcg2 significantly affects the systemic clearance of imatinib [18]. Collectively, it logically follows that patients with a rare genotype comprising two variant alleles at the *ABCG2* 421C>A locus may show markedly impaired ability to eliminate imatinib and subsequently demonstrate excessive toxicity. Clearly, additional investigation is required to unambiguously assess the relationship between the homozygous variant *ABCG2* 421C>A genotype and imatinib pharmacokinetics.

Houghton et al. have demonstrated previously that the accumulation and efflux of imatinib were not altered between ABCG2-expressing and non-expressing cells with drug concentrations higher than those used in the current experiments [19]. Although the discrepancies with our data are likely due to methodological issues, such as substantial dissimilarities in incubation time and temperature, it is also possible that this phenomenon is related in part to saturation or concentration-dependent inhibition of ABCG2 by imatinib, which can occur at pharmacologically relevant concentrations of the drug that are achieved in patient plasma [19]. Hence, there is the potential for imatinib to modulate its own absorption and transporter-mediated elimination regardless of *ABCG2* genotype status, as suggested previously [20]. Finally, it should be noted that, due to the rapid expansion of SNP discovery and the present lack of overlap amongst the various SNP databases[21], it cannot be entirely excluded that further functional polymorphisms in this

and other genes are still to be described. For example, recent data also suggest that molecular determinants of imatinib pharmacokinetics and response might include the organic cation transporter SLC22A1 (OCT1) [20], which is encoded by a gene that has various common allelic variants [22,23]. Incorporation of this knowledge may eventually provide further refinement of the predictive strategies for imatinib.

A sexual dimorphism in Abcg2 expression in mice and ABCG2 expression in humans has recently been demonstrated, with males having higher mRNA levels in the liver, but not in the small intestine or kidney [24]. This was shown to result in lower plasma concentrations of a variety of ABCG2 substrates, including the antibiotic nitrofurantoin, the antiulcerative drug cimetidine, the anticancer agent topotecan, and the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, in male mice compared with female mice after both oral and intravenous drug administration [24]. Consistent with these observations, the apparent oral clearance of imatinib has also been previously shown to exhibit significant sexual dimorphism, being on average 14.5 L/h [percent coefficient of variation (%CV), 67%] for males and 7.32 L/h (%CV, 40%) for females [1]. Similar sex-dependent differences were also observed in the currently studied cohort, even though these values did not reach statistical significance. Hence, the clinical importance of this sex effect and the role of ABCG2 in the context of treatment outcome with imatinib-containing regimens remain to be established.

It is of interest to note that the interindividual pharmacokinetic variability for imatinib as observed in this study was very substantial, apparently regardless of *ABCG2* 421C>A genotype status, and in spite of earlier reports indicating that the oral bioavailability of imatinib in healthy volunteers is approximately 98% [25]. The reasons for this extensive pharmacokinetic variability in cancer patients are not yet fully understood, but can likely be attributed to intersubject variations in the activity of other transporters and enzymes of putative relevance to the pharmacokinetic profile of imatinib. As mentioned previously, these include the ATP binding cassette transporters ABCG2 and ABCB1[6], and the cytochrome P450 isoforms CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5, [26]. Prior *in vitro* studies have shown that CYP3A4 is the major enzyme involved in the biotransformation of imatinib, and that the apparent oral clearance of imatinib is sensitive

to concurrent administration of typical inhibitors of this enzyme such as ketoconazole[27] as well as inducers such as rifampin [28] and St. John's wort [29]. Variability in CYP3A4 activity between cancer patients has been shown to be particularly large [30,31] and this may therefore, in part, contribute to the large intersubject variability in imatinib pharmacokinetic parameters observed here.

Previous work has shown that certain variants in the *ABCB1* gene are associated with reduced expression of its encoded protein [32], and that this may result in decreased intestinal and hepatobiliary secretion of substrate drugs. In this study, imatinib pharmacokinetics were not statistically significantly influenced by the common *ABCB1* 3435C>T allele. This observation is in line with the notion that the effect of *ABCB1* genotype is a relatively unimportant contributing factor to the pharmacokinetics of substrate drugs unless ABCB1-mediated transport is the crucial process in oral absorption [4]. The lack of effect of the evaluated variants in the *CYP3A4* and *CYP3A5* genes on imatinib pharmacokinetics is also consistent with the relatively low allelic frequencies in the Caucasian population and the available functional data indicating a limited role of such variants in the interindividual variability of CYP3A expression and activity in patients with cancer [31]. The lack of functional significance of the studied *CYP2C9* and *CYP2C19* variants in relation to imatinib pharmacokinetics is likely the result of the small relative contribution of these two isoforms to overall drug elimination [7].

One intriguing observation in the current study was that the median apparent oral clearance of imatinib was reduced in carriers of at least one *CYP2D6*4* allele, reaching borderline significance. Furthermore, patients with an oral clearance of imatinib higher than the median value were more likely to carry two copies of the reference *CYP2D6* sequence. Previous investigation has demonstrated that imatinib is excreted predominantly via the biliary-fecal route, but with only up to 28% of the dose in the excreta corresponding to parent drug. Since imatinib is a known potent inhibitor of CYP3A4 [33], it is theoretically plausible that, at steady-state, imatinib inhibits its own primary oxidation pathway and that metabolism is shunted to CYP2D6-mediated routes. In this scenario, the non-functional *CYP2D6*4* allele becomes of central interest as it is

carried by approximately 75% of CYP2D6 poor metabolizers in the Caucasian population [34].

In view of the presence of only one patient with homozygosity for this *CYP2D6* allele in our population as well as the relatively small sample size studied here, independent confirmation is needed to corroborate our findings. However, the magnitude of the observed effect of the *CYP2D6*4* genotype suggests that the overall impact might be of clinical importance with respect to pharmacodynamic outcome following treatment with imatinib. In this context, it is noteworthy that various studies have shown marked differences in genotype and allele frequencies for *CYP2D6* variants between different ethnic populations. Although the *CYP2D6*4* allele is rarely found in Asians [35], other alleles associated with low substrate affinity and low activity can play an important role in drug metabolism in this population. For example, the *CYP2D6*10* allele may be present in as much as 50% of Asians and is responsible for diminished enzyme activity and this may have therapeutic and prognostic implications [35]. This possibility is further exemplified by the observation that Asians have increased susceptibility to imatinib-induced hematological toxicity compared to Caucasians at the currently recommended drug doses [36]. For examples, there has been concern that dosing of imatinib at a dose of 400 mg, the recommended daily dose in the United States, may be too high for Japanese patients, based on the high incidence and severity of drug-induced thrombocytopenia in this population [37]. Additional analysis in patients of diverse ethnic background is clearly required to further resolve this issue.

In conclusion, this study indicates that the presently evaluated variant alleles in the *CYP2C9*, *CYP2C19*, *CYP3A4*, *CYP3A5*, *ABCB1* and *ABCG2* genes do not contribute substantially to explaining the extensive interindividual variability in imatinib pharmacokinetics. It is possible that additional genetic variants or haplotypes of importance to imatinib pharmacokinetics may yet be discovered. Further investigation, particularly with respect to homozygosity of variants in the *ABCG2* and *CYP2D6* genes, is needed to determine the relative role of genetic variation and environmental variables on the absorption and disposition of imatinib.

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Chapter 8

Summary, Conclusions and Future Perspectives

Imatinib mesylate, the first tyrosine kinase inhibitor to gain approval by the FDA, remains as a pivotal example of rational drug design. Initially, imatinib was found to target the bcr-abl fusion protein in CML and further targets have subsequently been identified, including c-kit in GIST. Though a great number of studies have elucidated underlying mechanisms to explain emerging resistance to this anti-cancer agent, many cases of resistance remain unexplained. Furthermore, patients exhibit high interindividual variability in imatinib pharmacokinetics, which may contribute to suboptimal drug exposure and response.

Chapter 2 provides a literature review on the role of two ABC transporter proteins, ABCB1 (P-glycoprotein) and ABCG2 (BCRP), both of which are expressed ubiquitously throughout the body and have been shown to transport imatinib.

Cytochrome P450 3A4 (CYP3A4) is considered to be the major enzyme involved in metabolism of imatinib. Midazolam, a benzodiazepine sedative, can be used as a probe to assess CYP3A4 and CYP3A5 activity *in vivo*. **Chapter 3** details the development and validation of a novel, sensitive assay employing LC-MS technology for the measurement of midazolam in human plasma. In **Chapter 4**, this assay was utilized to evaluate the pharmacokinetics of midazolam in a group of individuals with cancer, for assessment of CYP3A4 and CYP3A5 activity. The midazolam pharmacokinetic parameters were then correlated with polymorphisms in the *CYP3A4*, *CYP3A5* and *ABCB1* genes, to determine whether these mutations can account for the interindividual variability observed. In this population of 58 patients, no statistically significant correlations were found between any of the six polymorphisms and midazolam drug exposure (AUC), volume of distribution or drug clearance. As such, the highly variable activity of CYP3A4 and CYP3A5 does not appear to be directly due to genetic differences in the genes encoding them.

In **Chapter 5**, the effect of tariquidar, a dual inhibitor of both ABCB1 and ABCG2, on the pharmacokinetics of imatinib was evaluated *in vivo*. It was hypothesized that the addition of a transport inhibitor would increase drug concentrations in both plasma and tissues. It was of particular interest to determine whether increased penetration into the brain could be achieved. Oral administration of tariquidar, 30 minutes prior to imatinib administration, more than doubled exposure to imatinib in plasma. As anticipated, there

was no apparent change in the rate or extent of drug absorption, presumably due to the high bioavailability of imatinib. Though the brain exposure to imatinib also increased over 110% with tariquidar pretreatment, the overall brain-to-plasma ratio did not differ between the two groups. Presumably, tariquidar is inhibiting ABCB1 and ABCG2 expression in the liver and kidneys, leading to decreased drug elimination. Overall, these findings suggest that significant modulation of the blood brain barrier is not occurring with tariquidar. It is also possible that if used in a clinical setting, the increased plasma drug exposure may also increase the severity or frequency of side effects.

Clinical observations of increased imatinib clearance with long-term treatment brought forth the idea that the drug may be inducing its own elimination, through upregulation of transporter proteins or metabolizing enzymes. As increases in drug clearance could result in suboptimal drug exposure, this phenomenon may result in the development of pharmacokinetic resistance. Indeed, dose escalation has been shown to overcome resistance. **Chapter 6** details the effects of long-term treatment of normal mice with daily imatinib. Plasma concentrations of imatinib and expression of Abcb1 and Abcg2 were evaluated each week for four weeks. Plasma and liver concentrations of imatinib did not change significantly over the course of treatment. In contrast to in vitro findings published by Burger *et al.*, there was no apparent increase in Abcb1 or Abcg2 expression in liver or intestinal samples. As such, the mouse does not appear to develop pharmacokinetic resistance to imatinib as noted in humans and cannot be used to elucidate the causes of this resistance.

Finally, in **Chapter 7**, nine single nucleotide polymorphisms in seven genes involved in imatinib metabolism or transport were assessed in 82 patients treated with imatinib for gastrointestinal stromal tumors. *In vitro*, we demonstrated that the Q141K polymorpshim in ABCG2 results in significantly higher intracellular accumulation of imatinib. However, this finding was not confirmed *in vivo*. No significant correlations were found between polymorphisms in *ABCB1*, *ABCG2*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A4* or *CYP3A5* and the apparent oral clearance of imatinib.

The work presented in this thesis aimed to identify factors involved in the interindividual pharmacokinetic variability and development of pharmacokinetic

resistance to treatment with imatinib. Though CYP3A4 is the major enzyme responsible for metabolism of imatinib, polymorphisms in this gene did not correlate with steady-state pharmacokinetic parameters. Furthermore, it was demonstrated that polymorphisms in *CYP3A4* and *CYP3A5* are not surrogates for activity of the encoded enzymes in patients with cancer, as assessed using midazolam as a probe substrate.

Based on previous publications and our own *in vitro* findings, ABCG2 was hypothesized to play a major role in imatinib disposition, potentially increasing drug exposure for those with one or more Q141K alleles and/or leading to the increased drug clearance over time if imatinib was inducing *ABCG2*. However, pre-clinical and retrospective clinical studies did not confirm the *in vitro* results.

It is likely that the interindividual pharmacokinetic variability of imatinib cannot be attributed to a single transporter or enzyme, but that many factors in the disposition of this agent must be considered in combination. Furthermore, it appears that the mouse is not a suitable model for studying the development of pharmacokinetic resistance. A long-term, prospective clinical study of imatinib may offer considerable insight. Ideally, a study of this type would involve measurement of imatinib steady-state pharmacokinetics at monthly intervals. In addition, analysis of CGP74588, the major metabolite of imatinib formed by CYP3A4, coupled with repeated midazolam phenotyping to assess activity, would hopefully elucidate the role of this enzyme in variability, as well as changes over time. Along with pharmacogenetics, all of the data collected could be incorporated into a population pharmacokinetic model, allowing for assessment of many covariates which may be contributing to interindividual variability or long-term changes in drug clearance. It is only once the contributing factors are elucidated that successful mechanisms for overcoming these barriers to safe, yet highly effective treatment with imatinib can be explored.

Hoofdstuk 8

Samenvatting, Conclusies en Toekomstperspectieven

Imatinib mesylaat was de eerste tyrosinekinaseremmer die door de Amerikaanse 'Food and Drug Administration' (FDA) werd toegelaten tot de markt. Dit middel wordt thans beschouwd als een schoolvoorbeeld van de rationele geneesmiddelontwikkeling. Alhoewel aanvankelijk werd verondersteld dat het werkingsmechanisme van imatinib louter was gebaseerd op een interactie met het zogenaamde Bcr-Abl eiwit in chronische myeloïde leukemie (CML) zijn er later additionele aangrijpingspunten ontdekt, waaronder het c-KIT eiwit in gastrointestinale stromatumoren (GIST). Veel onderzoek is recent verricht naar de mechanismen die ten grondslag liggen aan het optreden van resistentie tegen imatinib. Echter, in vele gevallen blijft de exacte oorzaak voor het optreden van deze resistentie onopgehelderd. Eerder onderzoek heeft uitgewezen dat het farmacokinetisch gedrag van imatinib tussen patiënten onderling zeer sterk varieert, en het is mogelijk dat deze mate van variabiliteit bijdraagt aan een te lage blootstelling aan het middel, met als gevolg een verminderde kans op een respons.

In **hoofdstuk 2** wordt een literatuuroverzicht gegeven van recente ontwikkelingen in een aantal factoren die van invloed kunnen zijn op de farmacokinetiek van imatinib. In het bijzonder wordt aandacht geschonken aan twee transporteiwitten, met name ABCB1 (P-glycoproteïne) en ABCG2 (BCRP), en hun specifieke rol in de absorptie en eliminatie van diverse antikankergeneesmiddelen.

Over het algemeen wordt verondersteld dat het enzyme cytochrome P450 3A4 (CYP3A4) het belangrijkste metaboliserende eiwit is dat de eliminatie van imatinib reguleert. De fenotypische activiteit van dit CYP3A4 kan vastgesteld worden aan de hand van een test waarbij het benzodiazepinederivaat midazolam aan patiënten wordt toegediend. In **hoofdstuk 3** wordt de ontwikkeling en validatie beschreven van een analytische methode voor het bepalen van midazolamconcentraties in humaan bloedplasma, gebruikmakend van hogedruk-vloeistof chromatografie met massaspectrometrische (LC-MS) detectie. Deze methodiek werd vervolgens toegepast in het onderzoek beschreven in **hoofdstuk 4**, waarin de activiteit van CYP3A4 en het gerelateerde enzyme CYP3A5 is onderzocht in 58 kankerpatiënten. De farmacokinetische parameters voor midazolam werden vervolgens gecorreleerd aan een zestal polymorfismen in de *CYP3A4*, *CYP3A5* en *ABCB1* genen om vast te stellen of genetische

mutaties een bijdrage leveren aan de gevonden interindividuele variabiliteit in de klaring van midazolam. In de onderzochte populatie werden geen statistisch significante relaties gevonden tussen de 6 polymorfismen en de blootstelling aan midazolam (AUC), het distributievolume of de klaring. Derhalve kon worden geconcludeerd dat interindividuele variabiliteit in de activiteit van CYP3A4, CYP3A5 en ABCB1 waarschijnlijk niet in belangrijke mate wordt bepaald door genetische verschillen tussen patiënten in de genen die deze eiwitten tot expressie brengen.

In **hoofdstuk 5** is de invloed onderzocht van tariquidar, een remmer van de ABCB1 en ABCG2 transporteiwitten, op de farmacokinetiek van imatinib in een proefdiermodel. Dit onderzoek was gebaseerd op de hypothese dat behandeling met een dergelijke middel zal resulteren in verhoogde concentraties van imatinib in bloedplasma en weefsels, in het bijzonder de hersenen. Er werd gevonden dat tariquidar, wanneer oraal toegediend 30 minuten voorafgaand aan imatinib, de systemische blootstelling aan imatinib meer dan verdubbelde. Tariquidar had geen invloed op de snelheid en mate van de gastrointestinale absorptie van imatinib, hetgeen in overeenstemming is met de eerder beschreven hoge orale biologische beschikbaarheid van dit middel. Alhoewel de blootstelling aan imatinib in hersenweefsel met meer dan 110% toenam in aanwezigheid van tariquidar, werd geen verschil gevonden in de concentratieratio voor hersenen en plasma in de aan- of afwezigheid van tariquidar. Deze bevindingen zijn consistent met de gedachte dat tariquidar de functie van ABCB1 en ABCG2 efficient kan remmen in de lever maar niet in de hersenen. Dit proces heeft tot gevolg een vertraagde eliminatie van imatinib zonder dat de functie van de bloed-hersenbarriere wordt beïnvloed. Het ligt voor de hand dat, indien dit concept zou toegepast worden in de kliniek, de verhoogde bloedplasmaconcentraties van imatinib in aanwezigheid van tariquidar zullen resulteren in een toename in de mate en ernst van ongewenste bijwerkingen.

Eerder klinisch onderzoek heeft uitgewezen dat langdurige behandeling met imatinib kan leiden tot een verhoogde klaring van dit middel. Deze bevinding is mogelijk het gevolg van autoinductie, een proces waarbij een geneesmiddel zijn eigen eliminatie kan versnellen door het induceren van de expressie van enzymen en transporteiwitten. Aangezien een verhoogde klaring kan leiden tot suboptimale blootstelling aan een middel

kan dit proces uiteindelijk resulteren in de ontwikkeling van zogenaamde farmacokinetische resistentie, waarbij patiënten een hogere dosis imatinib behoeven om de beoogde therapeutische effecten te verkrijgen. In **hoofdstuk 6** is de invloed onderzocht van langdurige blootstelling aan imatinib, na dagelijkse toediening aan muizen, op de expressie van Abcb1 en Abcg2. Gedurende een behandelperiode van 4 weken werd gevonden dat de concentraties van imatinib in bloedplasma en de lever niet significant veranderden in de tijd. Er werd tevens geen significante toename gevonden in de expressie van Abcb1 en Abcg2 in lever- en darmweefsels, hetgeen niet in overeenstemming lijkt te zijn met eerder beschreven *in vitro* experimenten en klinisch onderzoek. Het is mogelijk dat de muis geen optimaal model is voor het verder ontrafelen van de specifieke oorzaken van deze vorm van geneesmiddelresistentie tegen imatinib.

Tot slot is in **hoofdstuk 7** de rol onderzocht van 9 zogenaamde SNPs (single-nucleotide polymorphisms) in 7 genen die betrokken zijn bij de eliminatie van geneesmiddelen in de farmacokinetiek van imatinib in 82 patiënten met GIST. Gebruikmakend van een *in vitro* modelsysteem werd aanvankelijk gevonden dat het Q141K polymorfisme in het *ABCG2* gen leidt tot een significant verhoogde intracellulaire accumulatie van imatinib. Echter, deze bevinding kon niet worden bevestigd *in vivo*. Significante correlaties werden tevens niet gevonden tussen polymorfismen in *ABCB1*, *ABCG2*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A4* of *CYP3A5* en de orale klaring van imatinib.

Het onderzoek beschreven in dit proefschrift had tot doel om factoren te identificeren die een rol spelen in de interindividuele farmacokinetische variabiliteit alsmede in de ontwikkeling van farmacokinetische resistentie bij de behandeling met imatinib. Alhoewel het enzyme CYP3A4 een belangrijkste plaats inneemt in het metabolisme van imatinib kon genetische variabiliteit in dit gen niet positief gecorreleerd worden met farmacokinetische parameters op steady-state. Bovendien kon worden aangetoond dat polymorfismen in de *CYP3A4* en *CYP3A5* genen niet fungeren als surrogaat voor de activiteit van de coderende eiwitten in kankerpatiënten, bepaald aan de hand van de klaring van het substraat midazolam.

Op basis van eerder gepubliceerd werk alsmede de alhier beschreven *in vitro* bevindingen was verondersteld dat ABCG2 een belangrijke rol zou kunnen spelen in de dispositie van imatinib. Met name lag het voor de hand dat het variante Q141K allel geassocieerd zou zijn met een verhoogde blootstelling aan imatinib en dat autoinductie zou leiden tot verhoogde expressie van ABCG2, met dientengevolge een versnelde klaring in de tijd. Echter, in preklinisch alsook retrospectief klinisch onderzoek konden deze hypotheses niet worden bevestigd.

Het ligt voor de hand dat de interindividuele farmacokinetische variabiliteit niet verklaard kan worden aan de hand van slechts één enkel transporteiwit of één enzyme, maar dat vele factoren tesamen in ogenschouw genomen moeten worden. Met betrekking tot de autoinductie van imatinib is het van belang zich te realiseren dat de muis waarschijnlijk geen bruikbaar model is voor verder onderzoek naar de mogelijke oorzaken van farmacokinetische resistentie. Een prospectief klinisch onderzoek waarbij imatinib langdurig wordt toegediend aan patienten zal noodzakelijk zijn om verder inzicht te verwerven in dit proces. Idealiter zou in een dergelijke studie tevens maandelijkse bloedbemonstering plaatsvinden voor het analyseren van steady-state concentraties van imatinib en de metaboliet CGP74588, die gevormd wordt vanuit imatinib via CYP3A4. Vervolgstudies waarin de fenotypische activiteit van CYP3A4 wordt bepaald aan de hand van de midazolamklaring zouden eveneens een bijdrage kunnen leveren aan het verder ontrafelen van de specifieke rol van dit enzyme in de farmacokinetische variabiliteit na behandeling met imatinib, alsmede in een mogelijk veranderde klaring in de tijd. Deze nieuw te vergaren informatie, tesamen met prospectief verzamelde gegevens aangaande farmacogenetica, kan dan geïncorporeerd worden in een zogenaamd populatie-farmacokinetisch model. Met behulp van een dergelijk model kan kwantitatief de bijdrage bepaald worden van covariabelen in de interindividuele farmacokinetische variabiliteit. Het verder verkennen en ontdekken van belangrijke covariabelen zal in de nabije toekomst resulteren in een beter voorspelbare en verlengde blootstelling van een tumor aan imatinib, en in een gunstiger behandelingsuitkomst.

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Curriculum Vitae

The author of this thesis was born in Palo Alto, California on May 31, 1980. In 1994, she moved to the United Kingdom and completed her International Baccalaureate Diploma at Sevenoaks School (Sevenoaks, Kent) in 1998. In 2002, she received a Masters in Chemistry from Keble College, Oxford University. Since 2002, she has worked within the Clinical Pharmacology Program headed by Dr. William D. Figg at the National Cancer Institute in Bethesda, Maryland. Research has included that contained in this thesis, in addition to *in vitro*, preclinical and clinical studies on a range of cancer therapeutics, including novel formulations of paclitaxel and numerous targeted agents. The work presented in this thesis originated from collaborations with Prof.dr. Jaap Verweij at Erasmus University, Daniel den Hoed Cancer Center, Rotterdam, the Netherlands.

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