Towards a Pharmacologically Guided Individualization of Imatinib and Sunitinib Therapy

Naar een individualisatie van imatinib en sunitinib therapie op geleide van farmacologische eigenschappen

Karel Eechoute

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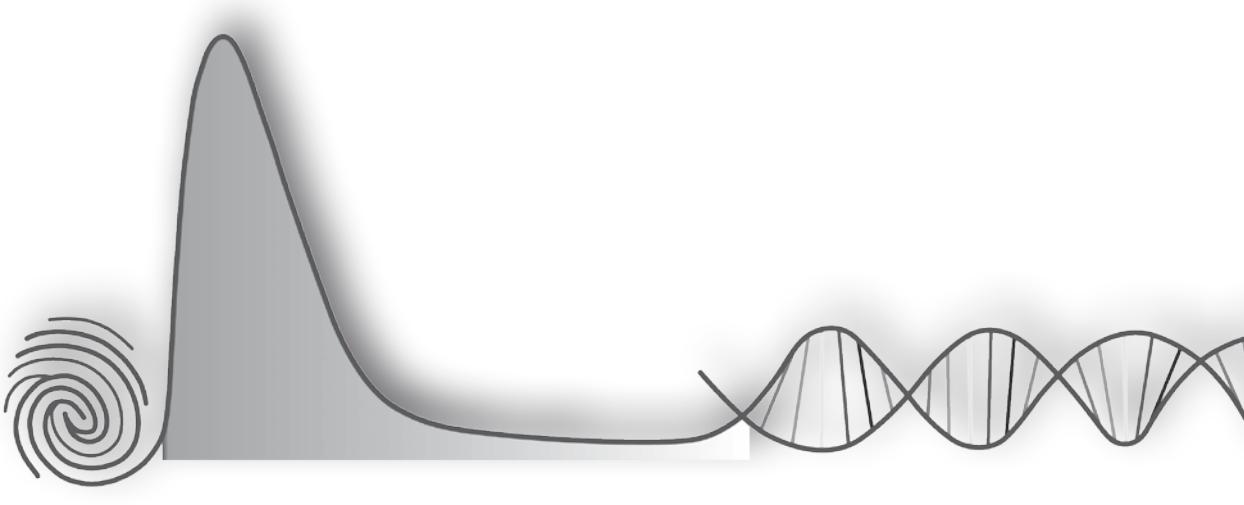
Let op, want ik ga iets genuanceerds zeggen
Louis Tobback

Voor Janneke, Sjors, Kamiel en Kaatje
Mijn maatjes

Contents

Chapter 1

Introduction to the thesis



The approval of imatinib mesylate (Gleevec™) in 2001 has added a new class of drugs to the systemic treatment of cancer: that of the tyrosine kinase inhibitors (TKIs). Imatinib inhibits autophosphorylation of specific proteins involved in oncogenesis such as the BCR-ABL fusion protein (expressed in Philadelphia chromosome positive chronic myeloid leukemia), c-KIT (expressed in gastrointestinal stromal tumors; GIST) and the platelet-derived growth factor receptor (PDGFR; i.e. expressed in GIST and several sarcomas).[1] After a decade of therapeutic use, imatinib has proven to be a highly effective targeted agent with a median overall survival in advanced GIST patients close to 5 years.[2]

Sunitinib malate (Sutent™) was developed in imatinib's foot steps and in 2006 it became the first anticancer drug that was simultaneously approved for two indications: as first-line treatment of locally advanced or metastatic renal cell carcinoma (mRCC) and as second-line therapy for GIST after disease progression or intolerance to imatinib therapy.[3] Recently, it was also registered for the treatment of advanced well-differentiated pancreatic neuroendocrine tumors, based on its proven efficacy.[4]

Sunitinib is a multi-targeted TKI with inhibitory effects on drug targets such as KIT and PDGFR, FMS-like tyrosine kinase 3 (FLT3; i.e. expressed in acute myeloid leukemia), RET (expressed in thyroid cancer) but also vascular endothelial growth factor receptor (VEGFR).[5] Activation of VEGFR will induce blood vessel growth in normal as well as in tumor tissue; a process called angiogenesis.[6,7] With a median overall survival of approximately 2 years, sunitinib-induced antiangiogenesis has proven to be an effective treatment modality in advanced mRCC patients.[8]

Both imatinib and sunitinib are orally administered drugs and show a large inter-individual variability in systemic exposure.[3,9] As clinical outcome may be correlated to exposure,[10,11] the elucidation of mechanisms underlying this pharmacokinetic variation may be of utmost clinical importance to the individual patient. Imatinib and sunitinib are extensively metabolized in the liver, mainly by cytochrome P450 enzyme isoform 3A4 (CYP3A4), to their active metabolites; CGP74588 and SU12662, respectively.[12,13] Biliary excretion accounts for the major part of imatinib and sunitinib clearance, with 60 – 70 % of the dose recovered in faeces.[12,14] Imatinib is a good substrate of several drug uptake and efflux transporters, making it a possible candidate for facilitated or active drug transport during absorption, distribution and elimination phases (**Chapter 2**). On the other hand, data on the effects of drug transporters on sunitinib exposure are limited.[15-18]

In the context of potential therapeutic relevance of imatinib plasma concentrations, it was the aim of the first part of this thesis to extensively analyse imatinib pharmacokinetics over time, and assess the possible contributing factors to the observed systemic exposure. Therefore, we analysed imatinib plasma concentrations in a long-term population pharmacokinetic study (**Chapter 3**) and evaluated the biliary secretion of imatinib during severe hepatic dysfunction (**Chapter 5**). In addition, in an *in vitro* and clinical set-

ting, we studied the role of an intestinally located solute carrier in imatinib transport and absorption (**Chapter 4**), in order to translate preclinical findings into clinical practice.

In the second part of the thesis, the clinical relevance of genetic variation in pharmacokinetic and pharmacodynamic pathways in sunitinib-treated patients was assessed. We therefore investigated single nucleotide polymorphisms in candidate genes involved in sunitinib exposure and therapeutic activity and assessed their association with drug related toxicities (**Chapter 6 and 8**) and survival (**Chapter 7**).

Finally, we investigated the suppression of allergic rhinitis symptoms in sunitinib-treated patients (**Chapter 9**), as this is a side-effect of sunitinib therapy that may hold therapeutic potential in inflammatory disorders like hay fever or asthma.

In conclusion, this thesis entitled "*Towards a pharmacologically guided individualization of imatinib and sunitinib therapy*" explores the pharmacokinetic, pharmacodynamic and pharmacogenetic factors that may clarify variability in outcome and toxicity in patients treated with either imatinib or sunitinib. The ultimate goal of these studies is to personalize dosing and treatment sequences of these rationally designed drugs in order to match an individual patient's needs.

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Chapter 2

Drug transporters and imatinib treatment: implications for clinical practice

Eechoute K., Sparreboom A., Burger H., Franke R.M., Schiavon G., Verweij J., Loos W.J., Wiemer E.A.C., Mathijssen R.H.J.

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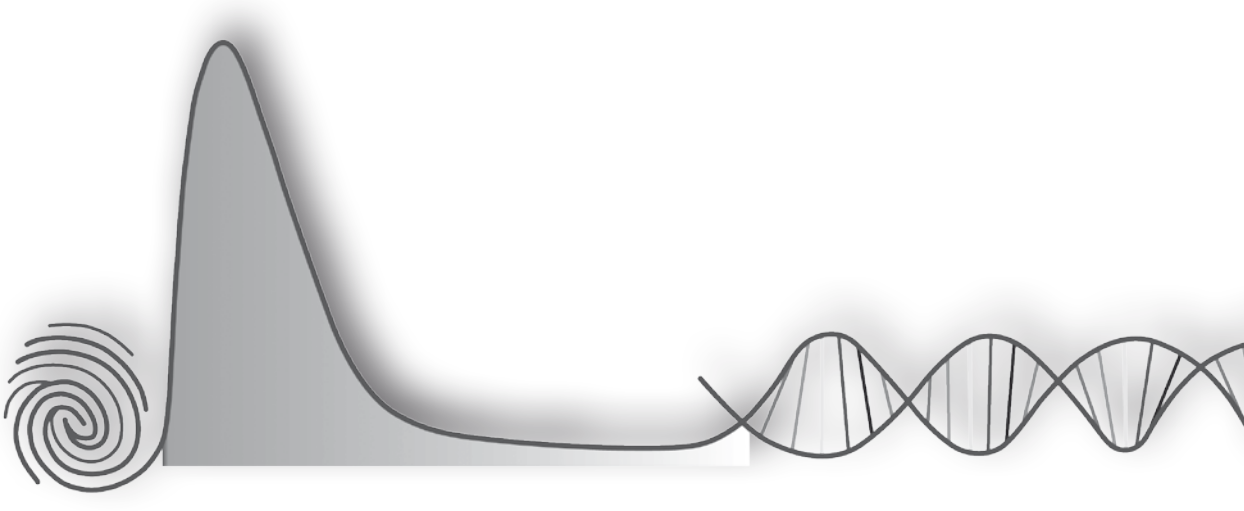

ABSTRACT

Imatinib mesylate is approved for the treatment of chronic myeloid leukemia (CML) and advanced gastrointestinal stromal tumors (GIST). Unfortunately, in the course of treatment, disease progression occurs in the majority of patients with GIST. Lowered plasma trough levels of imatinib over time potentially cause disease progression, a phenomenon known as "acquired pharmacokinetic drug resistance." This outcome may be the result of an altered expression pattern or activity of drug transporters. To date, the role of both efflux transporters (ATP-binding cassette transporters, such as ABCB1 and ABCG2) and uptake transporters [solute carriers such as organic cation transporter 1 (OCT1) and organic anion transporting polypeptide 1A2 (OATP1A2)] in imatinib pharmacokinetics and pharmacodynamics has been studied. In vitro experiments show a significant role of ABCB1 and ABCG2 in cellular uptake and retention of imatinib, although pharmacokinetic and pharmacogenetic data are still scarce and contradictory. ABCB1 and ABCC1 expression was shown in GIST, whereas ABCB1, ABCG2, and OCT1 were found in mononuclear cells in CML patients. Several studies have reported a clinical relevance of tumor expression or activity of OCT1 in CML patients. Further (clinical) studies are required to quantify drug transporter expression over time in organs involved in imatinib metabolism, as well as in tumor tissue. In addition, more pharmacogenetic studies will be needed to validate associations.

INTRODUCTION

Imatinib mesylate (Gleevec, Novartis International AG) is the first approved rationally designed inhibitor of specific protein tyrosine kinases. The drug inhibits ABL and the BCR ABL fusion protein [expressed in Philadelphia chromosome–positive chronic myeloid leukemia (CML)], c-KIT [expressed in gastrointestinal stromal tumors (GIST)], and the platelet-derived growth factor receptor (PDGF-R; i.e., expressed in some sarcomas[1-4]). Imatinib has become the standard treatment for patients with chronic myeloid leukemia[5,6] and GIST.[7-10] Although response rates in imatinib-treated patients are high, ranging between 70 to 90% of patients with GIST as well as CML [9,11,12], nonresponse or disease progression after a certain period of time may occur. Genetic mutations or gene amplification of the drug targets are known mechanisms for this observed (acquired) drug resistance.[12-15] Accumulating data, however, indicate a contributing role of pharmacokinetics in imatinib efficacy, as well as for the initial therapeutic response, and for the time to progression. Drug uptake and efflux transporters are likely to be involved in imatinib absorption, distribution, and excretion, thereby influencing pharmacokinetics. Imatinib is almost completely absorbed (>97%)[16] and is, then, extensively metabolized in the liver with CGP74588 as its most active metabolite, predominantly formed by cytochrome P450 isoform 3A4 and 3A5 (CYP3A4, CYP3A5) as shown in Fig. 1.[17] This

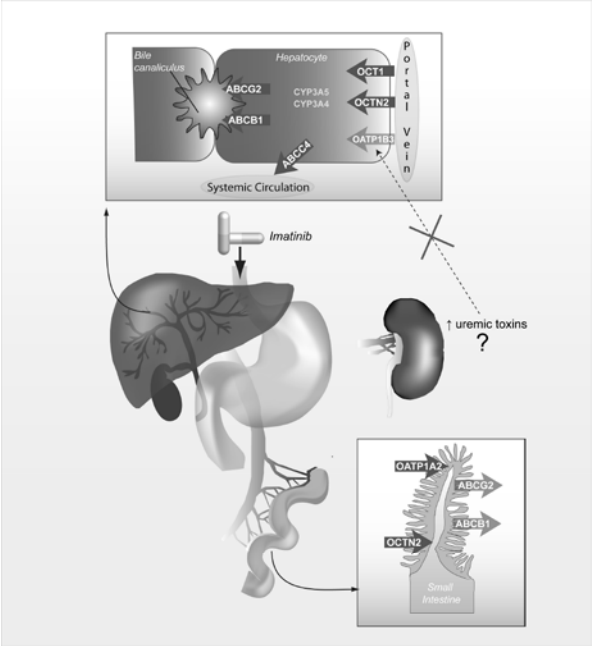

Figure 1. Scheme for the involvement of transporters in imatinib pharmacokinetics

metabolite is equipotent to its parental compound in vitro. Other cytochrome P450 isoforms (CYP1A2, CYP2D6, CYP2C8, CYP2C9, and CYP2C19) also play a (minor) role in imatinib metabolism.[17,18] At clinically relevant concentrations, imatinib is bound to plasma proteins, mainly albumin and α1-acid glycoprotein.[16,19] Both imatinib and its active metabolite are excreted in feces and, to a lesser extent, in urine. Initial drug resistance could be correlated with drug exposure. For instance, imatinib trough levels, the lowest drug concentration, right before administration of a new dose, in nonresponding patients are significantly lower (see Table 1) than in responding patients.[20-22] Moreover, imatinib pharmacokinetics may also contribute to acquired drug resistance. This finding was shown in a small-population pharmacokinetics study in patients with GIST.[23] After long-term treatment (>1 year), imatinib clearance increased by 33% and systemic exposure decreased by 42%, compared with the start of treatment, possibly suggesting involvement of pharmacokinetics in the development of resistance. This change in pharmacokinetics is likely also related to the reduced side effects over time and may also explain why dose escalation of imatinib at first progression results in clinical benefit in subsets of patients with advanced CML and GIST.[24-26] Despite a broad interpatient variability in imatinib plasma exposure[27], more recent data suggest the clinical significance of keeping imatinib plasma concentrations within a therapeutic range. That is, in GIST and CML, a lower response rate and/or shorter time to progression may occur when imatinib plasma levels drop below approximately 1,000 ng/mL.[21,28-30] As reported in the study with GIST patients, imatinib steady state trough levels were higher than mentioned in literature.[31] Therefore, an association with clinical benefit may be biased by the overestimation of trough levels. Nonetheless, steady state plasma concentrations above 1,000 ng/mL are often easily reached with a daily dose of 400 mg imatinib. Yet, in a subset of patients, this plasma concentration is not reached with this standard dose. Understanding the causes for this variability may be of clinical importance. One of the patient factors that is likely to be relevant for the observed differences in imatinib pharmacokinetics is the possible interpatient variability in drug transporter expression and activity. In this review, we give a detailed overview of the potential clinical relevance of recently characterized drug transporters for imatinib therapy.

INTERACTION OF IMATINIB WITH DRUG TRANSPORTERS

Efflux transporters.

Imatinib is a substrate of ATP-binding cassette (ABC) transporters such as the ABC subfamily B member 1 (ABCB1; formerly known as P-glycoprotein or MDR1[32-38]) and subfamily G member 2 [ABCG2; formerly known as breast cancer resistance protein (BCRP)[37,39,40], which are involved in its excretion process. These drug transporters use

Table 1. Imatinib trough levels (Cmin) in imatinib treated chronic myeloid leukaemia and gastrointestinal stromal cell tumor patients with or without clinical response.

Tumor type	Dose range (mg)	Number of patients	Number of responders	Mean or Median Cmin in responders (ng/mL)	Median free Cmin in responders (ng/mL)	Number of non-responders	Mean or Median Cmin in non-responders (ng/mL)	Median free Cmin in non-responders (ng/mL)[*]	P-value	Reference
CML[*]	400 - 800	351	297	1,009 (+/- 544)[†‡]	n.a.[§]	54	812 (+/- 409)[†]	n.a.[§]	P=0.01	(20)
CML[*]	50 - 800	254	218	1,057 (+/- 585)[†‡]	n.a.[§]	36	835 (+/- 524)[†]	n.a.[§]	P=0.033	(30)
CML[*]	50 - 800	254	166	1,107 (+/- 594)[†¶]	n.a.[§]	88	873 (+/- 528)[†]	n.a.[§]	P=0.002	(30)
CML[*]	400 – 600	68	56	1,123 (+/- 617)[†‡]	n.a.[§]	12	694 (+/- 556)[†]	n.a.[§]	P=0.03	(21)
CML[*]	400 – 600	68	34	1,452 (+/- 649)[†¶]	n.a.[§]	34	869 (+/- 427)[†]	n.a.[§]	P<0.001	(21)
CML[*]	400	40	20	2,340 (+/- 520)[†\\]	n.a.[§]	20	690 (+/- 150)[†]	n.a.[§]	P=0.002	(29)
GIST[**]	400 – 800	73	57	1,446 (414 – 3,336)[††‡‡]	n.a.[§]	16	1,155 (545 – 4,182)[††#]	n.a.[§]	P=0.25	(28)
GIST[¥]	not reported	33	14	n.a.[§]	25,7 (13,7 - 27)[∞]	19	n.a.[§]	10,1 (6,1 - 17,4)[∞∂]	P=0.013	(22)

Legend: [*] chronic myeloid leukemia; [†] mean Cmin (+/- standard deviation); [‡] response is defined as complete cytogenetic response; [§] not available; [¶]response is defined as major molecular response; [\\] response is defined as complete haematological response at 3 months or major cytogenetic response at 6 months; [**] gastrointestinal stromal cell tumor; [††] median Cmin (and range); [‡‡] response is defined by the response evaluation criteria in solid tumors (RECIST) as stable disease, partial response or complete response; [#] non-response is defined as disease progression by RECIST or not assessable; [¥] patients with exon 9 mutated or *wt KIT* GIST; [∞] median free Cmin (and range) deduced from imatinib and AGP levels; [∂] non-response is defined by RECIST as disease progression

the hydrolysis of ATP and subsequent phosphorylation of the transporter as an energy source, enabling active transport of substrates across various biomembranes.[41,42] Studies have reported imatinib as an inhibitor of ABC transporters[35,43,44], but there is growing consensus that ABC transporter inhibition by imatinib is dose dependent with inhibition only occurring at higher imatinib concentrations (Table 2).[35,37,38] ABCB1 and ABCG2 are expressed in a variety of tissues, including liver (at the sinusoidal basolateral membrane, as well as the apical bile canalicular membrane of hepatocytes)[1,37,45,46], intestine (at the apical membrane; see Fig. 1)[45-47], kidney, placenta[46,48], and the blood brain barrier.[41,49] The role of these efflux transporters in acquired drug resistance has been investigated more intensively than the role of uptake transporters because of the evident physiologic role of efflux transporters as a defense mechanism against penetration of xenobiotics.

Uptake transporters.

Meanwhile, more than one fourth of the present-day anticancer drugs are oral formulations, stressing the possible relevance of intestinal absorption through uptake transporters expressed on the apical membrane of enterocytes. These solute carriers (SLC) use electrochemical gradients of ions to transport substrates across a membrane. Mainly the role of organic cation transporter 1 (OCT1 or the SLC22A1 gene product) and to a lesser extent organic anion transporting polypeptide 1A2 (OATP1A2, the SLCO1A2 gene product) in imatinib uptake has been described.[50,51] Furthermore, imatinib proved to be a good substrate for the solute carriers OATP1B3 (SLCO1B3 gene product) and OCTN2 (SLC22A5 gene product), both expressed on the basolateral membrane of hepatocytes (Fig. 1).[51]

DRUG TRANSPORTERS AND IMATINIB PHARMACOKINETICS

Absorption

This knowledge raises the question whether altered pharmacokinetics can be (in part) the result of (over) expression of drug transporters. At duodenal pH 5 to 6, imatinib is mainly charged[52], implying active intestinal transport; this renders intestinally located solute carriers such as OATP1A2 and OCTN2 as good candidates for intestinal imatinib uptake.[53,54] However, to date, little is known about the influence of these uptake transporters on imatinib pharmacokinetics. Another good candidate for systemic imatinib uptake is ABCC4, expressed on the basolateral membrane of hepatocytes.[51] This efflux transporter could pump imatinib from the liver to the systemic circulation. As mentioned above, imatinib is absorbed very efficiently, which is somewhat surprising, considering the high affinity of imatinib for ABC transporters, expressed on the canalicular membrane of hepatocytes and on enterocytes. A possible explanation for this apparent

Table 2. Interaction between drug transporters and imatinib pharmacokinetics and pharmacodynamics

Drug transporter	Study design	Effects on imatinib PK*	Effects on imatinib PD†	Transporter interaction	Effect on imatinib IUR‡	Reference
ABCB1	*in vitro*				IUR‡ ↓	(34-35,39)
	in vitro		resistance ↑			(35)
	in vivo	systemic clearance ↑				(33,57)
	in vitro			inducer		(32,40)
	in vitro			inhibitor		(32,35,37)
	in vitro			substrate		(37-38)
ABCG2	*in vitro*				IUR‡ =	(44)
	in vitro				IUR‡ ↓	(39)
	in vitro		no resistance			(44)
	in vivo (mice)	systemic clearance ↑				(33,57)
	in vivo (mice)	plasma concentration =				(60)
	in vivo (mice)	liver concentration =				(60)
	in vitro			inhibitor		(37,43-44)
	in vitro			inducer		(40)
	in vivo (mice)			no inducer		(60)
	in vitro			substrate		(37-39)
OCT1	*in vitro*				IUR‡ =	(51)
	in vitro				IUR‡ ↑	(50,62)
	in vitro		resistance ↓			(50)
	clinical (CML§)		resistance ↓			(63,65-66)
				substrate		(50,62)
OATP1A2	*in vitro*			substrate		(51)
OATP1B3	*in vitro*			substrate		(51)
OCTN2	*in vitro*			substrate		(51)

Legend: * pharmacokinetics; † pharmacodynamics; ‡ intracellular uptake and retention; § chronic myeloid leukemia

contradiction could be local substrate inhibition of efflux transporters by imatinib, bearing in mind its dose-dependent interaction. Furthermore, absolute bioavailability could also be influenced by the balance between efflux and influx transport over the intestinal barrier, favoring active imatinib uptake.

Tissue distribution

Liver distribution.

Imatinib is actively cleared from the blood into the liver, where it is metabolized extensively. Possible candidates for this active transport are OATP1B3, OCTN2, and OCT1, predominantly located at the basolateral membrane of hepatocytes (Fig. 1).[16,51,55,56]. However, in vivo or clinical data, supporting the role of solute carriers in imatinib clearance, are not available.

Brain distribution.

Systemic treatment of brain tumors (primary as well as metastases) is limited because of low penetration of drugs into the brain tissue. Distribution to the brain is primarily prevented by the blood-brain barrier formed by the endothelial cells of brain capillaries. These endothelial cells also express ABCB1 and ABCG2[41,49], which actively prevent xenobiotics from diffusing into the brain. This finding was illustrated in Bcrp and Mdr1a/1b (rodent analogs of ABCG2 and ABCB1, respectively) knockout mouse models, showing that imatinib brain penetration significantly increased in knockout mice compared with wild-type mice, with a greater difference in Mdr1a/1b knockout.[57] Another study with a rodent model showed that combined Bcrp and Mdr1a/1b knockout proved to significantly increase brain penetration compared with individual Bcrp or Mdr1a/1b knockouts.[33] These data suggest that inhibition of efflux transporters at the blood-brain barrier may provide more tools in the treatment of brain metastases in imatinib-treated patients. A few obstacles remain, however. For instance, Gardner and colleagues showed that inhibition of ABCB1 and ABCG2 in mice resulted in a proportional increase in systemic exposure to imatinib in plasma and brain, leaving the brain-to-plasma concentration ratio unaltered.[58] This finding suggests that reduced systemic elimination of imatinib leads to the observed increase in imatinib exposure to the brain as a result of higher imatinib concentrations at the blood-brain barrier, rather than a modification of the barrier itself. Furthermore, it is still unclear if efflux inhibitors will increase imatinib levels in tumor cells located in the central nervous system, because these inhibitors may be merely increasing brain uptake of substrates but not necessarily uptake into brain tumors.[59]

Excretion

Biliary secretion.

Up until now, in vivo experiments on the importance of drug transporters for imatinib excretion have shown only minor effects. Systemic clearance of imatinib in Mdr1a/1b and Bcrp1 knockout mice was 1.3-fold and 1.6-fold lower than wild-type mice.[57] A combined Mdr1a/1b/Bcrp1 knockout showed a 1.8-fold reduction in imatinib plasma clearance compared with wild-type mice when imatinib was administered intravenously.[33] Interestingly, no differences in pharmacokinetic parameters were found between Mdr1a/1b/Bcrp knockouts and wild-type mice after oral administration of imatinib. Whether ABCB1 and ABCG2 contribute to imatinib clearance in humans to a similar degree and, more importantly, whether there is a possible upregulation of these efflux transporters in excretory organs during imatinib treatment is unknown. These murine data, however, suggest a minor contribution of efflux transporters to imatinib clearance, compared with the hepatic metabolism. Indeed, an extensive first-pass metabolism of imatinib could contribute more substantially to systemic clearance than Mdr1a/1b and Bcrp1 efflux. Furthermore, protein expression of Abcb1 and Bcrp1 in mice did not differ after long-term treatment with orally administered imatinib.[60] After daily administration for 4 consecutive weeks, no upregulation of Abcb1 and Bcrp1 in mouse liver and intestinal tissues was found. Also, no significant change in plasma and liver concentrations of imatinib was seen. Theoretically, however, the length of treatment required to induce upregulation of these drug transporters in mice might be longer or the activity of both efflux transporters over the course of time may change without a quantitative change in expression.

Renal excretion.

Although renal excretion accounts for less than 10% of imatinib excretion[31,61], increased plasma exposure and decreased clearance in imatinib-treated cancer patients with impaired renal function, were seen.[61] This finding may be due to increased levels of circulating uremic toxins. One such toxin inhibits OATP1B3 function in a rodent model[55], supporting the possibility that uremic toxins can directly reduce hepatic uptake of imatinib by OATP1B3. Further elucidation of this mechanism is needed.

DRUG TRANSPORTERS AND IMATINIB PHARMACODYNAMICS

Role of OCT1 in chronic myeloid leukemia blasts.

There is substantial evidence that tumor OCT1 expression or activity determines therapeutic outcome in imatinib-treated CML patients. Thomas and colleagues were the first

to show that inhibition of OCT1 in peripheral blood leukocytes from 6 CML patients, decreased intracellular imatinib uptake.[62] This finding was confirmed in another study, showing that imatinib uptake in aCMLcell line significantly correlated with OCT1mRNA-expression.[63] Furthermore, White and colleagues showed that in vitro sensitivity for imatinib strongly correlated with the intracellular uptake and retention of imatinib in mononuclear cells of untreated CML patients.[50] When prazosin, an OCT1 inhibitor, was added to these cells, the concentration needed to inhibit molecular drug targets was significantly increased. Furthermore, it was also shown that only the activity of OCT1 in mature CML blasts is associated with therapeutic outcome and not the OCT1 activity in immature CD34+ cells.[64] This could imply that effective tumoral uptake of imatinib by OCT1 may be decisive for therapeutic response in CML patients. On the other hand, Huand colleagues showed that intracellular uptake of imatinib in (nonleukemic) cells overexpressing OCT1 was only minimally higher than in control cells.[51] In a gene expression analysis, they showed that SLC22A1 is significantly interrelated with ABCB1, ABCG2, and SLCO1A2. Alternatively, SLC22A1 gene expression could, therefore, be a marker for expression and subsequent activity of other transporters involved in imatinib transport. SLC22A1 gene expression did prove to be a good predictor of clinical outcome in imatinib-treated CML patients.[63,65] Pretreatment OCT1 expression levels in 32 CML patients were 8 times higher in responders than in nonresponding patients.[65] This result was confirmed in 70 CML patients, in which high baseline OCT1 RNA expression levels correlated with better cytogenetic response at 6 months and prolonged progression-free and overall survival.[63] White and colleagues showed that chronic phase CML patients with low OCT1 activity showed clinical benefit from imatinib dose escalation, but they reported no correlation between clinical efficacy and OCT1 mRNA levels.[66]

ABC transporter expression in gastrointestinal stromal tumor and chronic myeloid leukemia.

ABCB1 and ABCC1 are expressed in approximately three quarters of GISTs, which is 2- to 3-fold more than the expression in leiomyosarcomas.[67-69] On the other hand, Western blot analysis of 21 GIST specimens showed no expression of ABCG2 .[68] So, in contrast to the possible influence of ABCG2 on the intestinal uptake of imatinib[33,40,57], there seems to be no role for ABCG2 on a tumoral level in GIST patients. Little can be said of the impact of ABCB1 and ABCC1 expression in stromal tumor cells on imatinib therapy in these GIST patients because only a very limited number of patients in these studies were treated with imatinib. Although preclinical data[34-36] show that cellular (over) expression of ABCB1 leads to a reduced intracellular accumulation of imatinib, it is not clear whether long-term treatment with imatinib induces overexpression of this transporter in tumor cells. Mahon and colleagues examined various cell lines and found no upregulation of the expression of the ABCB1 gene by imatinib in time.[70] In contrast, bone marrow mononuclear cells in CML patients resistant to imatinib showed an overexpression of

ABCB1 and ABCG2 (although not statistically significant)[65]. In addition, a gene expression analysis in CML patients showed that expression of ABCC3 in CML blasts was unique to patients with disease recurrence.[71] More studies with larger populations are needed to elucidate the possible tumoral upregulation of efflux transporters during imatinib therapy and its pharmacodynamic effects.

INTEGRATING KNOWLEDGE OF TRANSPORTERS IN IMPROVING IMATINIB THERAPY: PHARMACOGENETIC STUDIES

Pharmacokinetic impact of genetic variation in ABC transporters.

To date, pharmacogenetic association studies were predominantly done for ABCB1 and ABCG2 (Table 3). Associations between 2 single nucleotide polymorphisms, known to reduce the activity of ABCB1 and ABCG2, respectively, and steady state imatinib pharmacokinetics in 82 patients with mainly GIST, have been investigated.[72] Sixteen patients had a heterozygous variant (421 C > A) genotype for ABCG2 and 20 patients expressed a homozygous variant for ABCB1 3435C > T. No significant differences in imatinib pharmacokinetics were seen compared with the homozygous wild-type patients. On the other hand, Takahashi and colleagues recently showed that imatinib trough levels were significantly higher in CML patients carrying an ABCG2 421A allele (in homozygous as well as heterozygous variant genotypes).[73] Gurney and colleagues found that patients with a TTT haplotype in ABCB1 1236C > T, 2677G > T/A, and 3435 C > T loci had significant higher estimated imatinib clearances.[74] This finding is contradictory to reports showing lower mRNA and protein levels when a homozygous T allele for ABCB1 3435C > T was present[75], and to the findings of others who observed a decreased hepatic 99mTC-MIBI elimination rate, a phenotypic marker for ABCB1-mediated drug clearance, in patients with the TTT haplotype.[76] In addition, in CML patients, a TTT-haplotype was associated with higher imatinib trough levels.[77]

Clinical impact of genetic variation in ABC transporters.

Up until now, pharmacogenetic association studies assessing clinical efficacy were exclusively done in CML patients. A poor response was observed in CML patients who were homozygous for the G allele in ABCG2 34G > A.[78] As for ABCB1, a 1236T allele was associated with better response, whereas a 2677G allele or a CGC haplotype for the 1236, 677, and 3435 loci were associated with worse response in CML patients.[77] However, Kim and colleagues observed a reduced overall survival in CML patients carrying a TT genotype for 3435C > T locus, when analyzed univariately.[78] This finding was confirmed by another group, who observed more resistance in CML patients carrying T alleles at positions 1236 and 3435.[79] All in all, data on the role of pharmacogenetics in response

Table 3. Studied single-nucleotide polymorphisms in patients involved in imatinib pharmacokinetics and pharmacodynamics

Transporter gene	Polymorphism	Effects on imatinib PK*	Effects on imatinib PD†	No. of patients	Reference
ABCB1	3435 T	no		82	(72)
	3435 T	Cmin = §	response = \\	67	(73)
	TTT haplotype‡	Cmin ↑§		90	(77)
	TTT haplotype‡	clearance ↑		22	(74)
	3435 T		overall survival ↓#	229	(78)
	3435 T		resistance ↑ ¶	52	(79)
	1236 T		resistance ↑ ¶	52	(79)
	1236 T		response ↑\\	90	(77)
	1236 T	Cmin = §	response = \\	67	(73)
	2677 T/A	Cmin = §	response = \\	67	(73)
	2677 G		response ↓\\	90	(77)
	CGC haplotype‡		response ↓\\	90	(77)
ABCG2	421 A	no		82	(72)
	421 A	Cmin ↑§	response = \\	67	(73)
	34 A		response ↓**	229	(78)
ABCC2	-24 T	Cmin = §	response = \\	67	(73)
SLC22A1	181 T	SS†† imatinib plasma level =		73	(51)
	1393 A	SS†† imatinib plasma level =		73	(51)
	181 T	no		32	(80)
	480 G		response ↓‡‡	229	(78)
	480 G	Cmin = §	response = \\	67	(73)
	1022 T	Cmin = §	response = \\	67	(73)
	1222 G	Cmin = §	response ↑\\	67	(73)
	156 C	Cmin = §	response = \\	67	(73)
SLCO1B3	334 G	Cmin = §	response = \\	67	(73)

Legend: * pharmacokinetics; † pharmacodynamics; ‡ description haplotype: 1236C>T, 2677G>T/A, 3435C>T; § imatinib plasma trough level; \\ major molecular response; #defined as the period from initiation of imatinib therapy until the date of death from any cause or the date of last follow-up; ¶ non-response defined as absence of cytogenetic response; ** major or complete cytogenetic response; †† steady state; ‡‡ higher rate of loss of cytogenetic or molecular response

and survival in CML patients receiving imatinib therapy are scarce and poorly validated or reproduced.

Pharmacokinetic and clinical impact of genetic variations in solute carriers.

To date, studies assessing the possible role of genetic polymorphisms in solute carriers in imatinib therapy are limited to OCT1 and OATP1B3. Allelic variants of the SLC22A1 gene (encoding for the OCT1 protein) with known reduced function showed no effect on steady stateimatinib plasma levels (181C > T and1393G > A), in a group of GIST and CML patients compared with the reference genotype.[51] Furthermore, Zach and colleagues found no significant differences in response in patients heterozygous for the T allele in the SLC22A1 181C > T polymorphism.[80] Also, no correlation between SLCO1B3 334T > G polymorphism and imatinib exposure or clinical response was seen in CML patients.[73] On the other hand, CML patients carrying a homozygous GG genotype for the SLC22A1 480C > G polymorphism showed a lower response rate.[78] Furthermore, a higher response rate was seen in CML patients carrying a GG genotype for the 1222A >Glocus.[73] Unfortunately, imatinib trough levels did not significantly differ for these patients, carrying a 1222GG genotype, as compared with the reference allele.

FUTURE PERSPECTIVES

Although in vitro studies show that imatinib exposure leads to an upregulation of ABCB1 and ABCG2 in human colon carcinoma cells[40], currently no data are available on the expression of these drug transporters in human intestinal cells under imatinib therapy. Future studies should assess the possible correlation between their expression pattern in excretory organs over time with imatinib pharmacokinetics and clinical outcome. In order to study the role of these transporters in the observed decline in imatinib clearance, a series of intestinal biopsies at different time points are needed. Current studies also show that the majority of GIST expresses both ABCB1 and ABCC1, but its clinical importance is not yet elucidated. Although tumoral expression of OCT1 in CML patients has already been correlated with therapeutic outcome, more data are needed on the expression pattern of drug transporters in tumor cells in both CML and GIST patients and their possible pharmacodynamic impact. At least a quantification of these transporters over time should be made in GIST biopsies or mononuclear cells in CML patients in order to assess the possibility of an altered expression pattern of drug transporters as a mechanistic explanation for an altered sensitivity to the drug. Finally, pharmacogenetic association data will need to be validated or reproduced. At this point, no clear guidelines exist on the design of pharmacogenetic studies. Preventing selection bias, adequate power analysis, clear endpoints, correction for genetic and nongenetic covari-

ates, and other factors are often poorly implemented. Therefore, more pharmacogenetic studies assessing the association with imatinib pharmacokinetics and/or pharmacodynamics are desired, and study design will need to be uniform. In the present context of rapidly emerging promising compounds, the latter is of utmost importance if we want to personalize dosing and treatment sequences of rationally designed molecules to an individual patient's needs.

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Chapter 3

Correlations between imatinib plasma concentrations and clinical benefit in gastrointestinal stromal tumor (GIST) patients will be time point specific: results of a long-term prospective population pharmacokinetic study

Eechoute K., Fransson M.N., Reyners A.K., de Jong F.A., Sparreboom A., van der Graaf W.T.A., Friberg L.E., Schiavon G., Wiemer E.A.C., Verweij J., Loos W.J., Mathijssen R.H.J., De Giorgi U.

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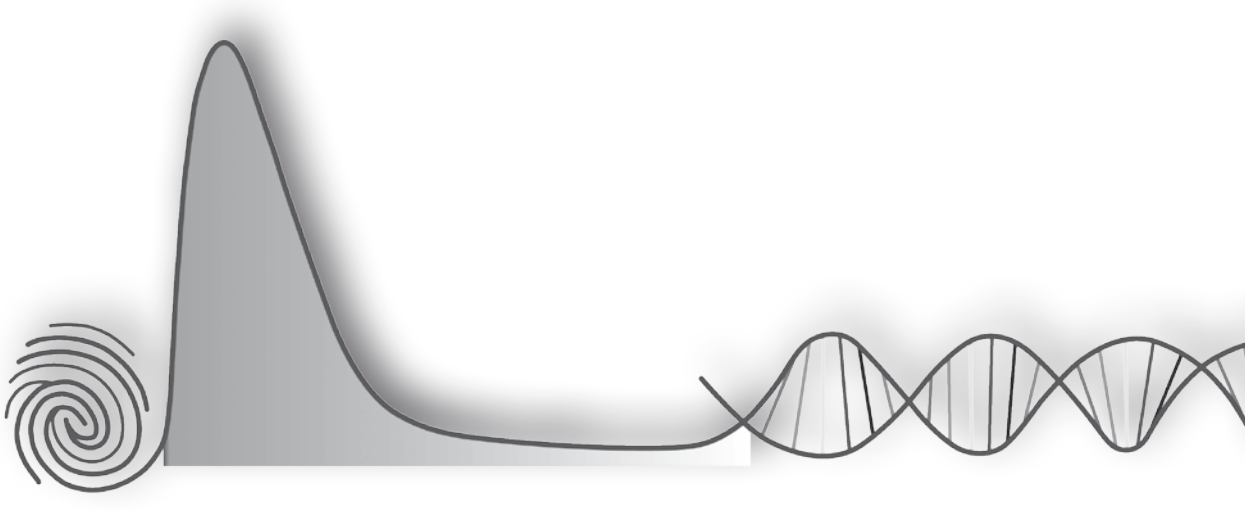


ABSTRACT

Purpose: Imatinib minimal (trough) plasma concentrations after 1 month of treatment have shown a significant association with clinical benefit in patients with gastrointestinal stromal tumors (GIST). Considering a retrospective pharmacokinetic (PK) analysis has also suggested that imatinib clearance increases over time in soft tissue sarcoma and GIST patients, the primary aim of this study was to assess systemic exposure to imatinib at multiple time points in a long-term prospective population PK study. As imatinib is mainly metabolized in the liver, secondary aim was to elucidate the potential effects of the volume of liver metastases on exposure to imatinib.

Patients and Methods: Full PK blood sampling was performed in 50 GIST patients on the first day of imatinib treatment, and after 1, 6 and 12 months. Additionally, on day 14, and monthly during imatinib treatment, trough samples were taken. PK analysis was performed using a compartmental model. Volume of liver metastases was assessed by CT imaging.

Results: After 90 days of treatment, a significant decrease in imatinib systemic exposure of 29.3 % compared to baseline was observed ($P < .01$). For every 100 cm^3 increase of metastatic volume, a predicted decrease of 3.81 % in imatinib clearance was observed ($P < .01$).

Conclusion: This is the first prospective PK study in GIST patients, demonstrating a significant decrease of ~30% in imatinib exposure after long-term treatment. This means that future "trough level – clinical benefit" analyses should be time-point specific. GIST liver involvement has a marginal effect on imatinib clearance.

INTRODUCTION

After a decade of therapeutic use, imatinib mesylate has proven to be a highly effective targeted agent in the treatment of advanced gastrointestinal stromal tumor (GIST) patients, with a median overall survival close to 5 years.[1] Imatinib mesylate is a small molecule that inhibits intracellular autophosphorylation of 2 tyrosine kinase receptors involved in the pathogenesis of GIST: mainly KIT and to a lesser extent platelet-derived growth factor receptor-α (PDGFR-α).[2-3] While a vast majority of GIST patients will demonstrate clinical benefit from imatinib therapy, approximately 10 – 15 % will experience progressive disease within 3 – 6 months after start of treatment.[4-6] Mechanisms behind this so-called early progression are not entirely elucidated. Although a majority of non-responding patients harbor mutations in the molecular drug targets,[3,7-8] early resistance may also result from imatinib plasma levels that are below a minimal effective threshold level. Although not significant, GIST patients not responding to imatinib treatment demonstrated lower imatinib plasma levels than responders.[9-10] In addition, in a retrospective pharmacokinetic (PK) side-study of the pivotal phase II B2222 trial, a significant shorter time to progression was observed in patients that demonstrated 1 month imatinib steady-state trough levels below 1,100 ng/ml.[10] Considering a retrospective PK analysis has also suggested that imatinib clearance increases over time in soft tissue sarcoma and GIST patients, the primary aim of this study was to assess systemic exposure to imatinib at multiple time points in a long-term prospective population PK study.[11]

Imatinib is extensively metabolized and cleared by the liver with 68 % of the dose recovered in faeces.[12] Its main metabolite CGP74588 is formed by hepatic oxidases from the cytochrome P450 superfamily (CYP) with isoforms 3A4 and 3A5 (CYP3A4 and CYP3A5, respectively) as its predominant members.[13] As a secondary objective, we therefore studied the potential metabolic effects of liver metastatic involvement on exposure to imatinib.

METHODS

Patients

Patients with histologically confirmed GIST were accrued at start of imatinib therapy for long-term imatinib PK assessment in two Dutch and two Italian medical centers. Patients treated with drugs known to show major interactions with cytochrome P450 isoforms 3A4 and 3A5 (CYP3A4 and CYP3A5) were excluded from the study if no alternative medication was available or if the patient was unwilling to change medication. Imatinib systemic treatment was initiated at the Erasmus University Medical Center (n = 31), University Medical Center Groningen (n = 14), San Giuseppe Hospital Empoli (n = 4)

and at the Santa Maria delle Croci Hospital Ravenna (n = 1). This study was approved by the medical ethics review boards and performed in accordance with the Declaration of Helsinki. This trial was registered at the International Standard Randomized Controlled Trials Number Register (ISRCTN63855172).

Pharmacokinetic sample collection

Blood samples for imatinib PK evaluation were collected on the first day of imatinib treatment and after 1, 6 and 12 months. On these days blood samples were collected immediately prior to imatinib administration and 30 minutes, 1, 2, 3, 4, 6 and 24 hours after imatinib intake. Additionally, on day 14, and monthly during imatinib treatment, trough samples were taken to assess minimal imatinib plasma concentrations. The trough samples were taken just prior to the next day administration.

Methods for blood sample processing and storage, as well as analytical imatinib quantification, were described previously.[14] A total of 1,820 imatinib plasma concentrations were considered for analysis. Ten samples were missing a concentration value (below the limit of quantification or assay error) and 67 trough samples were excluded since the time for sampling and/or the time for the preceding dose could not be determined exactly. Of the 1,743 observations that remained for PK model building, 512 observations from 69 occasions were in adjunction to measurements on volume of liver metastasis and on the metastasis/liver volume ratio.

Population pharmacokinetic model

Observed imatinib plasma concentrations were log transformed before being used for parameter estimation. Compartmental models with linear and non-linear processes were evaluated. As no intravenous data were available, the initial bioavailability (F) was set to 1, meaning that clearance and volume of distribution (V) should be interpreted as the clearance (CL/F) and apparent volume of distribution (V/F), respectively.

The population PK analysis was performed using nonlinear mixed effect modelling in the NONMEM software (version 7.1.2, ICON Development Solutions, Ellicott City, MD). The ADVAN5 subroutine combined with the first order conditional estimation method with interaction between random effects was used in the model building procedure. Perl-speaks NONMEM (version 3.2.12, http://psn.sourceforge.net/) and the R-package Xpose (version 4.3.0, http://xpose.sourceforge.net/) were used to automate model runs and for graphical analysis.

Presence of liver metastases was used as a dichotomous covariate (LIV) in the analysis. Time-dependency (TIME), body weight (WT), volume of liver metastasis (LIVM) and liver metastatic volume proportional to liver volume (LIVR) were evaluated as continuous covariates. Potential CYP-interactive drugs were highly restricted in this study. Hence, as major interactions were not expected, co-medication was not included as a covariate.

Recorded imatinib doses were used for modelling, and when information was missing it was assumed there was no change in the dosing until the next recorded dose.

Interindividual variability (IIV) for an individual (ind) was modelled exponentially, and the residual errors for observed concentrations (c_{obs}) versus predicted concentrations (c_{pred}) on the log scale were modelled using an additive error.

The NONMEM objective function value (OFV), which is proportional to –2 x log likelihood of the data, was used to evaluate different model structures. A difference in OFV of at least 6.63 (corresponding to $P < .01$) was used to discriminate between competing models. NONMEM standard errors were complemented with asymmetric confidence intervals by log-likelihood profiling (LLP) and a bootstrap (n=1,000, stratified on LIV) (http://psn.sourceforge.net/). A visual predictive check (VPC) was performed to evaluate the predictive performance of the model. The observed data was overlaid with a 90 % prediction interval based on 1,000 simulated data sets from the final model. The VPC was stratified on months of each 24-hour pharmacokinetic sampling in the following way; group 0 (day 1), group 1 (month 1, 2 and 3), group 2 (month 5 and 6) and group 3 (months ≥ 11).

In addition, to assess imatinib metabolic ratios (AUC_{tau} CGP74588 / AUC_{tau} imatinib) at start of therapy and after 6 and 12 months of treatment, a non-compartmental analysis of our steady-state imatinib and CGP74588 PK data after 1, 6 and 12 months was performed, using WinNonlin software (Phoenix WinNonlin version 6.1, Pharsight Corporation, St. Louis, MO).

Computed tomographic (CT) – guided volumetric assessment of liver metastases

Original Digital Imaging and Communications in Medicine (DICOM) files were imported in the open source OsiriX Imaging Software for MacOS X (OsiriX Foundation, Geneva, Switzerland). All available CT scans at baseline, at 6 and 12 months of treatment were studied. Two regions of interest (ROI) were assessed per CT slice: total liver area and liver metastasis area. Total liver areas were manually outlined by using the closed polygon selection tool and liver metastases lesions were drawn with the pencil selection tool, which allows a more precise drawing (**Figure 1A**). Volumes of all three-dimensional structures were then automatically calculated (**Figure 1B**). For metastases visualized in only one CT slice, the area was multiplied with slice thickness (5 mm) to estimate the volume. The gallbladder and the inferior vena cava were excluded from the ROI; intrahepatic biliary and vascular structures were included. The ratio of metastasis volume to liver volume was then calculated based on the above described volumetric assessments.

A B

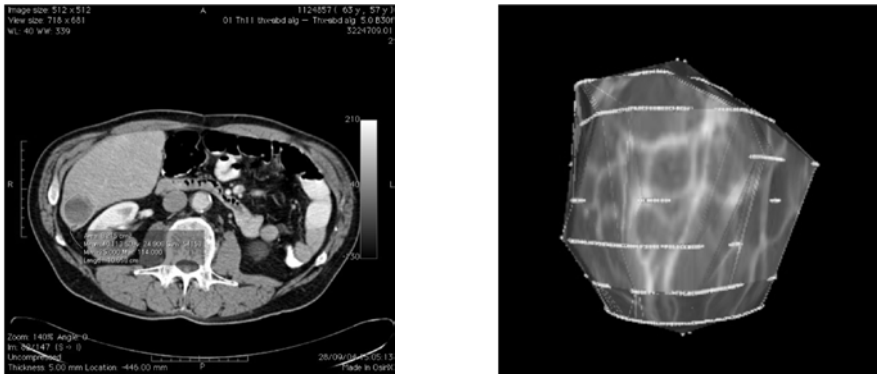


Figure 1A: Digital Imaging and Communications in Medicine (DICOM) files were imported in OsiriX Imaging Software for MacOS X (OsiriX Foundation, Geneva, Switzerland). Metastatic liver lesions were then drawn as areas of interest with the pencil selection tool.
Figure 1B: Volumes of three-dimensional structures were automatically calculated by OsiriX Imaging Software.

RESULTS

Patients

Twenty-nine male and 21 female patients were included in this population PK analysis. Median follow-up period was 366 days (range: 59 – 761 days); 26 patients (13 males, 13 females) had evaluable liver metastases. Median volume of liver metastases was 5.8 cm^3 (range 0.68 to 1,800 cm^3) and the median metastasis/liver volume ratio was 0.42 % (range 0.042 to 61 %). See **Table 1** for clinical characteristics.

Imatinib pharmacokinetics: time dependency

A 2-compartment model with linear elimination and 5 transit compartments to describe the absorption process (**Figure 2**) best fitted observed imatinib plasma concentrations. In addition, the *relative* bioavailability (F) and absorption rate (k_a) were both found to be significantly (ΔOFV = -246.4) time-dependent according to:

$$F = 1 + \theta_F * \exp(-\lambda * TIME/24)$$
$$ka,ind = (1+\theta_{ka} * \exp(-\lambda * TIME/24)) * k_{a,pop} * \exp(\eta_{ka,ind})$$

where TIME is the time in hours from first dose administration and λ the decay constant, which was found to have the same value for both parameters. θ_F and θ_{ka} were > 0 indicating that F and $k_{a,ind}$ start at a value of $1+ \theta_F$ and $k_{a,pop}*(1+\theta_{ka})$, respectively, and

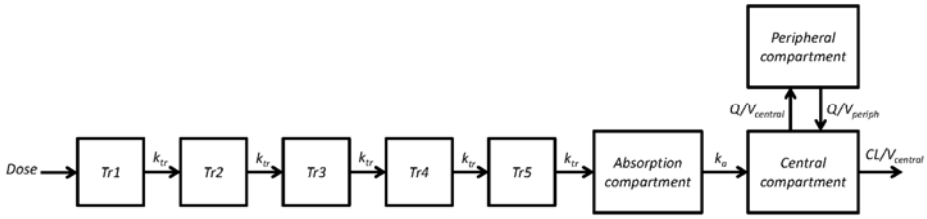
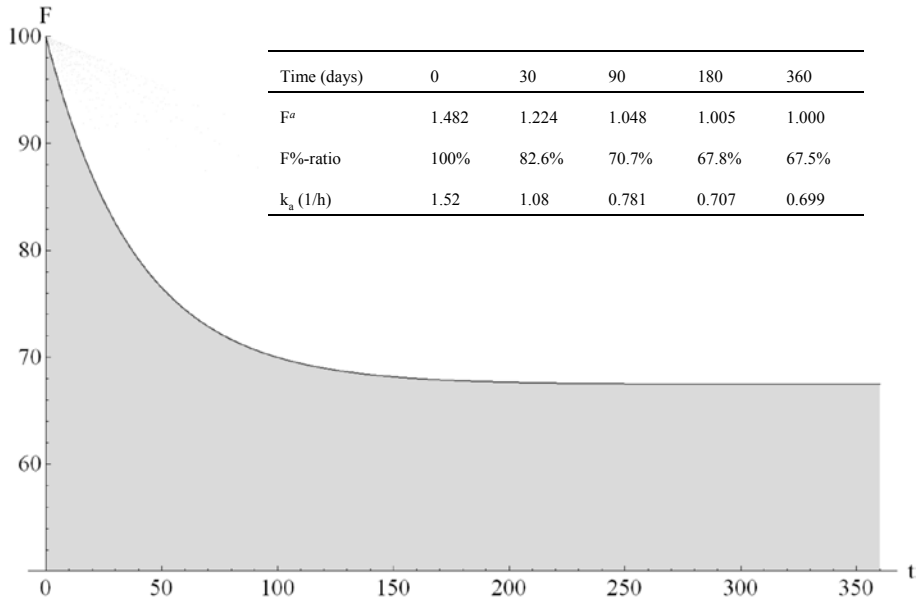


Figure 2: Imatinib population pharmacokinetic model.
A 2-compartment model with linear absorption and elimination and 5 transit compartments best fitted observed imatinib plasma concentrations.
Abbreviations: Tr1–5; transit compartments, k_a; absorption (L/h), Q; inter-compartmental clearance (L/h), CL; total clearance (L/h), V; volume of distribution (L)



Time (days)	0	30	90	180	360
F[a]	1.482	1.224	1.048	1.005	1.000
F%-ratio	100%	82.6%	70.7%	67.8%	67.5%
k_a (1/h)	1.52	1.08	0.781	0.707	0.699

Figure 3: Predicted reduction in the *relative* bioavailability, F, over time.
Assuming that the true bioavailability is equal to 100% at day 1, the predicted decrease can be understood by looking at percentage ratio (F%-ratio) after 30, 90, 180 and 360 days.
Abbreviations: F; relative bioavailability, F%-ratio; bioavailability percentage ratio, k_a; absorption (L/h), t; time (days)

both decrease with time to values of 1 and $k_{a,pop}$, respectively. Time-dependence on F and k_a could be replaced with time-dependence on CL and k_a (increasing CL with time) with an almost equally good fit (ΔOFV = +4.3). However, this model was not robust and it was highly sensitive to initial parameter estimates, and quantitative testing of covariate effects could not be performed. The predicted decrease in F and k_a as function of time after treatment start are demonstrated in **Figure 3**

Table 1. Patient characteristics (N=50)

Characteristic	No.	%
Median age (years) – range	61 (39 – 82)	
Sex –		
Male	29	58.0
Female	21	42.0
Primary cancer site –		
Stomach	28	56
Rectum	9	18
Small intestine	8	16
Unknown	5	10
Disease –		
Locally advanced	17	34
Metastatic	31	62
Liver	28	56
Intraperitoneal	22	44
Retroperitoneal	2	4
Other	4	8
Adjuvant setting	2	4
Histology –		
Spindle cell	28	56
Epitheloid	13	26
Mixed	5	10
Unknown	4	8
Imatinib dose –		
400 mg per day at start	50	100
Dose escalation during treatment –		
To 600 mg per day	1	2
To 800 mg per day	13	26
Median ASAT (U/L) – range	24 (12 – 145)	

Median ALAT (U/L) – range	22 (5 – 274)
Median ALP (U/L) – range	91 (47 – 847)
Median GGT (UL) – range	32 (10 – 613)
Median total bilirubin (µmol/L) – range	9 (3 – 39)

Abbreviations: ASAT; Aspartate aminotransferase, ALAT; alanine aminotransferase, ALP; Alkaline phosphatase, GGT; Gamma-glutamyltransferase

Non-compartmental analysis of our steady-state imatinib and CGP74588 PK data after 1, 6 and 12 months, showed that the metabolic ratio (AUC_{tau} CGP74588 / AUC_{tau} imatinib) remained stable over time; (mean metabolic ratios ± S.D. at 1, 6 and 12 months: 0.72 ± 0.12; 0.75 ± 0.11; 0.74 ± 0.11, respectively).

Imatinib pharmacokinetics: liver metastasis dependency

Covariates WT, LIV, LIVM and LIVR were tested on CL, $V_{central}$ and F. LIVM on CL gave the largest drop in OFV (ΔOFV = -21.8), while the dichotomous covariate LIV was not significantly correlated with CL (ΔOFV = -0.4). The effect of LIVM could not be associated with the parameters β_F or λ, governing time-dependence on F. Combining the effect of LIVM in CL with an effect of LIVM also in F was not significant (ΔOFV = 0.0). LIVR on CL was also significant (ΔOFV = -11.0) but was not significant (ΔOFV = -2.3) when combined with LIVM. While including LIVM reduced the proportional residual error ε from 35.4 % to 35.0 %, it did not reduce the IIV in CL.

WT on CL gave a significant drop in OFV (ΔOFV = -13.9), but was omitted in the final model because of too much data imputation.

The estimates of the final population PK model comprising the effect of LIVM on CL are presented in **Table 2** together with NONMEM relative standard errors (RSEs), 95 % CIs from the LLP and the median and 2.5 – 97.5 percentiles from 993 bootstrap replicates. The predicted decrease in CL as function of LIVM is expressed as:

$$CL_{pop} = 9.12 * (1 - 0.000381 * LIVM)$$

This means that for every 100 cm^3 increase in metastasis volume CL is decreased by 3.81 %. The effect is presented in **Table 3** for the minimum, median and maximum metastasis volume of the study population.

Table 2. Parameter estimates for the final population pharmacokinetic model.

Parameter	Estimate	RSE[a] (%)	Log-likelihood profiling	Estimates based on 993 bootstrap replicates stratified on LIV = 0/1	
			95% CI	Median	2.5–97.5 percentiles
CL (L/h)	9.12	7.5	7.94–10.5	9.13	7.76–10.6
$V_{central}$ (L)	128	18	95.8–165	130	96.0–165
Q (L/h)	24.9	13	19.0–31.9	25.3	18.2–34.9
V_{periph} (L)	197	15	145–265	202	142–276
k_a (1/h)	0.699	20	0.510–0.942	0.710	0.502–1.05
k_{tr} (1/h)	15.8	17	10.9–24.9	15.4	11.3–22.8
Residual error, ε (%)	35.0	6.4	33.6–36.5	34.5	30.3–39.2
θ_F	0.482	26	0.367–0.610	0.511	0.225–0.865
θ_{ka}	1.18	28	0.72–1.82	1.15	0.525–1.97
λ (1/day)	0.0256	37	0.0181–0.0357	0.0258	0.0143–0.0565
θ_{LIVM} (1/cm^3)	0.000381	3.9	0.000290–0.000440	0.000383	-0.000300–0.00124
IIV CL (CV %)[b]	49.5	26[c]	39.6–64.3	49.0	36.8–63.4
IIV $V_{central}$-k_a (CV %)[b]	70.9	26[c]	50.7–104	70.4	46.9–97.0
IIV k_{tr} (CV %)[b]	160	25[c]	104–314	153	97.0–263
IIV V_{periph} (CV %)[b]	65.9	33[c]	39.9–107	67.1	34.6–97.6

[a] Relative Standard Error given by NONMEM

[b] Coefficient of Variation, calculated as $(\exp(\omega^2) - 1)^{0.5}$

[c] RSE is related to the corresponding variance term, ω^2

Abbreviations: LIV; dichotomous covariate indicating presence or absence of liver metastasis, 95% CI; 95 percent confidence interval, CL; apparent oral clearance, $V_{central}$; volume of distribution for the central compartment, Q; inter-compartmental clearance, V_{periph}; volume of distribution for the peripheral compartment, k_a; absorption rate, k_{tr}; absorption rate between transit compartments, θ_F; change in bioavailability relative start of treatment, , θ_{ka}; change in absorption rate relative start of treatment, λ; decay constant, θ_{LIVM} ; variability based on liver metastatic volume, IIV CL; inter-individual variability in apparent oral clearance, IIV $V_{central-ka}$; inter-individual variability in volume of distribution for the central compartment or absorption rate, IIV k_{tr}; inter-individual variability in absorption rate between compartments, IIV V_{periph}; inter-individual variability in volume of distribution of the peripheral compartment

Table 3. The effect of liver metastasis volume on the apparent oral clearance.

Population metastasis[a]		Minimum	Median	Maximum
LIVM (cm^3)	0	0.68	5.8	1800
CL (L/h)	9.12	9.12	9.10	2.87

[a] The effect of liver metastasis volume using the minimum, median and maximum values from the study population.

Abbreviations: LIVM; liver metastasis volume, CL; apparent oral clearance

Validation of the final population pharmacokinetic model

NONMEM RSEs, LLP 95 % CIs and bootstrap 2.5 – 97.5 percentiles are in good agreement for most parameters, the notable exception being the covariate parameter θ_{LIVM}

for which the bootstrap percentiles contain the 0-value, while this is not the case for the corresponding LLP CI.

Visual predictive checks based on 1,000 simulations are shown in **Figure 4**. Observed imatinib plasma concentrations (in µmol/L) showed good agreement with the 95 % CIs for the 5th, 50th and 95th percentiles of the simulated predictions. Time after dose (in hours) was used as the independent variable.

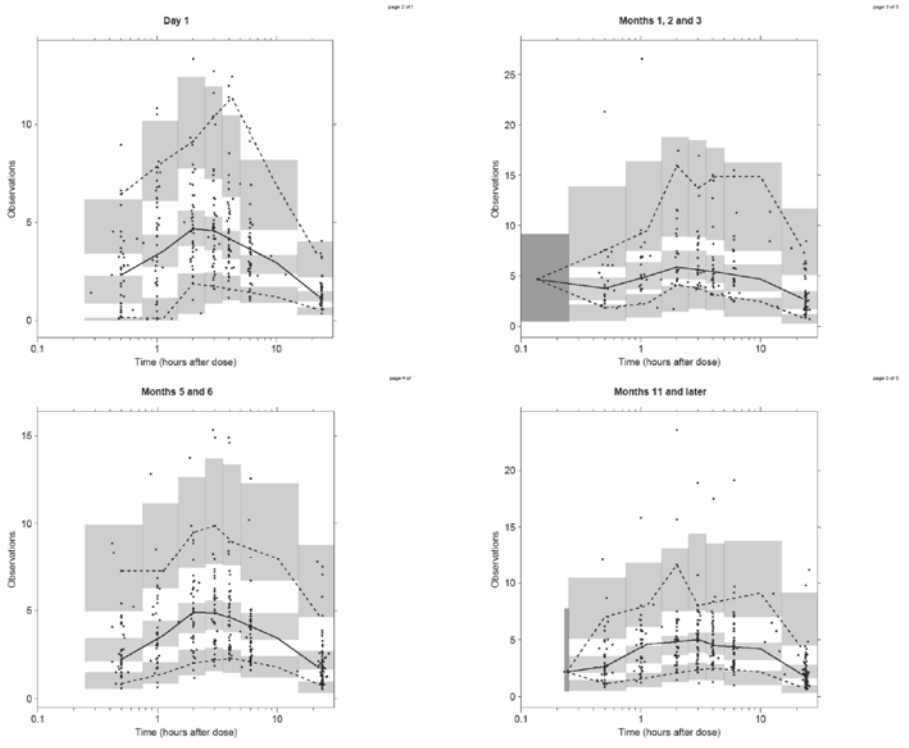


Figure 4: Visual predictive checks (VPC) based on 1,000 simulations for imatinib plasma concentrations in µmol/L as function of time after dose in hours.
The VPC was stratified on months after start of treatment for each 24-hour pharmacokinetic sampling in the following way; group 0 (day 1), group 1 (month 1, 2 and 3), group 2 (month 5 and 6) and group 3 (months ≥ 11). Legend: dots = real imatinib observations; solid and dashed lines = the 50th, 5th and 95th percentiles for all real observations in each bin; red and blue shaded areas = the 95% CI the 50th, 5th and 95th percentiles of the simulated data.

DISCUSSION

This is the first prospective population PK study in GIST patients, analyzing imatinib PK over an extensive median follow-up period of one year. During this study period, the reached number of follow-up PK observations was several times larger than in previous retrospective imatinib population PK studies in GIST patients, expressing the validity of the current data.[11,15] A multi-compartmental PK model was built by use of this PK data set, demonstrating a significant downward trend in systemic exposure to imatinib over time. From start of therapy up to 90 days, the initial imatinib exposure is reduced by approximately one third. From this time point on, the curve flattens, suggesting a further steady imatinib PK. Previous retrospective associations between imatinib trough levels at day 29 and clinical outcome benefit in GIST patients thus need to be put into this perspective.[10] As the predicted decrease in imatinib exposure at this time point is ~ 17 %, the distribution of patients in groups based on imatinib trough levels will differ from the distribution seen after 3 months, when predicted systemic exposure to imatinib has dropped by approximately 30 %. Within this 90-day period, significance of correlations between PK-based groups and clinical benefit will fluctuate. However, provided the proposed clinically relevant imatinib threshold level of 1,100 ng/ml is accurate, a number of patients will experience a drop below the efficacious plasma level after day 29 of imatinib therapy and may thus be underdosed when trough levels are only assessed after the first month of therapy. On the other hand, the proposed threshold may only be a marker for the actual clinically relevant imatinib plasma concentration cut-off value which is reached after three months. This is highly relevant if therapeutic drug monitoring should be applied in future imatinib dosing. Imatinib plasma level monitoring in GIST patients should therefore be time-point specific and repeated after the first quarter of the first year of imatinib treatment. This will have to be taken into account when designing randomized studies aimed to validate the use of imatinib plasma level monitoring, that up to that validation will have to be considered as investigational. Our data also provide a plausible explanation for the lack of decreased imatinib PK in GIST patients in a recent retrospective PK analysis, as the median time from start of therapy to first PK assessment in this study was 5.5 months.[15] In light of our findings, a major drop in imatinib plasma levels has already occurred during this lag time.

Currently, literature on mechanisms that may drive these acquired PK phenomena in imatinib-treated patients is scarce.[16] Visually, maximum imatinib plasma concentrations in our study population (C_{max}) are lower and time to reach C_{max} is longer after 3 months of dosing (**Figure 4**) as compared to the first 3 months of treatment. In addition, the final PK model fitted significantly better when adding a time-dependence in absorption rate as a covariate (OFV = -42.9). These observations suggest that the observed time-dependent drop in imatinib exposure is located at the absorption phase. Hence, there

may be a change in activity or expression of drug transporters involved in facilitated or active transport of imatinib. However, drug uptake and efflux transporters have shown a limited effect on imatinib absorption and excretion, [16-17] and *in vivo* data showed no up-regulation of drug efflux transporters after long-term treatment with imatinib.[18] So, up until now, key mediators of imatinib transport during absorption and elimination have not been identified. On the other hand, as imatinib is extensively metabolized by CYP3A4 to its main metabolite CGP74588,[13] upregulation of liver enzymatic function may also (in part) account for the observed drop in imatinib plasma levels. This PK mechanism would possibly have a minor impact on imatinib efficacy as CGP74588 is equipotent to its parent compound and has a longer terminal elimination half-life.[19] However, metabolic ratios (AUC_{tau} CGP74588 / AUC_{tau} imatinib) remained stable during the first year of treatment, implying that upregulation of metabolic activity does not occur over time. Finally, increasing patient non-adherence to imatinib treatment over time may also be involved in the observed decline in systemic exposure. This is less likely to be of large influence on exposure though, as full PK time-profiles show limited accumulation (a limited effect of single imatinib trough levels on total exposure). Occasional dosing delays will therefore have a limited effect on imatinib exposure. Moreover, an observational study evaluating compliance in 28 imatinib-treated GIST patients at 2 time points, detected no significant difference in non-adherence rates after 90 days of imatinib use.[20]

Secondary objective of this study was to evaluate if volume of liver metastasis in GIST patients is predictive for imatinib exposure. Liver metastasis volume appeared to have a minor effect on imatinib CL/F, rendering some clinical significance with massive liver involvement, as for every 100 cm^3 increase of metastatic volume, a predicted decrease of 3.81 % in CL/F is observed. This is in concordance with an earlier phase I side study that reported limited effect of liver dysfunction on imatinib exposure.[21] These data together with our present results indicate that neither liver metastatic involvement nor routine liver function testing highly correlate with hepatic CYP activity in imatinib treated patients.

To conclude, this observational population PK study demonstrates that imatinib PK in GIST patients stabilizes after approximately 3 months of dosing with a significant decrease in systemic exposure of ~30 % compared to baseline, most likely due to reduced absorption. This means that future "trough level – clinical benefit" analyses should be time-point specific and need to incorporate relevant tumor biology and patient characteristics in multivariate analyses. Such survival analyses based on imatinib PK should be performed in large (multicenter) patient populations and could, ultimately, lead to therapeutic fine tuning in which a minimal effective imatinib dose for an individual patient can be defined on accurate time points in a treatment course. Finally, volume of GIST liver metastases has a marginal effect on imatinib exposure.

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Chapter 4

Environmental and genetic factors affecting transport of imatinib by OATP1A2

Eechoute K., Franke R.M., Loos W.J., Scherkenbach L.A., Boere I., Rommel G.T., Verweij J., Gurney H., Kim R.B., Tirona R.G., Mathijssen R.H.J., Sparreboom A.

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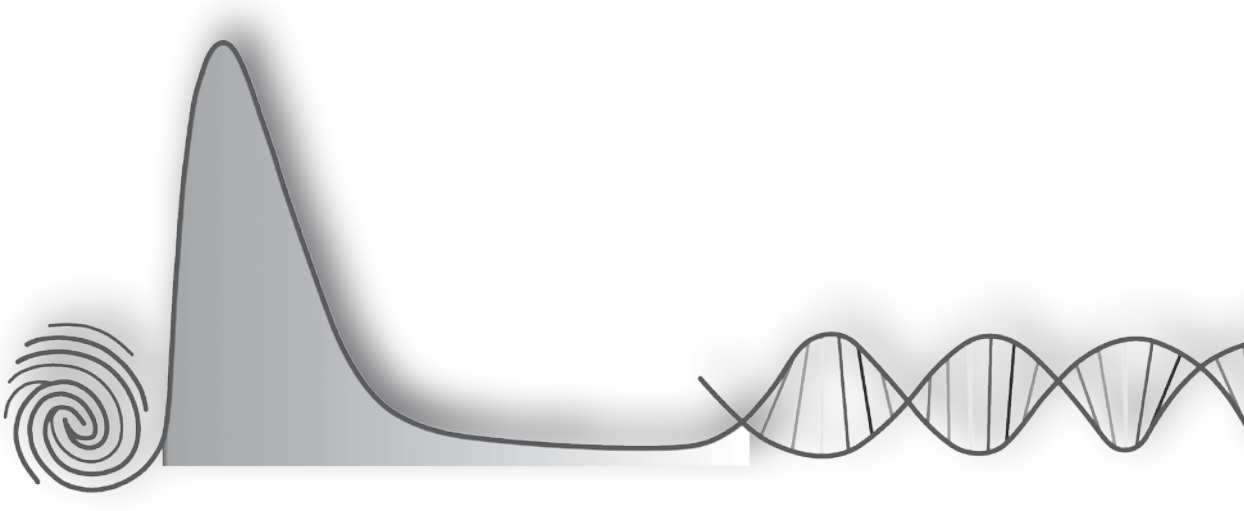

ABSTRACT

Imatinib has an oral bioavailability >90% despite being monocationic under the acidic conditions in the duodenum. *In vitro*, we found that imatinib is transported by the intestinal uptake carrier OATP1A2, and that this process is sensitive to pH, rosuvastatin, and genetic variants. In humans, however, imatinib absorption was not associated with OATP1A2 variants, and was unaffected by rosuvastatin. These findings highlight the importance of verifying drug-transporter interactions from *in vitro* tests in a clinical setting.

INTRODUCTION

The bioavailability of orally administered imatinib is >90%, although the drug is monocationic under the acidic conditions in the duodenum. *In vitro*, we found that imatinib is transported by the intestinal uptake carrier organic anion transporting polypeptide (OATP1A2) and that this process is sensitive to pH, rosuvastatin, and genetic variants. However, in a study in patients with cancer, imatinib absorption was not associated with OATP1A2 variants and was unaffected by rosuvastatin. These findings highlight the importance of verifying in a clinical setting the drug–transporter interactions observed in *in vitro* tests. Clinical use of the tyrosine kinase inhibitor imatinib is associated with a high interindividual pharmacokinetic variability. It was recently demonstrated that patients who do not respond to imatinib generally have lower systemic concentrations of imatinib as compared with those who do respond.[1,2] Furthermore, plasma imatinib concentration levels below a certain threshold value are possibly associated with a reduced response and reduced survival.[2,3] The results of previous studies indicate that imatinib is almost completely absorbed and has an oral bioavailability >90%.[4] This is somewhat unexpected, considering the affinity of imatinib for several intestinal efflux transporters, such as ABCB1 (Pgp) and ABCG2 (BCRP),[5,6] and the general assumption that, under the acidic conditions in the duodenum, imatinib is predominantly positively charged (~90% monocationic at pH 5–6).[7] These conditions may facilitate the uptake of imatinib across the intestinal wall by one or more carriers. Using data from preclinical studies, we previously identified imatinib as a substrate of the organic anion transporting polypeptide OATP1A2 (formerly OATP-A, OATP1, OATP),[8] which is expressed in duodenal enterocytes.[9] In the present study, we further characterized imatinib transport by OATP1A2 *in vitro* and explored the hypothesis that imatinib absorption in humans is affected by (i) common genetic variants in the gene encoding for *OATP1A2*, *SLCO1A2*, and (ii) concurrent administration of rosuvastatin, an inhibitor of OATP1A2.

RESULTS

Quantitative PCR analysis confirmed that the descending portion of the human duodenum showed the highest expression of OATP1A2 messenger RNA among all the tissues (including the tongue, stomach, small intestine, colon, and rectum) that are involved in the absorption of xenobiotics (Fig. 1a). Several other human tissues were found to express significant levels of OATP1A2, notably the spinal cord, intracranial artery, optic nerve, brain, lung, retina, uvula, and pituitary tissue (Fig. 1a). In *Xenopus laevis* oocytes expressing the human OATP1A2 or rodent Oatp1a4, but not rodent Oatp1a1, the uptake of imatinib at physiological pH was significantly higher than that observed in water-injected control oocytes (Fig. 1b). As compared with values at pH 7.4 (1.5-fold vs. control; $P = 0.0008$), a substantially higher uptake of imatinib by human OATP1A2 was seen at pH 5 (2.4-fold; $P = 0.0001$); a similar pH-dependent transport was seen with the Oatp1a4 complementary RNA (cRNA)-injected oocytes (Fig. 1b). When OATP1A2-expressing oo-

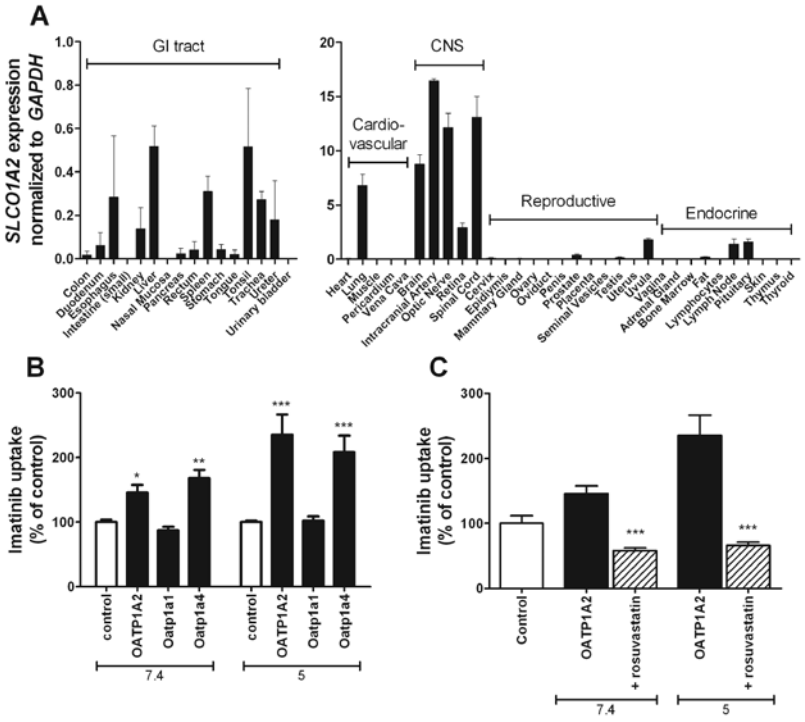

Figure 1 (**A**) Expression of the OATP1A2 gene *SLCO1A2* in a panel of 48 human tissues. (**B**) Uptake of [^{3}H] imatinib by cRNA-injected *Xenopus laevis* oocytes at pH 7.4 (left) and 5 (right). * $P = 0.01$; ** $P = 0.001$; *** $P = 0.0001$. (**C**) Influence of rosuvastatin on OATP1A2-mediated transport of [^{3}H]imatinib in *Xenopus laevis* oocytes.

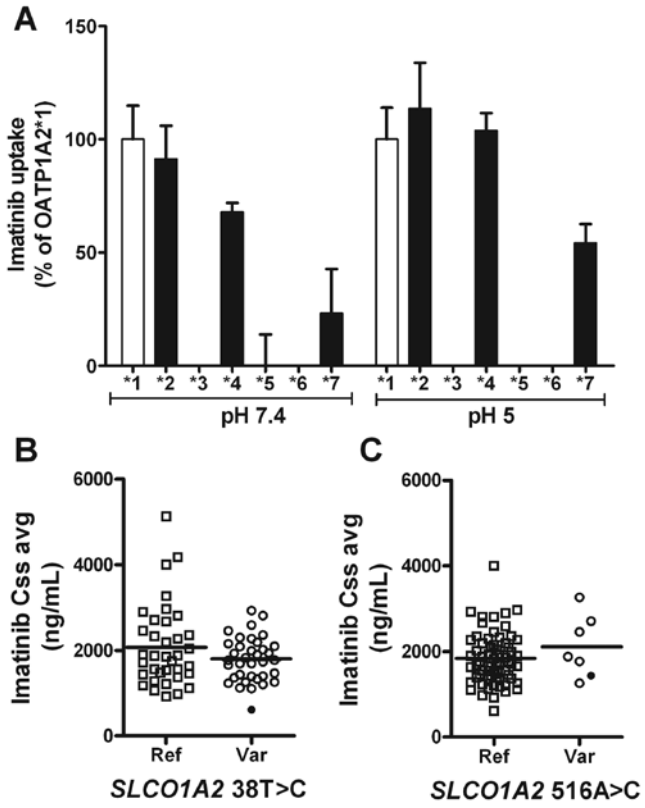

Figure 2 (**A**) Uptake of [^{3}H]imatinib in HeLa cells transfected with the OATP1A2 variants **2*, **3*, **4*, **5*, **6*, and **7* at pH 7.4 (left) and pH 5 (right). (**B**, **C**) Steady-state plasma concentrations of imatinib in a cohort of 94 white cancer patients, carrying OATP1A2 variants (Var) at the 38 (**B**) and 516 (**C**) nucleoside positions versus patients carrying two copies of the reference (Ref) allele (left panels). Each symbol represents an individual patient, and horizontal line denote the mean. The closed symbols represent the patients being homozygous for the variant allele.

cytes were coincubated with a mixture of imatinib and rosuvastatin (Fig. 1c), significant inhibition of imatinib transport occurred at both pH 7.4 and pH 5 ($P < 0.0001$ at both pH values). The uptake of imatinib in HeLa cells transiently transfected with six known *SLCO1A2* variants was reduced in four of the six variants, as compared to cells transfected with wild-type *OATP1A2*1*

(Fig. 2a). The uptake of imatinib uptake was completely abolished in HeLa cells expressing the *OATP1A2*3*, *OATP1A2*5*, and *OATP1A2*6* variants. In the case of *OATP1A2*7*, imatinib accumulation was reduced in a pH-dependent manner, with 77% reduction at pH 7.4 ($P = 0.0001$) and 46% reduction at pH 5 ($P = 0.033$) relative to *OATP1A2*1* at each respective pH value. As predicted from the *in vitro* data, the average steady-state

concentration of imatinib in 94 white patients was not significantly associated with the *OATP1A2*2* variant (38T>C) (Fig. 2b). However, it is noteworthy that the only patient who carried two copies of this variant had a steady-state plasma concentration of imatinib that was less than 1,000 ng/ml, which was previously identified as a threshold level associated with response to treatment.3 Although *OATP1A2*3* was associated with complete absence of imatinib transport *in vitro*, patients who carried this variant did not have altered levels of imatinib (Fig. 2c). Rosuvastatin significantly inhibited OATP1A2-mediated transport of imatinib *in vitro*, but this was not observed in a clinical setting; the concomitant administration of rosuvastatin had no influence on the steady-state pharmacokinetics of imatinib or its active metabolite CGP74588 in 12 patients (Fig. 3). There was a trend toward lower systemic concentrations of CGP74588 ($P = 0.15$) when imatinib was administered with rosuvastatin (Supplementary Table S1 online). As a result, a lower ratio of CGP74588-to-imatinib exposure was seen in almost all the patients; however, this difference did not reach statistical significance ($P = 0.065$). Treatment-related toxicity was generally mild (grade 2 edema and diarrhea were observed in one patient and grade 1 muscle cramps in two patients) and appeared to have no association with coadministration of rosuvastatin (data not shown).

DISCUSSION

Over the past few years, several studies have shown that, in the case of many drugs, transport across the intestinal epithelium may be mediated by solute carriers, including the human organic anion transporting polypeptide OATP1A2. This protein is highly expressed in the intestine, kidney, cholangiocytes, the blood–brain barrier, and certain cancers;[10] this pattern of localization suggests that OATP1A2 may be vitally important in the absorption, distribution, and excretion of a broad array of clinically important drugs. In this study, we evaluated the possible relevance of this uptake transporter in the intestinal absorption of imatinib, a known substrate of this carrier.[8] The findings of this study complement previous knowledge on the interaction of imatinib with organic ion transporters and provide further insights into the possible mechanisms underlying the effect of these proteins on the pharmacokinetic profile of imatinib. Using transfected *Xenopus laevis* oocytes, we found that human OATP1A2, as well as its rodent ortholog Oatp1a4, transports imatinib in a pH dependent manner, with increasing activity taking place at acidic pH, as is found in the duodenum. This result is consistent with the finding that imatinib occurs mainly as a monocationic isomer at duodenal pH.[7] A similar sensitivity to extracellular pH has been previously reported in relation to the *in vitro* transport of methotrexate by OATP1A2.[11] Using transfected HeLa cells, we also found that *in vitro* imatinib transport was completely absent or significantly reduced by several naturally

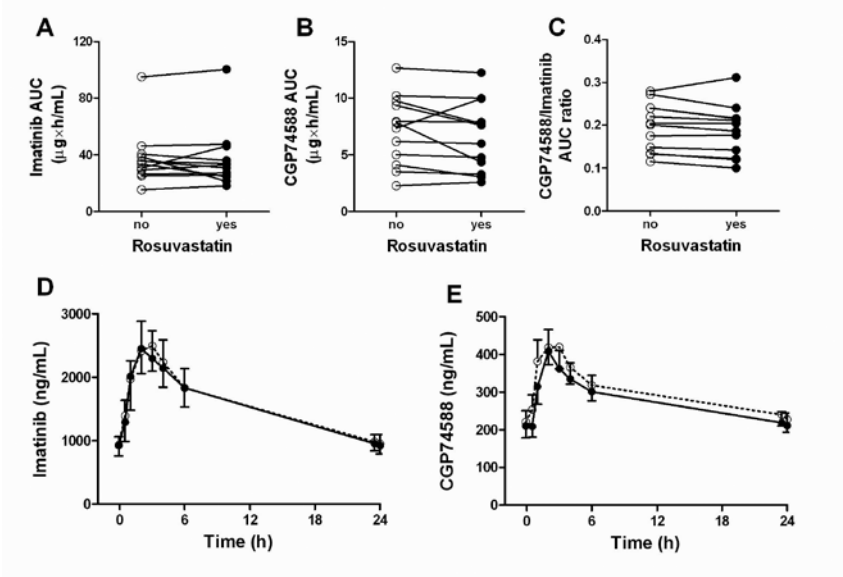

Figure 3 Individual paired areas under the plasma concentration time curves (AUC) of imatinib (**A**), CGP74588 (**B**), and CGP74588/imatinib AUC ratios (**C**) in 12 white cancer patients receiving concomitant administration of rosuvastatin (closed circles) or imatinib given alone (open circles). Average plasma concentration time profiles of imatinib (**D**) and CGP74588 (**E**) in the absence (open circles) and presence (closed circles) of rosuvastatin.

occurring protein variants of OATP1A2. Interestingly, the relevance of these genetic variants could not be confirmed from a pharmacogenetic association study in a group of 94 white patients with cancer who were receiving treatment with imatinib. However, in view of the fact that relatively few individuals with each of the variant genotypes were studied, the observed lack of significant relationships between the studied *SLCO1A2* variants and the steady-state pharmacokinetics of imatinib may be attributable to the study's limited statistical power. It is also theoretically possible that additional genetic variants or haplotypes of *SLCO1A2* of importance to the pharmacokinetics of imatinib in this population are yet to be discovered and/or that larger numbers of patients are needed to more precisely quantify genotype–phenotype associations. Nonetheless, in conjunction with the previous observation that common variants in the *ABCB1*, *ABCG2*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A4*, *CYP3A5*, and *SLC22A1* genes have only a limited effect on the pharmacokinetics of imatinib,[8,12,13] the findings of our study further support the possibility that intrinsic physiologic and environmental variables may have a more profound influence on the absorption and disposition of imatinib. On the basis of these considerations, and because recent data suggest that low circulating concentrations of imatinib may contribute to interindividual differences in clinical outcomes,[1,2,14,15] it was felt that an improved understanding of the possible contribution of OATP1A2 to the effects of drugs on imatinib absorption is of potential clinical significance. In order

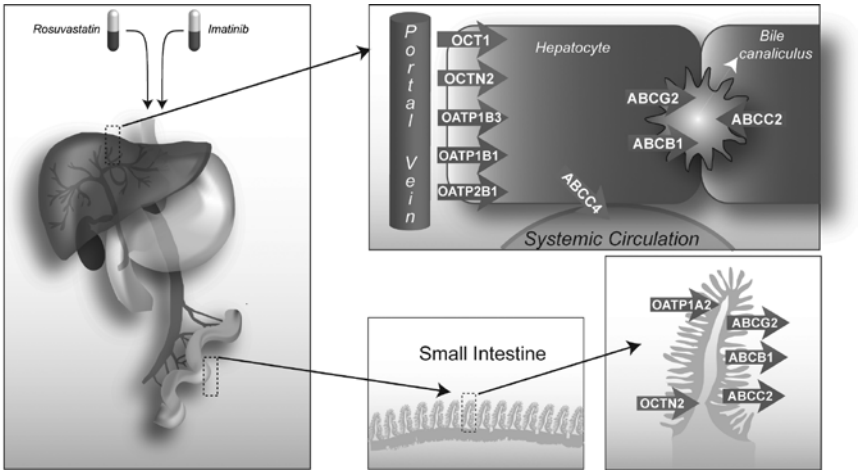

Figure 4 Possible transporter interactions between imatinib and rosuvastatin. Transporters involved in imatinib (red arrows) and rosuvastatin (blue arrows) pharmacokinetics are depicted, showing that imatinib and rosuvastatin share OATP1A2, ABCB1 and ABCG2 as a transporter at the intestinal level and OATP1B3, ABCB1 and ABCG2 at the hepatic basolateral and biliary membrane, respectively. Due to their involvement in the absorption, metabolism and excretion of both drugs, these transporters may affect imatinib and/or rosuvastatin pharmacokinetics during concomitant use.

to evaluate the impact of OATP1A2 inhibition on the pharmacokinetics of imatinib, a drug–drug interaction study was performed in patients concomitantly treated with imatinib and rosuvastatin. Unlike most statins, rosuvastatin is a hydrophilic compound[16] that undergoes limited metabolism and is mainly excreted into bile unchanged.[17,18] After 2 weeks of concomitant rosuvastatin use, no significant differences in imatinib pharmacokinetics or toxicity were observed. The collective results of this study illustrate the complications associated with translating preclinical pharmacological findings to the clinic. This recognition is particularly relevant in the context of the recent guidelines offered by the International Transporter Consortium regarding preclinical criteria needed to be met in order to trigger the conduct of clinical studies to evaluate drug–transporter interactions.[19] Theoretically, it is conceivable that the lack of a substantial effect of rosuvastatin on the steady-state concentrations of imatinib is the result of drug–drug interactions simultaneously taking place on several transporters (Figure 4).[8,20,21] For example, rosuvastatin is also known to interact with OATP1B3 transporters, which are expressed on the basolateral membrane of hepatocytes[22] and are involved in the regulation, in part, of the hepatocellular uptake of imatinib.[8] The combination of decreases in both intestinal and hepatic uptake transport by OATP1A2 and OATP1B3, respectively, could lead to a reduction in the oral bioavailability as well as the availability of imatinib for hepatic metabolism. These two mechanisms could balance out each other, thereby leaving systemic imatinib exposure unaltered. Indeed, we found a trend toward reduced

concentrations of the main CYP3A4-mediated metabolite of imatinib after 2 weeks of rosuvastatin dosing.

In conclusion, our results highlight the importance of verifying drug–transporter interactions from *in vitro* tests in a followup clinical study. Our study indicates that, although imatinib is a substrate for OATP1A2, this transporter by itself is unlikely to contribute substantially to the absorption profiles of imatinib in humans. Further investigation is warranted to determine the individual and collective contributions of additional, potentially redundant, intestinal carriers to the pharmacokinetics and pharmacodynamics of imatinib.

METHODS

Tissue expression of SLCO 1A2.

TissueScan Tissue qPCR Array plates (OriGene Technologies, Rockville, MD) were used to assess expression of *SLCO1A2* in 48 human tissues with a TaqMan Gene Expression Assay GEx probe 20× Mix for *SLCO1A2* (BD Biosciences, Rockville, MD) on an 0020 Applied Biosystems 7900HT Detection System (Applied Biosystems, Carlsbad, CA). The cDNA samples were derived from a pool of at least five donors. Expression levels analyzed in triplicate were normalized to *GAPDH*. *In vitro* transport studies. Uptake studies were performed in *Xenopus laevis* oocytes transfected with OATP1A2 cRNA, using [3H]imatinib as described8 at various pH values in the presence and absence of the OATP1A2 inhibitor rosuvastatin (1 mmol/l). The influence of *SLCO1A2* variants at the 38T>C (*2), 516A>C (*3), 559G>A (*4), 382A>T (*5), 404A>T (*6), and 2003C>G (T668S, *7) loci on imatinib transport was evaluated in transiently transfected HeLa cells. These cells were grown in 12-well plates (0.8 × 106 cells/well), infected with vaccinia in serum-free Opti-MEM I medium, and allowed to adsorb for 30 min at 37 °C. The cells in each well were then transfected with 1 ìg of wild-type or variant *SLCO1A2* cDNA packaged into a pEF6/V5-His-TOPO or pSPORT vector, along with Lipofectin, and incubated at 37 °C for 16 h. The parental plasmid without any insert was used as vector control.

Pharmacogenetic association studies.

DNA for genotyping was available from 94 white patients undergoing treatment with daily oral imatinib. Pharmacokinetic parameters were determined at steady state, as described.12 Genotypes of interest[23] were determined in each sample, using direct nucleotide sequencing of exon 1 (containing *SLCO1A2* 38T>C), exon 5 (containing *SLCO1A2* 502C>T and 516A>C), and exon 8 (containing *SLCO1A2* 968T>C and 1063A>G). The recorded genotype was termed "variant" if it differed from the reference sequence for the single nucleotide polymorphism position, as obtained from GenBank data. The

SLCO1A2 variants at the 559G>A (*4), 382A>T (*5), 404A>T (*6), and 2003C>G (T668S, *7) loci were previously shown to have an allele frequency of <0.5%; these were not analyzed in the samples.[24,25]

Clinical drug interaction studies.

Twelve patients with gastrointestinal stromal tumors were included in the interaction study. All of them had been treated with daily oral imatinib at a dose of 400 mg for at least 4 weeks (to guarantee steady-state conditions) before entering the study. With this sample size, the probability is 91% that a difference in oral drug availability caused by concurrent administration of the OATP1A2 inhibitor rosuvastatin will be detected at a two-sided 5% significance level, if the true difference is 0.3 units. This is based on a within-patient standard deviation of the response variable of 0.2 units. Additional eligibility criteria included age ≥18 years, World Health Organization performance ≤1, and adequate hematological, renal, and hepatic functions. The use of any medication or dietary supplement that could potentially inhibit or induce CYP3A4 or ABCB1 was prohibited. The study protocol was approved by the Erasmus University Medical Center review board, and all patients provided written informed consent before study entry. This clinical trial was registered with the Dutch trial registry (number NTR1504) and the European Clinical Trials Database (number 2008-002659-26). Blood samples for pharmacokinetic evaluation of imatinib and CGP74588 were taken during two 24-h periods at steady state: one period in the absence of rosuvastatin (i.e., day 1) and one period 14 days after the start of oral rosuvastatin at a dosage of 20 mg/day (i.e., day 16). Plasma was isolated and analyzed for imatinib and CGP74588 using a validated method based on liquid chromatography–tandem mass spectrometry.

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Chapter 5

Biliary excretion of imatinib and its active metabolite CGP74588 during severe hepatic dysfunction

Schiavon G., Eechoute K., Mathijssen R.H.J., de Bruijn P., van der Bol J., Verweij J., Sleijfer S., Loos W.J.

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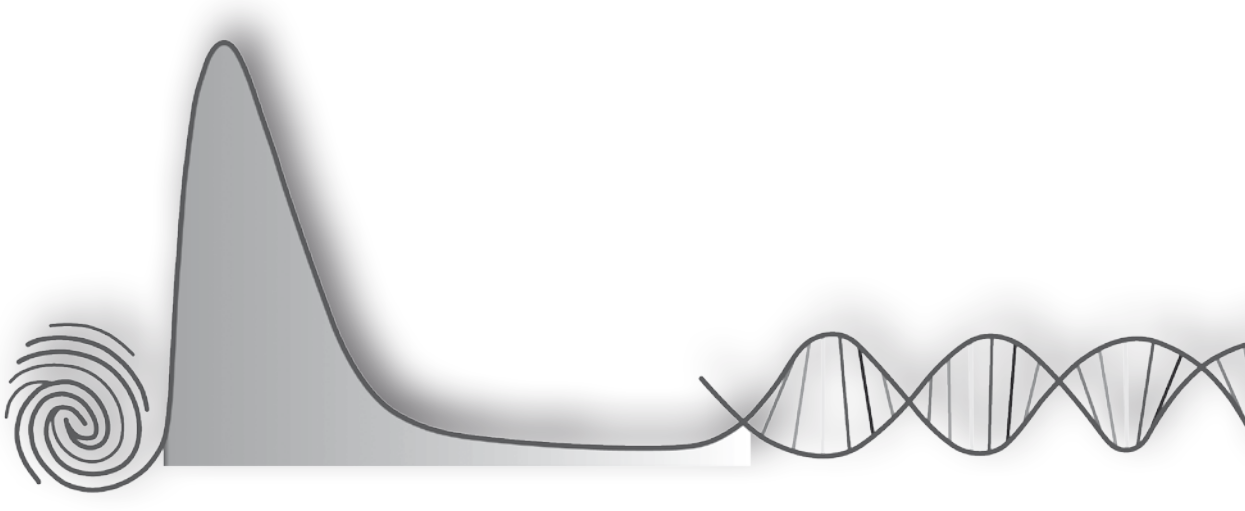

INTRODUCTION

Imatinib (STI571, Gleevec®) was the first approved rationally designed inhibitor of tyrosine kinases and is currently approved as standard care in patients with BCR-ABL-positive chronic myeloid leukemia (CML) and gastrointestinal stromal tumors (GIST).[1]

Imatinib is rapidly absorbed after oral administration, with a bioavailability of 98%. It is mainly metabolized in the liver predominantly by cytochrome P450 isoforms 3A4 and 3A5 (CYP3A4, CYP3A5) with CGP74588 as its most abundant (and equally active) metabolite.[2-4] CGP74588 represents approximately 10% of the imatinib area under the plasma concentration-time curve (AUC). The elimination half-life of imatinib and CGP74588 are approximately 18 and 40 hours (h) respectively, and imatinib is highly bound to proteins, predominantly to albumin and α1-glycoprotein. In patients with normal liver function, imatinib shows linear pharmacokinetics (PK) between the dose range of 25 mg/day and 1,000 mg/day.[5,6]

Imatinib is actively secreted into the bile by several drug efflux transporters from the ATP binding cassette superfamily, mainly ABCB1 and ABCG2.[3] In four healthy volunteers, an average of 80% of the radioactive dose of the drug was eliminated within 7 days (27% in 2 days). Approximately 67% of the dose was retrieved in feces and 13% in urine. In feces, 23% and 11% of the dose of unchanged imatinib and CGP74588 were found, respectively.[7] Imatinib plasma exposure, as measured by the AUC, was studied in a large cohort by the National Cancer Institute Organ Dysfunction Working Group (NCI ODWG) (89 patients, 24 of them with severe liver dysfunction). In this study, no differences in exposure were seen between patients with normal liver function and those with liver dysfunction (LD).[8] Despite these results, but based on dose-limiting toxicities occurring at an imatinib dose of 600 mg/day, the maximal recommended dose of imatinib for patients with mild LD was determined at 500 mg/day whereas for patients with normal liver function it is 800 mg/day. However, dosing guidelines for patients with moderate and severe LD could not be determined. Likewise, in two other phase-I/II pharmacokinetic trials with imatinib conducted in patients with impaired liver function and advanced hepatocellular carcinoma9 and in patients with advanced or metastatic hepatocellular cancer,10 no significantly different imatinib plasma PK was found compared to CML patients without liver disease. In the latter study an increased exposure to the metabolite was observed. However, in this study octreotide was co-administered and no control group (patients with normal function) was included. In addition, only patients with mild to moderate grade of LD were included in both studies (severe LD was an exclusion criterion). As a substantial number of GIST patients have liver enzyme disturbances and/or LD and since liver metastases represent the most frequent metastatic site in these patients, it is important to get a better insight in how LD impacts imatinib clearance.

To the best of our knowledge, a quantification of biliary excretion of imatinib has only been described in two patients so far; one patient with normal liver function and another with moderate LD.[11] In the patient with normal liver function, who underwent imatinib therapy at a dose of 400 mg/day, biliary excretion was studied for 24h at steady state. Biliary excretion of imatinib and CGP74588 accounted for 17.7% and 2.1% of the administered daily dose, respectively. In the patient with disturbed liver enzymes, biliary excretion was quantitated following the first administration of 300 mg of imatinib. During the first 24h, biliary excreted imatinib and CGP74588 accounted for 1.8% and 0.2% of the dose, respectively.[11] As for plasma exposure, biliary CGP74588 excretion represented approximately 10% of the biliary imatinib excretion in these two patients studied.

Here, we report on the biliary excretion of a patient with severe LD after 5 days of treatment with imatinib. The patient was sampled for plasma PK, as well as biliary PK for 48h, during therapy.

PATIENT AND METHODS

Patient and treatment

The patient was a 77-year-old caucasian man, who was treated with a total gastrectomy for a GIST of the stomach in 2006. Histological material showed a large epithelioid GIST (diameter 11 cm, MAI 6/10 HPF), with tumor involvement of the excision margins. Immunohistochemical and molecular analyses demonstrated weak CD117 positivity, CD34 negativity and a deletion in PDGFR alpha gene exon 18. No oncogenic mutations in KIT gene exons 8, 9, 11, 13 and 17 and in PDGFR alpha gene exons 10 and 12 were found. In 2008, this patient was referred to our hospital because of jaundice (bilirubin 166 µmol/L (Table 1)) and potbelly since one week. A computed tomography (CT) scan performed at admission showed the presence of multiple large liver metastases and signs of obstructed gall ducts. A Percutaneous Transhepatic Cholangiography (PTC) and complete biliary drainage were promptly performed, due to severe LD, defined according to the NCI ODWG Liver Function Classification.[8]

The patient had the following comorbitities: arythmia (chronic atrial fibrillation/flutter), rheumatoid arthritis and chronic obstructive pulmonary disease. There was no history of alcohol use or nicotine use, nor a positive serology for hepatitis B or C.

Imatinib-mesylate was started for the first time after biliary drain placement at a reduced dose of 200 mg/day (equivalent to 167 mg/day imatinib) for 4 days, which was increased to 300 mg/day (equivalent to 251 mg/day imatinib) from day 5 on. During the PK study, the patient was concomitantly treated with pantoprazole, prednisolone, metoprolole, furosemide, nadroparine and oxycodone. Methotrexate and folic acid were also administered for rheumatoid arthritis since 2004.

Table 1 Serum biochemical parameters, grade of liver dysfunction and biliary excretion of imatinib and GP74588

	Literature Case 1 [1,3]	Literature Case 2 [2,3]	Current Case
Serum biochemical parameters[4]			
ALT (U/L)	53 (21-72)	43 (21-72)	143 (0-40)
AST (U/L)	52 (17-59)	68 (17-59)	213 (0-36)
total bilirubin (µmol/L)	15.4 (3.4-22.2)	54.7 (3.4-22.2)	166 (0-16)
alkaline phosphatase (U/L)	262 (38-126)	133 (38-126)	349 (0-119)
gamma-glutamyltransferase (U/L)	–	–	303 (0-49)
lactate dehydrogenase (U/L)	–	–	1157 (0-449)
creatinine (µmol/L)	61.9 (70.7-132.6)	97.2 (70.7-132.6)	87 (65-115)
BUN (µmol/L)	6.1 (3.2-7.1)	10 (3.2-7.1)	7.3 (2.5-7.5)
LD grade conform NCI ODWG	Normal liver function	Moderate LD	Severe LD
Excretion data[5]			
bile excreted (ml)	1365	500	819 (417/402)
Imatinib-mesylate dose/day (mg)	400	300	300
% imatinib excreted	17.7	1.8	0.22 (0.27/0.17)
% CGP74588 excreted	2.1	0.2	0.42 (0.56/0.27)
Ratio imatinib/CGP74588 excreted	8.4	9	0.52 (0.48/0.63)

[1], patient with normal liver function (11); [2], patient with moderate LD (11); [3], Serum biochemical values for Literature Case 1 and 2 have been transformed from Conventional Unit to SI; [4], corresponding normal reference laboratory values of individual hospitals in parenthesis; [5], Excretion data are based on 24 h measurements for Literature Cases and 48 h measurements for Current Case. In parenthesis, 24h data for days 5 and 6 of the current case are presented.

Sample collection and bioanalysis

After written informed consent, blood samples for PK evaluation were collected daily prior to dosing from day 1 until day 14 via an indwelling intravenous catheter in the presence of lithium heparin as an anticoagulant. At day 1, 5 and 6 additional blood samples were collected at 0.5, 1, 2, 3, 4, 5, 6, 18 and 24 h after imatinib administration. Blood samples were centrifuged within 15 minutes after collection and the plasma supernatant was stored at T<-70°C until the analysis. Bile was quantitatively collected at days 5 and 6 at the same time-points as the blood samples. The volume of each portion was recorded and aliquots were stored at T<-70°C until analysis.

Imatinib and CGP74588 were quantitated by a validated liquid chromatography tandem triple quadruple mass spectrometry (LC-MS/MS) assay. The analytes were extracted by liquid-liquid extraction from 25 µL aliquots of plasma with stable labeled imatinib-d8 as internal standard. Multiple reaction monitoring settings (m/s) for the quantitation of imatinib, CGP74588 and imatinib-d8 were 494>394, 480>394 and 502>394, respectively. Peak area ratios were a function of the concentration from 20.0 to 5,000 ng/mL for

imatinib and CGP74588. For imatinib, a linear function was applied, while for CGP74588 a non-linear quadratic regression model was used. The within and between-run precisions at five tested concentrations were ≤12.6 and ≤3.7%, respectively, while the average accuracy ranged from 93.1 to 107.5%. Bile samples were processed following a 5-fold dilution in blank plasma.

RESULTS

Table 1 shows serum biochemical parameters, classification of liver function and biliary excretion data measured on the fifth and sixth day of imatinib treatment.

Plasma concentration time curves of imatinib and CGP74588 are presented in Figure 1A and 1B. Oral absorption on day 1 showed a long lag time, with imatinib detectable

Figure 1

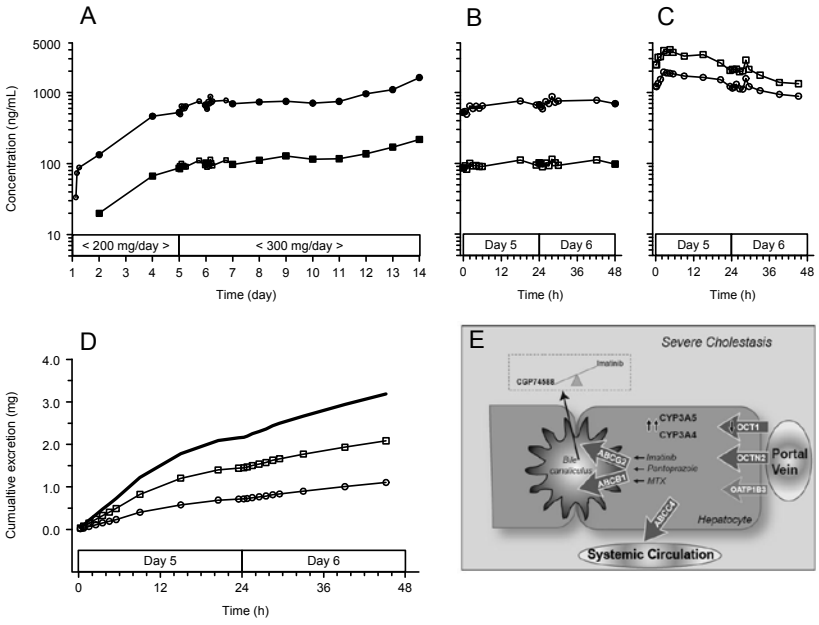

Figure 1 Plasma concentration time profiles of imatinib (circles) and CGP74588 (squares) in plasma (**A** and **B**) and in bile (**C**). Closed symbols in figure A and B represent trough concentrations, while open symbols represent concentrations observed during a 24h period. (**D**) Cumulative biliary excretion of imatinib (circles) and CGP74588 (squares) during day 5 and 6. The solid line represents the sum of imatinib and CGP74588. (**E**) Schematic illustration of the hypothesized processes in our patient with respect to imatinib metabolism and excretion. *Abbreviations:* ABC = ATP-binding cassette; CYP = cytochrome P450; MTX = methotrexate, OAT = organic anion transporting polypeptide; OCT = organic cation transporter.

at 2h after administration and quantifiable (i.e., >20 ng/mL) after 3h of administration. CGP74588 was detectable at 4h and 6h after administration and only quantifiable after 24h of administration.

Imatinib trough levels between day 5 and 10 were approximately 700 ng/mL and subsequently slowly increased from day 11 on to 1600 ng/mL at day 14 (last day of PK sampling).

The patient experienced no severe toxicities besides some well-known side effects, including grade 2-3 hypophosphatemia from day 5 and grade 1 fluid retention (according to Common Terminology Criteria for Adverse Events, version 4). Neither hematological toxicity nor other drug-related toxicities occurred.

In contrast to the plasma compartment, CGP74588 concentrations in bile at days 5 and 6 were higher compared to imatinib concentrations (Figure 1C). As shown in Table 1, a total of 819 mL of bile was collected through biliary drainage during the 48h study period, which is in the physiological range.[12]

Putting our findings into perspective to the previous report of two cases described recently (Table 1)[11], referred here as "Case 1" (patient with normal liver function) and "Case 2" (patient with moderate LD) some striking differences were seen. In comparison with Case 1 much lower percentages of imatinib excreted in bile were observed (Table 1). Cumulative biliary excretion (Figure 1D) of unchanged imatinib in our patient accounted for only 0.22% of the total dose of 600 mg administered, while CGP74588 accounted for 0.42%. Overall, only 0.64% (3.2 mg as imatinib base equivalents) of the total dose administered to our patient was excreted through the bile as unchanged imatinib plus CGP74588. Even in comparison with Case 2, who was sampled for only 24h after the first administration, low percentages of the administered dose were excreted through the bile. In addition, the biliary excretion ratio of imatinib/CGP74588 was reversed compared to both previously reported cases (Table 1).

In our patient, a CT scan after 4 weeks of imatinib therapy showed a minimal reduction in diameter of the largest liver metastasis, but hepatic function progressively worsened, leading to hypo-albuminemia and coagulation dysfunction. Treatment with imatinib was finally discontinued after 5 weeks of treatment due to deterioration of clinical performance.

DISCUSSION

Here we report plasma and bile PK data of imatinib and its main metabolite CGP74588 in a patient with metastatic GIST and severe LD.

Although imatinib absorption showed a long lag time, with imatinib concentrations quantifiable after 3h of administration, imatinib and CGP74588 plasma trough concen-

trations from day 5 and onward are within the range of values reported in the literature.[5] Our biliary data, however, appear to be in contrast. First of all, we observed low biliary excretion of imatinib as well as CGP74588 compared to the patient without LD, which was also at steady state therapy during the biliary drainage.[11] This observation could potentially be explained by a significant down regulation of the expression of OCT1, both at mRNA and protein level, as reported in liver tissue samples from cholestatic patients,[13] and in rats with induced cholestasis.[14] Reduced hepatic uptake transport of imatinib by OCT1 may subsequently impair the availability of the compound for biliary secretion (unchanged and metabolized) (Figure 1E). Of note, imatinib and CGP74588 bile excretion seems to decrease proportionally to liver function deterioration (Table 1). Also in comparison with the patient with moderate LD[11], the dose excreted as imatinib and CGP74588 was low in our patient. Although the plasma trough concentrations from day 5 and further are in agreement with literature data,[5] it cannot be excluded that the low percentage of the total dose of imatinib excreted via the bile is related to a low bioavailability in our patient. As shown (Figure 1A), a long lag time was observed after the administration on day 1 with peak plasma concentrations observed at 24h and a low oral availability is not unlikely. A combined effect of low oral availability and reduced hepatic uptake could balance each other out, resulting in plasma exposures of imatinib and CGP74588 comparable to the literature. Unfortunately, data on fecal excretion are not available.

With respect to the inverted biliary ratio of imatinib to CGP74588, both the high grade of LD/cholestasis and co-medication could have played a role. Adaptive phenomena occurring in the liver during cholestasis have been described, such as the activation of the Pregnane X receptor and Farnesoid X receptor.[15] These receptors regulate the expression of numerous transporters and enzymes (especially CYP3A family) and have a major role in maintaining bile acid homeostasis and protecting against cholestatic hepatotoxicity.[16] Mouse models of liver induced cholestasis revealed that enhanced expression of Cyp3a11 (the murine ortholog of human CYP3A4) is the major defense mechanism to detoxify cholestatic bile acids.[13-15,17] Based on this theory, enhanced enzymatic activity could therefore, at least partially, account for a higher proportion of imatinib metabolite in the bile of our patient.

In addition, the intake of potential competitive inhibitors for the ATP binding cassette transporters ABCB1 and ABCG2, the main efflux pumps for imatinib excretion, could have contributed to the low excretion and inverted ratio. The proton pump inhibitor (PPI) pantoprazole, for example, is an inhibitor of ABCB1 and ABCG2[18-21] and co-administration of benzimidazoles (e.g., anthelmintics as albendazole and PPIs) significantly inhibits ABCG2-mediated transport of methotrexate *in vitro* and reduces its clearance *in vivo*.[22] Therefore, competitive inhibition of imatinib by pantoprazole or methotrexate at the ABCG2 transport site may have contributed to the reversed ratio in our study

patient. However, data on different transporter specificity of imatinib and CGP74588 which could support this hypothesis are not available yet.

In addition, although a pharmacogenetic analysis was not performed in our patient, potential genetic variations may not explain our biliary findings. In fact, up until now, functional polymorphisms in genes involved in imatinib metabolism (e.g. genes coding for ABCB1, ABCG2, CYP2C9, CYP2C19, CYP2D6, CYP3A4, CYP3A5 and OCT1), have not shown a significant impact on systemic exposure to imatinib.3

In conclusion, biliary excretion of imatinib seems related to the severity of liver dysfunction. Tailored *in vivo* as well as clinical studies in patients with normal liver function and with different grades of cholestasis and liver involvement are warranted, to further elucidate the role of biliary excretion on the PK of imatinib.

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Chapter 6

Pharmacogenetic pathway analysis for determination of sunitinib-induced toxicity

van Erp N.P., Eechoute K., van der Veldt A.A.M., Haanen J.B., Reyners A.K.L., Mathijssen R.H.J., Boven E., van der Straaten T., Baak-Pablo R.F., Wessels J.A.M., Guchelaar H.-J. and Gelderblom H.

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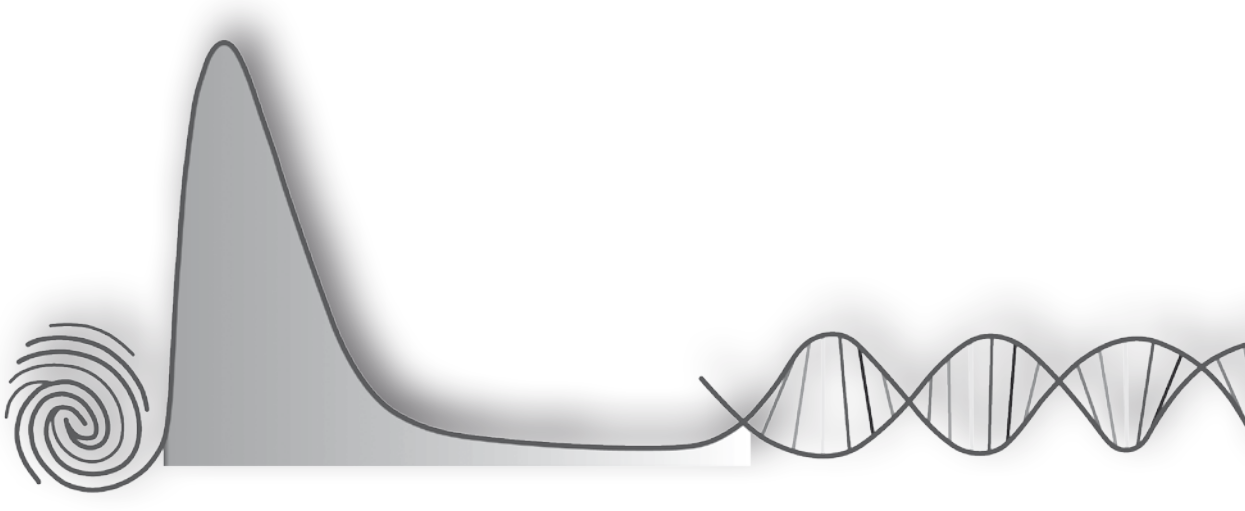

ABSTRACT

Purpose

To identify genetic markers in the pharmacokinetic and pharmacodynamic pathways of sunitinib that predispose for development of toxicities; thrombocytopenia, leukopenia, mucosal inflammation, hand-foot syndrome and any toxicity according to National Cancer Institute Common Toxicity Criteria higher than grade 2.

Patients and Methods

A multicenter pharmacogenetic association study was performed in 219 patients treated with single-agent sunitinib. A total of 31 single nucleotide polymorphisms in 12 candidate genes, together with several nongenetic variants, were analyzed for a possible association with toxicity. In addition, genetic haplotypes were developed and related to toxicity.

Results

The risk for leukopenia was increased when the G-allele in *CYP1A1* 2455A/G (odds ratio [OR], 6.24; $P = .029$) or the T-allele in *FLT3* 738T/C (OR, 2.8; $P = .008$) were present or CAG in the *NR1I3* (5719C/T, 7738A/C, 7837T/G) haplotype (OR, 1.74; $P = .041$) was absent. Any toxicity higher than grade 2 prevalence was increased when the T-allele of *VEGFR-2* 1191C/T (OR, 2.39; $P = .046$) or a copy of TT in the *ABCG2* (-15622C/T, 1143C/T) haplotype (OR, 2.63; $P = .016$) were present. The risk for mucosal inflammation was increased in the presence of the G-allele in *CYP1A1* 2455A/G (OR, 4.03; $P = .021$) and the prevalence of hand-foot syndrome was increased when a copy of TTT in the *ABCB1* (3435C/T, 1236C/T, 2677G/T) haplotype (OR, 2.56; $P = .035$) was present.

Conclusion

This exploratory study suggests that polymorphisms in specific genes encoding for metabolizing enzymes, efflux transporters, and drug targets are associated with sunitinib-related toxicities. A better understanding of genetic and nongenetic determinants of sunitinib toxicity should help to optimize drug treatment in individual patients.

INTRODUCTION

The oral, multitargeted tyrosine kinase inhibitor sunitinib (sunitinib malate; Sutent; Pfizer Pharmaceuticals Group, New York, NY) is known to inhibit vascular endothelial growth factor receptors (VEGFRs) 1, 2, and 3, platelet-derived growth factor receptor (PDGFR) α and β, KIT, Fms-like tyrosine kinase 3 receptor (FLT3), and the receptor encoded by the ret proto-oncogene (RET).[1-4] Sunitinib is approved for first-line treatment of metastatic renal cell carcinoma (mRCC) and imatinib-resistant metastatic gastrointestinal stromal tumors (GIST).[4-6] Targeted cancer therapies are generally considered to be less toxic than conventional chemotherapy since they specifically inhibit tyrosine kinase receptors that are frequently overexpressed or mutated in various types of tumor cells.[7] Tyrosine kinases, however, are also present in normal tissues and toxic effects are therefore difficult to eliminate. The 4 weeks on 2 weeks off dosing schedule of sunitinib was selected for the first phase I study on request of the health authorities to allow patients to recover from potential bone marrow and adrenal toxicity observed in animal models, indicating that toxicity was regarded as a serious problem.[3, 8] Although the proportion of patients with grade 3 to 4 adverse events was relatively low in the recent phase III studies, a dose interruption appeared to be necessary in 38% of patients with mRCC and in 28% of patients with GIST whereas a dose reduction was required in 32% and 11%, respectively. Similar percentages were reported in other studies.[2, 4, 9] Disease- and sunitinib-related toxicities can be distinguished based on results of a phase III trial in which the toxicity profile of sunitinib-treated patients has been compared with events in the placebo-treated patients.[2] Adverse events that preferentially occurred in the group treated with sunitinib were diarrhea, hand-foot syndrome, mucositis, vomiting, hypertension, leukopenia, neutropenia, and thrombocytopenia.[2-4, 9-13] Less common, but specific toxicities related to sunitinib were cardiotoxicity and hypothyroidism.[5, 14, 15]

Sunitinib is used as palliative therapy with no standard therapeutic options available after failure of the therapy. It is therefore relevant for patients to adhere to sunitinib therapy while their quality of life is not unnecessarily reduced by drug toxicity. To date, it is not completely clear which patient characteristics render an individual patient at risk for sunitinib-induced toxicity. The aim of the present study is to identify genetic markers in sunitinib disposition, metabolism, and mechanism of action that predispose for development of common sunitinib related toxicities: thrombocytopenia, leukopenia, mucosal inflammation, hand-foot syndrome and any higher than grade 2 National Cancer Institute Common Toxicity Criteria for Adverse Events (CTCAE) toxicity.

PATIENTS AND METHODS

A total of 219 patients from five Dutch medical centers were analyzed in this study. The study was approved by the medical ethics review board. Patients were treated at the Erasmus University Medical Center (n=74), the Netherlands Cancer Institute (n=51), Leiden University Medical Center (n=37), VU University Medical Center (n=36), and the University Medical Center Groningen (n=21). The collection of DNA and patient data was performed between June 2004 and May 2008. A total number of 159 mRCC, 50 GIST, and 10 patients with other tumors were included in this study. Of them, 77 patients with mRCC and 26 patients with GIST were treated according to an expanded access programme of sunitinib. Eligible patients were those treated with single agent sunitinib for at least one treatment cycle (4 consecutive weeks of 50 mg per day followed by a two-week period of rest).

Study design

Sunitinib toxicity was evaluated during the first treatment cycle by CTCAE version 3.0.[16] Toxicity scores were assessed by analysis of adverse events, physical examination and laboratory assessments carried out at baseline (before starting sunitinib), after 4 weeks of sunitinib therapy, and after 6 weeks (just before starting the second cycle). Demographic and clinical data of patients were reported on case record forms designed for data collection in this study. Patient characteristics considered relevant for experiencing toxicity were: age, gender, ethnicity, body-surface area (BSA), Eastern Cooperative Oncology Group (ECOG) performance status, tumor type, renal, liver and bone marrow function (serum creatinine, total bilirubin, albumin, ALT, AST, hemoglobin, leukocytes and thrombocytes). Residual blood or serum samples taken for routine patient care were stored at -20^{o}C at the local hospital laboratory. Of each patient one whole blood or serum sample was collected from the participating centers. All samples were anonymized by a third party, according to the instructions stated in the Codes for Proper Use and Proper Conduct in the Self-Regulatory Codes of Conduct (www.federa.org).

Definition of toxicity

All adverse events were graded by independent physicians of the participating medical centers. Four- and 6-week reported toxicities were compared to baseline conditions. The primary outcome measures of this study were thrombocytopenia, leukopenia, mucosal inflammation, hand-foot syndrome and any toxicity higher than grade 2. Toxicities were selected based on objectivity, clinical relevance and manageability of the symptoms. Thrombocytopenia and leukopenia were scored from blood cell counts and are thus objective endpoints. In case of any toxicity higher than grade 2, a dose interruption and, depending on the kind of toxicity, a resumed treatment with 25% dose reduction

is advised in the drug label of sunitinib. Moreover, mucosal inflammation and hand-foot syndrome are frequently reported and poorly manageable and therefore dose reduction is relatively soon considered. In addition, dose reduction of at least 25% according to the drug label (data complete for 187 patients) which is applied because of safety or tolerability issues, after cycle 1 to 3 was related to the toxicity outcomes.

Genetic Polymorphisms

Nineteen polymorphisms in seven genes involved in the pharmacokinetics and 12 polymorphisms in five genes involved in the pharmacodynamics of sunitinib were selected. Selection criteria for the polymorphisms were an allelic frequency higher than 0.2 in whites and an assumed clinical relevance based on previously reported associations or the assumption that nonsynonymous amino acid change leads to changed protein functionality. The selected polymorphisms are listed in Table 1.

Genotyping of selected polymorphisms

Germline DNA was isolated from 1 ml of serum or EDTA-blood with the Magnapure LC (Roche Diagnostics, Almere, The Netherlands). DNA concentrations were quantified on the nanodrop (Isogen, IJsselstein, The Netherlands). Taqman assays were obtained from Applied Biosystems (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands). All Single Nucleotide Polymorphisms (SNPs) were initially determined on the Biomark 48.48 Dynamic Array (Fluidigm, San Fransisco, CA, USA) according to the manufacturer's protocol. Failed samples were repeated on the TaqMan 7500 (Applied Biosystems), according to standard procedures. For serum samples, a pre-amplification step was necessary. Briefly, a dilution of all TaqMan assays in a total volume of 1.25 µL and 2.5 µL of pre-amplification mastermix (Applied Biosystems) was added to 1.25 µL of serum-DNA, and subsequently amplified by polymerase chain reaction. This mixture was 20 times diluted and 2.5 µL was used in the Biomark array according to the protocol.

Genotyping assay validity

The overall average success rate of the assays and the individual samples was 98%. The lowest success rate in our study was 93.5%. As a quality control, all DNA samples were genotyped in duplicate for 12 of 31 SNPs, and three DNA samples were genotyped in duplicate for all 31 SNPs. No inconsistencies were observed. In addition negative controls (water) were used. The allelic frequencies of the 31 single nucleotide polymorphisms were tested for Hardy-Weinberg equilibrium (HWE). Six genotype assay results did not meet HWE. However, of four of these, frequencies were compared with allelic frequencies as reported on the National Center for Biotechnology Information website (NCBI) for white population and found similar to the reported frequencies. Of the two remaining SNPs no frequencies were available on the NCBI website (www.ncbi.nlm.nih.gov). The

Table 1: Polymorphisms genotyped in the pharmacokinetic and pharmacodynamic pathway of sunitinib

	Gene	Polymorphism	rs-number
Pharmacokinetic pathway	NR1I2	-25385C/T	rs3814055
		-24113G/A	rs2276706
		7635A/G	rs6785049
		8055C/T	rs2276707
		10620C/T	rs1054190
		10799G/A	rs1054191
	NR1I3	5719C/T	rs2307424
		7738A/C	rs2307418
		7837T/G	rs4073054
	CYP3A5	6986A/G	rs776746
	CYP1A1	2455A/G	rs1048943
	CYP1A2	-163A/C	rs762551
	ABCG2	421C/A	rs2231142
		34G/A	rs2231137
		-15622C/T	*
		1143C/T	rs2622604
	ABCB1	3435C/T	rs1045642
		1236C/T	rs1128503
		2677G/T	rs2032582
Pharmacodynamic pathway	PDGFRα	1580T/C	rs35597368
		-1171C/G	rs1800810
		-735G/A	rs1800813
		-573G/T	rs1800812
	VEGFR2 (=KDR)	-604T/C	rs2071559
		-92G/A	rs1531289
		54T/C	rs7692791
		1191C/T	rs2305948
		1718T/A	rs1870377
	VEGFR3 (=FLT4)	1501A/G	rs307826
	RET	2251G/A	rs1799939
	FLT3	738T/C	rs1933437

* No rs-number assigned yet

homozygotic wildtype frequencies of both SNPs exceed the HWE and were therefore allowed for the analysis.

Haplotype estimation

Polymorphisms within a gene were tested to detect linkage disequilibrium (LD). If LD between SNPs was present, haploblocks (with several haplotypes) were determined. The uncertainty measure R_h^2 was calculated. R_h^2 gives us information on the uncertainty in the prediction of common haplotypes from unphased SNP genotypes [17]. A haplotype was considered to be present if the haplotype uncertainty measure R_h^2 was greater than 0.98 as tested with the software program CHAPLIN [18]. Haplotypes with an uncertainty measure $R_h^2 \leq 0.7$ in CHAPLIN were not considered for further analysis since the data provided no information on haplotypes in our population. All haplotypes with uncertainty ($0.7 < R_h^2 \leq 0.98$) and without uncertainty ($R_h^2 > 0.98$) were computed and assigned per individual using gPLINK [19]. Rare haplotypes (< 2%) were combined into one group of other haplotypes in the association analysis. The haplotypes used in this study had no phase uncertainty ($R_h^2 > 0.98$). The *VEGFR-2* gene had a large phase uncertainty ($R_h^2 \leq 0.7$) indicating that in our population *VEGFR-2* polymorphisms could not be defined as a haplotype. The following SNPs were combined for further analysis: *ABCG2*; 1143C/T and -15622C/T; *PDGFRa*; -573G/T, -1171C/G, -735G/A, 1580T/C; *NR1I3*; 5719C/T, 7738A/C, 7837T/G; *NR1I2* 10620C/T, 10799G/A and *ABCB1*; 3435C/T, 1236C/T, 2677G/T.

Statistical design and data analysis

For the analysis of toxicity, we used dichotomous end points expressed as increased toxicity (yes or no) or any toxicity higher than grade 2 (yes or no). All demographic and clinical variables were tested univariately against the selected primary outcomes using *t* test, the Mann-Whitney *U* test or the χ^2 test, depending on the tested variables. A χ^2 test was also used to detect linkage disequilibrium (LD). The polymorphisms were initially tested with 2 *df*. If the initial 2 *df* tests resulted in $P \leq .1$, the polymorphisms were fitted and the most appropriate model (multiplicative, dominant, or recessive) was selected. The number of copies of each haplotype was used as parameter in the analysis. The polymorphisms and haplotypes were tested univariately against the selected primary outcomes using a χ^2 test. Candidate variables with $P \leq .1$ were selected for the multiple logistic regression analysis with toxicity as depending variable. All multivariate logistic regression analyses were corrected for age, gender and ECOG performance status. Additional patient characteristics were introduced in the multivariate analyses based on univariate tested results if $P \leq .1$. Missing data were kept as missing data except for BSA and ECOG performance status. Missing BSA values (n=15) were replaced for the median BSA ($1.93m^2$) and missing ECOG performance status (n=7) were replaced for the median ECOG performance status (1). To test this action, the multivariate analyses were performed with and without the replacement of the patients with missing BSA and ECOG performance status. Similar results were generated, indicating that the replacement was legitimate. All statistical analyses were performed using SPSS 16.0 software

(SPSS, Chicago, IL, USA). With the sample size of our study, an increase in toxicity of 17% could be measured between two groups with a power of 80% and a confidence interval of 99%. All results from the multivariate analyses with P less than .05 were considered significant. Since this was an exploratory study, no correction for multiple testing was done.

RESULTS

Patients

Nineteen out of 219 patients had to be excluded from analysis for several reasons including progressive disease (PD) during the first treatment cycle resulting in early death (n=4), discontinuation of sunitinib in the first treatment cycle due to adverse events (hypertension grade 3, headache grade 3 and rash grade 3, respectively; n=3) and no acceptable genotyping success rate due to poor DNA quality (n=12). For toxicity analyses, a total of 200 patients were evaluable (Table 2). For the endpoint any toxicity higher than grade 2, the three patients who stopped therapy due to adverse events were included (n=203).

Toxicities

The hematological toxicities scored in this analysis were thrombocytopenia (40% any grade), leukopenia (59%, any grade). Non-hematological toxicities were primarily any toxicity higher than 2 (22%), mucosal inflammation (44%) and hand-foot syndrome (19%; Table 3). Dose reduction after cycle 1 to 3 was related to mucosal inflammation ($P = .002$) and any toxicity higher than grade 2 ($P < .001$)

Pharmacogenetic risk factors for sunitinib-induced toxicity

The results of the multivariate logistic regression analysis for the selected endpoints thrombocytopenia, leukopenia, mucosal inflammation, hand-foot syndrome and any toxicity higher than grade 2 are summarized in Table 4. For thrombocytopenia, an increase in age ($P = .030$) and ECOG performance status ($P = .050$) were independently significant in the multivariate logistic model. The factors associated with development of leukopenia were: *CYP1A1* 2455A/G; the presence of the G allele in an additive model was related to a 6.2-fold increase in the risk for leukopenia during the first treatment cycle ($P = .029$); the presence of the *FLT3* 738C allele (dominant model) was related to a 2.8-fold reduction in the risk for leukopenia ($P = .008$); the absence of the *NR1I3* CAG haplotype was related to a 1.7-fold increased risk for leukopenia ($P = .041$) and 4); one grade increase in ECOG performance status, implicating a worse clinical condition, was related to a 1.8-fold reduction in the risk of leukopenia ($P = .016$). The presence of the

VEGFR-2 1191T-allele (additive model) was related to an increased risk of 2.4-fold for the development of any toxicity higher than grade 2 ($P = .046$), while the risk for this toxicity was 2.6-fold higher when 1 or 2 copies of TT in the *ABCG2* haplotype were present ($P = .016$). For mucosal inflammation only *CYP1A1* 2455A/G was independently related; the G-allele (additive model) resulted in a 4.0-fold higher risk for mucosal inflammation ($P = .021$). The occurrence of hand-foot syndrome was related to the *ABCB1* haplotype; the absence of copies of the TTT haplotype was protective and was related to a 2.6-fold lower risk to experience hand-foot syndrome as compared to patients with copies of the TTT haplotype ($P=.035$). The explained variance (R^2) of the patient characteristics, without taking the polymorphisms into account, in the multivariate analyses was between the 2 to 10% of the total variance. After adding the selected polymorphisms the explained variance increased to 10 to 23% of the total variance.

DISCUSSION

To the best of our knowledge, this is the first study exploring the relationship between drug-induced toxicity and genetic polymorphisms in genes encoding for enzymes, efflux transporters and targets involved in the pharmacokinetics and pharmacodynamics of sunitinib.

Sunitinib is metabolized by cytochrome P450 3A4 (CYP3A4) and CYP3A5. In addition, affinity of sunitinib for the ATP-binding cassette transporters ABCG2 and ABCB1 has also recently been reported.[20] The transcription of CYP3A4 is regulated by members of the NR1I nuclear receptor subfamily.[21] Metabolism through CYP1A1 and CYP1A2 is hypothesized since these enzymes appear to be involved in the metabolism of multiple tyrosine kinase inhibitors (eg, imatinib, erlotinib).[22, 23] Both genes encoding the sunitinib targets, as well as genes encoding the enzymes (except for CYP3A4, in which no functional polymorphisms have been identified) and efflux transporters involved in sunitinib's disposition and metabolism are highly polymorphic and may be related to the differential toxicity response in patients treated with sunitinib.

Although the nature and incidence of adverse events related to sunitinib are currently well recognized and described, data regarding determinants of toxicity are still scarce.[2, 4, 5, 14, 24, 25] So far, only one study has described factors (low BSA, high age, female gender) that are associated with the development of severe toxicities, defined as dose reduction or permanent discontinuation of sunitinib therapy.[9] That study, however, was limited to patient characteristics and no genetic determinants were investigated. In our study, these patient characteristics, and another (performance status), were included as covariates in the data analysis. We should emphasize, however, that the definition of the

Table 2: Patient characteristics (N=203)

Characteristic	Value
Age (years)	
Median (range)	60 (20-84)
Sex	
Male	129 (63.5%)
Female	74 (36.5%)
Body Surface Area (square meters)	
Median (range)	1.93 (1.47-2.51)
ECOG performance status	
0	81 (39.9%)
1	90 (44.3%)
2	17 (8.4%)
3	8 (3.9%)
Missing	7 (3.4%)
Ethnicity	
Caucasian	190 (93.6%)
Blacks	6 (3.0%)
Asian	2 (1.0%)
Latin-American	2 (1.0%)
Middle East	3 (1.5%)
Tumor types	
Renal cell carcinoma	152 (74.9%)
Gastrointestinal stromal tumor	46 (22.7%)
Other	5 (2.5%)
Previous medical treatments	
Yes*	116 (57.1%)
No	87 (42.9%)
First treatment regimen (N=116)*	
Interferon-alpha (INF-α)	46 (39.7%)
Imatinib	46 (39.7%)
Sorafenib	5 (4.3%)
Others	19 (16.4%)

Dose reduction after sunitinib cycle 1 – 3		
Yes	Renal cell carcinoma	58 (28.6%)
	GIST	14 (6.9%)
	Other tumor	1 (0.5%)
No	Renal cell carcinoma	94 (46.3%)
	GIST	32 (15.8%)
	Other tumor	4 (2.0%)
Baseline chemistry and hematology		
Creatinine (μM)		
Median (range)		96.0 (40-176)
Total bilirubin (μM)		
Median (range)		7 (3-32)
Albumine (gram/L)		
Median (range)		40 (23-52)
ALT (units/L)		
Median (range)		18 (3-210)
AST (units/L)		
Median (range)		24 (9-190)
Hemoglobin (mM)		
Median (range)		7.6 (5.2-10.4)
Leukocytes (*10^9/L)		
Median (range)		7.5 (3.6-56.5)
Thrombocytes (*10^9/L)		
Median (range)		284.0 (92-864)

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ECOG, Eastern Cooperative Oncology Group

CR, complete response ; PR, partial response; SD stable disease ; PD, progressive disease

Table 3: Number (No) of patients (%) according to the distribution of increased toxicity grades

Toxicity	No/Yes	Grade	No (%)
Thrombocytopenia (n=198)	No		118 (59.0)
	Yes	1	58 (29.0)
		2	14 (7.0)
		3	7 (3.5)
		4	1 (0.5)
Leukopenia (n=198)	No		81 (40.5)
	Yes	1	91 (45.5)
		2	22 (11.0)
		3	4 (2.0)
Any toxicity > 2 (n=203)		0, 1, 2	158 (77.8)
		3, 4	45 (22.2)
Mucosal inflammation (n=199)	No		112 (56.0)
	Yes	1	57 (28.5)
		2	25 (12.5)
		3	5 (2.5)
Hand-foot syndrome (n=199)	No		162 (81.0)
	Yes	1	27 (13.5)
		2	8 (4.0)
		3	2 (1.0)

.

Table 4: Factors relevant for sunitinib-induced toxicity, defined as thrombocytopenia, leukopenia, any toxicity > grade 2, mucosal inflammation or hand-foot syndrome

	Data corrected for patient characteristics[1]				Uncorrected data[1]	
	Factor	Genotype	OR (95% CI)	*P-value*	OR (95% CI)*	*P-value*
Thrombocytopenia	Age		1.04 (1.00–1.07)	.030		
(n=177)	Gender		1.93 (0.97–3.85)	.063		
	ECOG		0.60 (0.37–1.00)	.050		
	NR1I2 -24113 G/A	GG *vs*	1		1	
		AG + AA	0.60 (0.28–1.28)	.187	0.51 (0.25–1.05)	0.067
	CYP1A2 -163 A/C	AA *vs*	1		1	
		AC + CC	0.67 (0.34–1.32)	.251	0.65 (0.34–1.23)	0.186

	ABCG2 421 C/A	CC *vs*	1		1	
		CA + AA	2.09 (0.88–4.96)	.096	1.93 (0.85–4.42)	0.118
	ABCG2 haplotype ξ	TT-TT + TT-other *vs*	1		1	
		Other-other	1.93 (0.96–3.86)	.065	1.77 (0.91–3.46)	0.093
	PDGFRα haplotype ψ	TGAT-TGAT + TGAT-other	1		1	
		vs Other-other	0.44 (0.17–1.18)	.103	0.36 (0.14–0.92)	0.033
Leukopenia	Age		1.01 (0.98–1.04)	.423		
(n=188)	Gender		0.81 (0.41–1.60)	.536		
	ECOG		0.57 (0.36–0.90)	.016		
	VEGFR-2 -92G/A	GG → GA → AA	0.74 (0.44–1.23)	.241	0.74 (0.45–1.22)	.235
	VEGFR-2 1718 T/A	TT → AT → AA	1.49 (0.84–2.66)	.172	1.47 (0.83–2.60)	.188
	CYP1A1 2455A/G	AA → AG → GG	6.24 (1.20–32.42)	.029	4.87 (1.06–22.29)	.042
	FLT 3 738T/C	TT *vs*	1		1	
		CT + CC	0.36 (0.17–0.77)	.008	0.41 (0.20–0.85)	.016
	NR1I3 haplotype Ω	CAG-CAG → CAG-other → other-other	1.74 (1.02–2.96)	.041	1.81 (1.07–3.04)	.026
	NR1I3 haplotype Ω	TCT-TCT → TCT-other → Other-other	0.54 (0.27–1.06)	.074	0.55 (0.28–1.06)	.075
Any toxicity > grade 2	Age		1.03 (0.99–1.07)	.140		

(n=183)	Gender		1.71 (0.69–4.26)	.248		
	ECOG		1.31 (0.79–2.19)	.299		
	BSA		0.22 (0.02–2.49)	.220		
	VEGFR-2 1191 C/T	CC → TC → TT	2.39 (1.02–5.60)	.046	2.31 (1.07–4.99)	.033
	ABCG2 haplotype ξ	TT-TT + TT-other *vs*	1		1	
		Other-other	0.38 (0.17–0.83)	.016	0.40 (0.19–0.84)	.016
Mucosal Inflammation	Age		1.00 (0.97–1.03)	.956		
	Gender		1.54 (0.82–2.88)	.177		
(n=193)	ECOG		1.31 (0.86–1.99)	.212		
	NR1I2 -24113G/A	GG *vs*	1		1	
		AG + AA	0.58 (0.30–1.13)	.110	0.55 (0.29–1.04)	.064
	CYP1A1 2455A/G	AA → AG → GG	4.03 (1.24–13.09)	.021	4.15 (1.29–13.36)	.017
	ABCG2 34G/A	GG → AG	2.45 (0.74–8.17)	.144	2.41 (0.75–7.76)	.140
	NR1I3 haplotype Ω	TCT-TCT → TCT-other → Other-other	0.78 (0.42–1.44)	.420	0.77 (0.42–1.42)	.404
Hand-Foot Syndrome	Age		0.99 (0.96–1.02)	.563		
(n=182)	Gender		1.22 (0.56–2.68)	.612		
	ECOG		0.76 (0.43–1.33)	.336		

ABCB1 haplotype ∞	TTT-TTT + TTT-other	1		1	
	vs Other-other	0.39 (0.16–0.94)	.035	0.39 (0.16–0.92)	.032
ABCB1 haplotype ∞	CTT-CTT → CTT-other → Other-other	0.38 (0.11–1.32)	.126	0.36 (0.10–1.27)	.114

Abbreviations: OR, odds ratio; CI, confidential interval; ECOG, Eastern Cooperative Oncology Group.
Multiplicative model is indicated with (→) between the genotypes
Dominant and recessive models are indicated with (*vs*) between the two groups of genotypes
1: All toxicity outcomes in the corrected analysis are corrected for; age, gender and ECOG performance status. Additional correction with body surface area (BSA) was done for Any toxicity > grade 2. Under the uncorrected data only the genotypes are included in the multivariate analysis.
Description haplotypes: ξ = ABCG2 -15622C/T and 1143C/T; ψ = PDGFRα -573G/T, -1171C/G, -735G/A and 1580T/C; Ω = NR1I3 5719C/T, 7738A/C and 7837T/G; ∞ = ABCB1 3435C/T, 1236C/T and 2677G/T
P-value < .05 is regarded as significant and printed bold.
Description of polymorphisms and rs-numbers: See table

endpoint severe toxicity is different in both studies as well as the observed study period (whole sunitinib treatment period *v* first treatment cycle in our study).

To our knowledge, we report for the first time herein that the *ABCB1* TTT haplotype was related to hand-foot syndrome. The TTT haplotype as well as the T genotype in 3435C/T and the T polymorphism in 1236C/T separately have been associated with higher exposures to drugs transported by ABCB1 due to a decreased expression of the ABCB1 transporter.[26-31] Also, for the other ABC-transporter investigated, ABCG2, the TT haplotype was related to the development of increased toxicity (eg, any toxicity > grade 2). This haplotype has been associated with increased erlotinib exposure, a tyrosine kinase inhibitor that uses metabolic and predisposition pathways similar to those of sunitinib.[32] Thus, our results concerning *ABCB1* and *ABCG2* are in line with previously reported functional consequences of the studied genetic variants and might lead to an increased systemic exposure to sunitinib resulting in dose-limiting toxicities. Certainly, to confirm our findings, further studies that relate pharmacogenetics to pharmacokinetics and pharmacodynamics are required.

Thus far, the extrahepatic CYP1A1 enzyme has not been described as being involved in the metabolism of sunitinib. For other receptor tyrosine kinase inhibitors, such as erlotinib, imatinib and gefitinib affinity for CYP1A1 has been demonstrated in in vitro studies.[22, 23] Therefore, we also included genetic variants of *CYP1A1* in the present study. The polymorphism studied in *CYP1A1* resulting in an amino acid change of isoleucine 462 Valine was found to be related to the occurrence of mucosal inflammation and leukopenia. This suggests that CYP1A1 may also play a role in the metabolism of sunitinib *in vivo*.

In addition, we investigated genetic polymorphisms in the *NR1I3* gene, encoding the constitutive androstane receptor. This nuclear receptor plays an important role in the regulation of multiple drug detoxification genes, such as *CYP3A4*. The functionality of polymorphisms in *NR1I3* is not yet fully elucidated, however we found a relationship between the absence of the CAG haplotype in this gene and an increased risk for leukopenia[33]. Obviously, it would be interesting to relate this polymorphism with sunitinib exposure levels in future studies.

The *VEGFR-2* 1191CT and TT genotypes were found to be predictive for the development of coronary heart disease due to a lower binding efficiency of VEGF to the polymorphic VEGFR-2.[34] In our study, these genotypes were related to the development of any toxicity higher than 2, which predominantly included fatigue, thrombocytopenia, and hypertension. The polymorphic receptor might therefore be involved in sunitinib-induced cardiac toxicity and the development of hypertension.

The importance of the FLT3 receptor has been described in relation to the development of several subtypes of leukemia such as acute myeloid leukemia, acute lymphocytic leukemia, and chronic myeloid leukemia, in which FLT3 is frequently overexpressed and/or mutated.[35, 36] However, the association between *FLT3* 738T/C polymorphism and a reduction in the risk of leukopenia has not previously been described. Since sunitinib-induced leukopenia could be regulated strongly by this polymorphic receptor the clinical relevance should be further investigated.

In our study, a large number of candidate polymorphic loci were evaluated and multiple analyses of each genetic polymorphism were performed. This introduces the potential problem of multiple testing which increases the risk to find false-positive relations. However, our study was designed to explore associations that should be confirmed in an independent group of patients. The presented odds ratios and CIs facilitate comparisons of replicate studies with our data.

The ECOG performance status was not consistently related to the occurrence of toxicities in our study. The quantified performance status is multifactorial and is dependent on subjective interpretation of the physician. Moreover, in our study patients with poor performance status had relatively high baseline thrombocyte and leukocyte counts resulting in a small number of reported leukopenia and thrombocytopenia in this group in the first treatment cycle.

Toxicities in the first treatment cycle of sunitinib were used as outcome measure. The rationale was that signs of clinical deterioration from disease progression in later cycles could be misinterpreted and would interfere with the drug-induced toxicity outcome. We hypothesized that patients that suffer from relatively mild (grade 1 or 2) toxicities in the first treatment cycle were at risk for developing more severe toxicity during further treatment cycles because the two weeks of rest would not be sufficient for patients to recover to baseline conditions. This cumulative effect is underscored by measured blood

cell counts and the observed dose reduction after cycle 1 to 3. Indeed, we found for leukocyte count and to a lesser extent also for thrombocyte count, that 91% and 73%, respectively, of the patients had not returned to baseline values (defined as > 90% of baseline counts) at cycle 2 day 1 (data not shown). In addition, we found that mucosal inflammation and any toxicity higher than grade 2 were strongly related to a dose reduction after cycle 1 to 3, indicating that these toxicities are regarded as clinically relevant to the treating physicians.

Together, the genetic, clinical and demographic determinants in this exploratory study explain between 10 and 23% of the total variance in toxicity response. Although it indicates that the major part of the variability is left unexplained, it also shows that pharmacogenetics may make a greater contribution to explaining variability in sunitinib toxicity as compared to the nongenetic determinants in our study. From this study we cannot conclude whether the genetic variants are prognostic or predictive markers, due to the absence of a placebo-treated control group of patients. However in the future, pharmacogenetics may help to select patients which need a priori dose reduction to prevent toxicities.

In conclusion, this study suggests a relationship between polymorphisms in the genes *CYP1A1*, *ABCB1*, *ABCG2*, *NR1I3*, *VEGFR-2* and *FLT3* and the development of sunitinib toxicity. The next step will be to validate our data with the aim to better understand the determinants of sunitinib toxicity.

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Chapter 7

Genetic polymorphisms associated with a prolonged progression-free survival in patients with metastatic renal cell cancer treated with sunitinib

van der Veldt. A.A.M.*, Eechoute K.* ., Gelderblom H., Gietema J., Guchelaar H.-J., van Erp N.P., van den Eertwegh A.J.M., Haanen J.B., Mathijssen R.H.J., Wessels J.A.M.

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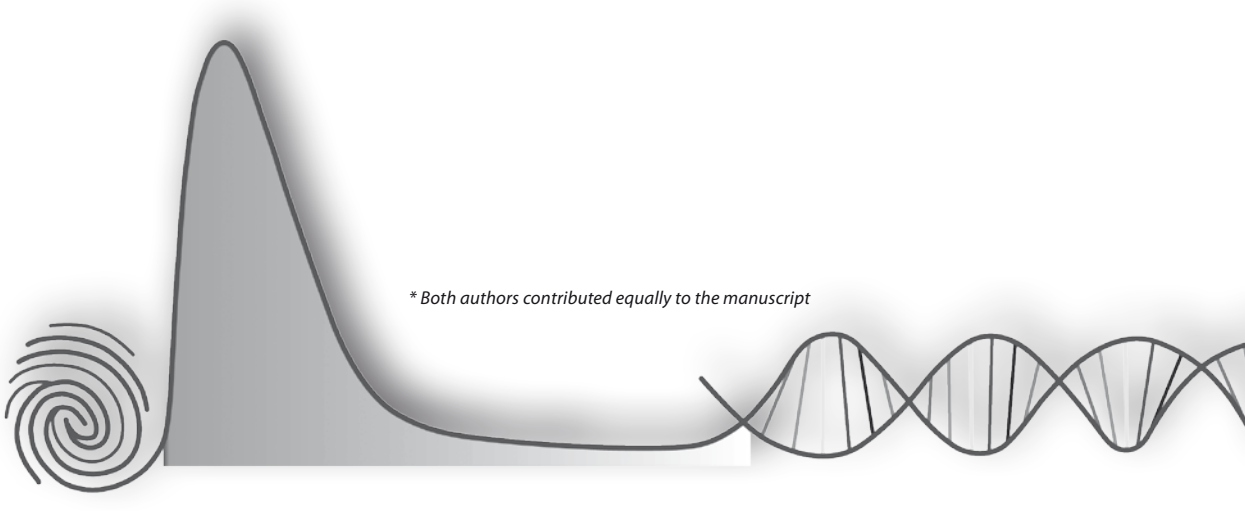

** Both authors contributed equally to the manuscript*

TRANSLATIONAL RELEVANCE

Currently, sunitinib is the most widely prescribed drug for the treatment of metastatic renal cell cancer. Unfortunately, only a part of treated patients will benefit from sunitinib therapy, despite the implementation of clinical prognostic criteria in the choice of therapy. As multiple systemic treatment modalities arise, a further refinement is needed to identify renal cell cancer patients who predispose to benefit from sunitinib treatment and patients who do not. One of the possible options to study the differential response to sunitinib treatment is to identify genetic polymorphisms related to the pharmacokinetics and pharmacodynamics of this drug. In the future, genetic variants may be added to the current prognostic criteria, enabling physicians to predict benefit from sunitinib in individual patients.

ABSTRACT

Purpose: The objective of this study was to identify genetic polymorphisms related to the pharmacokinetics and pharmacodynamics of sunitinib that are associated with a prolonged progression-free survival (PFS) and/or overall survival (OS) in patients with clear-cell metastatic renal cell cancer (mRCC) treated with sunitinib.

Experimental design: A retrospective multicenter pharmacogenetic association study was performed in 136 clear-cell mRCC patients treated with sunitinib. A total of 30 polymorphisms in 11 candidate genes, together with clinical characteristics were tested univariately for association with PFS as primary and OS as secondary outcome. Candidate variables with *P*-value <0.1 were analyzed in a multivariate Cox regression model.

Results: Multivariate analysis showed that PFS was significantly improved when an A-allele was present in *CYP3A5* 6986A/G (Hazard ratio [HR], 0.27;*P*=0.032), a CAT copy was absent in the *NR1I3* haplotype (5719C/T, 7738A/C, 7837T/G; HR, 1.76;*P*=0.017) and a TCG copy was present in the *ABCB1* haplotype (3435C/T, 1236C/T, 2677G/T; HR, 0.52;*P*=0.033). Carriers with a favorable genetic profile (n=95) had an improved PFS and OS as compared to non-carriers (median PFS and OS: 13.1 *vs.* 7.5 months and 19.9 *vs.* 12.3 months). Next to the genetic variants, the Memorial Sloan-Kettering Cancer Center prognostic criteria were associated with PFS and OS (HR, 1.99 and 2.27;*P* <0.001).

Conclusions: This exploratory study shows that genetic polymorphisms in three genes involved in sunitinib pharmacokinetics are associated with PFS in mRCC patients treated with this drug. These findings advocate prospective validation and further elucidation of these genetic determinants in relation to sunitinib exposure and efficacy.

INTRODUCTION

For decades, the treatment options of metastatic renal cell cancer (mRCC) have been limited and systemic treatment primarily consisted of immunotherapy with cytokines. Increasing knowledge of the underlying biology of renal cell cancer (RCC), in particular the clear-cell subtype, has expanded the treatment options for patients with mRCC (1). RCC is characterized by an inactivated *von Hippel-Lindau (VHL)* tumor suppressor gene. Inactivated *VHL* leads to elevated protein levels of hypoxia-induced factor-α (HIF-1α) which upregulates vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) genes and proteins. The development of targeted therapy against signaling of these proteins has significantly improved the perspectives of patients with mRCC.

Currently, sunitinib is the most widely prescribed drug for the treatment of mRCC and has been registered as first-line and second-line therapy. Sunitinib is an oral tyrosine kinase inhibitor (TKI) which targets several receptors including VEGF receptors-1,-2,-3, platelet-derived growth factor receptors-α and –β, c-KIT and FLT-3. In a randomized controlled trial, sunitinib significantly prolonged the progression-free survival (PFS) and overall survival (OS) as compared to interferon-α (2,3). Although sunitinib can achieve partial response rates of up to 40% (3-5), approximately 35% of mRCC patients do not benefit from sunitinib treatment (4,5). Since sunitinib treatment may also result in unnecessary toxicities (6,7), pre-treatment markers to identify mRCC patients with a favorable outcome to sunitinib treatment are warranted.

Sunitinib efficacy may be dependent on its exposure which is regulated by efflux pumps and metabolizing enzymes. After oral administration, the systemic exposure of sunitinib is initially determined by its absorption in the gastrointestinal tract (Figure 1). This process may be regulated by active drug transport over the intestinal wall, as sunitinib may be a substrate for polyspecific efflux transporters, expressed on enterocytes (8,9). The efflux transporters ABCB1 (ATP binding cassette member B1, formerly known as P-glycoprotein or MDR1) and ABCG2 (ATP binding cassette member G2, formerly known as breast cancer-resistance protein [BCRP] or mitoxantrone resistant protein [MXR]) are expressed in the intestine and liver, and are involved in the oral absorption and biliary secretion of several anticancer drugs (10). Therefore, expression levels and functionality of these drug transporters may have important consequences for the efficacy of sunitinib.

The cytochrome P450 (CYP) 3A (CYP3A) family is the predominant drug metabolizing enzyme and CYP3A4 is thought to be the key enzyme for the biotransformation of sunitinib (11). CYP3A4 is predominantly found in the liver and its expression is regulated by the ligand-activated nuclear receptors NR1I2 *(*pregnane X receptor [PXR]*)* and NR1I3 (constitutive androstane receptor [CAR]) (12). In addition, other enzymes of the cyto-

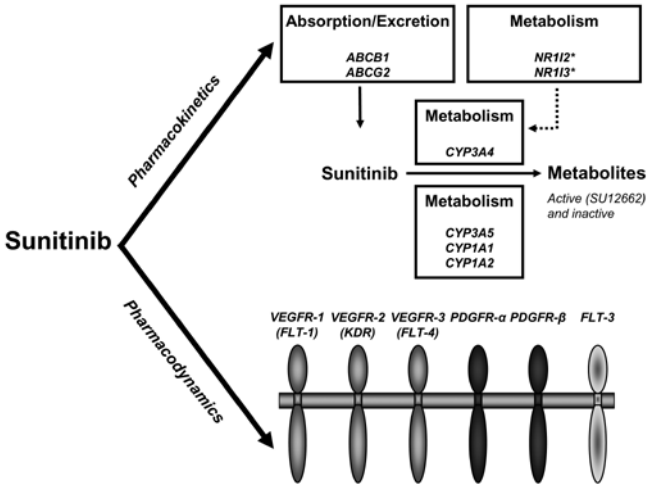

Figure 1 Schematic diagram of the pharmacokinetic and pharmacodynamic pathways that may be involved in the efficacy of sunitinib in mRCC.
* NR1I2 and NR1I3 regulate CYP3A4 expression

chrome P450 family (CYP3A5, CYP1A1 and CYP1A2) may metabolize sunitinib, as these enzymes are known to be involved in the metabolism of other TKIs (13).

Besides pharmacokinetic factors, pharmacodynamic factors may determine the efficacy of sunitinib. In RCC, sunitinib is thought to exert its major therapeutic effect by inhibition of the vascular endothelial growth factor receptor (VEGFR) on tumor-associated endothelium, leading to reduced tumor angiogenesis (14). In addition, inhibition of the platelet-derived growth factor receptor (PDGFR) might increase the antiangiogenic effects of sunitinib by targeting pericytes, which are able to protect endothelial cells from apoptosis (15). As the main targets for sunitinib are thought to be located in the microenvironment of tumor cells, the efficacy of sunitinib treatment may be related to the genetics of the surrounding microenvironment (16). Particularly, genetic variation in *VEGFR-2* may affect sunitinib activity, since VEGFR-2 is expressed in the normal endothelium (17) and the tumor vasculature may develop from pre-existing vessels of the host (18).

Single nucleotide polymorphisms (SNP) in genes encoding for efflux transporters, metabolizing enzymes, and drug targets may affect the efficacy of sunitinib in mRCC, as SNPs in specific genes have previously been associated with sunitinib-induced toxicities in patients with mRCC and gastrointestinal stromal tumors (19,20). Therefore, SNPs may be useful markers for personalized treatment planning and may be candidate markers for selecting mRCC patients for sunitinib treatment. The objective of the current study

was to identify SNPs involved in the pharmacokinetics and pharmacodynamics of sunitinib that are associated with a prolonged PFS and/or OS in mRCC patients.

PATIENTS AND METHODS

Study population

In our previous study, 219 sunitinib-treated patients with various maligancies were included to investigate the association between SNPs and sunitinib-induced toxicities (19). In the present study, a subset of patients with histologically proven clear-cell RCC were selected for the analyses. A total of 136 consecutive mRCC patients who initiated sunitinib treatment between December 2005 and May 2008 were included. Sunitinib was administered orally at a dose of 50 mg daily, consisting of 4 weeks of treatment followed by a 2-week rest-period in cycles of 6 weeks. Dose reductions of sunitinib were allowed depending on the type and severity of adverse events according to the current guidelines (21). The study was approved by the medical ethics review board.

Study design

Demographic and clinical data of patients were reported on case record forms designed for data collection for this study. Patient characteristics considered relevant for PFS and OS analysis were age, gender, Eastern Cooperative Oncology Group (ECOG) performance status, prior systemic therapy, prior radiotherapy, the number of metastatic sites, and the risk factors according to Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria, which is based on 5 risk factors including low Karnofsky performance status (< 80%), high lactate dehydrogenase (LDH, > 1.5 times the upper limit of normal), low hemoglobin level, high corrected serum calcium (> 10 mg/dL), and time from initial diagnosis to treatment < 1 year (22). Residual blood or serum samples taken for routine patient care were stored at -20^{o}C at the local hospital laboratory. Of each patient one whole blood or serum sample was collected from the participating hospitals. All samples were anonymized by a third party, according to the instructions stated in the Codes for Proper Use and Proper Conduct in the Self-Regulatory Codes of Conduct (www.federa.org).

Genetic Polymorphisms

Nineteen polymorphisms in 7 genes involved in the pharmacokinetics and 11 polymorphisms in 4 genes involved in the pharmacodynamics of sunitinib were selected (see Table 4). Selection criteria for the polymorphisms were a minor allelic frequency > 0.2 in Caucasians and an assumed clinical relevance based on previously reported associations

Table 1. Patient characteristics

Characteristic	No	%
Median age, years	60	
Range	25-84	
Sex		
Male	83	61.0
Female	53	39.0
Ethnicity		
Caucasian	130	95.6
Black	3	2.2
Asian	1	0.7
Latin-American	1	0.7
Arab	1	0.7
ECOG performance status*		
0	66	48.5
1	54	39.7
2	10	7.4
3	4	2.9
Missing	2	1.5
Previous medical treatments		
Yes	56	41.2
No	80	58.8
Previous treatment regimen		
Cytokine-based therapy	45	33.1
Anti-angiogenic therapy	4	2.9
Both	7	5.1
Previous nephrectomy		
Yes	105	77.2
No	31	22.8
Previous radiation therapy		
Yes	34	25.0
No	102	75.0
No. of metastatic sites		
1	31	22.8
2	47	34.6
≥ 3	58	42.6
Sites of metastases		
Lung	100	73.5
Liver	33	24.3
Bone	46	33.8
Lymph nodes	61	44.9

Brain	5	3.7
MSKCC risk factors**		
0 (favorable)	33	24.3
1 – 2 (intermediate)	81	59.6
≥ 3 (poor)	22	16.2

* ECOG, Eastern Cooperative Oncology Group

** Risk groups according to Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria [based on the 5 risk factors: low Karnofsky performance status (< 80%), high LDH (> 1.5 times the upper limit of normal), low serum hemoglobin, high corrected serum calcium (> 10 mg/dL), and time from initial diagnosis to treatment < 1 year] (22)

or the assumption that non-synonymous amino-acid change leads to changed protein functionality.

The 11 candidate genes were selected on their potential relation with the pharmacokinetics and pharmacodynamics of sunitinib (Figure 1). First, the candidate genotypes were selected by literature review. If there was no available data in the literature review, we referred to the SNPs from the dbSNP database (http://www.ncbi.nlm.nih.gov/sites/entrez). *ABCB1*, *ABCG2, NR1I2, NR1I3*, *CYP3A5*, *CYP1A1* and *CYP1A2* were selected for the pharmacokinetic pathways, whereas *VEGFR-2*, *VEGFR-3*, *PDGR-α*, and *FLT-3* were selected for the pharmacodynamic pathways. The most common functional SNPs in human *ABCB1* are the synonymous 3435C>T and 1236C>T changes and the non-synonymous 2677G>T change. As functional studies have shown that the haplotype of these three SNPs is a silent mutation and alters the function of the efflux transporter including its substrate specificity (23), the haplotype of *ABCB1*, instead of the three individual SNPs, was included in the analysis. Although VEGFR-1 is a target of sunitinib, and CYP3A4 is an important enzyme for metabolism of sunitinib (11), no polymorphisms of *VEGFR-1* and *CYP3A4* were analyzed, as no functional polymorphisms met the criteria for SNP selection.

Methods for genotyping assay validation and haplotype estimation have been described previously (19). Briefly, germline DNA was isolated from 1 mL of serum or EDTA-blood with the Magnapure LC (Roche Diagnostics, Almere, The Netherlands). Polymorphic sites in genomic DNA were analyzed with TaqMan assays (Applied Biosystems, Nieuwerkerk aan den IJssel, The Netherlands).

Statistical design and data analysis

For PFS and OS, data collection was closed on August 31, 2009. The primary outcome measures of this study, PFS, was defined as the time between the first day of sunitinib and the date of progressive disease (PD) according to Response Evaluation Criteria in Solid Tumors (24), clear clinical evidence of PD or death due to PD within 12 weeks after the last response evaluation. If a patient had not progressed, PFS was censored at the

Table 2. Univariate and multivariate analyses of progression-free survival in mRCC patients treated with sunitinib

		Univariate Analyses*			Multivariate Analyses		
Factors	**No**	**Median PFS (months)**	**95% CI**	***P***	**HR****	**95% CI**	***P***
Clinical factors							
MSKCC risk factors††				0.001	1.988	1.394-2.837	**< 0.001†**
0	33	24.3	11.3-37.2				
1-2	81	8.7	6.3-11.2				
≥ 3	22	7.0	0.0-14.4				
No. of metastatic sites				0.019	1.400	1.042-1.880	**0.025†**
1	31	19.4	7.8-31.0				
2	47	11.0	5.1-16.8				
≥ 3	58	8.1	7.6-8.6				
Age (HR = 1.024 per year increase)			1.000-1.048	0.047	1.031 per year increase	1.003-1.060	**0.029**
Genetic factors pharmacokinetic pathway							
CYP3A5 6986A/G				0.017			**0.032**
GG *vs.*	117	9.3	6.9-11.8		1		
AG + AA	11	Not reached	-		0.266	0.079-0.892	
NR1I3 haplotype‡				0.021			**0.017**
Other-other *vs.*	75	13.3	7.9-18.8		1		
CAT-other + CAT-CAT	60	8.0	7.5-8.6		1.758	1.108-2.790	
NR1I2 8055C/T				0.025			0.656
CC+ CT *vs.*	119	10.8	8.0-13.6		1		
TT	17	6.7	3.6-9.9		1.638	0.187-14.380	
NR1I2 -25385C/T				0.032			0.795
CC + CT *vs.*	118	10.8	8.0-13.6		1		
TT	18	6.7	2.8-10.7		0.755	0.091-6.273	

ABCB1 haplotype‡‡				0.072		**0.033**
Other-other *vs.*	100	8.4	7.0-9.7	1		
TCG-other	29	15.2	6.1-24.3	0.522	0.287-0.950	
ABCG2 34G/A				0.077		0.497
GG *vs.*	124	9.0	6.9-11.2	1		
AG	12	19.4	0.0-40.8	0.713	0.269-1.891	

* Only factors with *P*-value < 0.1 level are presented; factors with *P*-value < 0.1 in the univariate analyses were selected for multivariate analyses

** HR < 1.0 indicates that the factor associates with improved PFS, HR > 1.0 associates with worse PFS

† Multiplicative model, HR per increase in MSKCC class or number of metastatic sites class

†† Risk groups according to Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria [based on the 5 risk factors: low Karnofsky performance status (< 80%), high LDH (> 1.5 times the upper limit of normal), low serum hemoglobin, high corrected serum calcium (> 10 mg/dL), and time from initial diagnosis to treatment < 1 year] (22)

Description haplotypes: ‡ = *NR1I3* 5719C/T, 7738A/C and 7837T/G; ‡‡ = *ABCB1* 3435C/T, 1236C/T and 2677G/T

Abbreviations: 95% CI, 95% confidence interval; PFS, progression-free survival; HR, hazard ratio

time of the last follow-up. If the PD date was unknown or a patient died due to PD later than 12 weeks after the last response evaluation, PFS was censored at the last adequate tumor assessment. OS was the secondary outcome and was defined as the time between the first day of sunitinib treatment and the date of death or the date at which patients were last known to be alive.

All patient characteristics were tested univariately against the primary outcome using Kaplan-Meier and Cox-regression analysis, depending on the tested variables. The polymorphisms and haplotypes were tested univariately against PFS and OS using the Kaplan-Meier method. For this initial analysis, the general model was used. Given the explorative nature of this study, variables with a *P*-value ≤ 0.1 were selected as candidate variabeles for multivariate Cox-regression analyses. Data were fitted to the most appropriate model (multiplicative, dominant or recessive) and tested in the multivariate Cox regression survival analysis with PFS and OS as depending variables. Additional patient characteristics were introduced in the multivariate analyses based on univariately tested results if *P*-value < 0.1. Hazard ratios (HR) were generated considering patients with the most common clinical factor or genotype as the reference group. Missing data were kept as missing except for factors in the MSKCC score and the ECOG performance status. Patients with missing performance status (n = 2), LDH (n = 2), hemoglobin (n = 1) and baseline calcium values (n = 2) were assumed to be part of the worse prognosis scores. Accordingly, MSKCC scores were increased with one risk factor in 5 patients and with

3. Table Univariate and multivariate analyses of overall survival in mRCC patients treated with sunitinib

		Univariate Analyses*			Multivariate Analyses		
Factors	**No**	**Median OS (months)**	**95% CI**	***P***	**HR****	**95% CI**	***P***
Clinical factors							
MSKCC risk factors				<			<
0	33	Not reached	-	0.001	2.273	1.595-	**0.001**†
1-2	81	14.8	11.8-17.7			3.238	
≥ 3	22	10.9	7.2-14.7				
No. of metastatic sites				0.058			0.097†
1	31	28.8	15.3-42.2		1.273	0.957-	
2	47	15.6	13.5-17.7			1.693	
≥ 3	58	13.2	9.5-16.9				
Genetic factors pharmacokinetic pathway							
NR1I2 -25385C/T				0.017			0.178
CC + CT *vs.*	118	17.1	12.9-21.2		1		
TT	18	10.2	7.4-13.1		1.490	0.834-	
						2.660	
ABCB1 haplotype‡				0.097			0.078
Other-other *vs.*	100	15.4	12.4-18.3		1		
TCG-other	29	23.9	9.0-38.7		0.593	0.332-	
						1.061	
ABCG2 34G/A				0.072			0.069
GG *vs.*	124	15.4	12.5-18.3		1		
AG	12	39.9	16.7-63.1		0.416	0.162-	
						1.070	
Genetic factors pharmacodynamic pathway							
PDGFR-α haplotype‡‡				0.002			0.108
GCGT-GCGT *vs.*	78	24.2	17.2-31.2		1		
GCG-other + other-other	56	14.8	11.6-17.9		1.458	0.920-	
						2.310	
VEGFR-2 -1718T/A				0.022			**0.016**
AA + AT *vs.*	125	16.3	12.4-20.2		1		
TT	8	9.4	7.2-11.7		2.907	1.224-	
						6.903	

* Only factors with *P*-value < 0.1 level are presented; factors with *P*-value < 0.1 in the univariate analyses were selected for multivariate analyses

** HR < 1.0 indicates that the factor associates with improved OS, HR > 1.0 associates with worse OS

† Multiplicative model, HR per increase in MSKCC class or number of disease sites class

†† Risk groups according to Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria [based on the 5 risk factors: low Karnofsky performance status (< 80%), high LDH (> 1.5 times the upper limit of normal), low serum hemoglobin, high corrected serum calcium (> 10 mg/dL), and time from initial diagnosis to treatment < 1 year] (22).

Description haplotypes: ‡ = *ABCB1* 3435C/T, 1236C/T and 2677G/T; ‡‡ = *PDGFR-α* 1580T/C -1171C/G -735G/A -573G/T

Abbreviations: 95% CI, 95% confidence interval; OS, overall survival; HR, hazard ratio

two risk factors in one patient. As a result 3 patients were categorized into the intermediate risk group, whereas 3 other patients were categorized into the poor risk group. Patients with missing ECOG performance statuses (n = 2) were scored as ECOG = 1. To test these assumptions, the multivariate analyses were performed with and without the replacement of the patients with missing factors in the MSKCC score. Similar results were generated, indicating that the replacement was legitimate. All statistical analyses were performed using SPSS 16.0 software (SPSS, Chicago, IL). A 20% improvement (HR, 0 = 0.44) in PFS at ~1 year in patients with sunitinib was judged to be clinically meaningful by the investigators designing the study. Forty-six events with disease progression were estimated to be needed to detect such an improvement using a two-sided, unstratified log-rank test with an overall significance level of 0.05 and power of 0.80. All results from the multivariate analyses with *P*-values < 0.05 were considered significant. Since this is an explorative study, no correction for multiple testing was made.

RESULTS

Study Population

The main patient characteristics are presented in Table 1. Thirty-one (22.8%) patients had one metastatic site, 47 (34.6%) patients had two metastatic sites and 58 (42.6%) patients had at least 3 metastatic sites. According to the MSKCC prognostic criteria most patients (59.6%) were categorized into the intermediate risk group, whereas 24.3% and 16.2% of the patients were categorized into the favorable and poor risk group, respectively. At the time of the analysis, 47 (34.6%) patients were alive and 92 (67.6%) patients had disease progression. Overall, the median PFS time was 10.0 months (range, 7.6-12.4 months) and the median OS time was 16.3 months (range, 13.5-19.2 months). Of the clinical characteristics, the MSKCC risk factors had the largest contribution to PFS and OS (P = 0.001 and P < 0.001, respectively) (Tables 2 and 3). In addition, the number of metastatic sites, age and the ECOG performance status were prognostic for PFS (P = 0.019, 0.047 and 0.049, respectively), whereas only the ECOG performance status and the number of metastatic sites were also prognostic for OS (P = 0.004 and 0.058, respectively).

Table 4: Polymorphisms genotyped in the pharmacokinetic and pharmacodynamic pathways of sunitinib

	Gene	Polymorphism	rs-number
Pharmacokinetic pathway	*ABCG2*	421C/A	rs2231142
		34G/A	rs2231137
		-15622C/T	*
		1143C/T	rs2622604
	ABCB1	3435C/T	rs1045642
		1236C/T	rs1128503
		2677G/T	rs2032582
	NR1I2	-25385C/T	rs3814055
		-24113G/A	rs2276706
		7635A/G	rs6785049
		8055C/T	rs2276707
		10620C/T	rs1054190
		10799G/A	rs1054191
	NR1I3	5719C/T	rs2307424
		7738A/C	rs2307418
		7837T/G	rs4073054
	CYP3A5	6986A/G	rs776746
	CYP1A1	2455A/G	rs1048943
	CYP1A2	-163A/C	rs762551
Pharmacodynamic pathway	*VEGFR-2 (= KDR)*	-604T/C	rs2071559
		-92G/A	rs1531289
		54T/C	rs7692791
		1191C/T	rs2305948
		1718T/A	rs1870377
	VEGFR-3 (= FLT-4)	1501A/G	rs307826
	PDGFR-α	1580T/C	rs35597368
		-1171C/G	rs1800810
		-735G/A	rs1800813
		-573G/T	rs1800812
	FLT-3	738T/C	rs1933437

* No rs-number assigned yet

Baseline characteristics entered into the multivariate Cox models included the MSKCC risk factors, the number of metastatic sites, and age for PFS analyses, and the MSKCC risk factors and the number of metastatic sites for OS analyses. The ECOG performance status was excluded from the multivariate analyses due to co-linearity with the MSKCC prognostic criteria (22).

Pharmacogenetic Factors for Sunitinib and Progression-free survival

Among the 30 studied polymorphisms, only polymorphisms related to the pharmacokinetics of sunitinib were predictive of PFS (Table 2). A prolonged PFS was found in the univariate analysis of patients with presence of the A-allele in *CYP3A5* 6986A/G ($P = 0.017$), absence of a CAT copy in the *NR1I3* haplotype ($P = 0.021$), presence of the C-allele in *NR1I2* 8055C/T ($P = 0.025$), presence of the C-allele in *NR1I2* -25385C/T ($P = 0.032$), presence of a TCG copy in the *ABCB1* haplotype ($P = 0.072$) and presence of the A-allele in *ABCG2* (34G/A) ($P = 0.077$). Together with the MSKCC risk factors, the numbers of metastatic sites and age, these polymorphisms were entered with into the multivariate Cox model.

Multivariate analysis confirmed the following factors as significant (< 0.05) predictors of improved PFS: the MSKCC risk factors (HR: 1.988; 95%CI, 1.394-2.837), the number of metastatic sites (HR: 1.400; 95%CI, 1.042-1.880), age (HR: 1.031 per year increase; 95%CI, 1.003-1.060), presence of the A-allele in *CYP3A5* 6986A/G (HR: 0.266; 95%CI, 0.079-0.892), absence of a CAT copy in the *NR1I3* haplotype (HR: 1.758; 95%CI, 1.108-2.790), and a TCG copy in the *ABCB1* haplotype (HR: 0.522; 95%CI, 0.287-0.950).

Pharmacogenetic Factors for Sunitinib and Overall Survival

In univariate analysis, polymorphisms related to the pharmacokinetics and pharmacodynamics of sunitinib were predictive of OS (Table 3). Of the pharmacokinetic polymorphisms, presence of the C-allele in *NR1I2* -25385C/T ($P = 0.017$), presence of a TCG copy in the *ABCB1* haplotype ($P = 0.097$) and presence of the A-allele in *ABCG2* 34G/A ($P = 0.072$) were associated with a prolonged OS. In addition, univariate analyses identified two pharmacodynamic polymorphisms including two GCGT copies in the *PDGFR-α* haplotype and presence of the A-allele in *VEGFR-2* 1718T/A as factors for a prolonged OS ($P = 0.002$ and 0.022, respectively).

Multivariate analysis confirmed the MSKCC risk factors (HR: 2.273; 95%CI, 1.595-3.238) and the presence of the A-allele in *VEGFR-2* 1718T/A (HR: 2.907; 95%CI, 1.224-6.903) as significant (< 0.05) predictors of a prolonged OS. In multivariate analysis, there was a trend towards an improved OS for patients with a TCG copy in the *ABCB1* haplotype (HR: 0.593; 95%CI, 0.332-1.061; $P = 0.078$) or presence of the A-allele in *ABCG2* 34G/A (HR: 0.416; 95%CI, 0.162-1.070; $P = 0.069$).

Favorable Genetic Profiles and Outcome

Polymorphisms that were associated with an improved PFS were combined in a predictive model. Patients were categorized as carriers of the favorable genetic profiles when they had at least an A-allele in *CYP3A5*, a TCG copy in the *ABCB1* haplotype or a missing CAT copy in the *NR1I3* haplotype. Carriers with a favorable genetic profile (n = 95) had an improved PFS and OS as compared to non-carriers (median PFS: 13.1 *vs.* 7.5 months, $P =$

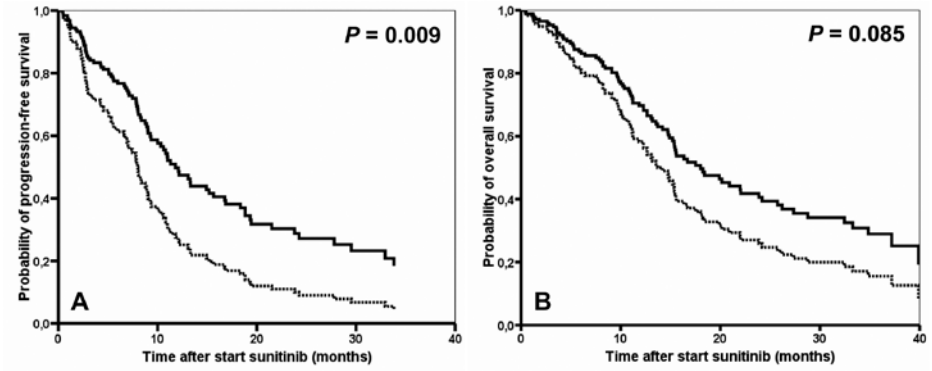

Figure 2 Favorable (_____) and non-favorable genetic profile (-----) in mRCC patients treated with sunitinib for progression-free (A) and overall survival (B) using multivariate Cox regression analysis. Patients were categorized as carriers of the favorable genetic profiles when they had at least an A-allele in *CYP3A5*, a TCG copy in the *ABCB1* haplotype or a missing CAT copy in the *NR1I3* haplotype.

0.001 and median OS: 19.9 *vs.* 12.3 months, $P = 0.009$). Multivariate analysis including the clinical factors showed consistent predictive value of the model for PFS and showed a trend for OS (HR: 0.541; 95%CI, 0.340-0.860, $P = 0.009$ and HR: 0.667; 95%CI, 0.420-1.058, $P = 0.085$, respectively) (Figure 2).

DISCUSSION

In mRCC patients treated with sunitinib, the MSKCC risk groups and the number of metastatic sites are clinical factors that are usually associated with PFS and OS (2,3,7). However, these clinical factors are prognostic criteria that are associated with the extent of the disease and do not necessarily predict antitumor efficacy of a specific drug. As an increasing number of drugs is currently available for the treatment of mRCC (25), tools are needed to identify patients who predispose to benefit from sunitinib treatment and to select individual mRCC patients for treatment with this drug. The efficacy of sunitinib may be influenced by multiple genes encoding for enzymes, efflux transporters and targets related to the pharmacokinetics and pharmacodynamics of sunitinib. Therefore, we analyzed whether SNPs in the pharmacokinetic and pharmacodynamic pathways of sunitinib were predictive of PFS and OS in patients with clear-cell mRCC. Our study showed that next to 3 clinical characteristics (MSKCC prognostic criteria, number of metastatic sites and age), 3 genetic variants in the *CYP3A5*, *NR1I3* and *ABCB1* genes were predictive factors for PFS. In addition, a role of an A-allele in *VEGFR-2* 1718T/A for a prolonged OS as a secondary outcome was found.

Clinical benefit from sunitinib treatment may depend on systemic exposure to sunitinib. Sunitinib is metabolized primarily to the active N-de-ethylated metabolite SU12662,

which reaches similar plasma concentrations and has equipotent biochemical activity as the parent compound (26). Thereafter, SU12662 undergoes a second N-de-ethylation step, which occurs at a slower rate, to the inactive metabolite SU14335. Recently, a meta-analysis of pharmacokinetic data in 443 patients treated with sunitinib, showed that higher plasma levels of sunitinib and its active metabolite SU12662 were associated with a prolonged time-to-tumor progression and OS (27). Currently, it is not clear which underlying factors account for the observed inter-individual differences in plasma levels of sunitinib and its active metabolite SU12662. Inter-individual differences in sunitinib exposure may be the result of variations in sunitinib absorption, metabolism, distribution and excretion through metabolizing enzymes and transporter proteins. Concerning the pharmacokinetics of sunitinib, the present study identified variants in three genes (*CYP3A5*, *NR1I3* and *ABCB1*) as predictive factors for PFS in mRCC patients treated with sunitinib. Although these polymorphisms were not predictive of OS, there was a trend towards a prolonged OS for patients with a TCG copy in the *ABCB1* haplotype. Additional treatment after discontinuation of sunitinib treatment may explain the discrepancy between the results of the PFS and OS analyses, as 26% of patients were subsequently treated with at least one other agent, including sorafenib (22%), temsirolimus (2%) and everolimus (4%).

CYP3A4 is the major key enzyme for the biotransformation of sunitinib (11). However, no polymorphisms of *CYP3A4* were analyzed in the present study, as there are no functional polymorphisms in *CYP3A4* that meet our described criteria for SNP selection yet. The CYP3A5 enzyme is another important enzyme for the metabolism of several TKIs including erlotinib, gefitinib and imatinib (13). Similarly, the CYP3A5 enzyme may metabolize sunitinib and was therefore included in the analysis, though the sunitinib-metabolizing capacity of CYP3A5 has to be confirmed. In the current study, presence of the A-allele in *CYP3A5,* a SNP which leads to the CYP3A5 expressor phenotype (28,29), was a predictive factor for a prolonged PFS. As the CYP3A5 expressor phenotype may lead to increased metabolism of sunitinib, these findings suggest that the prolonged PFS in patients with presence of the A-allele in *CYP3A5* may be caused by increased levels of the active metabolite SU12662, which has a longer half-life than the parent compound (80-100 hours *vs.* 40-60 hours) (26). Furthermore, polymorphisms in other genes (*NR1I2 and NR1I3*) that regulate the expression of CYP3A4 (12) were identified as predictive factors for outcome in sunitinib-treated mRCC patients.

The efflux transporters ABCB1 and ABCG2 play an important role in drug absorption, excretion, cellular accumulation and resistance (10). Consequently, polymorphisms in *ABCB1* and *ABCG2* may affect drug absorption and excretion of sunitinib. In the present study, the found associations between polymorphisms in the *ABCB1* haplotype (a TCG copy) and the *ABCG2* gene (presence of the A-allele), and improved outcome suggest that these polymorphisms may lead to reduced efflux transport of sunitinib into the

gastrointestinal lumen and bile, resulting in increased systemic exposure of sunitinib. In vitro studies have reported conflicting data on the affinity of sunitinib for ABCB1 and ABCG2 (8,9). Hu et al (8) found a moderate affinity of sunitinib for ABCB1 and a negligible transport of sunitinib in cells over-expressing ABCG2, whereas another study reported higher sunitinib affinity for ABCG2 compared with ABCB1 (9). Furthermore, sunitinib reversed ABCG2-mediated multidrug resistance by inhibiting the drug efflux function of ABCG2 (30). This inhibitory capacity of sunitinib on ABCG2 appeared to be sensitive for the *ABCG2* 1291T>C genotype (31). In addition, another SNP in *ABCG2* (421C>A) was associated with increased sunitinib exposure (32).

The ABC transporters may contribute to multidrug resistance in tumors by actively extruding drugs from cancer cells. In RCC, an increase in ABCB1 expression (33,34) and activity (35) has been reported, suggesting a contribution of ABCB1 to the resistance of RCC to some anticancer drugs. Although polymorphisms in *ABCB1* and *ABCG2* may be associated with the development of RCC (36,37), it is currently not known whether polymorphisms in *ABCB1* and *ABCG2* are associated with the expression and function of these transporters at the somatic level in renal cancer cells (38). Nevertheless, the role of efflux transporters in tumor cells may be limited for acquired resistance to sunitinib, which may develop after an initial response to sunitinib, as acquired resistance to sunitinib may be more related to physiological changes in the microenvironment of tumors, allowing reestablishment of angiogenesis during sunitinib treatment (16).

Clinical efficacy of treatment with TKIs may also be related to specific mutations in drug targets, as was shown for imatinib and gefitinib (39,40). Currently, it is not known which targets in RCC predict response to sunitinib or whether the somatic polymorphisms of targets in RCC correlate with genetic polymorphisms obtained from germline cells. Of the studied pharmacodynamic polymorphisms of sunitinib, only a polymorphism of *VEGFR-2* 1718T/A was associated with a decreased OS in multivariate analysis, whereas the presence of two GCGT copies in the *PDGFR-α* haplotype was associated with a prolonged OS in univariate analysis. However, no significant association between these polymorphisms and PFS was found. These findings may suggest that polymorphisms in *VEGFR-2* and *PDGFR-α* may be associated with the nature of the disease and may therefore be prognostic instead of predictive. However, prospective validation in an independent mRCC cohort that is not treated with sunitinib is necessary to determine whether the associated polymorphisms of the present study are predictive markers of sunitinib activity or prognostic markers of mRCC disease.

In our previous study, several polymorphisms in genes involved in the pharmacokinetic and pharmacodynamic pathways of sunitinib were associated with sunitinib-induced toxicity (19). Polymorphisms of *NR1I3* (absence of a CAG copy in the haplotype), *ABCB1* (presence of a TTT copy) and *VEGFR-2* (T allele in 1191 C/T) were significantly related with an increased risk for leucocytopenia, hand-foot syndrome and any toxicity > grade

2, respectively (19). In the present study however, other genotypes or haplotypes in *NR1I3*, *ABCB1* and *VEGFR-2* were associated with clinical outcome. It is currently not clear how the different genotypes and haplotypes between these studies are related, but it is conceivable that severity and prevalence of some sunitinib-induced toxicities may basically depend on inhibition of a specific molecular pathway rather than variation in exposure, whereas other sunitinib-induced toxicities may mainly depend on exposure to the drug. In addition, the discrepancy between these two studies may be the result of different study populations, as patients with different malignancies were included in our previous study. Recently, Houk et al (27) have shown a relationship between sunitinib exposure and the probability of grade ≥ 1 fatigue, the absolute neutrophil count and the changes in diastolic blood pressure. However, these sunitinib-induced toxicities cannot be extrapolated to our previous toxicity study (19), as different toxicities and grades of toxicity were analyzed in both studies.

A potential limitation of the study is the retrospective design. As a result, pharmacokinetic data were not available to correlate polymorphisms of *CYP3A5*, *NR1I3* and *ABCB1* with plasma levels of sunitinib and its active metabolite SU12662. If future studies reveal a relation between sunitinib exposure and presence of an A-allele in *CYP3A5* 6986A/G, absence of a CAT copy in the *NR1I3* haplotype or presence of a TCG copy in the *ABCB1* haplotype, the sunitinib starting dose may be adjusted to dose-escalation of sunitinib > 50 mg/daily for patients without these genotypes and haplotypes. The non-beneficial genetic profile may be used to select patients who may be eligible for alternative dosing schedules with intensive monitoring of plasma levels of sunitinib and its active metabolite SU12662. Before this genetic profile can be implemented, prospective validation in an independent patient population is necessary.

In conclusion, pharmacokinetic but not pharmacodynamic polymorphisms were independent predictive factors for PFS in patients with clear-cell mRCC who were treated with sunitinib. Patients with an A-allele in *CYP3A5* 6986A/G, absence of a CAT copy in the *NR1I3* haplotype or presence of a TCG copy in the *ABCB1* haplotype had a prolonged PFS. These polymorphisms may be valuable factors to identify patients with reduced exposure to sunitinib in order to improve treatment strategies in these patients. The findings of this study advocate more pharmacokinetic studies in patients treated with sunitinib to further elucidate the role of these genetic determinants in sunitinib exposure and efficacy.

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Chapter 8

Single nucleotide polymorphisms (SNPs) in the endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor (VEGF) genes independently predict sunitinib-induced hypertension

Eechoute K.*, van der Veldt A.A.M.*, Oosting S., Kappers M.H.W., Wessels J.A.M., Gelderblom H., Guchelaar H.-J., Reyners A.K.L., van Herpen C.M.L., Haanen J.B., Mathijssen R.H.J., Boven E.

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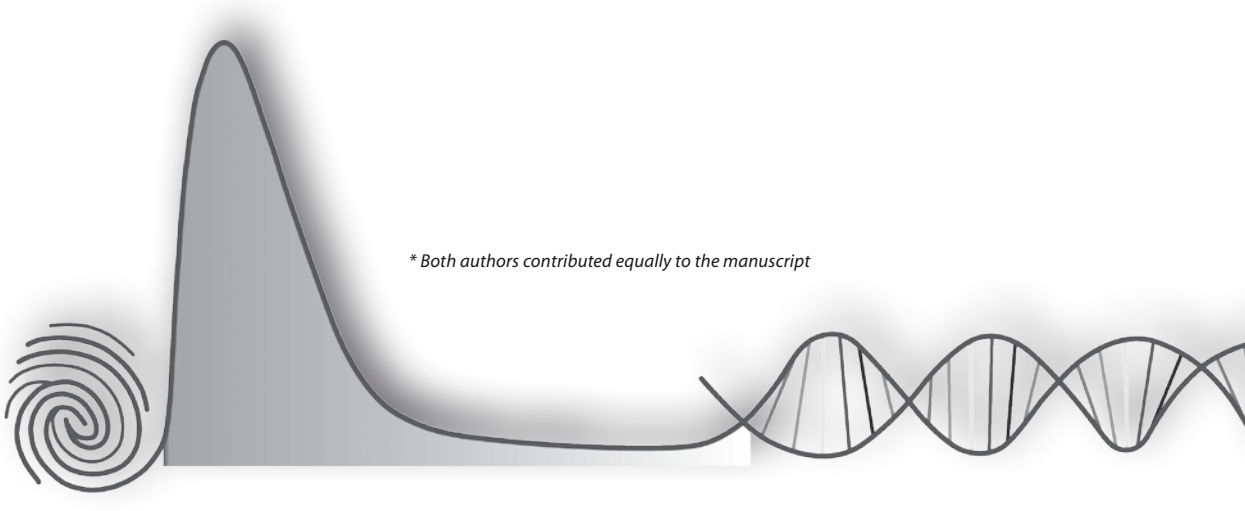

** Both authors contributed equally to the manuscript*

ABSTRACT

Purpose: Hypertension is a common side-effect in patients treated with sunitinib. We investigated whether polymorphisms in genes involved in regulation of blood vessel tone can predict blood pressure change and hypertension during sunitinib treatment. In addition, possible association of hypertension with survival was evaluated in the subset of patients with metastatic renal cell cancer (mRCC).

Patients and Methods: In 291 sunitinib-treated patients, single nucleotide polymorphisms in *VEGFA* (rs2010963, rs833061, rs3025039, rs699947), *VEGFR-2* (rs1870377), *ET-1* (rs5370) and *eNOS* (rs2070744) were multivariately tested against the maximal change in systolic (SBP), diastolic (DBP) and mean arterial (MAP) blood pressure and hypertension grades during the first treatment cycle. Overall survival (OS) was assessed in mRCC patients of the clear-cell type (n = 158) for a possible relation with hypertension.

Results: A higher rise in SBP and MAP was associated with presence of an ACG haplotype in *VEGFA* rs699947, rs833061 and rs2010963 ($P = .014$ and $P = .036$). Development of hypertension grade 3 was associated with the same haplotype and with a C allele in *eNOS* rs2070744 ($P = .031$ and $P = .045$; respectively). Mean OS in sunitinib-treated mRCC patients suffering from hypertension was prolonged with 7.2 months *(*$P = .035$; $P = .026$; for SBP and DBP, respectively*)*.

Conclusion: Hypertension during sunitinib treatment in mRCC patients of the clear-cell type is an independent factor for OS and is potentially suitable as a biomarker for outcome. Genetic polymorphisms in *VEGFA* and *eNOS* independently predict blood pressure rise and/or severe hypertension in sunitinib-treated patients.

INTRODUCTION

Sunitinib is a multi-targeted tyrosine kinase inhibitor, known to inhibit autophosphorylation in several drug targets such as the vascular endothelial growth factor receptor (VEGFR) and KIT.[1] VEGFR and its ligand VEGF play a key role in the regulation of angiogenesis,[2] which is essential for growth and metastatic spread of malignant tumors.[3-4] The introduction of this agent in the clinic has improved the perspectives of several patient populations including patients with metastatic renal cell cancer (mRCC), advanced gastrointestinal stromal cell tumors (GIST) and neuroendocrine tumors.[5-8]

Treatment with VEGF inhibitors, such as sunitinib, is associated with an increase in blood pressure in a substantial proportion of patients. Although hypertension induced by drugs targeting the VEGF pathway is usually manageable, a number of patients require dose reductions of these drugs or even treatment discontinuation. Recent studies, however, have shown that the development of hypertension during VEGF/VEGFR-2 inhibition is associated with improved clinical outcome in mRCC patients.[9-14] The exact mechanism by which VEGF inhibitors induce hypertension has not been completely clarified. Previous studies have shown that VEGFR-2 is involved in the regulation of vascular tone. Activation of VEGFR-2 via phosphoinositide 3-kinase (PI3K) and its down-stream serine protein kinase Akt stimulates endothelium-derived nitric oxide synthase (eNOS), subsequently leading to the production of the potent vasodilator nitric oxide (NO) (**Figure 1**). In addition, decreased NO bioavailability favors the production and/or activity of endothelin-1 (ET-1) through the loss of inhibitory effects of NO on ET-1.[15-16] Indeed, plasma ET-1 concentrations have been described to increase in subjects treated with sunitinib.[17-18] Consequently, it could be hypothesized that inhibition of VEGFR-2 causes an imbalance between the vasodilator NO en the vasoconstrictor ET-1, favouring ET-1 and thereby resulting in the development of hypertension.

Currently, predisposing factors for the development of hypertension during treatment with VEGF inhibitors have not been identified.[19] Polymorphisms in genes that are associated with the regulation of blood pressure may be involved in the differential occurrence of this side-effect in individual patients. In a previous study in patients with mRCC and GIST, we have demonstrated that several single nucleotide polymorphisms (SNP) in specific genes encoding for metabolizing enzymes, efflux transporters and drug targets are associated with sunitinib-induced toxicities.[20] Polymorphisms in *VEGFA* and *VEGFR-2* and down-stream mediators *eNOS* and *ET*-1 may be important factors in blood pressure changes, since inhibition of the VEGFR-2 signalling route may decrease the production of NO leading to peripheral vasoconstriction[21-22] and subsequent development of hypertension. The primary objective of the present study was to evaluate the predictive value of genetic polymorphisms in *VEGFA*, *VEGFR-2*, *eNOS* and *ET-1* for the development of hypertension in patients treated with sunitinib. The secondary objective was to evaluate

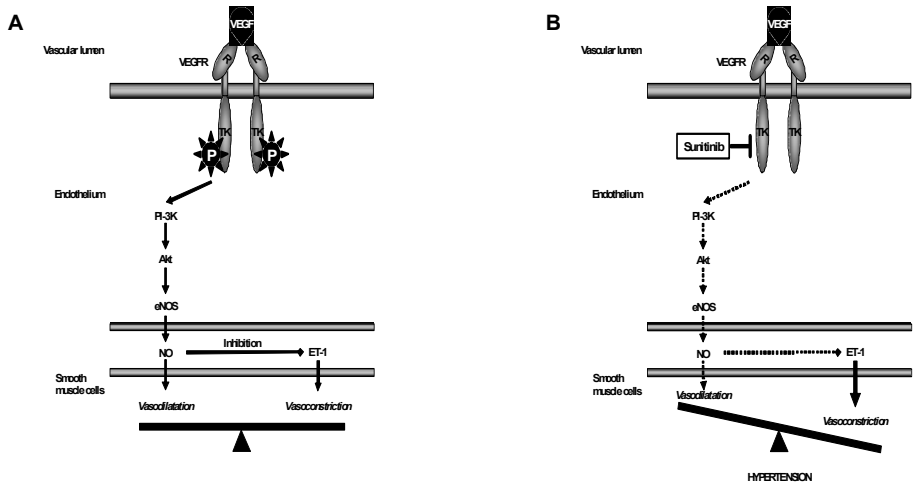

Figure 1 Role of vascular endothelial growth factor receptor in blood vessel tone in patients (A) without or (B) with sunitinib-induced hypertension.
Activation of VEGFR-2 via phosphoinositide 3-kinase (PI3K) and its downstream serine protein kinase Akt stimulates endothelium-derived nitric oxide synthase (eNOS), subsequently leading to the production of the potent vasodilator nitric oxide (NO). NO also exerts an inhibitory effect on the vasoconstrictor endothelin-1 (ET-1) (1A). In patients treated with sunitinib, inhibition of VEGFR-2 causes an imbalance between the vasodilator NO en the vasoconstrictor ET-1, favouring ET-1 and thereby resulting in the development of hypertension (1B).

the predictive value of hypertension for survival outcome in the subset of sunitinib-treated mRCC patients of the clear-cell type in order to validate recent findings.

METHODS

Patients

Patients were considered eligible when treated with sunitinib for at least four consecutive weeks. DNA from 291 cancer patients was collected in six Dutch medical centers between June 2004 and October 2010 at the Erasmus University Medical Center (n = 105), the Netherlands Cancer Institute (n = 49), Leiden University Medical Center (n = 47), VU Medical Center (n = 42), University Medical Center Groningen (n = 40) and Radboud University Nijmegen Medical Center (n = 8). Thirty-six of 291 patients had to be excluded from analysis due to lack of sufficient blood pressure measurements. For all endpoints, 255 patients were available for analysis (See **Table 1** for clinical characteristics). For the primary outcomes (change in SBP and DBP), the following baseline clinical characteristics were entered into the multivariate linear regression analysis: weight, performance

Table 1: Characteristics of evaluable patients (N=255)

Characteristic	No. (range)	%
Median age (years)	60 (20 – 89)	
Sex – no. (%)		
Male	161	63.1
Female	94	36.9
Ethnicity		
Caucasian	232	91
Other	15	5.9
Unknown	8	3.1
Tumor type		
Renal cell carcinoma	193	75.7
Clear cell	167	65.5
Non clear cell	22	8.6
Unknown	4	1.6
GIST	53	20.8
Other tumor types	9	3.5
Median weight (kilograms)	77.0 (43.9 – 135.0)	
ECOG performance status		
0	104	40.8
1	123	48.2
2	20	7.8
3	7	2.7
Unknown	1	0.4
Median glomerular filtration rate (milliliter/minute)	76.5 (21 – 176)	
Antihypertensive therapy at baseline		
Yes	110	56.1
No	143	43.1
Unknown	2	0.8
Sunitinib dose during first treatment cycle (milligrams)		
12.5	3	1.2

25	1	0.4
37.5	6	2.4
50	242	94.9
Unknown	3	1.2
Pre-existing hypertension		
Yes	76	29.8
No	179	70.2
Pre-existing hypercholesterolemia		
Yes	17	93.3
No	238	6.7
Pre-existing diabetes mellitus		
Yes	7	2.7
No	248	97.3
Prexisting other cardiovascular event/disease		
Yes	39	15.3
No	216	84.7
Median systolic blood pressure at start of therapy	135 (90 – 210)	
Median diastolic blood pressure at start of therapy	80 (43 – 105)	

Abbreviations: GIST, gastrointestinal stromal tumor; ECOG, Eastern Cooperative Oncology Group

status, SBP and DBP at start of therapy. For change in MAP, weight and SBP and DBP at start of therapy were tested in a multivariate linear regression analysis. For the secondary endpoint, hypertension grade 0-2 versus ≥ 3, no clinical characteristics were entered into a multivariate logistic regression. Due to a lack of proportionality among classes, performance status was recoded into dummy variables. This study was approved by the institutional medical ethics review boards and performed in accordance with the Declaration of Helsinki.

Study design

Residual blood or serum samples taken for routine patient care were stored at -20°C or lower temperature at the local hospital laboratory. At least one whole blood or serum sample was available for this study from each patient. All samples were anonymized by

a third party, according to the instructions stated in the Codes for Proper Use and Proper Conduct in the Self-Regulatory Codes of Conduct (www.federa.org).

Office systolic (SBP) and diastolic blood pressures (DBP) were measured manually or electronically (with oscillometric devices) during the first treatment cycle with sunitinib at start of therapy (baseline), day 14 and day 28. Baseline patient characteristics that were considered relevant for the development of hypertension were: age, sex, ethnicity, tumor type, weight, Eastern Cooperative Oncology Group (ECOG) performance status, estimated creatinin clearance rate (Cockcroft-Gault formula), pre-existing hypertension, hypercholesterolemia and diabetes mellitus, a history of cardiovascular disease, and SBP and DBP at start of therapy. Mean arterial pressure (MAP) was estimated by using the formula: MAP ≈ 2/3 DBP + 1/3 SBP. Maximum change in blood pressure during the first treatment cycle measured either on day 14 or day 28 was calculated by subtracting SBP, DBP and MAP at baseline from the highest measured SBP, DBP and MAP, respectively, without initiation of antihypertensive therapy or dose modification of antihypertensive agents in use at baseline. In addition, hypertension during the first treatment cycle was assessed by using Common Terminology Criteria for Adverse Events version 3.0 (CTCAE v3.0).

Genetic polymorphisms

Seven polymorphisms in four genes were selected which were considered to be involved in blood pressure regulation possibly affected by sunitinib. Selection criteria were a minor allele frequency of more than 0.2 in a Caucasian population as found in literature or the dbSNP database (http://www.ncbi.nlm.nih.gov/snp) and an assumed clinical relevance based on previous literature.[23-29] The selected polymorphisms are listed in **Table 2**. Detailed methods for genotyping assay validation and haplotype estimation have been described previously.[20]

Table 2: selected polymorphisms.

Gene	Polymorphism	rs-number
VEGFA	405C>G	rs2010963
	-460C>T	rs833061
	936C>T	rs3025039
	-2578A>C	rs699947
VEGFR-2	1718T>A	rs1870377
EDN1	594G>T	rs5370
eNOS	-786T>C	rs2070744

Data analysis and statistical design

Primary endpoints for the pharmacogenetic association analysis were the maximal change in SBP, DBP and MAP in mmHg during the first treatment cycle. For the analysis of hypertension grades, dichotomous endpoints were used, expressed as grade 0-2 versus ≥ grade 3. All demographic and clinical variables were tested univariately against the selected primary outcomes using a χ^2 test, *t* test or *ANOVA* test, depending on the tested variables. A χ^2 test was also used to detect linkage desequilibrium (LD). Polymorphisms were initially tested with 2 *df*. If the initial 2 *df* tests resulted in $P \leq .1$, these polymorphisms were fitted into the most appropriate model (multiplicative, dominant, recessive). Polymorphisms were tested univariately against the primary outcomes, using a *t* test or *ANOVA* test for the change in SBP, DBP and MAP and by using a χ^2 test for the association analysis with hypertension grades. Genetic variables associated with the outcome variables (*P* value ≤ .1) were selected for a multivariate linear regression analysis with change in SBP, DBP and MAP or a multivariate logistic regression analysis with hypertension grades as depending variables. Post-hoc analysis showed that with the sample size in our study, a difference in SBP and DBP of 7.5 mmHg and 5.8 mmHg, respectively, could be detected with a power of 91%. All results from the multivariate analyses with $P \leq .05$ were considered significant. Since this was an exploratory study, no correction was applied for multiple testing.

In our survival analysis, the occurrence of hypertension during the first cycle of sunitinib was univariately (Kaplan-Meier analysis) and multivariately (Cox-regression

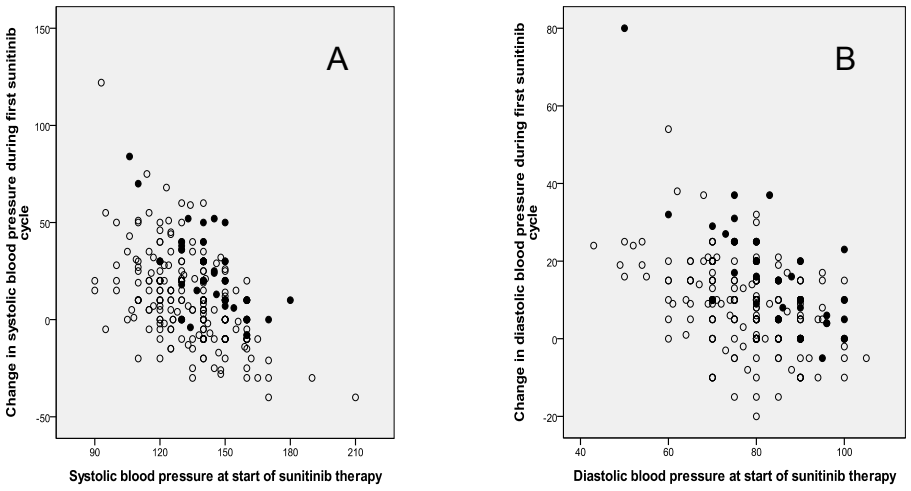

Figure 2 Closed symbols represent patients that experience hypertension grade 3 during the first cycle of sunitinib treatment. Open symbols represent patients that experience hypertension grade 0–2. Systolic blood pressure values are expressed in mmHg.

analysis) tested for association with progression-free and overall survival (PFS and OS, respectively). Baseline characteristics relevant for PFS were: age, Memorial Sloan-Kettering Cancer Center (MSKCC) prognostic criteria and number of disease sites and for OS were: MSKCC risk groups and number of disease sites, as defined previously.[30] Hypertension as a depending variable was defined as a recorded SBP > 140 mmHg or DBP > 90 mmHg or MAP > 110 mmHg during first sunitinib treatment cycle. All results in this study are presented as mean values (± standard deviation), unless stated otherwise.

RESULTS

Sunitinib-induced blood pressure changes during the first treatment cycle

Blood pressure values at start of sunitinib therapy (n = 255) were 135 ± 18 mmHg, 79 ± 11 mmHg and 98 ± 12 mmHg for SBP, DBP and MAP, respectively. During the first cycle of sunitinib therapy, 166 patients (65%) showed an increase in SBP (25 ± 18 mmHg), while a rise in DBP (15 ± 9 mmHg) was measured in 186 (73%) and a rise in MAP (16 ± 11 mmHg) in 193 (76%) patients. Thirty-three patients (13%) developed hypertension grade 3 during the first treatment cycle, while 222 patients (87%) showed hypertension grade 0-2 (162, 27 and 33 patients with grade 0, 1 and 2, respectively). For the change in blood pressure during the first sunitinib cycle, following clinical characteristics were significantly associated in a univariate analysis: SBP at baseline ($P < .001$) for change in SBP; and SBP and DBP at baseline for change in MAP ($P < .001$, $P = .001$, respectively).

For patients with low blood pressure at baseline, the rise in both SBP and DBP during treatment was higher than for patients with higher blood pressures at baseline (ANOVA, $P < .001$ and $P < .001$, for SBP and DBP, respectively; see **Figure 2**).

Pharmacogenetic risk factors for sunitinib-induced hypertension

Factors that were predictive for blood pressure change in the univariate and multivariate regression analyses are presented in **Table 3**. In a univariate analysis, an ACG haplotype in *VEGFA* rs699947 (-2578A>C), rs833061 (-460C>T) and rs2010963 (405C>G) was significantly associated with a higher increase in SBP during the first treatment cycle ($P = .026$). No significant associations were found between selected polymorphisms and DBP change during first cycle of sunitinib treatment. For change in MAP, presence of a *VEGFA* ACG haplotype in a dominant model was selected for multivariate analysis ($P = .053$). Presence of a *VEGFA* ACG haplotype was an independent predictor of change in SBP and MAP, indicating that the presence of an ACG copy increased the risk for higher rises in SBP and MAP. In order to confirm the independent prediction of blood pressure rise by presence of an ACG haplotype, further analysis showed that an ACG haplotype was not associated with higher baseline blood pressure or with higher incidence of

Table 3: Univariate and multivariate analyses of blood pressure in 255 evaluable study patients

Factor	Univariate analyses			Multivariate analyses		
	No.	Genotype	*P*	OR*	95% CI	*P*
Rise in systolic blood pressure						
Systolic blood pressure at start of therapy	255	N.A.	<.001	N.A.	N.A.	<.001
VEGFA haplotype**	182 65	ACG-ACG + ACG-other *versus* Other-other	.026	N.A.	N.A.	.014
Rise in mean arterial pressure						
Systolic blood pressure at start of therapy	255	N.A.	<.001	N.A.	N.A.	.004
Diastolic blood pressure at start of therapy	255	N.A.	.001	N.A.	N.A.	<.001
VEGFA haplotype	182 65	ACG-ACG + ACG-other versus Other-other	.053	N.A.	N.A.	.036
Hypertension CTCAE grade 3						
VEGFA haplotype	247	ACG-ACG → ACG-other → other-other	.083	0.59	0.34 – 1.03	.031
eNOS rs2070744	101 153	TT *versus* CC + CT	.051	1 2.62	1.08 – 6.35	.045

* OR < 1.0 indicates that the factor associates with lower incidence of hypertension grade 3 as compared to the reference genotype (most prevalent genotype or haplotype), OR > 1.0 indicates that the factor associates with higher incidence of hypertension grade 3 as compared to the reference genotype (most prevalent genotype or haplotype)

** Description haplotype: *VEGFA* rs699947 (-2578A>C), rs833061 (-460C>T), rs2010963 (405C>G)

Abbreviations: OR, odds ratio; 95% CI, 95 percent confidence interval; CTCAE, Common Terminology Criteria for Adverse Events; N.A, not applicable

antihypertensive drug treatment at baseline (data not shown). Multivariate linear regression analysis confirmed all selected clinical characteristics as inversely associated with change in SBP or MAP.

Concerning the incidence of hypertension during the first treatment cycle, presence of an ACG haplotype in *VEGFA* or presence of a C-allele in *eNOS* rs2070744 (-786T>C),

in a multiplicative or dominant model, respectively, was univariately associated with hypertension grade 3 (P = .083, and P = .051, respectively). When these polymorphisms were tested in a multivariate logistic regression analysis, an ACG haplotype significantly predicted grade 3 hypertension (OR: 1.69, confidence interval (CI) 0.97 – 2.94, P = .031). In addition, presence of a C-allele in *eNOS* -786T>C in a dominant model was independently associated with grade 3 hypertension (OR 2.62, CI 1.08 – 6.35, P = .045).

Blood pressure and survival in mRCC patients

To elucidate the possibly predictive value of high blood pressure during sunitinib treatment on survival in the subpopulation of 167 mRCC patients of the clear-cell type, outcome variables (PFS and OS) were compared between patients with and without hypertension during the first cycle of sunitinib, using Kaplan-Meier survival analyses (see **Figure 3**). Seven patients were excluded from analysis due to lack of sufficient survival data and/or data on clinical co-variables, resulting in 158 evaluable patients. No significant association with PFS was seen in patients with hypertension (see **Table 4**). Patients with a MAP, SBP or DBP above the defined threshold had a significant reduction in relative risk of death as compared to patients with blood pressure below the defined threshold (see **Table 4**). OS was prolonged with 7.2 months in patients suffering from

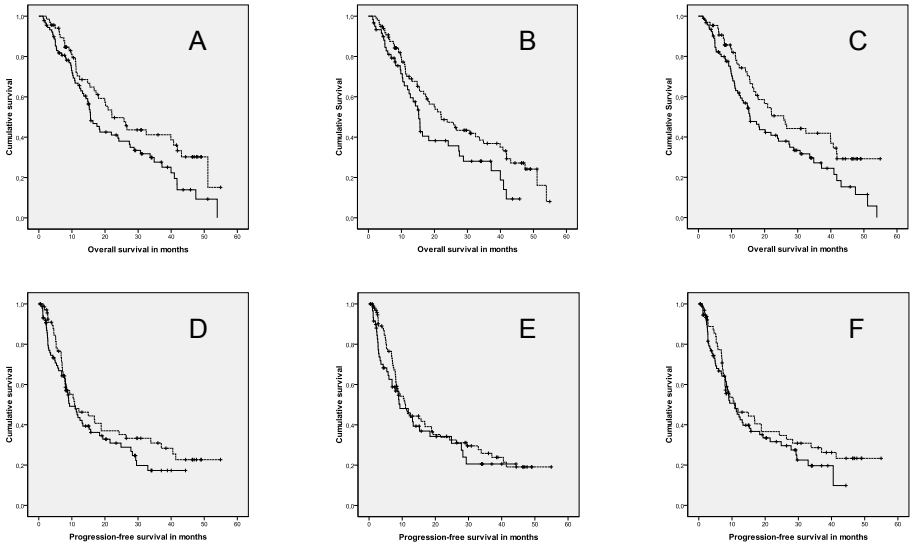

Figure 3 Kaplan-Meier survival curves for sunitinib-treated metastatic renal cell carcinoma patients according to blood pressure during first cycle of treatment. Dotted lines represent cumulative survival in patients with MAP >110 mmHg (3A/3D), or with SBP > 140 mmHg (3B/3E), or with DBP > 90 mmHg in (3C/3E). Continuous lines represent cumulative survival in patients with MAP ≤ 110 mmHg (3A/3D), or with SBP ≤ 140 mmHg (3B/3E), or with DBP ≤ 90 mmHg (3C/3F).

Table 4: Survival in 158 sunitinib-treated clear-cell mRCC patients with or without hypertension

Factors		Kaplan-Meier analysis			Multivariate Cox-regression analysis*		
	No. (%)	PFS	95% CI	*P*	HR	95% CI	*P*
MAP > 110 mmHg	68 (43)	22.6	13.2 – 20.3	.214	.756	.504 – 1.134	.177
≤ 110 mmHg	90 (57)	16.8	17.3 – 27.8				
SBP > 140 mmHg	98 (62)	21.1	16.9 – 25.4	.363	.691	.457 – 1.045	.080
≤ 140 mmHg	60 (38)	17.1	12.6 – 21.7				
DBP > 90 mmHg	65 (41)	22.3	16.9 – 27.6	.332	.828	.551 – 1.244	.363
≤ 90 mmHg	93 (59)	17.0	13.5 – 20.5				
	No. (%)	OS	95% CI	*P*	HR	95% CI	*P*
MAP > 110 mmHg	68 (43)	29.1	24.1 – 34.1	.037	.644	.426 – .975	.038
≤ 110 mmHg	90 (57)	22.8	19.0 – 26.6				
SBP > 140 mmHg	98 (62)	27.9	23.9 – 31.9	.035	.532	.349 – .810	.003
≤ 140 mmHg	60 (38)	20.7	16.6 – 24.8				
DBP > 90 mmHg	65 (41)	29.9	24.8 – 35.0	.026	.633	.417 – .961	.032
≤ 90 mmHg	93 (59)	22.7	18.9 – 26.6				

* Relevant baseline characteristics included in the multivariate analysis were: for PFS: age, Memorial Sloan-Kettering Cancer Center (MSKCC) risk groups, and number of disease sites and for OS: MSKCC risk groups and number of disease sites, as defined previously

Abbreviations: PFS, mean progression-free survival in months; 95% CI, 95 percent confidence interval; HR, Hazard ratio; MAP, Mean arterial pressure; SBP, Systolic blood pressure; DBP, Diastolic blood pressure; OS, Mean overall survival in months

high SBP or DBP *(P* = .035; *P* = .026; for SBP and DBP, respectively*)* and 6.3 months in patients suffering from high MAP (*P* = .037). Multivariate analysis confirmed that high MAP, SBP and DBP during the first cycle of sunitinib treatment are predictive markers for improved OS, independently of MSKCC criteria and number of disease sites (see **Table 4**).

DISCUSSION

The present results demonstrate an increase in blood pressure in approximately 70% of patients treated with sunitinib, resulting in grade 3 hypertension in 13% of all treated patients. Persistent hypertensive disease can lead to serious cardiovascular events, and in rare cases malignant hypertension may be complicated by reversible posterior leukoencephalopathy.[31-32] Prediction and adequate treatment of these adverse reactions are therefore clinically relevant. The aim of this study was to investigate if selected genetic differences in pathways involved in the regulation of blood vessel tone, are independently predictive for the occurrence of high-grade hypertension or for the extent of blood pressure rise during sunitinib treatment.

The current analysis shows that an ACG haplotype in *VEGFA* is a significant factor for a higher rise in SBP and MAP as well as for the incidence of hypertension grade 3 during the first cycle of sunitinib therapy. More than one out of five study subjects who were homozygous for the ACG haplotype developed hypertension grade 3 versus only 7.7% of patients who carried no copy of ACG.

In addition, presence of an *eNOS* -786C allele is related to a 2.6-fold increase in the risk of hypertension grade 3 during the first cycle of sunitinib treatment. These pharmacogenetic associations demonstrate the clinical importance of polymorphisms in genes coding for VEGF and its downstream mediator NO during sunitinib treatment. Inhibition of VEGFR-2 pathways in these patients combined with possibly reduced VEGF levels and decreased NO production may enhance the development of hypertension.

The biological effect of an ACG haplotype at position -2578, -460 and 405 is subject of debate as consensus has yet to be reached on the correlation between these SNPs and VEGF expression. In various patient populations -2578A, -460C and 405G carriers showed reduced circulating VEGF levels,[33-35] while other studies reported no association between VEGF plasma levels and haplotypes including the -2578A and 405G alleles.[34,36] In contrast, clinical have shown a correlation of the ACG haplotype with ischemic heart disease and myocardial infarction[37] and of the -2578AA genotype with coronary artery disease and atherosclerosis.[38-39] All these conditions are closely related to pre-existing chronic hypertensive disease.

Concerning the *eNOS* -786T>C polymorphism, there is accumulating evidence that presence of a C allele has functional consequences with reports on decreased transcriptional activity and lowered NO plasma levels.[40-44] In addition, a correlation has been found between the -786C allele and the incidence of diabetic retinopathy, coronary spasms and preeclampsia.[45-46] The latter finding may also be complementary to our results as it was recently shown that sunitinib-induced hypertension ressembles part of a preeclampsia-like syndrome.[18]

Early identification of patients at risk to develop hypertension on the basis of these genetic polymorphisms could yield specific monitoring methods, timely selection of antihypertensive agents and ultimately optimize sunitinib treatment. For instance, in our study population, an inverted association was observed between blood pressure at start of therapy and blood pressure increase during the first cycle of sunitinib treatment. This association may be explained by a phenomenon called "white-coat hypertension" in which (hospital / doctor-induced) stress-related factors may result in higher blood pressure at start of anti-cancer treatment compared to follow up measurements.[47] Alternatively, it may also reflect a physiological maximum in blood vessel tone due to VEGF inhibition. Thus, it can be concluded that patients with a high blood pressure at start of therapy are not at risk of developing higher increases in blood pressure during sunitinib treatment than patients with blood pressure within the normal range. In contrast, in patients developing grade 3 hypertension during the first cycle of sunitinib, both the rise in blood pressure as well as blood pressure at start of therapy were higher as compared to patients with grade 0-2 hypertension (31 ± 17 vs 24 ± 19 mmHg, $P = .032$; and 20 ± 15 vs 13 ± 8 mmHg, $P = .027$; for change in SBP and DBP, respectively; and 140 ± 15 vs 128 ± 15 mmHg, $P < .001$; 82 ± 11 vs 76 ± 11 mmHg, $P = .005$; for baseline SBP and DBP, respectively; see **Figure 2**). Pharmacogenetic screening may therefore help to distinguish these patients among subjects with high baseline blood pressure who are predisposed for the development of severe hypertension requiring (adjustment of) antihypertensive medication.

Interestingly, hypertension in mRCC patients treated with sunitinib has recently been correlated with survival benefit.[12-13] In our study, mRCC patients who suffered from drug-related hypertension after start of sunitinib treatment showed a significantly longer OS than patients with a lower blood pressure. In line with recent literature, the most significant reduction in risk of death was seen in patients with high SBP.[13] Hypertension in our population showed no significant associations with PFS. This is in accordance with Rini *et al.* who have demonstrated a much larger reduction in HR for the risk of occurrence of death than a reduction in HR for the occurrence of progressive disease when patients suffered from hypertension during sunitinib treatment.[13]

In conclusion, genetic polymorphisms in *VEGFA* and *eNOS* independently predict blood pressure rise and/or severe hypertension in sunitinib-treated patients and may therefore be helpful in early diagnosis and initiation or adjustment of antihypertensive therapy. In addition, for Caucasian mRCC patients of the clear-cell type treated with sunitinib, we confirmed hypertension as an independent factor for OS and as a potential biomarker for outcome.

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Chapter 9

Suppressing effects of sunitinib on allergic rhinitis: previously undefined side effects with therapeutic potential

Eechoute K., van Zonneveld M., van Daele P.L.A., Gerth van Wijk R., Mathijssen R.H.J.

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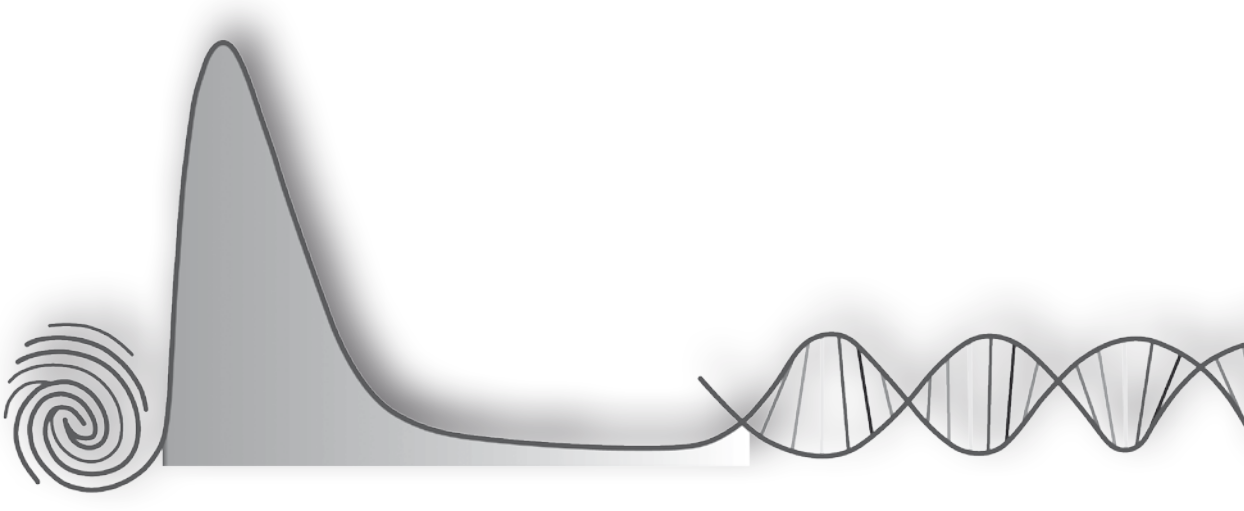

INTRODUCTION

We observed two female patients (51 and 59 years old, respectively) who were known with serious pollen allergy (hay fever) since childhood, expressed by rhino-conjunctivitis and asthma. For many years, these conditions were treated with antihistamines, β2 agonists and pulmonary corticosteroids. In 2007, both patients were diagnosed with metastatic renal cell cancer for which they were treated with sunitinib mono-therapy since October and November 2007, respectively. Since the start of anti-cancer treatment allergic rhinitis related co-medication was not allowed, to prevent unwanted drug-drug interactions. To their surprise, and despite this prohibition, during the pollen season of 2008 both patients experienced no allergic rhinitis complaints at all. In this study, we tried to elucidate the underlying mechanism for this remarkable observation.

Sunitinib malate is an anti-cancer drug, currently used in the palliative treatment of metastatic renal cell carcinoma and gastro-intestinal stromal cell tumors [1-2]. Treatment regimens exist of once daily dosing of 50 milligram for four consecutive weeks, followed by two weeks of rest [3]. Sunitinib acts as a multi-targeted tyrosine kinase inhibitor, known to inhibit the intrinsic tyrosine kinase activity of several specific proteins, including KIT, vascular endothelial growth factor receptor (VEGF-R) and the platelet-derived growth factor receptor (PDGF-R)[4], resulting in reduced tumor vessel growth or carcinogenesis. Sunitinib-induced toxicities may be serious and quite diverse. They include hypertension, diarrhoea, hand-foot syndrome, mucositis, vomiting, leukopenia, cardiotoxicity, and hypothyroidism [5].

Of all these molecular targets inhibited by sunitinib, KIT is most likely to be involved in the pathogenesis of allergic rhinitis. KIT is encoded by the *c-kit* gene. It is a transmembrane protein, expressed on a variety of cells, including mast cells, haematopoietic progenitor cells, melanocytes, germ cells and gastro-intestinal pacemaker cells. Heterozygous loss of function of KIT in mice results in macrocytic anaemia, hair depigmentation, sterility and reduced numbers of gastrointestinal pacemaker cells [6]. Moreover, in mast cells, KIT acts as a receptor for the stem cell factor. Binding of stem cell factor on KIT is essential for the survival and differentiation, chemotaxis and functional activity of mast cells [7]. Mast cells on their part play a vital role in the symptomatology of allergic rhinitis.

Allergic reactions usually occur when an individual who has been sensitized to an allergen (e.g. grass pollen), produces allergen-specific immunoglobulin isotype E antibodies (IgE) and subsequently encounters this particular allergen again. The allergen will then trigger the activation of IgE-binding mast cells in the exposed tissue. As a result, these mast cells degranulate, releasing a variety of mediators causing clinical symptoms. Therefore, inhibition of mast cell activity could reduce allergic symptoms.

METHODS

During all outpatient visits (usually at the beginning and at the end of a four-weekly treatment cycle), we measured total serum IgE and specific IgE antibodies in our study patients. The resting period was occasionally prolonged with a couple of days due to common sunitinib-related toxicity. These serological tests are used routinely in the diagnosis and management of allergic disease. Skin tests and nasal challenge tests [8] with birch and/or grass pollen extract were used to characterise the allergic status of the patients. Myelosuppression as a mechanism of impaired immune response in our study patients was also investigated by measuring total white blood cell counts before start of therapy and during all consecutive courses of sunitinib treatment.

Nasal provocation tests were performed at the beginning and at the end of one treatment cycle to evaluate the nasal response to birch pollen (patient 1) or grass pollen (patient 2). Nasal provocation tests were performed according to standard protocol with three consecutive doses of 100, 1000 and 10.000 biological units per milliliter (BU/ml) of the allergen. Symptoms of rhinoconjunctivitis were scored after each dose according to Lebel (maximum total score 33) [8].

In order to assess the effects of sunitinib on the mast cell population, we measured serum tryptase levels. Total serum tryptase levels are used as indicators of mast cell number and activation [9].

As sunitinib treatment was already given for several months to our two study patients, we could not measure tryptase concentrations from the start of therapy. As a result we cannot exclude that tryptase levels in these patients were already extremely low prior to the start of anti-cancer treatment. Therefore, in a separate cohort of patients with renal cell carcinoma, we measured tryptase levels before the start of their first sunitinib courses and during the successive courses. The non-parametric Friedman test was used to assess fluctuations in this cohort. The Wilcoxon signed ranks test was used for comparison between two time points (SPSS 15.0 for Windows).

RESULTS

Total serum IgE levels were moderately elevated, but showed no significant changes during the four weeks treatment with sunitinib compared to the values measured straight after the two-week resting period (Table 1). Specific serum IgE antibodies (Table 1) and skin tests (data not shown) fluctuated, but showed no significant alterations over the observed study period. White blood cell counts in our study patients were within the normal range before start of sunitinib treatment and during all the following treatment cycles. Table 1 depicts white blood cell counts during two treatment cycles.

Table 1. Imatinib trough levels (Cmin) in imatinib treated chronic myeloid leukaemia and gastrointestinal stromal cell tumor patients with or without clinical response.

Patients	Cycles studied				
	Start cycle 9	End cycle 9	Start cycle 10	End cycle 10	Start cycle 11
Study patient No 1					
Tryptase (< 11.4 mg/La)	<1.0d	<1.0d	<1.0d	<1.0d	<1.0d
Total IgEb (<100 u/mLa)	258	307	139	306	341
Birch pollen IgEb (<0.35 u/mLa)	80.5	>100e	87.0	>100e	>100e
Grass pollen IgEb (<0.35 u/mLa)	14.5	29.0	28.5	33.7	32.1
Nasal provocation total scorec	n.a.f	8	0	n.a.f	n.a.f
WBCg (4.1-10.9x109/L)	5.4	8.4	4.8	5.8	3.9
Study patient No 2	Start cycle 9	End cycle 9	Start cycle 10	End cycle 10	Start cycle 11
Tryptase (< 11.4 mg/La)	<1.0d	<1.0d	<1.0d	<1.0d	<1.0d
Total IgEb (<100 u/mLa)	200	329	288	398	211
Birch pollen IgEb (<0.35 u/mLa)	21.0	50.8	41.3	52.1	28.7
Grass pollen IgEb (<0.35 u/mLa)	11.7	21.5	17.9	21.9	12.9
Nasal provocation total scorec	n.a.f	5	2	n.a.f	n.a.f
WBCg (4.1-10.9x109/L)	4.3	n.a.f	7.6	5.0	5.2
Tryptase in control patients (< 11.4 mg/La)	Start cycle 1	End cycle 1	Start cycle 2	End cycle 2	Start cycle 3
Patient No 1	4.4	<1.0d	1.2	<1.0d	<1.0d
Patient No 2	9.5	2.0	2.4	<1.0d	n.a.h
Patient No 3	3.6	<1.0d	1.4	<1.0d	n.a.h
Patient No 4	7.5	<1.0d	n.a.h	n.a.h	n.a.h
Patient No 5	5.1	2.1	5.7	1.1	6.6
Patient No 6	4.9	<1.0d	<1.0d	<1.0d	2.7
Patient No 7	2.9	<1.0d	2.4	<1.0d	1.9
Patient No 8	6.4	<1.0d	4.6	<1.0d	n.a.f
Patient No 9	4.3	1.9	5.3	1.9	4.4

aNormal values in microgram or units per (milli)liter; bImmunoglobulin type E; cAccording to standard protocol (total score may range between 0 and 33); dBelow threshold values; eAbove detectable values, fNot available; gWhite blood cell count; h Not available due to cessation treatment because of worsened clinical performance.

Surprisingly, the four nasal provocation tests (two per patient) were negative at three occasions and marginally positive (8 out of 33 points maximally) at one occasion.

Also of interest: during the entire observation period of three months, total serum tryptase levels were extremely low (below threshold values) in both study patients. As shown in Table 1, tryptase levels in the separate cohort of control patients with renal cell carcinoma changed significantly from normal pre-treatment values to levels below the threshold, in most cases within the observation period of two treatment cycles ($\chi^2 = 20.24$; $p<0.0001$). A drop was already seen after one treatment cycle ($p = 0.008$).

Although these patients were not known for allergic rhinitis, we have no reason to suppose another mechanistic effect on mast cells in the control patients.

DISCUSSION

Up till now, the clinical benefit of mast cell inhibition through inhibition of KIT autophosphorylation has been investigated in patients with systemic mastocytosis, where it showed little effect [10-11]. However, this lack of effect is caused by a specific mutation in the catalytic domain, which is typical for the majority of neoplastic mast cells in human mastocytosis, making these cells refractory to tyrosine kinase inhibitors. Cell growth of human mast cells carrying wild type KIT is potently inhibited by inhibition of KIT [11]. Also, preclinical data in a murine model with allergen-induced airway inflammation showed that both induced airway hyperresponsiveness and chronic airway inflammation were significantly inhibited when these mice were treated with sunitinib [12]. These sunitinib-treated mice showed a lower expression of KIT in the airways (measured in bronchoalveolar lavage fluid) compared to non-treated mice. The clinical benefit of KIT inhibition in the treatment of asthma was confirmed in thirty-three severe corticoid-dependent asthmatic patients, receiving masitinib, a KIT and PDGFR tyrosine kinase inhibitor [13]. These patients showed a significant symptomatic relief as compared to eleven placebo-treated patients. In this report the resolution of allergic symptoms in two cancer patients is correlated with a decreased mast cell population and activity, most probably through inhibition of KIT. This mechanism of reduced mast cell population and activity is confirmed in our sunitinib treated control patients.

However, as sunitinib is a myelotoxic compound [5, 14], sunitinib-induced leukopenia may account for an impaired immune response in both our study patients, next to the above-mentioned mechanism. This is relevant because lymphocytic cell lines mediate the IgE response in allergic rhinitis. However, our study patients had normal white blood cell counts during the entire observation period. Moreover, recent data show that sunitinib may act as an immunomodulator that enhances T-lymphocytic function [15-16]. These results plead against sunitinib-induced myelosuppression as a possible mechanism of reduced symptoms of allergic rhinitis in our patients.

Although the costs and unfavourable safety profile do not justify treatment of allergic disorders with sunitinib, further elucidation of this sunitinib-related mechanism on allergic rhinitis may help to develop more effective medication for allergic disorders. For example, locally produced KIT-ligand in the airways is responsible for the recruitment and adhesion of mast cells to the airway epithelium [17-18]. Therefore, topical application of KIT inhibitors through inhalation or nasal spraying may be an efficient and safe treatment of local inflammation in allergic disorders. Furthermore, little is known about the threshold

dose of sunitinib, needed to effectively inhibit mast cell function. If lower dosages than those used in cancer treatment are sufficient to potently inhibit mast cell numbers, this could also improve the safety profile of sunitinib treatment in allergic disorders. In conclusion, along the known pathways responsible for allergic reactions, this observation suggests that sunitinib effectively inhibits mast cell proliferation and activity, resulting in a total absence of allergic rhinitis in our patients. This sunitinib-related mechanism may ultimately lead to the development of new therapeutic pathways in the treatment of (corticosteroid-resistant) allergic disorders.

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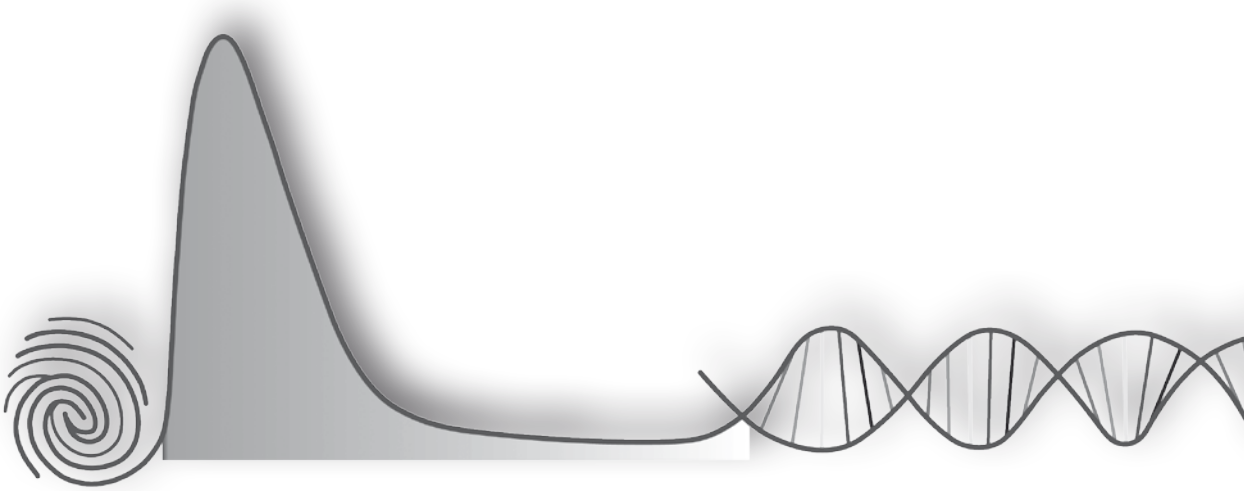
Chapter 10

Summary



IMATINIB STUDIES

Most patients that suffer from an advanced or metastatic gastrointestinal stromal tumor (GIST) gain benefit from systemic treatment with imatinib. However, in 10% to 15% of patients, disease will progress within three to six months. Mechanisms behind this ´early´ disease progression have not been entirely clarified. A majority of these non- responding patients harbor specific mutations in molecular drug targets.[1-3] However, early drug resistance may also result from imatinib plasma levels that drop below a minimally effective threshold level, a phenomenon called "*acquired pharmacokinetic drug resistance*". GIST patients not responding to imatinib treatment appeared to demonstrate lower imatinib plasma levels than responders.[4,5] In addition, in a retrospective pharmacokinetic side-study of the pivotal phase II B2222 trial, median time to progression was approximately twenty months shorter in patients that demonstrated one month imatinib steady-state trough levels below 1,100 ng/mL.[5]

Therefore, in the first part of this thesis, an overview that focuses on the pharmacokinetic and pharmacodynamic relevance of drug transporters in imatinib therapy is given (**Chapter 2**). Although *in vitro* data indicate that imatinib is a good substrate of efflux transporters such as ABCB1 and ABCG2, and solute carriers such as OCT1, OATP1A2 and OATP1B3, clinical studies that investigate the impact of these transporters on imatinib pharmacokinetics and efficacy in humans are scarce. Pharmacogenetic studies assessing associations between single nucleotide polymorphisms (SNPs) in *ABCB1* and *ABCG2* genes with imatinib pharmacokinetics in GIST patients have generated contradictory findings. Furthermore, allelic variants in the *SLC22A1* and *SLCO1B3* gene (encoding for the proteins OCT1 and OATP1B3, respectively) had no clear effects on imatinib exposure compared to the reference allele. To date, the most prominent pharmacodynamic effects of drug transporters have been described in imatinib-treated chronic myeloid leukemia (CML) patients who demonstrated a correlation between OCT1 expression and activity in CML blasts and therapeutic outcome.

As stated earlier, minimal imatinib trough levels of approximately 1,100 ng/mL may be required to achieve therapeutic success in GIST patients. It is therefore of clinical importance to assess acquired pharmacokinetic phenomena in imatinib-treated patients over time. Consequently, in **Chapter 3**, we designed a prospective population pharmacokinetic study in fifty GIST patients in order to assess systemic exposure to imatinib at multiple time points during a median follow-up period of one year. As imatinib is extensively metabolized by the liver, the secondary aim of this study was to evaluate the metabolic effects of GIST metastatic liver involvement on imatinib exposure. Full pharmacokinetic sampling was performed at start of therapy and after one, six and twelve months of therapy. In addition, at several time-points, trough samples were taken to assess minimal imatinib plasma concentrations. These data were analysed in a

compartmental pharmacokinetic model, which showed a significant downward trend in imatinib systemic exposure during the first three months of treatment to approximately thirty percent of the exposure at the beginning, with stabilization in imatinib pharmacokinetics from this time point on. Previous retrospective associations between imatinib trough levels at day 29 and clinical outcome benefit in GIST patients thus should be put into perspective.[5] Concerning the secondary objective of this study, volume of liver metastasis had a minor impact on imatinib clearance as for every 100 cm^3 increase in volume, a decrease of less than four percent clearance was observed.

Due to the discrepancy between previously reported results from imatinib *in vitro* transport assays and clinical studies, the aim of **Chapter 4** was to translate preclinical findings concerning a specific solute carrier; the organic anion transporting polypeptide 1A2 (OATP1A2), directly into clinical practice. Imatinib uptake in cells expressing human OATP1A2 or its rodent orthologue Oatp1a4, was significantly higher than in water-injected control cells. In addition, as imatinib uptake transport by OATP1A2 was higher in an acidic environment as found in the jejunum, and as OATP1A2 messenger RNA expression in the descending part of the human duodenum was the highest among all tissues, OATP1A2 was a promising candidate for the intestinal uptake of imatinib. In order to translate this to clinical practice, we performed a drug-drug interaction study and a pharmacogenetic association study in imatinib-treated patients. Rosuvastatin significantly inhibited OATP1A2-mediated transport of imatinib *in vitro* but concomitant administration of imatinib and rosuvastatin in GIST patients had no effect on steady-state pharmacokinetics of imatinib. Moreover, an allelic variant of the *SLCO1A2* gene that was associated with complete absence of imatinib transport *in vitro*, had no effect on imatinib plasma levels in patients carrying this genotype. This study therefore indicates that, although imatinib is a substrate for OATP1A2, this transporter by itself is unlikely to contribute substantially to the absorption profiles of imatinib in humans. Further investigation is warranted to determine the individual and collective contributions of additional, potentially redundant, intestinal carriers to the pharmacokinetics and pharmacodynamics of imatinib.

In **Chapter 5** we (further) elucidated the role of liver dysfunction in imatinib clearance by quantifying biliary secretion in the case of severe hepatic dysfunction. Full plasma pharmacokinetic sampling occurred at days one, five and six. In addition, trough samples were taken from days one to day fourteen of imatinib therapy. Bile was also collected for pharmacokinetic analysis. The major finding of this study was that the biliary secreted imatinib dose was a mere fraction of previous reported concentration data and that the ratio of biliary imatinib to metabolite CGP74588 concentrations was inverted. On the other hand, imatinib plasma exposure was within the normal range as reported in literature. This indicates that, although biliary excretion of imatinib seems related to severe liver dysfunction, no major alterations in imatinib exposure were observed. This

may be due to adaptive phenomena, in which a low bioavailability and low systemic clearance balance each other out, resulting in normal imatinib plasma concentrations. Consequently, these data further support the need to investigate the collective contribution of drug transporters to imatinib pharmacokinetics.

SUNITINIB STUDIES

The second part of this thesis is dedicated to the inter-individual variability in sunitinib pharmacodynamics. Sunitinib is directed against multiple molecular drug targets, which are involved in normal physiological processes. Therefore, unlike imatinib, sunitinib has a broad toxicity profile, which will lead to a dose modification in one out of three patients.[6] In addition, clinical response and survival in sunitinib-treated patients show a large variability.[6] Accurate prediction of the appropriate sunitinib dose and/or prediction of sunitinib's proper place in the sequence of systemic treatment lines are thus highly relevant for the individual patient.

Therefore, in **Chapters 6 and 7** we analysed potential genetic factors for sunitinib-induced toxicity and survival. A total of 31 SNPs in twelve candidate genes involved in sunitinib metabolism and pharmacodynamics together with several non-genetic variables were tested for associations with toxicity and survival in 219 patients treated with single-agent sunitinib. An interesting finding of these analyses was that genetic factors for variation in sunitinib-induced toxicity and survival were mainly located in pharmacokinetic pathways. SNPs in genes encoding for drug efflux transporters (ABCB1 and ABCG2), cytochrome P450 oxydases (CYP3A5 and CYP1A1) or nuclear receptors involved in cytochrome P450 regulation (NR1I3) proved to be independent factors for toxicity and/or survival in sunitinib-treated patients. The risk for leukopenia increased when the G allele in *CYP1A1* 2455A/G or the T allele in *FLT3* 738T/C were present, or CAG in the *NR1I3* (5719C/T, 7738A/C, 7837T/G) haplotype was absent. Any toxicity higher than grade 2 prevalence was increased when the T allele of vascular endothelial growth factor receptor 2 1191C/T or a copy of TT in the *ABCG2* (-15622C/T, 1143C/T) haplotype were present. The risk for mucosal inflammation was increased in the presence of the G allele in *CYP1A1* 2455A/G and the prevalence of hand-foot syndrome was increased when a copy of TTT in the *ABCB1* (3435C/T, 1236C/T, 2677G/T) haplotype was present.

In metastatic renal cell carcinoma (mRCC) patients of the clear-cell subtype treated with sunitinib, multivariate analysis showed that progression-free survival was significantly improved when an A-allele was present in *CYP3A5* 6986A/G, a CAT copy was absent in the *NR1I3* haplotype (5719C/T, 7738A/C, 7837T/G) and a TCG copy was present in the *ABCB1* haplotype (3435C/T, 1236C/T, 2677G/T). Carriers with a favorable genetic profile in these three genes involved in sunitinib pharmacokinetics had a significantly

improved progression-free (13.1 versus 7.5 months) and overall (19.9 versus 12.3 months) survival as compared to non-carriers.

Among sunitinib-induced toxicities, hypertension is a common side-effect. Approximately one third of patients treated with sunitinib will develop hypertensive disease and one third of this subgroup will need intensive medical treatment. In **Chapter 8** we identified genetic factors for this rise in blood pressure and/or for the development of severe hypertension during sunitinib therapy. In addition, associations of hypertension with survival were assessed in the subset of mRCC patients. In almost three-hundred sunitinib-treated patients, single nucleotide polymorphisms in *VEGFA* (405C/G, -460C/T, 936C/T, -2578A/C), *VEGFR-2* (1718T/A), *ET-1* (594G/T) and *eNOS* (-786T/C) were multivariately tested against the maximal change in systolic, diastolic and mean arterial blood pressure and hypertension grades during the cycle of therapy. Overall survival was evaluated in mRCC patients of the clear-cell type for a possible relation with hypertension. A higher rise in systolic blood pressure and mean arterial pressure was associated with presence of an ACG haplotype in *VEGFA* -2578A/C, -460C/T and 405C/G. Development of hypertension grade 3 was associated with this haplotype and with a C allele in *eNOS* -786T/C. Mean overall survival in sunitinib-treated mRCC patients suffering from hypertension appeared more than seven months longer.

As stated earlier, sunitinib is a multitargeted agent, directed against several proteins that are involved in a diversity of (patho-)physiological processes. Among these drug targets, KIT is responsible for the proliferation and functional activity of mast cells, involved in allergic rhinitis.[7] Consequently, in **Chapter 9**, inhibition of KIT activity in sunitinib-treated mRCC patients was investigated as a potential therapeutic pathway in inflammatory processes such as allergic rhinitis. Allergic reactions occur when an individual who has been sensitized to an allergen such as grass pollen, produces allergen-specific immunoglobulin isotype E antibodies (IgE) and subsequently encounters this particular allergen again. The allergen will then induce the activation of IgE-binding mast cells in the exposed tissue. As a result, these mast cells degranulate, releasing a variety of mediators causing clinical symptoms. Metastatic RCC patients who were known with severe pollen allergy since childhood, showed complete absence of symptoms of allergic rhinitis during treatment with sunitinib. Total serum tryptase levels, which are used as indicators of mast cell number and activation, were below threshold values during the entire observation period of three months. Moreover, in a separate cohort of control mRCC patients serum tryptase changed significantly from normal pre-treatment values to levels below the threshold, in most cases within the observation period of two treatment cycles. No significant changes were observed in serum IgE levels. Hence, resolution of allergic symptoms during sunitinib therapy is correlated to decreased mast cell activity, most probably through inhibition of KIT, as confirmed in a control population.

CONCLUSIONS AND PERSPECTIVES

Clinical research in this thesis extensively describes long-term imatinib pharmacokinetics in GIST patients. A significant drop in imatinib exposure during the first year of therapy is observed, which may account for early tumor progression, a process called "acquired pharmacokinetic drug resistance". Up until now, responsible alterations in imatinib pharmacokinetic pathways are not fully elucidated. A change in drug transporter activity or expression may account for the observed descent in systemic exposure. We performed an *in vitro* and clinical study to assess the potential role of a solute carrier, OATP1A2, in imatinib pharmacokinetics, but found no significant impact on imatinib absorption in imatinib-treated patients. Future (pre)clinical studies that assess mediators of imatinib pharmacokinetics, will have to evaluate the involved pathways at a systemic level. Drug transporters and enzymes involved in imatinib absorption, metabolism and excretion, are most probably subject to a complex interplay with each other. Study design should therefore aim at evaluation of the collective contribution of potentially involved drug transporters and enzymes. This balance between drug transporters involved in imatinib absorption on the one hand and hepatic uptake and excretion on the other, could also explain why no major effect of liver dysfunction or liver metastases on imatinib exposure was observed in GIST patients, although biliary excretion of imatinib seems to be affected by severe liver dysfunction.

Clinical significance of these acquired pharmacokinetic processes should be assessed in "trough level – clinical benefit" analyses. These assessments should be time-point specific and need to incorporate relevant tumor biology and patient characteristics in a multivariate analysis in order to be able to define a minimal effective imatinib dose for an individual patient on accurate time points in a treatment course.

Genetic variation in genes involved in sunitinib pharmacokinetics proved to be an independent factor for toxicity and survival. Future clinical studies should therefore prospectively assess the correlations between these polymorphisms and sunitinib exposure. At present, a study that integrates genotypic and phenotypic data together with clinical characteristics and data on sunitinib pharmacokinetics, is being performed at our institution.

This thesis also presents genetic factors for sunitinib-induced hypertension in a large cohort of patients and validates hypertension as a biomarker for survival in mRCC patients of the clear-cell subtype treated with sunitinib.

Finally, along the pharmacodynamic pathways that are inhibited by sunitinib, KIT inhibition lead to downregulation of mast cell activity and consequently to the resolution of allergic symptoms. These findings should be validated in a prospective clinical study and may eventually hold therapeutic potential in inflammatory diseases such as asthma and allergic rhinitis.

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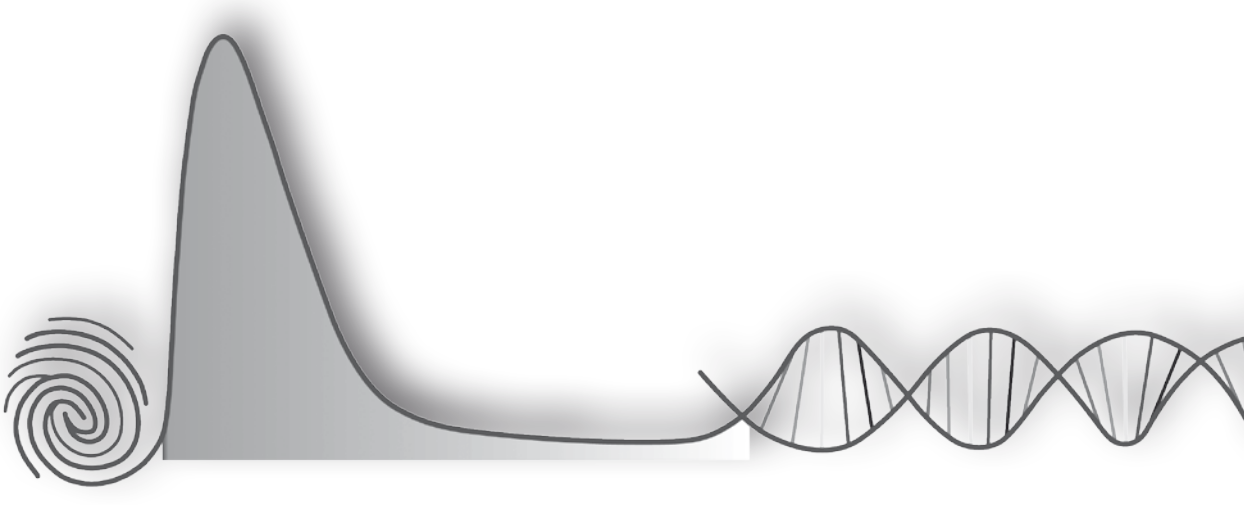
APPENDIX



SAMENVATTING EN CONCLUSIES

Imatinib studies

Het merendeel van de patiënten die lijden aan een gevorderde of uitgezaaide gastroïntestinale stromale tumor (GIST) zullen een therapeutisch voordeel hebben van een behandeling met imatinib. Echter, in 10 tot 15% van deze patiënten zal er een ziekteprogressie optreden binnen drie tot zes maanden. De mechanismen achter deze zogenaamde "vroege" ziekteprogressie zijn niet geheel opgehelderd. Een meerderheid van deze niet-responderende patiënten zullen drager zijn van specifieke mutaties in de moleculaire doelen. Echter, vroege geneesmiddelenresistentie kan ook het resultaat zijn van imatinib plasmaspiegels die onder een minimaal effectieve drempelwaarde zakken, een fenomeen dat gekend is als "*verworven farmacokinetische geneesmiddelenresistentie*". GIST patiënten die geen therapeutische respons vertonen op behandeling met imatinib lijken dan ook lagere imatinib plasmawaarden te vertonen dan responderende patiënten. Daarenboven werd in een retrospectieve studie aangetoond dat de mediane tijd tot progressie ongeveer 20 maanden korter is in patiënten die na één maand behandeling een steady-state imatinib dalspiegel vertonen die lager is dan 1.100 ng/ml.

Om die redenen, wordt er in het eerste deel van dit proefschrift een overzicht gegeven van de farmacokinetische en farmacodynamische relevantie van drugpompen tijdens behandeling met imatinib (Hoofdstuk 2). Hoewel *in vitro* data aangeven dat imatinib een goed substraat is van effluxpompen zoals ABCB1 en ABCG2, en van opnamepompen zoals OCT1, OATP1A2 en OATP1B3, zijn er weinig klinische studies die het effect van deze pompen analyseren. Bovendien hebben farmacogenetische associatie analyses in *ABCB1 en ABCG2* tegenstrijdige bevindingen opgeleverd. Ook allelische varianten in het *SLC22A1* en *SLCO1B3* gen (die respectievelijk coderen voor OCT1 en OATP1B3) hadden geen duidelijke effecten op de systemische blootstelling aan imatinib.

Op de achtergrond van mogelijk therapeutisch relevante imatinib dalspiegels, hebben we in Hoofdstuk 3 een prospectieve opgezet om de systemische blootstelling aan imatinib te volgen over een mediane periode van 1 jaar. Omdat imatinib in hoge mate gemetaboliseerd wordt in de lever, was de secundaire doelstelling van deze studie de evaluatie van de eventuele metabole effecten van GIST levermetastasen op imatinib blootstelling. Alle farmacokinetische data werden geanalyseerd in een compartimenteel model, dat een significante neerwaartse trend liet zien in imatinib blootstelling gedurende de het eerste kwartaal van de behandeling met daaropvolgend een plateaufase. Na 3 maanden behandeling is de blootstelling gedaald met ongeveer 30 procent. Wat betreft de secundaire doelstelling, werd een verwaarloosbaar effect van levermetastasering gezien op de kinetiek van imatinib.

Omwille van de discrepantie tussen eerder gerapporteerde resultaten van imatinib *in vitro* transport assays en klinische studies, werd in Hoofdstuk 4 getracht om preklinische

bevinden rond een specifieke opnamepomp (OATP1A2) onmiddellijk te vertalen naar de kliniek. Opname van imatinib in cellen die het humane OATP1A2 of het ortholoog bij de rat (Oatp1a4) tot expressie brengen, was significant hoger dan in water-geïnjecteerde controlecellen. Daarenboven was imatinib opnametransport hoger in een zuur milieu zoals dat gevonden wordt in het proximale gedeelte van de darm, waar de expressie van OATP1A2 hoog is. Dit alles maakte OATP1A2 een goede kandidaat voor imatinib opnametransport in de darm. Om dit te vertalen naar de kliniek, hebben we een interactiestudie en een farmacogenetische associatie-analyse uitgevoerd in patiënten behandeld met imatinib. Rosuvastatine liet een significante inhibitie zien van *in vitro* imatinib transport maar concomitante toediening van rosuvastatine en imatinib in GIST patiënten had geen effect op de steady-state farmacokinetiek. Daarenboven liet een allelische variatie in *SLCO1A2* geen effect zien op imatinib plasmaconcentratie. Deze studie illustreert daarmee dat OATP1A2 waarschijnlijk weinig bijdraagt aan het absorptieprofiel van imatinib in mensen.

In Hoofdstuk 5 hebben we verder getracht de rol van leverdysfunctie op te helderen door biliaire secretie te kwantificeren in het geval van ernstige hepatische dysfunctie. Famacokinetische monsterafname en galverzameling gebeurden op dag 1, 5, 6 en 14. De belangrijkste bevinding van deze studie was dat de dosis imatinib die gesecreteerd werd in de gal, een fractie was van eerdere gerapporteerde bevindingen. Daarenboven was de verhouding van biliaire concentraties imatinib op de metaboliet CGP74588, omgekeerd. Dit wijst aan dat, hoewel biliaire secretie van imatinib gerelateerd lijkt te zijn aan leverdysfunctie, er geen belangrijke veranderingen gezien worden in de blootstelling. Dit kan het gevolg zijn van adaptieve verschijnselen, waarbij de lage biologische beschikbaarheid en lage systemische klaring elkaar in evenwicht houden, wat resulteert in plasmaconcentraties binnen de normale range.

Sunitinib studies

Het tweede deel van dit proefschrift is gewijd aan de inter-individuele variabiliteit in sunitinib pharmacodynamiek. Sunitinib is gericht tegen meerdere moleculaire doelen die betrokken zijn bij normale fysiologische processen. Sunitinib zal daarom, in tegenstelling tot imatinib, een breder toxisch profiel vertonen, dat zal leiden tot een dosisaanpassing in ongeveer een derde van de patiënten. Daarnaast, wordt een grote variatie gezien in klinische respons en overleving. Een accurate voorspelling van de gepaste dosis sunitinib en/of voorspellen van de gepaste plaats van sunitinib in de sequentie van systemische behandelingenslijnen, zijn daarom hoogst relevant voor de individuele patiënt.

In Hoofdstuk 6 en 7 hebben we dan ook potentiële genetische factoren geanalyseerd voor toxiciteit en overleving. Een interessante bevinding van deze analyses was dat de genetische factoren voor variatie in sunitinib-geïnduceerde toxiciteit of overleving,

voornamelijk gelokaliseerd waren in de farmacokinetische genen. Single nucleotide polymorfismen (SNPs) in genen die coderen voor effluxpompen (ABCB1 en ABCG2), leverenzymen (CYP3A5 en CYP1A1) en nucleaire receptoren (NR1I3) die betrokken zijn bij de regulatie van leverenzymen, bleken onafhankelijke factoren te zijn voor toxiciteit en overleving. Zo liet een multivariate analyse in patiënten met een uitgezaaid niercelcarcinoom (mRCC) van het heldercellige subtype zien dat de progressievrije overleving significant beter was indien patiënten drager waren van een gunstig genetisch profiel in *ABCB1*, *CYP3A5* en *NR1I3* (13,1 versus 7,5 maanden).

Hoge bloeddruk of hypertensie is gekende bijwerking van sunitinibtherapie. Eén derde van de patiënten, behandeld met sunitinib zal hypertensie ontwikkelen en ongeveer een derde van deze patiënten zal intensieve behandeling nodig hebben. In Hoofdstuk 8 hebben we dan ook geprobeerd om genetische factoren te identificeren voor bloeddrukstijging tijdens behandeling met sunitinib en om de potentiële predictieve waarde van hypertensie voor overleving aan te tonen. Deze studie liet zien dat een haplotype in *VEGFA* (een gen dat codeert voor de ligand van het primaire therapeutische doel van sunitinib) en een SNP in *eNOS* (gen dat codeert voor een enzym betrokken bij bloeddrukregulatie) onafhankelijke factoren zijn voor ernstige hypertensie tijdens behandeling met sunitinib. Wat betreft het secundaire doel van de studie, zagen we dat de algehele overleving in mRCC patiënten die hypertensie vertoonden tijdens behandeling met sunitinib meer dan 7 maanden langer was dan in patiënten zonder hypertensie.

Zoals eerder gesteld, is sunitinib een geneesmiddel dat gericht is tegen meerdere eiwitten die betrokken zijn bij diverse (patho)fysiologische processen. Onder deze doelen, is KIT betrokken bij de proliferatie en functionele activiteit van mestcellen, die betrokken zijn bij de pathofysiologie van allergische rhinitis of hooikoorts. In Hoofdstuk 9, wordt de inhibitie van KIT-activiteit in sunitinib-behandelde mRCC patiënten onderzocht als een potentiële therapeutische modaliteit bij inflammatoire processen zoals hooikoorts. Allergische reacties ontstaan wanneer een individu die gesensibiliseerd is voor een allergeen, allergeen-specifieke immunoglobuline isotype E antilichamen (IgE) produceert en daaropvolgend opnieuw in aanraking komt met dit allergeen. Dit zal een activatie van de IgE-bindende mestcellen veroorzaken in het blootgestelde weefsel. De mestcellen zullen dan degranuleren wat een variëteit aan symptomen tot gevolg heeft.

Tijdens onze observatie lieten mRCC patiënten die gekend waren met ernstige hooikoorts een complete afwezigheid van hooikoortssymptomen zien tijdens behandeling met sunitinib. Totale tryptase serumspiegels, die gebruikt worden als indicator van mestcelactiviteit en -aantal, waren onmeetbaar laag tijdens de gehele observatieperiode van 3 maanden. Daarenboven werd in een tweede cohort van controlepatiënten gezien dat bij de meeste individuen tryptasespiegels significant daalden van normale waarden voor start van de behandeling naar onmeetbaar lage spiegels binnen twee behandelingscycli. Er werden geen significante veranderingen waargenomen in serum

IgE spiegels. De resolutie van hooikoortssymptomen tijdens behandeling met sunitinib is dus gecorreleerd aan een verlaagde mestcelactiviteit, meest waarschijnlijk door inhibitie van KIT.

DANKWOORD

De bevindingen die in dit proefschrift beschreven staan, zijn het resultaat van klinisch onderzoek en dus het werk van vele mensen. In de eerste plaats dragen alle patiënten die, ondanks hun ernstige ziekte, deelnamen aan deze studies mijn dankbaarheid en bewondering.

Verder was dit werk niet mogelijk geweest zonder de dagelijkse inzet op zowel de klinische afdelingen als in "ons" lab Translationele Farmacologie. Een aantal van de mensen die daar werken, wil ik graag in het bijzonder bedanken.

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En dan nu: NUNC EST BIBENDUM!

CURRICULUM VITAE

Karel Eechoute werd geboren op 22 februari 1980 te Gent, België. In 1998 voltooide hij het algemeen secundair onderwijs, richting wetenschappen-wiskunde, aan het Koninklijk Atheneum te Wetteren, België. In 2008 behaalde Karel het diploma arts aan de Universiteit Gent, België. Aansluitend werkte hij als arts niet in opleiding tot specialist aan het Erasmus Medisch Centrum, afdeling Interne Oncologie en dit gedurende één jaar. Gelijktijdig werd gestart met onderzoek aan het laboratorium Translationele Farmacologie van de afdeling Interne Oncologie in het Erasmus Medisch Centrum, onder supervisie van dr. A.H.J. Mathijssen en prof.dr. J. Verweij. Dit onderzoek werd afgerond in november 2011 en resulteerde in het huidig proefschrift. Per 1 januari 2012 begon Karel aan de opleiding tot internist in het Ikazia Ziekenhuis te Rotterdam (opleiders prof.dr. J.L.C.M. van Saase en dr. A.A.M. Zandbergen).

In 2010 en 2011 ontving Karel voor zijn onderzoekswerk een Merit Award van de American Society of Clinical Oncology.

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PhD Portfolio

Summary of PhD training and teaching

Name PhD student: Karel Eechoute	PhD period: 2008-2011
Erasmus MC Department: Medical Oncology	Promotor: prof dr. J. Verweij
	Co-promotor: dr. A.H.J. Mathijssen

	Year	Workload
General courses		
BROK ('Basiscursus Regelgeving Klinisch Onderzoek')	2008	1 ECTS
Specific courses		
SNP's and Human Disease (MolMed)[a]	2009	1.8 ECTS
NVKF&B "Leren doen van farmacologisch onderzoek"	2009	1 ECTS
Seminars and workshops		
OMBO cursus Daniel den Hoed	2008 - 2010	1 ECTS
Refereerbijeenkomst dept. Medical Oncology		
Refereerbijeenkomst dept. Medical Oncology	2008 - 2010	1.2 ECTS
Refereerbijeenkomst dept. Medical Oncology		
NIHES cursus "Principles of Clinical Pharmacology"[b]	2010	1 ECTS
OIO overleg	2010 - 2011	3 ECTS
Urology Tour d'Europe	2011	0.5 ECTS
Presentations		
Scientific meetings dept. Medical Oncology Rotterdam	2009 + 2011	1 ECTS
FIGON Dutch Medicine Days[c]	2009	0.5 ECTS
Josefine Nefkens Institute Lab Meetings	2009	0.5 ECTS
ASCO poster discussion presentation[d]	2010	1.2 ECTS
Clinical Research Meeting dept. Medical Oncology	2010	1 ECTS
EORTC-NCI-AACR poster presentation[e]	2010	1 ECTS
ASCO poster discussion presentation[d]	2011	1.2 ECTS
Urology Tour d'Europe oral presentation	2011	1 ECTS
(Inter)national conferences		
FIGON Dutch Pharmacology Days[c]	2008 - 2010	2.4 ECTS
Annual Meetings American Society Clinical Oncology[d]	2010 + 2011	2 ECTS
EORTC-NCI-AACR International Symposium on Molecular[e] Targets and Cancer Therapeutics	2009 + 2010	0.8 ECTS
Continuüm Oncologie Jaarsymposium	2010	0.3 ECTS
Multidisciplinair symposium niercelkanker (WIN-O)[f]	2010	0.3 ECTS
Mededelingendag NVKF&B Jaarbeurs Utrecht	2010	0.3 ECTS
Other		
Wetenschapsdag Interne Oncologie	2009 - 2011	1.2 ECTS
IKNL (Zuid-Nederland) netwerkdagen	2010 + 2011	1.6 ECTS
Teaching		
3e Nascholing "Targeted Therapy"	2009	1 ECTS
Lecturing		
Guest lecture carcinogenesis, master in molecular life sciences, HAN University of Applied Science, Nijmegen	2011	2 ECTS

[a]MolMed: Molecular Medicine Postgraduate School
[b]NIHES: Netherlands Institute for Health Sciences
[c]FIGON: Federatie voor Innovatief Geneesmiddelonderzoek Nederland
[d]ASCO: American Society of Clinical Oncology
[e]EORTC-NCI-AACR: European Organisation for Research and Treatment of Cancer-National Cancer Institute-American Association for Cancer Research.
[f]WIN-O: Werkgroep Immunotherapie Nederland voor Oncologie