Personalized medicine of targeted anti-cancer drugs

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ISBN/EAN: 978-90-3935738-5

Graphic design: CO3, Toon van Lieshout

Personalized medicine of targeted anti-cancer drugs

Geïndividualiseerde geneeskunde van gerichte kanker behandeling

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr.G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op woensdag 21 maart 2012 des middags te 2.30 uur

door
Heinz-Josef Klümpen
geboren op 5 juni 1969 te Sevelen (Duitsland)

Promotoren:
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The research described in this thesis was performed at the department of medical oncology of the Westmead Hospital, Sydney West Area Health Service, University of Sydney, Australia, and the department of medical oncology of the Academic Medical Centre, Amsterdam, The Netherlands.

Publication of this thesis was financially supported by:

Bayer
Pfizer
Ipsen
Jansen-Cilag
Amgen
Novartis
Glaxo-Smith-Kline
Roche
Abbott

Für meine Eltern

"Always laugh when you can. It is cheap medicine. "
Lord Byron

Contents

Chapter 1
General introduction

Since 2008 cancer is the most common cause of death in The Netherlands.[1] In 2009 the incidence of cancer rose to 91400 cases in the Dutch population[2], and more than 44000 people died of cancer in 2010.[3] On the other side, the risk to die of cancer in The Netherlands fell over the last decade by 15% in men and by 5% in women.[4] This is most likely due to early detection of cancer and more effective cancer treatment.

Over the last decades, studies on mechanisms of cancer cell proliferation have led to a better understanding of the drivers of cancer growth. It is now common knowledge that the first step of signal-translation between the outside of a cancer cell and the activation of signal transduction cascades is mediated by activation of tyrosine kinases. In 1988 the first proof of principle of blocking Epidermal Growth Factor (EGF) dependent cell proliferation by EGF receptor tyrosine kinase inhibitors (TKI) was obtained.[5] In most cancers one or more signal transduction cascades are activated or mutated and act as pathological drivers of cell-proliferation. In depth discovery of the specific drivers of each cancer type and the development of specific targeting drugs (TKIs or non-TKIs), inhibiting the mutated function, has been the main focus of cancer research since 1988. Well-known examples of diseases previously only treated with chemotherapy for which now effective TKIs exist are chronic myeloid leukemia, gastrointestinal stromal tumors and renal-cell cancer. Many more drugs targeting specific cell-proliferation pathways are under preclinical and clinical investigations and are doubtless the main focus of cancer treatment research in academic institutes and pharmaceutical companies.

In the era of targeted therapy in cancer care, there are currently 11 orally available TKIs with proven efficacy and EMA and Food and Drug Administration approval. However, the treatment effect is still palliation rather than cure. This probably is due to the high grade of heterogeneity of mutations in advanced solid and haematological malignancies in addition to the pharmacokinetic variability in the disposition of anticancer drugs. Individualized treatment is a potential way to overcome treatment resistance.

Research of this thesis is focussed on two different strategies to individualise treatment.

Strategy 1: Dose-individualization

One strategy is individualization of dosing. Most of the targeted therapy agents are given in a fixed daily dose, despite the wide interpatient variation of drug exposure described in pharmacokinetic trials. By determination of factors influencing drug-exposure the ‘right’ dose might be predicted per individual. The major objective of the herein described research is to find predictive factors for dose individualization to

optimize treatment outcome. To investigate these predictive factors a literature search was performed to collect all data on pharmacokinetic and -dynamic factors of TKIs. Chapter 2 summarises the current knowledge of eight of the clinically used targeted agents in cancer treatment. The spotlight is on the question if blood drug levels can predict antitumor activity or whether there are any other predictive surrogate markers of antitumor activity and toxicity. In the same chapter current literature on alternative drug dosing procedures was reviewed.

It is estimated that genetics can account for 20 to 95 percent of variability in drug disposition and effects.[6] Although many non-genetic factors can influence the pharmacological effects of medications, including age, organ function, concomitant therapy, drug interactions, and the nature of the disease there are now numerous examples of cases in which interindividual differences in drug response are due to pharmacogenomic factors.[7] Pharmacogenomics (PG) is the study of the role of inherited and acquired genetic variation in drug response.[8] For the purpose of PG dose individualization the germline genome of the patient is aim of investigations.

Clinical studies described in this thesis explore PG of imatinib and sunitinib, two commonly used TKIs.

In a study of patients treated with imatinib, summarised in chapter 3, the pharmacogenetic profile was retrospectively investigated to find significant single-nucleotide-polymorphisms (SNPs) to predict the need for dose-adjustments, toxicity and treatment outcome.

A second trial examining sunitinib, which is in daily use as first line treatment for advanced renal-cell-cancer is described in the appendix of this thesis. In this study of a prospective investigation not only the PG of the patient but also drug levels of sunitinib and the phenotypical activity of drug metabolism and elimination are analysed. The combination of these investigations is thought to reflect more realistically the complexity of drug metabolism and exposure than PG alone.

Strategy 2: target individualisation

Another strategy of treatment individualization is to find the right target of anti-cancer treatment in an individual. Each cancer in an individual may have different main drivers of disease proliferation. Mutations of the mammalian target of rapamycin (mTOR) are common drivers of disease proliferation due to its central role in tumor suppression. Inhibition of mTOR has proven anti-proliferative activity in several tumor types, which is thought to be mainly cytostatic (chapter 4). To enhance the cytotoxic properties of mTOR inhibitors combination therapies are proposed.

The major objective was to clinically investigate the feasibility and antitumor activity of combination treatment of mTOR inhibition with chemotherapy but also with other targeted therapies. In the first study cancer patients were treated with temsirolimus (chapter 5), an intravenously used mTOR inhibitor, in combination with nelfinavir, a potent Akt inhibitor. Akt is an enzyme which may be activated by mTOR inhibition and may

induce drug resistance. The additional inhibition of Akt is thought to increase anti-cancer activity of temsirolimus and overcome drug resistance. Taking into account that nelfinavir also is a strong CYP3A4 inhibitor, a major metabolising enzyme of temsirolimus, increased toxicity of temsirolimus was anticipated. Translational endpoints were studied in peripheral blood mononuclear cells to investigate the intended Akt and mTOR inhibition.

In chapter 6 the orally available mTOR inhibitor everolimus was combined with capecitabine an orally-administerd prodrug of 5-fluorouracil (5FU). Preclinical synergistic potential of 5FU and mTOR inhibition has been described, but the combination was not feasible in a clinical setting using similar drug dosages and schedules.[9][10] A dose escalation Phase I study was performed to find the maximum tolerated dose of capecitabine combined with a standard dose of everolimus. To examine potential drug-drug interactions drug levels of both drugs independently and combined were analysed.

The drug level found in the above mentioned phase I study was used in a Phase II study in pancreatic cancer patients (chapter 7). Advanced pancreatic cancer has a poor prognosis with only a minimal chance of response to anti-tumoral therapy. 5FU is one of the few drugs used as a monotherapy with a chance of tumor response. Antitumor activity and feasibility of the combination of everolimus and capecitabine were investigated. This is the first ever in human initiated Phase II study of an mTOR inhibitor combined with a 5FU prodrug.

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The compounds investigated in this thesis

Name	Target(s)	Mechanism of Action	Chapter
Targeted Agents			
Axitinib (AG013736)	VEGFR 1-3, PDGFR, c-Kit	Angiogenesis inhibition	2
Dasatinib (Sprycel®)	Src, Bcr-Abl, Stat5	Inhibition of tyrosine phosphorylation of SFKs	2
Everolimus (Zortress®/Certican®/Afinitor®)	mTOR	Serine/threonine kinase inhibitor	2, 4, 6, 7
Erlotinib (Tarceva®)	EGFR	Inhibition of ErbB-1	2
Gefitinib (Iressa®)	EGFR	Serine/threonine kinase inhibitor	2
Imatinib (Gleevec®/Glivec®)	c-KIT, PDGF, Bcr-Abl	Serine/threonine kinase inhibitor	2, 3
Lapatinib (Tykerb®/Tyverb®)	Her2	Serine/threonine kinase inhibitor	2
Nilotinib (Tasigna®)	Bcr-Abl	Serine/threonine kinase inhibitor	2
Pazopanib (Votrient®)	VEGFR-1,-2,-3, PDGFR a/b, c-KIT	Multikinase inhibitor	2
Sorafenib (Nexavar®)	Raf kinase, VEGF-2,-3, c-KIT, PDGF	Multikinase inhibitor	2
Sunitinib (Sutent®)	VEGFR-1,-2, FLT3, KIT, PDGFR	Multikinase inhibitor	2, Appendix
Temsirolimus Vandetanib (ZD6474)	mTOR VEGFR, EGFR, RET	Inhibition of mTOR Serine/threonine kinase inhibitor	5 2
Vatalanib (PTK787)	VEGFR-1,-2,-3, PDGFR, c-KIT, c-Fms	Multikinase inhibitor	2
Conventional cytotoxic agents			
Capecitabine (Xeloda®)	Thymidylate synthase, RNA, DNA	Inhibition of DNA synthesis	6, 7
Others			
Nelfinavir (Viracept®)	Akt, HIV-1 and HIV-2 proteases, CYP3A4	Protease inhibiton, Akt inhibition	5

Part 1

Pharmacogenetics of small molecules

Chapter 2

Moving towards dose individualization of tyrosine kinase inhibitors

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Cancer Treatment Reviews. 2011; 37:251–260

ABSTRACT

Molecular targeted therapies with tyrosine kinase inhibitors (TKIs) have been a recent breakthrough in cancer treatment. These small molecules are mainly used at a fixed dose ignoring the possible need for dose individualization. Fixed dosing may indeed result in suboptimal treatment or excessive toxicity considering the high inter-individual variability in the pharmacokinetics (PK) of these therapies. The PK, toxicity and efficacy of ten commonly used molecular targeted anti-cancer therapies were reviewed in order to optimize their prescription. A wide interpatient variability in the pharmacokinetics of these small molecules is demonstrated. Moreover associations between certain toxicities and the treatment efficacy have also been demonstrated for some agents, such as erlotinib and skin rash, that may be used as a surrogate marker. Other biomarkers intended to substitute for a clinical endpoint have been described for some TKIs and may be useful for dose individualization. Promising alternatives to fixed dosing were explored such as therapeutic drug monitoring, genotype and phenotype adjusted dosing, and toxicity-adjusted dosing. Prospective studies are needed to validate these methods so that dosing algorithms may be developed in the near future in order to personalize therapeutics to the individual needs of each cancer patient.

INTRODUCTION

Until the late 1990s, anti-cancer drugs were directed towards metabolic enzymes (e.g. methotrexate and 5-fluoruracil), to DNA (cisplatin, gemcitabine, cyclophosphamide), with increasing success to DNA topoisomerases (doxorubicin, etoposide, irinotecan) and to hormonal signaling pathways via the nuclear hormone receptors for breast and prostate cancer [1] Nowadays, protein kinases, particularly tyrosine kinases, have become the prime targets for drug intervention in malignant disease. Protein kinases are enzymes that phosphorylate proteins leading to the activation of signal- transduction pathways that play a critical role in a variety of biologic processes, including cell growth, differentiation and death [2][3]. The tyrosine kinase inhibitors (TKI), also called small molecules due to their size, are biologically active compounds (biomolecules) that are not polymer in contrast to nucleic acids, proteins and polysaccharides. All small molecules approved by the FDA are currently prescribed at a fixed dose. All, apart from temsirolimus, are given orally. Oral formulation may be convenient for the patient and relieve the heavy burden of day-stay infusion units and oncology pharmacy, but this practice has particular concerns. Oral bioavailability of some TKI is highly dependent on gastrointestinal absorption and first-pass hepatic metabolism, two processes that vary considerably among individuals. Most TKI are also eliminated by a machinery that normally varies considerably between healthy individuals, and even more so by illness [4][5]. Chronic administration of these drugs may also induce cellular drug efflux and upregulate elimination mechanisms, in the same way as cancer cells develop drug resistance in vitro [6]. Finally, patient compliance to therapy with oral antineoplastic agents is quite variable, with reported adherence rates ranging from 20% to 100% [7]. This review will focus on currently approved TKI by the Food and Drug Administration (FDA) as well as promising ones (Table 1), and describes their pharmaco-kinetics (PK), the correlations between their PK and pharmacodynamics (PD) where available, and putative biomarkers that may be used to assess clinical response. Alternative dosing methods tested to individualize prescription are then presented.

Table 1 Small molecules, indications, targets and pharmacokinetic parameters

Drug	Indication	Target	Dosis per day (mg)	Half-life (h)	Protein binding (%)	Bioavail-ability (%)	T_{max} (h)	Elimination	Ref
Axitinib	RCC Phase III	VEGFR 1-3, PDGFR c-Kit	10	2-5	NA	NA	2-6		[91]
Dasatinib	Second line CML	Src, Bcr-Abl	100	3-5	96	14-34	0,5-6	Fecal 85% renal 4%	[92]
Everolimus	Second line RCC	mTOR	10	38	73	16	1	Fecal 98% renal 2%	[79][80]
Erlotinib	NSCLC, pancreatic cancer	EGFR	100	36	a	60	1.4	Fecal 83% renal 8%	[31]
Gefitinib	NSCLC	EGFR	250	48	90	60	3-7	Fecal 86% renal 4%	[93]
Imatinib	First line CML, GIST	c-KIT, PDGF, Bcr-Abl	400	18 (40*)	95 α a	98	2.5	Fecal 68% renal 13%	[10][94]
Lapatinib	Second line HER2 + breast cancer	Her2	1250	24	α + a >99	?	3-4	Fecal 27% unchanged	[95]
Nilotinib	CML	Bcr-Abl	800	15	98	?	3		[24]
Pazopanib	Advanced RCC, sarcomas (phase III)	VEGFR-1, 2, 3, PDGFR a/b, c-Kit	800	31-35	>99	?	2-4		[96][97]
Sorafenib	Second line RCC, first line HCC	Raf kinase, VEGF 2+3, c-Kit, PDGF	800	25-48	99.5	29-49		Fecal 77% renal 19%	[26][98]
Sunitinib	First line RCC	VEGFR-1, 2, FLT3, KIT, PDGFR	50	40-60	95	?	6-12	Fecal 61% renal 16%	[32][99]
Vandetanib	NSCLC (phase III)	VEGFR, EGFR, RET	100-300	105		?	6.5-7.5		[100][101]
Vatalanib	Colorectal (phase III)	VEGFR 1-3, PDGFR, c-Kit, c-Fms	100-300	4.6	90	35	1.8	Fecal 60% renal 23%	[102]

α	α 1-acid glycoprotein	c-FMS	colony stimulating factor 1 receptor or macrophage colony-stimulating factor receptor (M-CFSR) or CD115 (Cluster of Differentiation 115)	mTOR	mammalian target of rapamycin
a	albumin			PDGFR	platelet derived growth factor receptor
CML	chronic myeloid leukemia			Raf	serine/threonine-specific kinase
GIST	gastro-intestinal stromal tumor			RET	proto-oncogene receptor protein kinase
HCC	hepatocellular carcinoma	c-Kit	cytokine receptor CD117		
NA	not available	EGFR	epidermal growth factor receptor	SRC	Sarcoma proto-oncogenic tyrosine kinase
NSCLC	non small cell lung carcinoma	FLT3	cytokine receptor CD135		
RCC	renal cell carcinoma.	Her2	Human Epidermal growth factor Receptor 2	VEGFR 1-3	vascular endothelial growth factor receptor.
BCR-ABL	oncogene fusion protein				

Importance of understanding drug disposition

Drug disposition is the combination of the processes resulting in a particular drug exposure within an individual. The four major processes describing drug disposition are: Absorption, Distribution, Metabolism and Elimination (ADME). They will all influence the level and kinetics of drug exposure to the tissue. The example of imatinib will illustrate the complexity and consequences of drug disposition.

Imatinib is an inhibitor of c-KIT, PDGF and Bcr-Abl, that was approved by the FDA in May 2001 as an effective treatment of chronic myeloid leukemia (CML) and CD14 expressing gastro-intestinal stromal cell tumors (GIST). After oral administration, imatinib reaches bioavailability of nearly 100%. It is mainly bound (96%) to plasma proteins mostly to alpha 1 acid glycoprotein (AGP). It is extensively metabolized by the liver. Only 10% is cleared by the kidneys and more than 80% of imatinib and metabolites can be recovered from the feces. Imatinib is metabolized in the liver by the cytochrome P450 (CYP) 3A to the active metabolite CGP74588 [8] Imatinib is also a substrate for the hepatocyte efflux transporters, in particular the ATP-binding cassette sub-family G member 2 (ABCG2 or BCRP) and sub-family B member 1 (ABCB1 or MDR1) which excrete both unmetabolized and metabolized imatinib into the bile [9]. Imatinib exposure varies 3-fold in healthy individuals and over 4-fold in patients with CML [10]. Imatinib is nevertheless still given at a fixed dose, even though the wide interindividual PK variability may have an impact on the likelihood of response [11] Non-responders of a fixed standard dose of imatinib have indeed significantly lower plasma levels of imatinib than responders in patients with CML [12][13][14] and GIST [15][16]. Significant and wide inter-individual variations in the activity and expression of the enzyme (CYP3A), transporter and nuclear receptors involved in imatinib disposition have been described due to genetic and environmental factors. Several studies have tried to link individual patient characteristics with the inter-individual variability of the pharmacokinetics of imatinib such as the variability of the enzymes and transporters involved in imatinib disposition. Studies have assessed the impact of CYP3A inhibition, ABCB1, ABCG2, CYP3A genotype, AGP levels on imatinib PK. Another well-described cause of variation in response to imatinib is acquired PK resistance after prolonged treatment [6]. Mutations of the tyrosine kinase receptor may result in reduced affinity for imatinib, a common cause of resistance in both CML and GIST [17][18]. Another hypothesis is that imatinib clearance increases after chronic exposure due to the auto-induction of transport proteins and/or metabolizing enzymes. In GIST patients, imatinib clearance increased by 33% over time resulting in a 42% decrease in systemic exposure [19]. Dose escalation can overcome imatinib resistance in some cases in CML [20] and GIST patients [21].

However, doubling the daily dose of imatinib (from 400 to 800 mg) in 934 GIST patients did not result in increased overall survival (OS) in a population without known tumor mutations which supports the need for dose individualization.

Inter-individual variability in the pharmacokinetics of TKIs

Variability in the PK of most TKI is described (Table 2). Extreme variations have been demonstrated in axitinib, imatinib, gefitinib and lapatinib AUCs. The variability in drug exposure to TKIs may contribute to the variation in anti-cancer PD effect. Causes for this variability are manifold during the different processes of ADME. Identified factors affecting drug disposition are genetic polymorphisms, age, gender, diet, smoking, alcohol consumption, renal and liver function, concomitant diseases, co-medication, but also other unknown causes [11]. The genetic factors determining drug disposition are also called pharmacogenetic factors. The frequency of pharmacogenetic polymorphisms differ among ethnic groups (e.g. CYP2D6 duplications frequency is 10-fold higher in Northern Spain than Sweden) [22]. Nevertheless, a natural clustering scheme is difficult to develop due to the complexity of human demographic history [67].

Table 2 Pharmacokinetic variability of small molecules

Drug	Variability of pharmacokinetics expressed in %CV of difference in fold (x)					
	Dosage per day	C_{max}	AUC	Half-life	C_{min}	Ref
Axitinib	10mg	36-93%	39-94%	13-101%		[91]
Dasatinib	50mg/m^2	4.5x		1.4x		[103]
Everolimus	10mg	28%	45%		60%	[104]
Erlotinib	100 mg	28%	36%	43%		[31]
	150mg		64%		51%	[52]
Gefitinib	250 mg	9.6x	15x		18x	[105][106]
	500 mg	19x	16x		11x	
	300 mg MD	75%	113%			
Imatinib	400mg	30-80%	25-55 %	18-39%		[94][107-111]
	600mg	47-70%	40-83%	18-35%		[94][108][110]
	400mg bid	27-39%	19-39%	13-22%	43%	[94]
Lapatinib	1200-1500mg	2.5x	6x		6.2x	[112-114]
	250mg	2.7x	5.2x	1.8x		[95]
Nilotinib	800mg		32-64 %			[155]
Sorafenib	400mg	13-26%	15-18%	5-26%		[26]
	800mg	41-107%	24-91%	22-24%		[26][98][115]
Sunitinib	50 mg	2x				[116]
	50mg	46%	41%		54%	[117]
Vandetanib	100mg	73%	58%	21%		[101]
Vatalanib	300mg	56%	47%		20%	[49]

AUC area under the curve
C_{max} maximal concentration
C_{min} minimal concentration
CV coefficient of variation

Table 3
Enzymes and transporters involved in the PK of small molecules and influence of drug interactions and food

Drug	Active metabolites	Enzyme phase I and II	CYP inhibitor/ inducer	Drug-drug interactions	Fasting/fed	In/efflux transporters
Axitinib	No	CYP3A4, 1A2[91], UGT	?	Rabeprazole: ↑ C_{max} Phenytoine: ↓ 10 x AUC_{24} + C_{max} [91]	↑ C_{max} 49%, ↑AUC, ↓ T_{max} (fasted state)[91]	
Dasatinib	M4, M5, M6, M20 and M24	CYP3A4, UGT	CYP3A4 inhibitor	CYP3A4 inducers: ↓AUC 80% [92][118] ketoconazole: ↑AUC (5x)[119] [120]	No influence	ABCB1, ABCG2, not OCT-1 [121]
Erlotinib	O-desmethyl erlotinib OSI-420	CYP3A[31], 1A2	PXR/CYP3A4 inducer[122]	Rifampin: ↓AUC 67% phenytoin: ↓AUC[30] ketoconazole: ↑AUC 86%[123]	AUC + C_{max} ↑33% (high fat)	ABCB1
Gefitinib	O-desmethyl gefitinib M523595	CYP3A4 and 2D6	CYP2D6 and 2C19 inhibitor	Rifampin: ↓AUC 85%[111] ranitidine: ↓AUC 44% itraconazole: ↑AUC 78% [106] [111]	No influence	
Imatinib	CGP74588	CYP3A (major), CYP1A2, 2D6, 2C9 (minor)	CYP3A, 2D6 and 2C9 inhibitor[124]	Ketoconazole: ↑AUC 40%[125], St. John's Wort ↑CL44%[126], rifampin: ↓AUC68%[8]	No influence [25]	ABCB1[75][127], ABCG2[29], OATP1A2[48], hOCT-1[46]
Lapatinib	GW690006	CYP3A4/5 (major), CYP2C8 and 2C19 (minor)	CYP2C8 and 3A4 inhibitor	Ketoconazole: ↑AUC (3.6x), paclitaxel: ↑AUC (21%), carbamazepine: ↓AUC (72%)[128]	↑absorption 4x (high or low fat) [129]	ABCB1, ABCG2, SLC22A1, OATP1B1 inhibitor, ABCB1, ABCG2 inhibitor[44]
Nilotinib	No	CYP3A4[130]	CYP3A4, 2C9, CYP2D6 and UGT1A1[131] inhibitor	Ketoconazole: ↑AUC (x3)[132] rifampin: ↓AUC(80%)[133]	Biodisp ↑82% (high-fat)[132]	ABCB1, ABCG2[134]
Pazopanib	GSK1268992, GSK1268997, GSK1071306	CYP3A CYP1A2, 2C8 (minor)	CYP3A4 inhibitor CYP3A4 inductor (higher dose)	Ketoconazole: ↑AUC (220%) and ↑ C_{max} (150%) lapatinib ↑AUC and C_{max} (50-60%)	↑x 2 AUC and C_{max} (high or low fat meal)	ABCB1, BCRP
Sorafenib	Pyridine N-oxide BAY 673472	CYP3A4, UGT1A9	CYP2C9 inhibitor, UGT1A1/9 inhibitor	No effect of ketoconazole[38]	Recom. without food	ABCB1 substrate and inhibitor
Sunitinib	SU12662	CYP3A4		Rifampin: ↓AUC (51%)[135] ketoconazole: ↑AUC (50%)[136]	No influence	ABCB1 substrate and inhibitor, ABCG2
Vandetanib	ZD6474			No effect of CYP3A4 inhibitors [22]	No influence [137][138]	
Vatalanib	PTK787	CYP3A4 [102]				

Absorption

Variability in intestinal absorption and entero-hepatic circulation will contribute to the interpatient variation in drug disposition. The influences of fasting, concomitant food intake and medication ingestion for the different TKIs are presented in Table 3. The impact of a high fat meal vary with the molecule considered and may have clinical implications. For example, after a high fat meal, lapatinib exposure increased by 3-fold [23] and nilotinib exposure increased by 82% [24]. Inversely, fasting state increased axitinib drug exposure by 49% as compared to fed state. Food intake had no significant influence on dasatinib, gefitinib, imatinib [25], sorafenib [26] and sunitinib [27] absorption.

Distribution

Following absorption, TKIs bind to plasma proteins, such as albumin and a-1-acid glycoprotein (AGP) (Table 1), and distribute from the intravascular space to their targets in the extra-vascular space. Only unbound (free) drugs are pharmacologically active. Hypoalbuminaemia secondary to malignant cachexia or liver metastases can increase the amount of free drug, leading to increased toxicity. Data assessing the effect of albumin and AGP variation on the studied TKIs are sparse except for imatinib and erlotinib. Plasma AGP concentrations had a marked influence on total imatinib concentrations [28]. In 67 patients with GIST and other solid malignancies, imatinib oral clearance (CL/F) was also positively correlated with body weight and albuminaemia and negatively correlated with AGP [29]. For erlotinib, AGP was one of the most important factors affecting clearance, with a linear correlation between steady-state concentration and AGP levels [30].

Metabolism

The majority of small molecules are metabolized by hepatic phase I and/or II drug metabolizing enzymes (Fig. 1). Phase I oxidative or reductive reactions result in the loss of pharmacological activity of some drugs (e.g. erlotinib) [31] or the activation of others (e.g. temsirolimus, everolimus) (Table 1). For some molecules, both the mother molecule and the major metabolite(s) are active (e.g. imatinib, sunitinib) [32]. Phase II reactions conjugate phase I products or parent drug that usually form inactive polar products readily available for renal and biliary elimination. All studied TKIs are metabolized by hepatic CYP3A, the most abundant CYP in the human liver and intestine, whose protein expression varies up to 20-fold among individuals within the liver [33][34]. The expression of CYP3A is regulated at the transcriptional level by the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) [35]. The variable CYP3A4 activity can have a profound effect on clinical outcomes. Factors that may contribute to this variability are drug–drug interactions (inhibition and induction), environmental factors, genetic polymorphisms and certain disease states [36][37].

Drug–drug interactions

Drug–drug interactions with CYP3A4 inhibitors and inducers need to be taken into account for all TKIs and have to be used with caution as advised in the drug information of most small molecules. For example, sorafenib is metabolized through two pathways: oxidation to the main active metabolite sorafenib N-oxide mediated by CYP3A4; and conjugation to a minor metabolite mediated by UGT1A9. The co-administration of the potent CYP3A inhibitor ketoconazole did not lead to the predicted increase in sorafenib plasma concentration, even though the formation of the oxidative metabolite, sorafenib N-oxide, decreased [38] This suggests that conjugation mediated by UGT1A9 is able to compensate when the oxidation pathway is suppressed. Other relevant examples of drug–drug interactions with small molecules are presented in Table 3.

Genetic polymorphism

Most of the genes encoding for drug metabolizing enzymes are polymorphic which may contribute to the inter-individual PK variability of small molecules. Ethnic background should therefore be taken into account as a contributor to variation in drug disposition and response [11]. A number of studies have outlined the role of ethnicity for cytotoxic chemotherapy in the past. Even though data, remain purely experimental for most of TKIS, some genetic changes have also been associated with response to treatment such as imatinib [29] and gefitinib [39]. However, the clinical utility of pharmacogenetics still needs to be proven in large prospective randomized controlled trials of patients with difference ethnic background.

Elimination

Small molecules are excreted mainly through the biliary tract, while a minority of the dose (~10%) is excreted by the kidney. In the liver, many transport systems are involved in drug uptake by hepatocytes and biliary elimination, such as the solute carrier (SLC) and the ATP-binding cassette (ABC) transporters. Liver metastasis, concomitant medications and other diseases can affect transporter systems and reduce biliary elimination of anticancer drugs. Indeed the half-life of erlotinib was prolonged in patients with hepatic dysfunction possibly due to the increase of the volume of distribution by reduced protein binding [40]. Impaired renal function may potentially decrease clearance and increase the toxicity associated with these drugs. Gibbons et al. found an increased imatinib exposure in patients with mild or moderate renal dysfunction even though less than 13% of imatinib is excreted in the urine [25]. Reduction in hepatic metabolism is however demonstrated in patients with chronic renal failure. Moreover, serum AGP concentrations are increased in patients with renal dysfunction. This results in increased total imatinib plasma concentrations, but decrease the free fraction or unchanged free drug level, leading to an apparent increase in imatinib clearance [25]. Dasatinib, erlotinib, everolimus, nilotinib and imatinib are substrates for the efflux pump ABCB1 (encoded by MDR1) [41]. Lapatinib is a substrate of ABCB1 and BCRP1 [42] and these two transporters had a synergistic effect on the uptake of

lapatinib in knock out mice [43]. Lapatinib is also a direct inhibitor of ABCB1 and ABCG2 transport function [44]. Only limited (preclinical) data is available in regard to ABCB1 and other small molecules [45]. Sparse data is available for other efflux pumps such as ABCG2, a carrier for imatinib, nilotinib and dasatinib. Observations on other influx pumps such as OCT-1 and imatinib [46] and dasatinib [47] and OATP1A2 [48] and imatinib are presented in Table 3. Interpatient variation in these drug transporter activities determined by genotypes is an important recent discovery. However, imatinib is so far the only drug for which clinically significant data is available. Studies are ongoing to assess the impact of other gene polymorphisms on the pharmacokinetic of sorafenib and sunitinib.

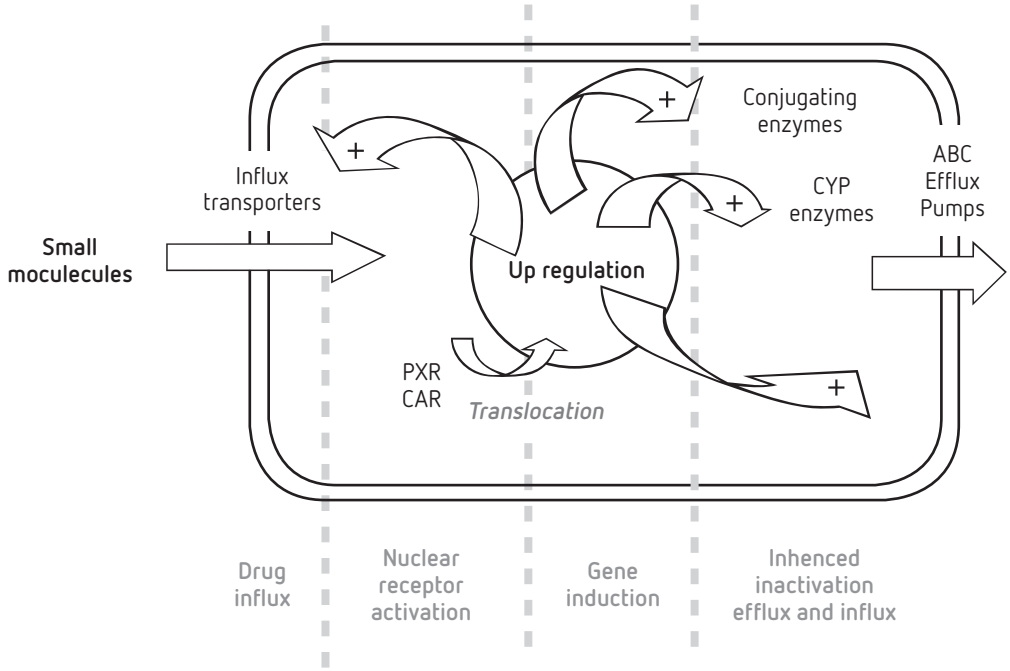

Fig 1 Schematic drug disposition in hepatocytes

Table 4 Pharmacokinetic and pharmacodynamic correlations

Drug	Correlation	PK parameter	Outcome	Ref
Erlotinib	Yes	AUC and C_{max}	Rash	[53]
	No	AUC and C_{max}	Diarrhea	[53]
	Yes	AUC_{24}	Cutaneous toxicity	[52]
	Yes	Css_{min}	Time to progression (and ↓ pEGFR)	[139]
	Yes	C_{ss} and C_{min}	Survival	[140][141]]
Gefitinib	Yes	unbound gefitinib AUC	Diarrhea	[74]
	No	unbound gefitinib AUC	Rash	
	Yes	unbound gefitinib AUC	↓pMAPK	
Imatinib	Yes	Plasma C_{min}	Response	[13]
	No	Serum levels	Tissue levels in tumor	[142]
	Yes	Higher dose (800mg *vs* 400mg)	Progression free survival (GIST with *KIT* exon 9 mutants)	[143]
	Yes	AUC	Neutropenia	[144]
	Yes	Higher dose (800mg *vs* 400mg)	Cytogenetic and earlier molecular responses (CML)	[145]
	Yes	Free AUC	Response (GIST)	[28]
Lapatinib	Yes	C_{min}	Response	[112]
Sorafenib	No	C_{ss}	DCE-MRI response	[146]
Sunitinib	Yes	AUC	Response	[147]
Vatalanib	Yes	C_{min}, AUC and C_{max} (day 2)	Outcome	[49][50][51]

AUC area under the curve
C_{max} maximal concentration
C_{min} minimal concentration
CML chronic myeloid leukemia
C_{ss} concentration steady state
CV coefficient of variation
DEC-MRI dynamic contrast-enhanced resonance imaging
GIST gastrointestinal stromal tumor.

Pharmacodynamic consequences of inter-individual variability

The available literature on the PK-PD correlations of small molecules was examined in order to assess whether the interpatient variability impacts on drug antitumor activity, safety and outcomes. A certain set of PK parameters have been correlated to response to TKIs, either response or toxicity.

Efficacy

Indeed, plasma trough levels of imatinib were correlated to response in the treatment of CML [13]. The AUCs of dasatinib, erlotinib, lapatinib, sorafenib and sunitinib were also positively correlated with the likelihood response (Table 4). Vatalanib trough plasma concentration, AUC and maximal concentration (on day 2) were positively correlated with likelihood of response in metastatic liver lesions on imaging [49–51].

Toxicity

Some PK parameters have also been positively correlated with toxicities for some TKIs. Indeed, erlotinib AUC was positively correlated with the occurrence of skin toxicity in two independent studies [52][53]. Some adverse drug reactions to small molecules are called 'group effects', due to their relationship to the supposed mechanism of action. Cutaneous toxicity (rash) is a common example of toxicity of EGFR inhibitors. An association has been found between erlotinib-induced rash, reduction in EGFR levels on skin biopsy and survival in patients with lung cancer [54]. Rash has also been correlated to gefinitib response in several studies [55]. Hypertension is another example of group effect of VEGF inhibitors, which might be due to inhibition of vascular relaxation, decreased production and rarefaction of nitric oxide [56][57]. Indeed, the rise in diastolic blood pressure during axitinib treatment was a predictor of longer survival in patients with various malignancies [58]. Thyroid dysfunction may be another specific toxicity of VEGF tyrosine kinase receptor inhibitors, and its occurrence has some predictive value in the response to sunitinib [59] and axitinib [60]. Despite these associations have been demonstrated between PK parameters and outcomes/toxicities, the challenge remains to find markers of biological activity (so called biomarkers), including non-invasive PK techniques, to ensure that adequate active doses are administrated. Surrogate markers on the other hand are a measure of the biological activity inside the body that indicates the effect of treatment on the state of disease. Some surrogate markers of biological activity have been described in the literature but to date only a few have been found to be therapeutically relevant (Table 5).

Table 5 Surrogate markers of clinical response

Drug	Correlation	Surrogate marker	Outcome	Ref
Axitinib	Yes	↑ dBP > 90mmHg (on therapy)	↑ survival	[58]
Erlotinib	Yes	Skin toxicity	↑ response ↑ survival	[148][149]
	No	Diarrhea		
	Yes	Severity of skin toxicity	TTP, ↑ Overall survival	[139]
	Yes	↓ p-EGFR expression	TTP	
	Yes	Severity of skin toxicity (> grade 2)	↑ Overall survival	[140]
	Yes	UGT1A1*28	Hyperbilirubinemia	
	Yes	↓ p-EGFR level (skin biopsy)	↑ clinical outcome	[150]
Everolimus	Yes	S6K1 inhibition in tumors or PBMCs	Antitumor effect	[55]
Gefitinib	Yes	Skin toxicity	TTP	[151]
	Yes	Midazolam oral clearance	Gefitinib exposure	[105]
	Yes	ABCG2 (421C>A)	Diarrhea	[39]
Imatinib	Yes	NK cell activation	TTP	[152]
	Yes	CYP3A5 rs776746 AA	Cytogenic and molecular response	[29]
	Yes	SLC22A1 rs683369 CC	Molecular response	[29]
	Yes	CYP2D6*4	Decreased clearance	
	Yes	Cytogenetic response	↑ Survival	[153]
Lapatinib	No	Diarrhea		
Sunitinib	Yes	Circulating sKIT	TTP	[154]
	Yes	Thyroid dysfunction	PFS	[59]

ABCG2 ATP-binding cassette sub-family G member 2
dBP diastolic blood pressure
ERK extracellular signal-regulated kinase
NK natural killer
PBMCs Peripheral blood mononuclear cells
p-EGFR phosphor-epidermal growth factor receptor
PFS Progression free survival
S6K1 ribosomal protein S6 kinase beta 1
TTP time to progression
sKIT soluble cytokine receptor CD117

Guidance based on preclinical data

In order to inhibit an oncogene target sufficiently, a critical drug concentration is required, which may be reflected by the blood drug levels in cancer patients. In the case of dasatinib, in a human CML xenograft mice model, pharmacokinetic/biomarker modeling predicted that the total plasma concentration of dasatinib required for inhibiting 90% of phospho-BCR-ABL in vivo was 10.9 ng/mL in mice and 14.6 ng/mL in humans, which is within the range of concentrations achieved in CML patients who responded to dasatinib treatment in the clinic (61). Inhibition of mTOR by everolimus inactivates the downstream targets p70S6 kinase and elF4E of the Akt pathway, resulting in G1 phase cell cycle arrest and subsequent inhibition of tumor growth. In a rat model, the hypophosphorylation of S6 kinase 1 (S6K1), a downstream target of activated mTOR, in peripheral blood mononuclear cells (PBMCs) has been shown to correlate with the antitumor effect of everolimus. This was confirmed in humans and can potentially be used as a pharmacodynamic (PD) marker in patients [55][62][63].

Dose individualization alternatives

The challenge of dose individualization goes back to the 19th century, where Sir William Osler wrote in 1892: "If it were not for the great variability among individuals, medicine might as well be a science and not an art". More recently Dr. Gerhard Levy wrote in 1998; "Emphasis should not be focused on population averages, but rather on providing prescribers with the tools to determine the most effective and safest dosage for individual patients with a minimum of trial and error" [64]. In the era of fast development of new anti-cancer treatments, mainly small molecules, the challenge of dose individualization has not been solved. Clinical evaluation of these new promising drugs has been performed in the same way as it has been done for intravenous classical chemotherapy in the past, with cohort dose escalation phase I studies. Furthermore, the chronic prescription of TKIs makes the importance of dose adjustments even more central. Alternative concepts such as intra-patient dose escalation have been proposed for classical chemotherapy by Simon et al. in 1997, but have not been used in the development of small molecules [65]. Some authors have even stated that the use of traditional phase I studies to assess maximum tolerated dose (MTD) and dose limiting toxicities are not suitable to new anti-cancer drugs. Indeed, the MTD might not be reached by those agents with highly specific targets if their therapeutic ratio is wide. Alternative endpoints such as molecular drug effect (target effect) through PK-PD could help to identify a minimum target-inhibiting dose (MTID) and therefore the optimal dose schedule [66]. Cytotoxic chemotherapy is traditionally dosed according to body surface area (BSA), even though body size does not substantially reduce inter-patient variability [67]. Other dosing methods have been examined, such as GFR-based dosing for carboplatin [68]. However, renal elimination is a minor route of elimination of TKIs and is unlikely to be helpful in dose calculation. Other investigated dosing methods are based on tests investigating the individual ability to handle drugs according to their phenotype (activity of the enzyme or transporter) called phenotype-guided dosing. Genotypic dosing is based on the individual 'fingerprint' of drug disposition genes. Dose

adjustment methods of therapeutic drug monitoring, toxicity- adjusted dosing and ramp-dosing are also reviewed.

Phenotype-guided dosing

There are several representative phenotype tests for CYP and ABC activities readily available, such as midazolam clearance that assesses CYP3A activity [69], cocktail approaches[70] or sestamibiliver-scans assessing ABCB1 efflux-pump [71][72]. Probe phenotyping drugs are now used as microdoses ensuring that none of the side effects generally observed appears which makes it a minor burden for patients and is easy to perform. The use of midazolam as a single oral microdose (75 µg) that is 100-fold lower than a therapeutic dose (7.5 mg) is now used routinely to assess CYP3A activity [73]. Several studies have investigated the predictive value of phenotype tests on drug disposition. The midazolam clearance test was for example a good predictor of drug exposure in the treatment with gefitinib, explaining about 40% of the inter-individual variability [74]. However, investigations of these probes in the treatment with imatinib did not show a correlation with imatinib drug exposure in a small study [75]. Another study assessing the impact of ABCB1 and CYP3A phenotypes on sunitinib response and PK is ongoing (Clear Sun study ClinicalTrials.gov Identifier NCT01098903).

Genotype-guided dosing

Genotype studies performed with small molecules have focused mostly on single nuclear polymorphisms (SNPs) analysis instead of haplotypes or whole gene array analysis (see Table 3). Despite the growing knowledge of the human genome and haplotypes, there are only few data on genotypic predictive markers. Recently van Erp et al. were able to find significant correlations between sunitinib toxicities (thrombocytopenia, leukopenia, mucosal inflammation, hand-foot syndrome, and any toxicity higher than grade 2) and certain SNPs among 12 candidate genes encoding for drug metabolizing enzymes, efflux transporters and drug targets [76]. In addition, Eechoute et al., found a relationship between polymorphisms involved in sunitinib pharmacokinetics (CYP3A5, NR1I3, and ABCB1) and progression-free survival in patients with renal-cell cancer [77]. Other haplotypes determination studies are ongoing for several small molecules in order to assess correlation with PK, and/or outcome. We demonstrated in a multicentric pharmacogenetic study (n = 158) that higher doses of imatinib were associated with polymorphisms of some influx transporters (SLC1A2, 10A1 and 22A1) as well as CYP3A5 (78).

Therapeutic drug monitoring (TDM)

TDM is a measure of plasma drug concentrations to individualize dosage in order to achieve a target blood concentration. As a general rule, TDM has been proven useful in situations where a drug has a narrow therapeutical window and/or a large PK variability, but also when a direct relationship between plasma concentrations/effects (efficacy or toxicity) has been demonstrated. TDM is widely used for a number of medications including antibiotics, anticonvulsants, and cancer chemotherapy [72]. In oncology, TDM is routinely used

to prevent overdosing of methotrexate. In the case of TKIs, trough level measurements are not routinely used but could give the potential to individualize dosing. There is some evidence that TKIs might benefit from a TDM. Imatinib concentrations were associated with TKIs response to standard-dose treatment in a retrospective study [13][16]. Kirchner et al. demonstrated a high correlation between steady-state trough concentration and the exposure to everolimus [79][80]. This simple and reliable parameter for monitoring everolimus exposure is however not used in the treatment of renal-cell cancer routinely. In non-oncology fields the same problems are seen, such as in the use of anticonvulsants, where in spite of the evidence for the benefit of TDM for treatment individualization, TDM is considered unfavourable for the marketing of a new drug [81]. The benefits of TDM should be studied in well designed prospective studies. The utility and cost effectiveness of TDM should however be first validated in prospective studies. Another impediment to the widespread implementation of TDM is the need for staff, instruments and skills to perform the commonly used liquid chromatography–tandem mass spectrometry.

Toxicity-adjusted dosing (TAD)

Another approach to achieve dose individualization is the use of toxicity-adjusted dosing, as proposed with classic chemotherapy regimen more than 10 years ago [68][72][82][83]. This method is based on the theory that specific toxicities are predictors of drug bioavailabilities, such as neutropenia in adjuvant chemotherapy for breast cancer [84] or skin toxicity in cetuximab therapy [85]. Given the heterogeneity of tumor sensitivity to toxic drugs as compared with normal tissue, it is not surprising that positive correlations are more readily found for toxicities than for tumor responses. This has lead to the use of toxicity as a guide for achieving maximally tolerable drug levels. In order to ensure that a safe non-subtherapeutic dose is prescribed, some authors now suggest that pharmacological endpoints should be used to adjust dose for each patient [68][72][82][83]. For example, grade 3 hypertension could potentially be used as a pharmacological endpoint for response to treatment of sunitinib [86] and axitinib [58]. However, not all toxicities have been proven useful for this approach. Some toxicity such as diarrhea after treatment with lapatinib is not associated with likelihood of response. It has been suggested that diarrhea is due to unabsorbed lapatinib, as it is better correlated with dose than with plasma concentration [23]. A low-tech, albeit rather blunt form of individualized dosing is ramp-dosing, defined as intra-individual dose escalation (weekly or biweekly) until dose limiting toxicity (e.g. Grade 3–4 toxicity as defined by the CTC criteria) occurs. This simple dosing method was effective with sorafenib in a small phase II study, where an increase in overall response was demonstrated [87]. The same pattern and intensity of toxicities were observed after each dose escalation step. Interestingly, after 2–3 weeks a decrease in the toxicity is demonstrated. This was seen within the first 3–4 dose escalations until dose limiting toxicities were reached. A larger phase II study is ongoing to examine this dosing method for sorafenib (Clinical- Trials.gov Identifier: NCT00618982).

Compliance

An important factor to explain part of the inter-individual variability of TKI is finally compliance. Orally prescribed drugs offer unprecedented convenience with greater autonomy and easier administration without venopuncture but the hazards of patient compliance issues need to be taken into consideration. Compliance and safety issues have been extensively discussed for oral chemotherapy in the past. A recent American study examining the current safety practices for the use of oral chemotherapy demonstrated no consensus about safe practices of these drugs, and that few of the safeguards suggested for parenteral drugs are used for oral agents in the ambulatory setting [7]. DeMario and Ratain concluded in 1998 that for self-administered oral regimens, quantifying compliance rates are essential for the accurate determination of regimen efficacy [88]. Non-compliant rates of 43% even in a curative setting of breast cancer treatment has been reported, and is demanding novel approaches of patient compliance. Electronic activated tablet bottles have been suggested to cope with this problem. However they have not been implemented broadly. Ethical questions need to be addressed such as respecting the autonomy of the patients in making their own choices, safety issues with the use of ambulatory oral small molecules or the need for drug level measurements to ensure patient's compliance.

Future directions

There is not only a challenge to find the right starting dose but also to carry out any proper subsequent dose adjustments. Three methods to achieve dose titration were assessed based on laboratory and clinical concepts. One is TDM where regular trough levels are measured during treatment. The second is TAD based on the concept that a lack of toxicity may be a sign of under dosing and exceeding toxicity a sign of overdosing. The third is ramp-dosing (a variation of TAD) where doses are increased stepwise over time and adjusted depending on tolerability. All three methods have advantages and disadvantages and need validation by prospective studies with each small molecule. In TDM, the optimal drug level or minimal target inhibiting drug level needs to be found. The question remaining is whether such a level, found in a group of patients, is specific enough to be translated to the individual patient. Ideally, biomarkers of target inhibition would be used to find the individual target drug levels. For TAD, the importance to find surrogate toxicities to predict the optimal dose is crucial and also need to be confirmed in larger prospective studies. Common toxicities of a group of small molecules such as rash and EGFR inhibitors, or hypertension and VEGFR inhibitors may be useful surrogate markers of efficacy. Given the complexity of potential factors influencing drug disposition, the development of predefined dosing algorithms are very promising. An algorithm would be able to combine pharmacogenetic factors with clinical determinants such as performance status and co-medication as already developed for irinotecan [89] and suggested for imatinib, combining the plasma trough levels and gastric resection in GIST patients [90].

CONCLUSIONS

Tyrosine kinase inhibitors are new promising tools available to medical oncologists in the treatment of cancer. However, fixed dosing is still standard practice, even though the problems associated with interpatient variations in the treatment response, known from classical chemotherapy, have not vanished and may even be greater because of variability in exposure due the bioavailability variation of these oral drugs. This review demonstrated the wide inter-individual variability of the PK of the tyrosine kinase inhibitors and some preliminary evidence of its consequences on treatment response. Alternatives to fixed dosing were described such as TDM, TAD and ramp-dosing. These alternatives should be systematically explored in prospective randomized studies. Dose individualization should become a standard of care and a priority in research for new small molecules in development. The development of predefined dosing algorithms should allow answering the question of the 'right dose'.

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Chapter 3

Pharmacogenetics of imatinib in CML patients, prediction of toxicity, dose adjustments and response

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Submitted for publication

ABSTRACT

Background

In patients treated with imatinib (Gleevec®) a 4-fold interpatient variability in drug exposure exists, partly as a result of pharmacogenetic variability, which implies that some patients may be underdosed if a fixed dose is used. In this study we identified genetic markers to predict the ability to tolerate a higher dose of imatinib and also examined the relationship of these markers with imatinib-induced toxicity.

Patients and Methods

A multicenter pharmacogenetic association study was performed in chronic myeloid leukemia patients treated with single agent imatinib. A total of 385 single nucleotide polymorphisms (SNPs) in 129 genes, known to be involved in drug absorption, disposition, metabolism and elimination, were tested for their association with three dose-level/ toxicity categories. These categories were defined based on starting dose, toxicity profile and dose adjustments at 3 and 12 months after start of treatment.

Results

Seventy-seven patients were accrued. At 3 and 12 months, 26% and 47% of patients fitted the category tolerating higher-doses. Between 3 and 12 months of treatment 5 patients stopped treatment. Tolerability of a higher dose at 3 months and 12 months were significantly associated with SNPs in CYP3A5 (rs 776746) and a novel gene, ABCC12, a member of the multi-resistance protein subfamily. Imatinib-induced non-hematological toxicity was highly correlated with SNPs in CYP2B6 (p=00002) and a glutathione transferase gene (GSTM5, p=00002), Hematological toxicity was associated with multiple SNPs including those in CYP8B1, and a number of ABC transporters.

Conclusion

In this exploratory analysis on imatinib disposition, several novel relationships between metabolizing enzymes and drug transporters and tolerability of imatinib were found. These genetic biomarkers need to be confirmed in prospective studies.

INTRODUCTION

Imatinib mesylate (imatinib) is the first tyrosine kinase inhibitor that has significant activity in chronic myeloid leukemia (CML) and gastro-intestinal stromal tumours (GIST).[1-3] This oral drug is known to inhibit BCR-ABL, the receptor for platelet-derived growth factor (PDGF), and c-kit tyrosine kinases.[4] After enteral absorption, imatinib is mainly bound to plasma proteins with a bioavailability of nearly 100%.[5] Only 10% is renally cleared, with the main route of elimination by hepatic metabolism and biliary excretion. More than 80% of unchanged or metabolized imatinib can be recovered from faeces. Conversion of imatinib to the equally active metabolite CGP74588 takes place in the liver by cytochrome P450 (CYP) family of enzymes,[6] of which, CYP3A4 and CYP3A5 appear to be the most important, although CYP1A2, CYP2D6, CYP2C9 and CYP2C19 may also participate.[5][7][8] The expression of CYP3A is regulated at the transcription level by the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR).[9]

Excretion of both unchanged and metabolized imatinib into the bile is an active process undertaken by a number of efflux pumps, particularly the ATP-binding cassette transporters, sub-family G member 2 (ABCG2, formerly called BCRP) and sub-family B member 1 (ABCB1, formerly called multi-drug resistance 1 [MDR1] or P-glycoprotein [P-gp]).[10] Active uptake of imatinib in the small intestine, and by the kidneys and liver, has been found to be facilitated by solute carriers such as organic cation transporter 1 (OCT1 also called SLCO22A1) and organic anion transporter 1A2 (OATP1A2 also called SLCO1A2).[11-13]

There is a wide interpatient variation in drug exposure. A fixed dose of imatinib is associated with a four-fold variation of imatinib blood levels between patients treated within the conventional dose range.[2][14] Despite this, imatinib is usually given at a fixed dose of 400 mg for GIST and CML, or 600mg in advanced phases of CML. It is possible that this wide inter-individual variability in drug exposure impacts on response and toxicity.[5][15] For example, non-responders have significantly lower plasma levels of imatinib compared to responders, in both CML[16-20] as well as GIST.[21] Another known cause of variation in response is so-called acquired pharmacokinetic (PK) resistance[22] where, despite a good initial response to imatinib, drug resistance may appear after prolonged treatment. This resistance is partly due to new mutations in the tyrosine kinase receptor which have a reduced affinity for imatinib[23][24] but may also result from auto-induction of transport proteins and/or metabolizing enzymes. Imatinib clearance increases within individuals over time, resulting in a decrease in systemic exposure.[25] Subsequently, dose escalation may overcome therapy resistance in some cases of CML[26][27] and GIST.[28] On the other hand, the unselected increase in the dose of imatinib was not associated with an increase in overall survival (OS) in GIST patients (400 vs. 800 mg /day).[29] These data suggest the need for dose individualization for this drug.

There are several single nucleotide polymorphisms (SNPs) described that may partly explain the inter-individual variability of imatinib drug disposition; namely those in ABCB1[30-33], ABCG2[34][35], SLC22A1[11][12] and CYP3A4/3A5[6][8][36]. However, it is likely that other variations in drug metabolism and elimination (DME) genes are also involved. In a previous study, we showed a correlation between polymorphisms of ABCB1 and CYP3A5 and tolerability to the administered dose after three months of treatment.[33]

The primary purpose of the present study was to identify possible new genetic markers for imatinib disposition associated with the ability to tolerate a higher starting dose of imatinib in patients with CML. We speculate that there is a correlation between drug exposure and toxicity for imatinib. If so, the level of toxicity has the potential to act as a surrogate marker for drug disposition. Those patients with no or minimal toxicity at standard doses, may have enhanced drug elimination and thus need a higher-than-standard dose of imatinib. The standard dose of imatinib in GIST is 400 mg daily and infrequently requires dose reduction due to toxicity. For this pharmacogenomic study we therefore focused on patients with CML due to the fact that they are usually treated with upfront doses of higher than 400 mg daily. The most commonly reported adverse events of imatinib are edema (including peripheral and periorbital edema) (60%), muscle cramps (49%), diarrhea (45%), nausea (50%), musculoskeletal pain (47%), rash and other skin problems (40%), abdominal pain (37%), fatigue (39%), joint pain (31%), and headache (37%). Grade 3 or 4 adverse events of imatinib in a phase 3 study in CML are: neutropenia (17%), thrombocytopenia (9%), anemia (4%), elevated liver enzymes (5%), and other drug-related adverse events (17%).[37]

Patients and Methods

This was a retrospective study of patients treated with imatinib for CML correlating toxicity and dose, with a panel of drug metabolising and elimination (DME) SNPs in those patients. Study participants were identified at participating hospitals and tissue banks. To be eligible patients needed to have received single agent imatinib for at least 10 weeks (to ensure steady state drug levels) and to have given informed consent prior to study participation. Additional eligibility criteria included: (a) Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1 or 2 at start of treatment with imatinib; (b) Availability of medication records of the first 3 months ± 14 days of treatment; (c) Starting dose of 400 mg imatinib or higher. The study was approved by the Westmead Hospital institutional review board (HREC2006/3/4.17[2317]).

Data collection focused on the following time frames of assessments: (a) the start of imatinib (b) after 4-8 weeks of treatment (c) after 10-14 weeks of treatment, and (d) whenever available, 11-13 months after start of treatment. Data collected at these time frames included laboratory tests of hematology, liver and renal function, defined as serum bilirubin, AST, ALT, and serum creatinine. Further clinical data included patient demographics, ethnicity and histopathology. Also collected were concomitant medications and imatinib dosage at the above time points. Response was assessed by bone marrow evaluation for morphology, conventional cytogenetic analysis and BCR/ABL mRNA reverse transcriptional PCR at baseline and follow-up and interpreted using the National Cancer Institute-Working Group 1996 guidelines.[38]

Definition of Toxicity-adjusted-dosing

Toxicity scores of imatinib were assessed by analysis of adverse events, physical examination and laboratory assessments. All adverse events were graded using National Cancer Institute- Common Terminology Criteria of Adverse Events version 3.0 by independent physicians of the participating medical centers.[39]. Toxicities were grouped as hematological or non-hematological toxicities.

The primary outcome measures of this study were toxicity-adjusted-dosing at 3 months as defined by the dose the patient was taking 3 months after commencement and allowing for dose adjustment due to toxicity. Dose adjustments were made at the discretion of the treating specialist or according to study protocols in case of treatment within a clinical study.

For the purposes of this study three 'dose tolerability' groups were inferred by a pre-defined definition based on the actual dose received (at 3 and 12 months) and the toxicity experience at that dose by individual patients. (Table 1).

Table 1 Inferred dose tolerability categories of imatinib

Dose and toxicity experienced	Dose tolerability category
< 400 mg or 400 mg with toxicity (grade 2 or higher)	Low
400 mg without toxicity 500 mg any toxicity 600 mg with toxicity (grade 2 or higher)	Medium
600 mg without toxicity 700 mg any toxicity 800 mg any toxicity	High

Genetic Polymorphisms

In all patients, about 5-10 mL of whole blood was collected into EDTA tubes and stored at minus 20-80°C at respective sites. Samples were anonymized according to the instructions stated in the Codes for Proper Use and Proper Conduct in the Self-Regulatory Codes of Conduct (www.federa.org). Genomic DNA was extracted from whole blood using the QIAamp DNA Blood Midi Kit (Qiagen, Valencia, CA), according to manufacturer's instructions. Genotyping was performed using Goldengate technology (Illumina).

Twenty-one SNPs in 9 candidate genes known to be involved in the pharmacokinetics of imatinib were selected from the literature. Another 364 SNPs in 129 candidate DME genes were also selected. The criteria for selection of the polymorphisms were those DME SNPs that an allelic frequency higher than 0.2 in CEU population of the HapMap project (www.hapmap.org) and an assumed clinical relevance based on previously reported associations or the assumption that non-synonymous amino acid change leads to changed protein functionality. The selected SNPs are listed in supplementary appendix B.

Statistical Design and Data Analysis

All SNPs were analyzed for a possible association with the three defined categories of inferred dose tolerability (low, medium, high; table 1) using chi-square-tests with one degree of freedom. A one-way analysis of variance was used to evaluate associations between imatinib dose and ABCB1 genotype. A P-value of less than 0.05 was considered statistically significant. Because of the small sample of only 77 patients we did not perform a multiple test correction such as the Bonferroni method because that would mean that the power of the comparisons would be reduced greatly. We therefore accepted the increased type-I error rate and interpreted the results cautiously and as hypothesis generating only. We did however report the exact p-values so that readers are able to adjust the significance level for multiple testing; the Bonferroni adjusted significance level was about 0.0001.

All statistical analyses were performed using SPSS version 16.0 software (SPSS, Chicago, IL) and R, an object oriented free and open source statistical computing environment (http://www.r-project.org).

RESULTS

A total of 77 patients were enrolled at 13 centres in Australia (supplementary appendix A) between May 2006 and June 2008. Between 3 and 12 months after start of imatinib treatment 5 patients stopped their therapy (due to toxicity or progression of disease).

Patient characteristics

Patient's demographics and disease characteristics prior to imatinib therapy are summarized in Table 2. The majority of patients had a good performance status (>90% ECOG 0 or 1). A total of 11 patients were started with an imatinib dose of 400 mg daily, 55 patients were started with a dose of 600 mg, and 11 with a dose of 800 mg. Dose reductions within the first 3 months of treatment were necessary in 18% of patients. Eighteen patients (23%) had a dose escalation within the first 12 months due to disease resistance to imatinib therapy. The proportion of patients fitting in the predefined category of 'high dose' tolerability nearly doubled from 26% (n=20) at 3 months to 47% (n=36) at 12 months. Table 2: Demographic characteristics of patients included in the study at baseline (N=77)

Toxicity induced by imatinib

Non-hematological grade 1 and 2 toxicities were most commonly observed (Table 3), which is comparable to literature data.(37) Over the 12 months of treatment the frequency of grade 3/4 toxicity diminished. The rate of grade 3/4 hematological toxicity within the first 3 months was 29% and decreased to 12% between 3 and 12 months (P = 0.002). Similarly, the incidence of non-hematological grade 3/4 toxicities reduced from 17% to 4% over the same period (P = 0.001). Sixteen patients (20%) had a toxicity-induced dose reduction in the first 3 months, but later fourteen (18%) of those tolerated a higher dose at

Table 2 Patient characteristics at baseline (N=77)

Characteristic		Median	Range	No	%
Age (years)		50	21-75		
Body Surface Area m^2		1.90	1.4-2.4		
Sex	Male			45	59
	Female			31	40
	Missing			1	1
Ethnicity	Caucasian			49	64
	Asian			4	5
	Polynesian			1	1
	Missing			23	30
Imatinib treatment within a clinical trial	Yes			65	84
	No			12	16
Previous medical treatments	Yes			9	12
	No			68	88
ECOG performance status	0			61	79
	1			11	14
	2			2	3
	3			0	0
	Missing			3	4
Starting dose of imatinib	400mg			11	14
	600mg			55	72
	800mg			11	14

Baseline chemistry and hematology	Median	Range
Creatinine, µM	83	44-160
Total bilirubin, µM	10	2-27
Albumine, gram/L	41	33-47
ALT, units/L	24	8-63
AST, units/L	24	1-67
Hemoglobin, g/L	123	65-169
Leukocytes, $\times 10^9$/L	46	2-297
Thrombocytes, $\times 10^9$/L	400	90-1216

ECOG Eastern Cooperative Oncology Group
CML chronic myeloid leukemia.

Table 3 Distribution of imatinib doses and toxicity grades (N=77)

			No	%
Mean imatinib dose in mg per day at	Day1	(N=77)	600	
	6 weeks	(N=75)	553	
	3 months	(N=76)	562	
	12 months	(N=72)	621	
Dose changes within 3 months of imatinib treatment	increase		1	1
	no change		60	78
	decrease		16	20
	stop		1	1
Dose changes between 3 and 12 months compared with start dose	increase		18	23
	no change		43	56
	decrease		11	14
	stop		5	7
Imatinib Category at 3 months of treatment	Low		13	17
	Medium		44	57
	High		20	26
Imatinib Dose Category at 12 months of treatment	Low		7	9
	Medium		29	38
	High		36	47
	Stopped treatment		5	6
Toxicity within first 6 weeks (n = 77)	Nonhematological			
	Grade 1/2		55	71
	Grade 3/4		12	16
	Hematological			
	Grade 1/2		39	51
	Grade 3/4		14	18
ECOG performance status	0		61	79
	1		11	14
	2		2	3
	3		0	0
	Missing		3	4
Starting dose of imatinib	400mg		11	14
	600mg		55	72
	800mg		11	14

12 months of whom three patients tolerated a dose higher than their starting dose. Median starting dose of imatinib was 600mg and at 12 months was 621mg (P=0.131). Five patients stopped treatment during the first 12 months; four patients due to toxicity despite dose reduction to 400mg, and one patient due to progressive disease.

Best response to treatment within the first 12 months

All of the 77 patients were evaluable for analysis and had a complete hematological response. Of those 86% had a complete cytogenetic response, but only 48% had a major molecular response (MMR). Twelve of the 18 patients with a dose increase between 3 and 12 months did not achieve MMR. The mean time to best response was 7 months (range 2-13 months) for all patients.

Table 4 Best Response within the first 12 months of treatment with imatinib

Best Response		No	%
CML (n = 77)			
Complete hematological response	Yes	76	99
	No data	1	1
Cytogenetic response	No response	1	1
	Major response	8	10
	Complete response	66	86
	No data	2	3
Major molecular response	Yes	37	48
	No	37	48
	No data	3	4
Mean time to best response in months after start of treatment	7		
Range	2-13		

CML chronic myeloid leukemia.

Table 5 **Results of the univariate analyses of all literature-described SNPs in regards to the tolerability of a higher or lower dose at 3 and 12 months, and grade3/4 toxicity.**
Boxes with gray background show the significant associations defined as p-value < 0.05.

					Tolerability of higher dose at 3 months		
					MAF		
SNPs	Chromosome	Position	N	Gene-symbol	controls	cases	P-value
rs2231142	4	89052323	77	ABCG2	0.89	0.825	0.27
rs274558	5	131721174	77	SLC22A5	0.39	0.4	0.89
rs274548	5	131730807	77	SLC22A5	0.77	0.8	0.70
rs1045020	5	131730011	77	SLC22A5	0.90	0.875	0.75
rs628031	6	160560845	77	SLC22A1	0.67	0.65	0.85
rs683369	6	160551204	77	SLC22A1	0.78	0.9	0.11
rs1128503	7	87179601	77	ABCB1	0.55	0.65	0.23
rs2032582	7	87160618	77	ABCB1	0.51	0.65	0.094
rs1045642	7	87138645	77	ABCB1	0.5	0.625	0.13
rs28365094	7	99250475	77	CYP3A5	0.12	0.125	0.97
rs28365067	7	99272310	77	CYP3A5	0.53	0.525	0.96
rs28371764	7	99277593	77	CYP3A5	0.96	1	0.22
rs776746	7	93906909	77	CYP3A5	0.14	0.0	0.017
rs10841795	12	21487544	77	SLCO1A2	0.11	0.1	0.92
rs11568563	12	21457434	77	SLCO1A2	0.07	0.025	0.28
rs4149117	12	21011480	77	SLCO1B3	0.82	0.95	0.044
rs7311358	12	21015760	77	SLCO1B3	0.67	0.55	0.044
rs212090	16	16236004	77	ABCC1	0.38	0.35	0.75
rs35587	16	16139714	77	ABCC1	0.31	0.3	0.94
rs212091	16	16236650	77	ABCC1	0.14	0.125	0.81
rs2277624	17	48761105	77	ABCC3	0.81	0.775	0.67
rs11568591	17	48761053	77	ABCC3	0.91	0.9	0.81

Tolerability of higher dose at 12 months			Grade 3/4 nonhematological toxicity at 3 months			Grade 3/4 hematological toxicity at 3 months		
MAF			MAF			MAF		
controls	cases	P-value	controls	cases	P-value	controls	cases	P-value
0.89	0.85	0.48	0.88	0.875	0.99	0.89	0.83	0.27
0.35	0.39	0.65	0.38	0.625	0.22	0.39	0.4	0.89
0.78	0.78	1	0.79	0.625	0.27	0.77	0.8	0.70
0.90	0.92	0.78	0.90	0.75	0.23	0.89	0.875	0.75
0.64	0.67	0.73	0.66	0.75	0.59	0.67	0.65	0.85
0.81	0.79	0.84	0.80	1	0.18	0.78	0.9	0.11
0.53	0.64	0.13	0.58	0.625	0.76	0.55	0.65	0.23
0.5	0.60	0.20	0.54	0.625	0.61	0.51	0.65	0.093
0.52	0.56	0.59	0.53	0.625	0.55	0.5	0.625	0.13
0.14	0.11	0.59	0.12	0.125	0.99	0.12	0.125	0.97
0.51	0.54	0.30	0.52	0.625	0.067	0.53	0.525	0.96
0.97	0.97	1	0.97	1	0.63	0.96	1	0.22
0.18	0.044	0.013	0.09	0.50	0.0088	0,096	0,12	0,72
0.11	0.097	0.77	0.11	0	0.29	0.11	0.1	0.92
0.056	0.069	0.72	0.062	0	0.45	0.070	0.025	0.28
0.82	0.88	0.34	0.86	0.875	0.88	0.82	0.95	0.044
0.68	0.61	0.19	0.64	0.625	0.92	0.67	0.55	0.044
0.33	0.40	0.38	0.38	0.25	0.45	0.38	0.35	0.75
0.31	0.26	0.58	0.30	0.375	0.67	0.31	0.3	0.94
0.18	0.097	0.16	0.14	0	0.26	0.14	0.125	0.81
0.81	0.83	0.69	0.79	0.875	0.59	0.81	0.775	0.67
0.93	0.89	0.36	0.91	0.875	0.72	0.91	0.9	0.81

Pharmacogenetic risk factors for imatinib-induced toxicity

Two types of toxicity categories were used at 3 and 12 months of imatinib: inferred dose tolerability categories ('high' versus 'medium' combined with 'low' [tables 6 and 7]), and toxicity according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3 grade (table 8 and 9). [40]

Higher dose at 3 months

Inferred tolerability of a higher dose of imatinib at 3 months was associated with a SNPs in ABCC12 (rs16945874, p = 0.0074), CYP8B1 (rs735320, p = 0.013), CYP2D6 (rs3892097, p = 0.017) and CYP3A5 (rs776746, p = 0.017), (Table 6).

Higher dose at 12 months

At 12 months two SNPs remained significant, namely ABCC12 (rs16945874, p = 0.0085) and CYP3A5 (rs776746, p = 0.014) (Table 7). Other significant SNPs at 12 months included ABCB5 (rs23001641, p =0.0030), SLC10A2 (rs7987433, p=0.0039), PON3 (paraoxonase-3, rs 1053275, p = 0.0014), CYP3A4 (rs2740574, p = 0.020) and several SNPs of enzymes of the glucuronidation pathway (UGT1A4, UGTA1 and three SNPs of UGT1A6) and the aldehyde dehydrogenase gene (two SNPs of ALDH3B2 and one SNP of ALDH9A1) (Table 7).

SNPs associated with CTCAE toxicity grade

Table 8 and 9 show significant SNPs associated with imatinib-induced grade 3/4 toxicities (hematological and non-hematological) measured by CTCAE grade.

Non-hematological toxicity

Two SNPs were highly correlated with grade 3/4 non-hematological (table 8); one in a glutathione transferase gene (GSTM5, gstm5_x1, p = 0.000017) and a one in the CYP2B6 gene (rs2279341, p = 0.000017). Other significant SNPs were in CYP2A13 (rs1709082, p = 0.0021), the influx gene SLC22A4 (rs10479002, p = 0.0024), the efflux gene ABCC11 (rs17822931, p = 0.0077), and CYP3A5 (rs776746). Several SNPs of the NR2A1 gene (nuclear receptor subfamily 2, group A, member 1, also known as hepatocyte nuclear factor 4 alpha [HNF4A]) are also significantly associated with non-hematological toxicity. NR2A1 is required for the pregnane X receptor (PXR, also known as NR1I2) and CAR-mediated transcriptional activation of CYP3A4.(9) More additional significant SNPs found, such as of the Cytochrome P450 system (CYP2C9, CYP1B1, CYP3A4, CYP4F3, CYP2B6) are described in Table 8.

Hematological toxicity

Table 9 shows all SNPs significantly associated with grade 3/4 hematological toxicity. Significant SNPs included those in CYP8B1 (rs 735320, p = 0.00083), ABCB1 (rs 3842, p = 0.0010), ABCC13 (rs17274058, p = 0.0014) and 6 other members of the ABC family. Other SNPs included genes that are involved in the transcriptional activation of CYP3A4 (six SNPs of NR1I2 and NR2A1, with p values between 0.042 and 0.0082, Table 9).

Table 6 Results of the univariate analyses for the tolerability of a higher dose of imatinib in CML patients at *3 months* according to the candidate genotypes.

SNPs	Chromo-some	Position	N	Gene-symbol	MAF		P-value
					controls	cases	
rs16945874	16	48175235	77	ABCC12	0.930	0.775	0.007
rs735320	3	42915878	77	CYP8B1	0.763	0.950	0.013
rs3892097	22	25490965	69	CYP2D6	0.390	0.158	0.017
rs776746	7	93906909	69	CYP3A5	0.140	0.000	0.017
rs1065411	1	110233138	77	GSTM1	0.158	0.025	0.018
rs1065852	22	25492712	69	CYP2D6	0.440	0.211	0.020
rs28399435	19	41356246	77	CYP2A6	0.991	0.925	0.022
rs3764006	12	21054369	77	SLCO1B3	0.149	0.025	0.024
rs2301157	13	103698363	77	SLC10A2	0.465	0.675	0.027
rs2230707	1	19202896	77	ALDH4A1	0.605	0.425	0.031
rs4149253	8	27396208	77	EPHX2	0.956	0.850	0.034
rs28624811	22	42527533	77	CYP2D6	0.702	0.525	0.036
rs7311358	12	21015760	77	SLCO1B3	0.667	0.550	0.044
rs4149117	12	21011480	77	SLCO1B3	0.825	0.950	0.044
rs2053098	12	21036411	77	SLCO1B3	0.825	0.950	0.044
rs4633	22	19950235	77	COMT	0.404	0.575	0.044
rs4680	22	19951271	77	COMT	0.404	0.575	0.044
rs4803418	19	41511803	77	CYP2B6	0.193	0.350	0.050
rs1135612	7	75609677	77	POR	0.254	0.425	0.050

SNPs Single nuclear polymorphisms
MAF Minor allele frequency

Table 7 Results of the univariate analyses for the tolerability of a higher dose of imatinib in CML patients at *12 months* according to the candidate genotypes.

SNPs	Chromo-some	Position	N	Gene-symbol	MAF		P-value
					controls	cases	
rs7987433	13	103719056	72	SLC10A2	0.264	0.083	0.004
rs16945874	16	48175235	72	ABCC12	0.958	0.819	0.009
rs776746	7	93906909	64	CYP3A5	0.183	0.044	0.013
rs1053275	7	95001555	72	PON3	0.417	0.625	0.014
rs2740574	7	99382096	72	CYP3A4	0.069	0.000	0.020
rs592792	1	110211956	72	GSTM2	0.833	0.944	0.023
rs2011404	2	234627937	72	UGT1A4	0.889	0.736	0.027
rs4124874	2	234665659	72	UGT1A1	0.528	0.347	0.030
rs16961281	13	103718824	72	SLC10A2	0.861	0.958	0.032
rs4646823	11	67434416	72	ALDH3B2	0.097	0.222	0.032
rs34603556	7	20691047	72	ABCB5	0.278	0.153	0.032
rs7947978	11	67442043	72	ALDH3B2	0.903	0.778	0.032
rs2276299	11	62766431	72	SLC22A8	0.889	0.764	0.035
rs762551	15	75041917	72	CYP1A2	0.250	0.403	0.039
rs1143659	1	165652273	72	ALDH9A1	0.611	0.764	0.041
rs1105880	2	234601965	72	UGT1A6	0.403	0.264	0.041
rs2070959	2	234602191	72	UGT1A6	0.403	0.264	0.041
rs1105879	2	234602202	72	UGT1A6	0.403	0.264	0.041
rs735320	3	42915878	72	CYP8B1	0.736	0.875	0.046

SNPs Single nuclear polymorphisms
MAF Minor allele frequency

Table 8 Results of the univariate analyses for the chance of grade 3/4 *non-hematological* toxicity of imatinib in CML patients at 3 months according to the candidate genotypes.

SNPs	Chromo-some	Position	N	Gene-symbol	MAF		P-value
					controls	cases	
gstm5_x1	1	110257500	77	GSTM5	0.00	0.13	0.00002
rs2279341	19	41509950	77	CYP2B6	0.00	0.13	0.00002
rs1709082	19	41601609	77	CYP2A13	0.06	0.38	0.00211
rs10479002	5	131663072	77	SLC22A4	0.98	0.75	0.00240
rs17822931	16	48258198	77	ABCC11	0.86	0.50	0.00773
rs776746	7	93906909	69	CYP3A5	0.09	0.50	0.00881
rs2302948	19	49096065	77	SULT2B1	0.78	0.38	0.01030
rs12514417	5	125887715	77	ALDH7A1	0.05	0.25	0.01431
rs2501873	1	161204538	77	NR1I3	0.57	0.13	0.01705
rs4918758	10	96697252	77	CYP2C9	0.40	0.00	0.01751
rs1056837	2	38298150	77	CYP1B1	0.57	1.00	0.01888
rs1056836	2	38298203	77	CYP1B1	0.57	1.00	0.01888
rs6103731	2	43047293	77	NR2A1	0.58	1.00	0.02167
rs1028583	2	43050761	77	NR2A1	0.58	1.00	0.02167
rs3212198	2	43044362	77	NR2A1	0.42	0.00	0.02188
rs2851069	11	74862356	77	SLCO2B1	0.66	0.25	0.02253
rs2287622	2	169830328	77	ABCB11	0.65	1.00	0.02721
rs1050152	5	131676320	77	SLC22A4	0.61	1.00	0.03220
rs1143659	1	165652273	77	ALDH9A1	0.72	0.38	0.03324
rs7796976	7	17338430	77	AHR	0.72	0.38	0.03324
rs2230709	1	19201928	77	ALDH4A1	0.87	0.63	0.03477
rs2241894	4	100266133	77	ADH1C	0.23	0.50	0.03744
rs2242480	7	99361466	77	CYP3A4	0.88	0.63	0.03846
rs1126670	4	100052733	77	ADH4	0.31	0.00	0.04150
rs4646904	19	15763721	77	CYP4F3	0.70	0.38	0.04282
rs3211371	19	41522715	77	CYP2B6	0.36	0.13	0.04283
rs2230707	1	19202896	77	ALDH4A1	0.54	0.88	0.04349
rs1126673	4	100045616	77	ADH4	0.32	0.00	0.04377
rs1126671	4	100048414	77	ADH4	0.68	1.00	0.04377
rs1229984	4	100239319	77	ADH1B	0.95	0.75	0.04574

SNPs Single nuclear polymorphisms
MAF Minor allele frequency

Table 9 Results of the univariate analyses for the chance of grade 3/4 *hematological* toxicity of imatinib in CML patients at 3 months according to the candidate genotypes.

SNPs	Chromo-some	Position	N	Gene-symbol	MAF		P-value
					controls	cases	
rs735320	3	42915878	77	CYP8B1	0.87	0.61	0.0008
rs3842	7	87133366	77	ABCB1	0.08	0.31	0.0010
rs17274058	21	15664040	77	ABCC13	1.00	0.92	0.0014
rs2071645	7	87105276	77	ABCB4	0.87	0.64	0.0018
rs17064	7	87133470	77	ABCB1	0.02	0.14	0.0060
rs2515641	10	135351362	77	CYP2E1	0.81	1.00	0.0062
rs8187858	16	16162039	77	ABCC1	0.92	0.75	0.0077
rs2235015	7	87199564	77	ABCB1	0.87	0.69	0.0080
rs2461818	3	119523872	77	NR1I2	0.97	0.83	0.0082
rs2109505	7	87079406	77	ABCB4	0.14	0.33	0.0083
rs2302387	7	87092185	77	ABCB4	0.90	0.72	0.0138
rs8187707	10	101610533	77	ABCC2	0.71	0.56	0.0150
rs7987433	13	103719056	77	SLC10A2	0.14	0.31	0.0170
rs6784598	3	119533256	77	NR1I2	0.67	0.44	0.0170
rs6785049	3	119533733	77	NR1I2	0.33	0.56	0.0170
rs1885088	2	43039040	77	NR2A1	0.78	0.94	0.0215
rs2276707	3	119534153	77	NR1I2	0.85	0.67	0.0268
rs4148807	7	87105812	77	ABCB4	0.97	0.89	0.0268
rs3814058	3	119537291	77	NR1I2	0.16	0.33	0.0355
rs4646823	11	67434416	77	ALDH3B2	0.19	0.06	0.0366
rs7947978	11	67442043	77	ALDH3B2	0.81	0.94	0.0366
rs2056822	19	15739597	77	CYP4F8	0.71	0.53	0.0374
rs7216	17	19578873	77	ALDH3A2	0.36	0.56	0.0418
rs3814055	3	119500035	77	NR1I2	0.75	0.56	0.0424
rs17856219	11	67431914	77	ALDH3B2	0.82	0.94	0.0470
rs3741172	11	67430698	77	ALDH3B2	0.81	0.94	0.0474
rs1551886	11	67430762	77	ALDH3B2	0.81	0.94	0.0474

SNPs Single nuclear polymorphisms
MAF Minor allele frequency

Discussion

Even though this is a retrospective study, most of the patients (84%) were treated prospectively in other clinical trials of single agent imatinib, resulting in reliable clinical data. We have shown that toxicity as well as the ability to tolerate a higher dose of imatinib in patients with CML, may be predicted by certain SNPs in 129 DME genes. Some of these SNPs have been previously identified, but many have not until now been associated with imatinib metabolism. Given the patient numbers in our study coupled with the relatively large number of SNPs examined, these findings can only be hypothesis generating, given the risk of false positive associations. We purposefully have not used the Bonferroni method to correct for multiple comparisons in this broad analysis because of the probability of producing false negatives.[41] Instead, we use these results to indicate possible SNPs of interest for future studies.

In the current study, the tolerability of higher doses of imatinib was significantly associated with SNPs for CYP3A5 (rs776746), both at 3 months ($p = 0.017$), and at 12 months of imatinib ($p = 0.013$). We also found that this same CYP3A5 SNP was significantly associated with the development of grade 3/4 hematological toxicity ($p = 0.008$). CYP3A5 is known to be involved in the metabolism of imatinib. Kim et al. previously found that this same SNP was associated with response to imatinib.[42] The CYP3A5 SNP (rs776746) is not in linkage disequilibrium with other SNPs previously described in the literature as being of importance for imatinib metabolism.

Our study also suggested that several SNPs of the efflux pump ABCB1 (rs3842, rs17064 and rs2235015) were associated with grade 3/4 hematological toxicity. The role of ABCB1 in the metabolism and elimination of imatinib has been intensively investigated over the last few years but its relevance remains controversial. Gurney et al. previously found that another SNP of ABCB1 (rs1045642) was significantly associated with toxicity.[33] Imatinib is a substrate of ABCB1 and Burger et al. showed, in cell lines, that ABCB1 was upregulated when exposed to imatinib.[22] However, Gardner et al failed to confirm upregulation of ABCB1 in mice during long-term treatment.[43] Additionally, in 82 Caucasian GIST patients, ABCB1 genotypes had no influence on the PK of imatinib.[8]

An example of a novel finding in this descriptive analysis is the association between CYP8B1 (rs735320) and the risk of developing grade 3/4 hematological toxicity and the ability to tolerate a higher dose of imatinib. CYP8B1 (Sterol 12alpha-hydroxylase) is an obligatory enzyme for the synthesis of cholic acid and regulation of liver bile acid synthesis and intestine cholesterol absorption.[44] The relationship of imatinib with this gene is uncertain. However, a fall in cholesterol has been described in patients receiving imatinib although the mechanism is unknown.[45] Our result raises the possibility of an interaction between enzymes involved with bile salt metabolism and imatinib which may be worthy of further investigation Another previously undescribed SNP is that in ABCC12. This gene is a member of the MRP subfamily which is involved in multi-drug resistance.[46] To date, little is known about this gene apart from its proximity to ABCC11, which encodes the biliary salt excretory pump (BSEP).[47]

SNPs in two metabolising genes were highly associated with non-hematological toxicity; GSTM5 and CYP2B6. GSTM5 is a glutathione transferase gene not previously associated with imatinib. However, recently genotype of an associated gene, GSTT1, has been associated with poor response in CML patient who have received a dose escalation of imatinib.[48] Likewise, CYP2B6 has not previously been associated with imatinib. This enzyme is involved with metabolism of a large number of drugs, including cytotoxic agents, as well as cholesterol and other lipids.[49][50]

In our population, the majority of patients were Caucasian. The genetic profile of patients depends partly on their ethnic background.(51) For example, clinical response in Asian CML patients was found to be associated with the ABCG2 421C>A SNP which is more prevalent among Japanese and Han Chinese individuals, and less common among Africans and Caucasians.[52]

In summary, our study suggests that CYP3A5 (rs776746) and CYP8B1 (rs735320) may be associated with the ability of CML patients to tolerate a higher dose of imatinib, and also the chance of developing toxicity from that drug, both at start of treatment and after chronic exposure. Our results also suggest several novel genes that may also be associated with imatinib tolerability and these findings may help in the design of prospective studies of individualised dosing of imatinib.

Appendix A

Participation of patients according to site

Site	N
Westmead Hospital, Sydney	19
The Alfred Hospital, Melbourne	14
Royal Adelaide Hospital	11
Royal Melbourne Hospital	8
Royal Brisbane Hospital	6
St. Vincent Hospital Melbourne	5
Princess Alexandra Hospital Brisbane	4
Peter MacCallum Cancer Centre Melbourne	4
Albury Base Hospital	1
Royal Perth Hospital	2
Mater Hospital Brisbane	1
Freemantle Hospital Perth	1
St Vincent Hospital Sydney	1

Appendix B

List of selected investigated candidate genes and their relevant SNPs
Highlighted with a grey background are the 21 SNPs, which are described in published literature of pharmacogenetic studies.

SNP	reference SNP ID number	Gene Symbol	Poly-morphism	SNP	reference SNP ID number	Gene Symbol	Poly-morphism
[T/C]	rs10276036	ABCB1	C/T	[A/G]	abcc1_x1	ABCC1	A/G
[T/C]	rs2235033	ABCB1	A/G	[A/G]	rs4148380	ABCC1	A/G
[A/G]	rs1128503	ABCB1	A/G	[T/C]	rs2125739	ABCC10	C/T
[T/C]	rs1045642	ABCB1	A/G	[T/C]	rs12443685	ABCC11	C/T
[A/C]	rs2235015	ABCB1	A/C	[T/C]	rs8047091	ABCC11	C/T
[A/G]	rs3842	ABCB1	C/T	[A/G]	rs17822931	ABCC11	C/T
[A/G]	rs2235040	ABCB1	C/T	[A/G]	rs11866251	ABCC11	A/G
[T/C]	rs9282564	ABCB1	C/T	[T/C]	rs16945916	ABCC11	C/T
[T/C]	rs2214102	ABCB1	C/T	[T/C]	rs17822471	ABCC11	A/G
[A/T]	rs17064	ABCB1	A/T	[A/G]	rs16945988	ABCC11	C/T
[T/C]	rs2032588	ABCB1	A/G	[T/C]	rs16945974	ABCC11	C/T
[A/G]	rs3213619	ABCB1	A/G	[T/C]	abcc11_x1	ABCC11	C/T

SNP	reference SNP ID number	Gene Symbol	Poly-morphism	SNP	reference SNP ID number	Gene Symbol	Poly-morphism
[T/C]	rs2032582	ABCB1	C/T	[T/C]	rs12149826	ABCC12	C/T
[A/C]	rs2032582_1	ABCB1	C/A	[T/C]	rs7193955	ABCC12	A/G
[T/C]	rs3738187	ABCB10	A/G	[T/G]	rs16945874	ABCC12	G/T
[T/C]	rs497692	ABCB11	C/T	[A/G]	rs2822558	ABCC13	A/G
[A/G]	rs496550	ABCB11	C/T	[T/C]	rs17274058	ABCC13	C/T
[T/C]	rs473351	ABCB11	C/T	[T/C]	rs3740066	ABCC2	C/T
[A/G]	rs2287622	ABCB11	G/A	[A/G]	rs717620	ABCC2	C/T
[T/C]	rs3770602	ABCB11	A/G	[A/G]	rs2273697	ABCC2	A/G
[T/C]	rs7602171	ABCB11	A/G	[T/C]	rs8187707	ABCC2	C/T
[T/C]	rs4668115	ABCB11	C/T	[A/T]	rs17222723	ABCC2	A/T
[A/G]	rs4148768	ABCB11	A/G	[A/C]	rs1137968	ABCC2	G/T
[A/G]	rs3770603	ABCB11	C/T	[A/G]	rs2277624	ABCC3	C/T
[A/G]	rs1202283	ABCB4	A/G	[T/C]	rs11568591	ABCC3	A/G
[T/A]	rs2109505	ABCB4	A/T	[A/G]	rs1059751	ABCC4	A/G
[G/C]	rs2071645	ABCB4	C/G	[T/C]	rs4148553	ABCC4	C/T
[A/G]	rs2302387	ABCB4	A/G	[T/C]	rs2274406	ABCC4	C/T
[T/C]	rs4148807	ABCB4	G/A	[T/C]	rs4148551	ABCC4	C/T
[T/C]	rs4148808	ABCB4	C/T	[A/G]	rs2274405	ABCC4	C/T
[T/C]	rs8187799	ABCB4	C/T	[A/G]	rs1751034	ABCC4	C/T
[A/G]	rs10254317	ABCB5	A/G	[A/C]	rs2274407	ABCC4	A/C
[T/C]	rs2301641	ABCB5	A/G	[T/C]	rs1678339	ABCC4	C/T
[A/G]	rs2893006	ABCB5	C/T	[T/C]	rs1189466	ABCC4	A/G
[T/C]	rs34603556	ABCB5	C/T	[A/G]	rs1132776	ABCC5	A/G
[A/G]	abcb5_x1	ABCB5	A/G	[T/C]	rs939336	ABCC5	A/G
[T/C]	rs6461515	ABCB5	A/G	[A/G]	rs7636910	ABCC5	C/T
[A/G]	rs1109866	ABCB6	C/T	[T/C]	rs3749442	ABCC5	A/G
[T/G]	rs1109867	ABCB6	G/T	[T/C]	rs12931472	ABCC6	A/G
[A/T]	rs2303926	ABCB8	A/T	[T/G]	rs8058694	ABCC6	G/T
[T/C]	rs17545756	ABCB8	C/T	[G/C]	rs8058696	ABCC6	C/G
[T/G]	abcb8_x1	ABCB8	G/T	[T/C]	rs9940825	ABCC6	C/T
[A/T]	rs212090	ABCC1	A/T	[A/G]	rs2238472	ABCC6	C/T
[A/G]	rs35587	ABCC1	C/T	[T/C]	rs9924755	ABCC6	A/G
[A/G]	rs2239330	ABCC1	A/G	[T/C]	rs2856585	ABCC6	A/G
[T/C]	rs35605	ABCC1	C/T	[A/G]	rs1048099	ABCC8	A/G
[A/G]	rs212091	ABCC1	C/T	[T/C]	rs1799857	ABCC8	A/G
[T/C]	rs8187858	ABCC1	C/T	[T/G]	rs757110	ABCC8	A/C
[T/C]	rs1799858	ABCC8	C/T	[G/C]	rs1573496	ADH7	C/G
[T/C]	rs1801261	ABCC8	A/G	[T/C]	rs971074	ADH7	C/T

SNP	reference SNP ID number	Gene Symbol	Poly-morphism	SNP	reference SNP ID number	Gene Symbol	Poly-morphism
[T/G]	rs2231142	ABCG2	G/T	[T/C]	rs1060242	ADHFE1	C/T
[A/G]	rs1229984	ADH1B	C/T	[C/G]	rs2555588	ADHFE1	C/G
[T/C]	rs1693425	ADH1C	C/T	[T/C]	rs7796976	AHR	A/G
[A/G]	rs1693482	ADH1C	C/T	[A/G]	rs17779352	AHR	C/T
[A/G]	rs698	ADH1C	C/T	[A/G]	rs2066853	AHR	A/G
[T/C]	rs1789915	ADH1C	A/G	[A/G]	rs13959	ALDH1A1	A/G
[T/C]	rs2241894	ADH1C	C/T	[T/G]	rs2073478	ALDH1B1	G/T
[A/C]	rs1800759	ADH4	G/T	[T/C]	rs2073477	ALDH1B1	C/T
[A/T]	rs1800760	ADH4	A/T	[T/C]	rs886205	ALDH2	A/G
[A/G]	rs1126671	ADH4	C/T	[T/A]	rs2072330	ALDH3A1	A/T
[A/G]	rs1042364	ADH4	C/T	[C/G]	rs2228100	ALDH3A1	C/G
[A/G]	rs1126672	ADH4	A/G	[T/G]	rs887241	ALDH3A1	C/A
[A/C]	rs1126670	ADH4	A/C	[A/G]	aldh3a1_x1	ALDH3A1	C/T
[T/C]	rs1126673	ADH4	C/T	[T/A]	rs7216	ALDH3A2	A/T
[T/C]	rs1154400	ADH5	C/T	[T/C]	rs2286163	ALDH3B1	C/T
[T/C]	rs17537595	ADH7	A/G	[A/G]	rs308341	ALDH3B1	A/G
[G/C]	rs1573496	ADH7	C/G	[A/G]	rs3741172	ALDH3B2	A/G
[T/C]	rs971074	ADH7	C/T	[A/C]	rs7947978	ALDH3B2	A/C
[T/C]	rs1060242	ADHFE1	C/T	[A/C]	rs4646823	ALDH3B2	A/C
[C/G]	rs2555588	ADHFE1	C/G	[T/C]	rs1551886	ALDH3B2	C/T
[T/C]	rs7796976	AHR	A/G	[T/C]	rs17856219	ALDH3B2	A/G
[A/G]	rs17779352	AHR	C/T	[T/C]	rs2230707	ALDH4A1	A/G
[A/G]	rs2066853	AHR	A/G	[A/G]	rs7550938	ALDH4A1	C/T
[A/G]	rs13959	ALDH1A1	A/G	[G/C]	rs2230705	ALDH4A1	C/G
[T/G]	rs2073478	ALDH1B1	G/T	[T/C]	rs2230708	ALDH4A1	A/G
[T/C]	rs2073477	ALDH1B1	C/T	[A/G]	rs2230709	ALDH4A1	C/T
[T/C]	rs886205	ALDH2	A/G	[T/G]	rs12514417	ALDH7A1	G/T
[T/A]	rs2072330	ALDH3A1	A/T	[T/G]	rs3813342	ALDH8A1	A/C
[C/G]	rs2228100	ALDH3A1	C/G	[T/C]	rs1143659	ALDH9A1	A/G
[T/G]	rs887241	ALDH3A1	C/A	[T/C]	aox1_x1	AOX1	A/G
[A/G]	aldh3a1_x1	ALDH3A1	C/T	[T/C]	rs1048977	CDA	C/T
[T/A]	rs7216	ALDH3A2	A/T	[A/C]	rs2072671	CDA	A/C
[T/C]	rs2286163	ALDH3B1	C/T	[G/C]	rs4818	COMT	C/G
[A/G]	rs308341	ALDH3B1	A/G	[A/G]	rs4633	COMT	C/T
[A/G]	rs3741172	ALDH3B2	A/G	[A/G]	rs4680	COMT	A/G
[A/C]	rs7947978	ALDH3B2	A/C	[T/C]	rs2470890	CYP1A2	T/C
[T/C]	rs1693425	ADH1C	C/T	[A/C]	rs762551	CYP1A2	C/A
[A/G]	rs1693482	ADH1C	C/T	[A/C]	rs2069526	CYP1A2	G/T

SNP	reference SNP ID number	Gene Symbol	Poly-morphism	SNP	reference SNP ID number	Gene Symbol	Poly-morphism
[A/G]	rs698	ADH1C	C/T	[T/C]	rs1056837	CYP1B1	A/G
[T/C]	rs1789915	ADH1C	A/G	[G/C]	rs1573496	ADH7	C/G
[T/C]	rs2241894	ADH1C	C/T	[T/C]	rs971074	ADH7	C/T
[A/C]	rs1800759	ADH4	G/T	[T/C]	rs1060242	ADHFE1	C/T
[A/T]	rs1800760	ADH4	A/T	[C/G]	rs2555588	ADHFE1	C/G
[A/G]	rs1126671	ADH4	C/T	[T/C]	rs7796976	AHR	A/G
[A/G]	rs1042364	ADH4	C/T	[A/G]	rs17779352	AHR	C/T
[A/G]	rs1126672	ADH4	A/G	[A/G]	rs2066853	AHR	A/G
[A/C]	rs1126670	ADH4	A/C	[A/G]	rs13959	ALDH1A1	A/G
[T/C]	rs1126673	ADH4	C/T	[T/G]	rs2073478	ALDH1B1	G/T
[T/C]	rs1154400	ADH5	C/T	[T/C]	rs2073477	ALDH1B1	C/T
[T/C]	rs17537595	ADH7	A/G	[T/C]	rs886205	ALDH2	A/G
[G/C]	rs1573496	ADH7	C/G	[A/G]	rs4803419	CYP2B6	C/T
[T/C]	rs971074	ADH7	C/T	[G/C]	rs4803418	CYP2B6	C/G
[T/C]	rs1060242	ADHFE1	C/T	[A/G]	rs3211371	CYP2B6	C/T
[C/G]	rs2555588	ADHFE1	C/G	[T/C]	rs8192719	CYP2B6	C/T
[T/C]	rs7796976	AHR	A/G	[T/G]	rs3745274	CYP2B6	G/T
[A/G]	rs17779352	AHR	C/T	[A/G]	rs2054675	CYP2B6	C/T
[A/G]	rs2066853	AHR	A/G	[T/C]	rs3760657	CYP2B6	A/G
[A/G]	rs13959	ALDH1A1	A/G	[T/A]	rs2279342	CYP2B6	A/T
[T/G]	rs2073478	ALDH1B1	G/T	[C/G]	rs2279341	CYP2B6	C/G
[T/C]	rs2073477	ALDH1B1	C/T	[A/G]	rs2860840	CYP2C18	C/T
[T/C]	rs886205	ALDH2	A/G	[T/C]	rs2281891	CYP2C18	C/T
[T/A]	rs2072330	ALDH3A1	A/T	[A/G]	rs3740367	CYP2C18	A/G
[C/G]	rs2228100	ALDH3A1	C/G	[T/C]	rs12248560	CYP2C19	C/T
[T/G]	rs887241	ALDH3A1	C/A	[T/C]	rs3758580	CYP2C19	C/T
[A/G]	aldh3a1_x1	ALDH3A1	C/T	[A/G]	rs4244285	CYP2C19	A/G
[T/A]	rs7216	ALDH3A2	A/T	[T/C]	cyp2c19_x1	CYP2C19	C/T
[T/C]	rs2286163	ALDH3B1	C/T	[A/G]	rs10509681	CYP2C8	C/T
[A/G]	rs308341	ALDH3B1	A/G	[A/C]	rs17110453	CYP2C8	A/C
[A/G]	rs3741172	ALDH3B2	A/G	[A/G]	rs4918758	CYP2C9	C/T
[A/C]	rs7947978	ALDH3B2	A/C	[T/C]	rs4917636	CYP2C9	A/G
[A/C]	rs4646823	ALDH3B2	A/C	[T/G]	rs9332100	CYP2C9	G/T
[T/C]	rs1551886	ALDH3B2	C/T	[A/G]	rs1799853	CYP2C9	C/T
[T/C]	rs17856219	ALDH3B2	A/G	[A/G]	rs9332098	CYP2C9	A/G
[T/C]	rs2230707	ALDH4A1	A/G	[T/C]	cyp2c9_x1	CYP2C9	A/G
[A/G]	rs7550938	ALDH4A1	C/T	[T/C]	rs9332092	CYP2C9	C/T
[G/C]	rs2230705	ALDH4A1	C/G	[A/T]	rs1057911	CYP2C9	A/T

SNP	reference SNP ID number	Gene Symbol	Poly-morphism	SNP	reference SNP ID number	Gene Symbol	Poly-morphism
[T/C]	rs2230708	ALDH4A1	A/G	[T/G]	rs1057910	CYP2C9	C/A
[A/G]	rs2230709	ALDH4A1	C/T	[G/C]	rs1135840	CYP2D6	C/G
[T/G]	rs12514417	ALDH7A1	G/T	[A/G]	rs16947	CYP2D6	A/G
[T/G]	rs3813342	ALDH8A1	A/C	[T/C]	rs28624811	CYP2D6	G/A
[T/C]	rs1143659	ALDH9A1	A/G	[A/G]	rs769258	CYP2D6	C/T
[T/C]	aox1_x1	AOX1	A/G	[A/T]	rs2070673	CYP2E1	A/T
[T/C]	rs1048977	CDA	C/T	[T/C]	rs2515641	CYP2E1	T/C
[A/C]	rs2072671	CDA	A/C	[T/C]	rs305968	CYP2F1	A/G
[G/C]	rs4818	COMT	C/G	[C/G]	rs338599	CYP2S1	C/G
[A/G]	rs4633	COMT	C/T	[T/C]	rs2242480	CYP3A4	C/T
[A/G]	rs4680	COMT	A/G	[A/G]	rs800667	CYP3A43	C/T
[T/C]	rs2470890	CYP1A2	T/C	[T/C]	rs17342647	CYP3A43	C/T
[A/C]	rs762551	CYP1A2	C/A	[C/G]	rs680055	CYP3A43	C/G
[A/C]	rs2069526	CYP1A2	G/T	[T/C]	rs28365094	CYP3A5	C/T
[T/C]	rs1056837	CYP1B1	A/G	[A/G]	rs28365067	CYP3A5	A/G
[C/G]	rs1056836	CYP1B1	C/G	[A/G]	cyp3a5_x1	CYP3A5	C/T
[A/G]	rs1800440	CYP1B1	C/T	[C/T]	rs776746	CYP3A5	T/C
[T/G]	rs1709082	CYP2A13	G/T	[T/C]	rs28371764	CYP3A5	A/G
[T/G]	rs8192726	CYP2A6	A/C	[C/G]	rs2257401	CYP3A7	C/G
[A/C]	rs28399433	CYP2A6	A/C	[T/C]	rs12564525	CYP4A22	C/T
[T/C]	rs28399435	CYP2A6	C/T	[T/C]	rs2297810	CYP4B1	G/A
[T/C]	rs3815711	CYP2A7	A/G	[A/G]	rs2297809	CYP4B1	T/C
[T/G]	cyp2a7_x1	CYP2A7	A/C	[T/C]	rs4646487	CYP4B1	C/T
[T/C]	rs3869579	CYP2A7	A/G	[T/C]	rs2108622	CYP4F2	C/T
[T/C]	rs2279344	CYP2B6	A/G	[T/C]	rs2074900	CYP4F2	A/G
[A/G]	rs7254579	CYP2B6	C/T	[A/G]	rs3093114	CYP4F2	A/G
[A/G]	rs3093106	CYP4F2	C/T	[T/C]	rs6195	NR3C1	C/T
[A/G]	rs4646904	CYP4F3	A/G	[T/A]	rs854560	PON1	A/T
[A/G]	rs1805041	CYP4F3	A/G	[T/C]	rs662	PON1	C/T
[A/G]	rs1053037	CYP4F3	A/G	[C/G]	rs7493	PON2	C/G
[A/C]	rs2056822	CYP4F8	A/C	[C/G]	rs12026	PON2	C/G
[T/C]	rs4808326	CYP4F8	A/G	[A/G]	rs1053275	PON3	C/T
[A/G]	rs8192879	CYP7A1	C/T	[A/G]	rs13226149	PON3	A/G
[T/C]	rs735320	CYP8B1	C/T	[T/C]	rs1057870	POR	A/G
[A/G]	rs1801265	DPYD	A/G	[T/C]	rs1135612	POR	A/G
[A/G]	rs1801159	DPYD	C/T	[A/G]	rs17685	POR	A/G
[A/G]	rs2297595	DPYD	C/T	[A/G]	rs4646285	SLC10A1	C/T
[A/G]	rs1801160	DPYD	C/T	[A/G]	rs2301157	SLC10A2	A/G

SNP	reference SNP ID number	Gene Symbol	Poly-morphism	SNP	reference SNP ID number	Gene Symbol	Poly-morphism
[T/C]	rs1051740	EPHX1	C/T	[A/G]	rs2301159	SLC10A2	A/G
[T/C]	rs2292568	EPHX1	C/T	[G/C]	rs279942	SLC10A2	C/G
[G/C]	rs4149230	EPHX1	C/G	[A/G]	rs3803258	SLC10A2	C/T
[T/C]	rs2292566	EPHX1	A/G	[A/G]	rs7987433	SLC10A2	C/T
[T/C]	rs1051741	EPHX1	C/T	[T/C]	rs16961281	SLC10A2	A/G
[A/C]	rs1126452	EPHX2	A/C	[T/G]	rs279941	SLC10A2	A/C
[A/G]	rs751141	EPHX2	A/G	[A/C]	rs188096	SLC10A2	A/C
[A/G]	ephx2_x1	EPHX2	A/G	[T/C]	rs7169	SLC16A1	A/G
[A/G]	rs13439459	EPHX2	C/T	[T/A]	rs1049434	SLC16A1	A/T
[T/C]	rs4149253	EPHX2	A/G	[A/G]	rs12659	SLC19A1	A/G
[A/G]	rs1800668	GPX1	G/A	[A/G]	rs628031	SLC22A1	G/A
[A/G]	rs8177412	GPX3	C/T	[G/C]	rs683369	SLC22A1	C/G
[G/C]	rs2180314	GSTA2	C/G	[T/C]	rs316003	SLC22A2	C/T
[A/C]	rs6577	GSTA2	G/T	[T/G]	rs316019	SLC22A2	A/C
[A/G]	rs405729	GSTA4	C/T	[A/G]	rs2292334	SLC22A3	A/G
[A/C]	rs367836	GSTA4	G/T	[A/G]	rs1050152	SLC22A4	C/T
[T/C]	rs1802061	GSTA4	C/T	[T/C]	rs272893	SLC22A4	C/T
[G/C]	rs1065411	GSTM1	C/G	[G/C]	rs10479002	SLC22A4	C/G
[A/G]	rs592792	GSTM2	C/T	[A/G]	rs274558	SLC22A5	A/G
[A/C]	rs1332018	GSTM3	G/T	[T/C]	rs274548	SLC22A5	C/T
[A/G]	rs7483	GSTM3	C/T	[A/G]	rs1045020	SLC22A5	C/T
[G/C]	rs1010167	GSTM4	C/G	[T/G]	rs11568628	SLC22A6	A/C
[T/C]	gstm5_x1	GSTM5	C/T	[T/C]	rs11568629	SLC22A6	C/T
[A/G]	rs17596954	GSTM5	C/T	[A/T]	rs2276299	SLC22A8	A/T
[T/G]	rs4925	GST01	A/C	[T/C]	rs4115170	SLC01A2	C/T
[A/G]	rs1695	GSTP1	A/G	[T/A]	rs7957203	SLC01A2	A/T
[T/C]	rs1138272	GSTP1	C/T	[T/C]	rs10841803	SLC01A2	A/G
[A/G]	rs35953316	HNMT	C/T	[T/C]	rs10841804	SLC01A2	A/G
[T/C]	rs1137070	MAOA	C/T	[T/G]	rs11568563	SLC01A2	G/T
[T/G]	rs1800464	MAOA	A/C	[A/G]	rs4337089	SLC01A2	C/T
[T/C]	rs1801280	NAT2	C/T	[A/G]	rs10841795	SLC01A2	A/G
[A/G]	rs1208	NAT2	A/G	[A/G]	rs4149057	SLC01B1	C/T
[A/G]	rs1799929	NAT2	C/T	[T/C]	rs2306283	SLC01B1	A/G
[A/G]	rs1041983	NAT2	C/T	[T/C]	rs2291075	SLC01B1	C/T
[A/G]	rs1799930	NAT2	A/G	[A/G]	rs4149056	SLC01B1	C/T
[T/C]	rs1800566	NQ01	A/G	[T/C]	rs2053098	SLC01B3	A/G
[A/G]	rs10517	NQ01	A/G	[A/G]	rs7311358	SLC01B3	A/G
[T/C]	rs689453	NQ01	C/T	[A/C]	rs4149117	SLC01B3	G/T

SNP	reference SNP ID number	Gene Symbol	Poly-morphism	SNP	reference SNP ID number	Gene Symbol	Poly-morphism
[A/G]	rs2307424	NR1I3	A/G	[C/G]	slco1b3_x1	SLCO1B3	C/G
[T/C]	rs6196	NR3C1	A/G	[A/G]	rs3764006	SLCO1B3	A/G
[A/G]	rs2851069	SLCO2B1	C/T	[G/C]	rs6784598	NR1I2	
[A/G]	rs12422149	SLCO2B1	A/G	[T/C]	rs2461823	NR1I2	
[A/G]	rs2292954	SPG7	A/G	[A/G]	rs6785049	NR1I2	
[A/G]	rs2077412	SULT1A1	T/C	[T/C]	rs3814058	NR1I2	
[C/G]	rs1402467	SULT1C4	C/G	[A/G]	rs2276707	NR1I2	
[A/G]	rs2302948	SULT2B1	C/T	[A/G]	rs7643645	NR1I2	
[T/C]	rs2544794	SULT2B1	C/T	[T/C]	rs3814055	NR1I2	
[A/G]	rs138057	SULT4A1	A/G	[A/G]	rs2461818	NR1I2	
[A/C]	rs138056	SULT4A1	A/C	[A/G]	rs1054190	NR1I2	
[A/G]	rs1057141	TAP1	C/T	[A/G]	rs2501873	NR1I3	
[T/C]	rs1135216	TAP1	C/T	[A/C]	rs4073054	NR1I3	
[A/G]	rs2071552	TAP2	A/G	[T/G]	rs2307418	NR1I3	
[T/C]	rs241448	TAP2	A/G	[A/C]	rs3818247	NR2A1	
[T/C]	rs241441	TAP2	C/T	[A/G]	rs6130615	NR2A1	
[A/G]	rs1800454	TAP2	C/T	[A/C]	rs1028583	NR2A1	
[T/G]	rs2228397	TAP2	A/C	[A/G]	rs6103731	NR2A1	
[A/G]	rs4576294	TAP2	A/G	[A/G]	rs6017335	NR2A1	
[A/G]	rs2842934	TPMT	A/G	[A/C]	rs2425637	NR2A1	
[T/G]	rs4124874	UGT1A1	G/T	[T/C]	rs3212198	NR2A1	
[A/G]	rs7574296	UGT1A3	A/G	[A/G]	rs2425640	NR2A1	
[A/G]	rs6706232	UGT1A3	A/G	[A/G]	rs2071197	NR2A1	
[A/G]	rs2011404	UGT1A4	C/T	[A/G]	rs745975	NR2A1	
[A/C]	rs6759892	UGT1A6	G/T	[C/G]	rs6031558	NR2A1	
[A/G]	rs1105880	UGT1A6	A/G	[T/C]	rs1800961	NR2A1	
[T/G]	rs1105879	UGT1A6	A/C	[T/C]	rs1885088	NR2A1	
[A/G]	rs2070959	UGT1A6	A/G	[A/G]	rs28399499	CYP2B6	
[A/C]	rs7577677	UGT1A7	A/C	[A/G]	rs4986893	CYP2C19	
[G/C]	rs1042597	UGT1A8	C/G	[T/C]	rs28371706	CYP2D6	
[A/G]	rs7697037	UGT2B11	A/G	[A/G]	rs2740574	CYP3A4	
[A/C]	rs4148269	UGT2B15	G/T	[T/C]	rs1801133	MTHFR	
[A/G]	rs7668258	UGT2B7	C/T	[A/C]	rs1801131	MTHFR	
[A/G]	rs7662029	UGT2B7	A/G				

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Acknowledgements: This study was made possible thanks to the Australian Lymphoma and Leukaemia Group (ALLG) tissue bank samples, a Clinical Fellowship Grant by the Australian New South Wales Cancer Institute and a research grant by Novartis.

Part 2

mTOR inhibition in cancer treatment

Chapter 4

Inhibitors of mTOR

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The Oncologist. 2010; 15(12):1262–9.

ABSTRACT

Inhibitors of mammalian target of rapamycin (mTOR) have been approved for the treatment of renal cell carcinoma and appear to have a role in the treatment of other malignancies. The primary objective of this drug review is to provide pharmacokinetic and dynamic properties of the commonly used drugs everolimus and temsirolimus. Additionally information on the clinical use, mechanism of action, bioanalysis, drug-drug interaction, alterations with disease or age, pharmacogenetics and drug resistance is given. This overview should assist the treating medical oncologist to adjust the treatment with mTOR inhibitors to the individual patient circumstances.

INTRODUCTION

Inhibitors of the mammalian Target Of Rapamycin (mTOR) have anticancer activity. Recently two mTOR inhibitors have been registered in adults for the treatment of cancer: (a) Temsirolimus (Figure 1a) for the treatment of advanced renal cell cancer (RCC) as first line treatment for patients of the unfavorable prognostic group (Temsirolimus is also indicated for the treatment of mantle cell lymphoma [orphan designation]). (b) Everolimus (Figure 1b) for the treatment of RCC as second line treatment. Everolimus has already undergone extensive clinical testing in the renal and cardiac transplantation setting, being well tolerated and effective with daily dosing. [1][2] Everolimus is a derivative of sirolimus (rapamycin), a macrolide antibiotic produced by Streptomyces hygroscopicus, an actinomycete, isolated in 1975 from soil of Rapa-Nui (Easter Island). [3][4] In 1991, the target of rapamycin (TOR) was discovered in yeast. [5] The only known homolog in mammals was subsequently cloned and called mammalian TOR, or mTOR. [6] mTOR has different functions depending on whether it binds to regulatory associated protein of mTOR (Raptor; mTOR complex 1[mTORC1]) or rapamycin-insensitive companion of mTOR (Rictor; mTOR complex 2mTORC2]). mTORC1 controls translation, suppresses autophagy, and regulates transcription and response to DNA damage through the phosphorylation of its downstream substrates 4E-BPs and S6Ks.[7] Inhibition of mTORC1 by rapalogs leads to hyperphosphorylation of Akt(Ser473) in many cancer cell lines. Importantly, activation of Akt may lead to survival when mTORC1 is inhibited. mTORC2 regulates actin cytoskeleton and activates Akt through phosphorylation of Ser-473. [8][9] Inhibitors of mTOR predominantly work through their effect on mTOR complex 1.[10] Ongoing studies investigate the potential anticancer activity of mTOR inhibitors in lymphomas, neuroendocrine tumors, gastric, breast, lung and hepatocellular cancer (www.clinicaltrials.gov). Other mTOR inhibitors under investigation are ridaforolimus (formerly deforolimus, AP23573) and sirolimus itself in combination with grapefruit juice. We focus on the EMEA and FDA approved drugs temsirolimus and everolimus.

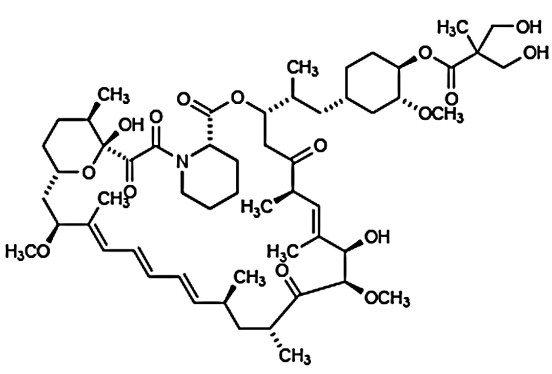

Figure 1b Chemical structure of everolimus [40-O-(2-hydroxy)ethyl-rapamycin]. Positions of ^{3}H- and ^{14}C-labeling indicated by number signs and asterisks, respectively.

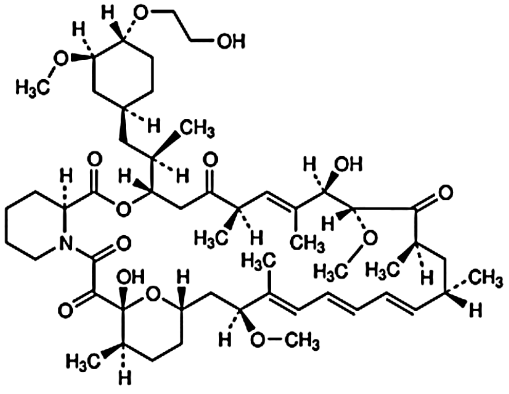

Figure 1a Chemical structure of temsirolimus, 42-[3-hydroxy-2 -(hydroxymethyl)-2-methylpropanoate]

Clinical use

Temsirolimus, a more water-soluble ester derivative of its parent compound sirolimus, is available as a concentrate for solution for intravenous injections (25 mg/ml). The recommended dose is 25 mg weekly. [9][11] Everolimus is available as oral tablets of 1.5 mg, 5 mg and 10 mg. The daily recommended dose is 10 mg, either with or without food.[12] Preliminary dose-finding studies suggest that the dose of everolimus may need to be reduced in combinationtherapy, mainly because of cumulative toxicity. [13][14] Optimal dosing of mTOR inhibitors may be difficult to define based on toxicity. [15]

Mechanism of action

mTOR is a highly conserved serine-threonine kinase and a key regulatory protein in cancer that recognizes stress signals (eg, nutrient and energy depletion, oxidative or hypoxic stress, and proliferative and survival signals) via the PI3K/Akt pathway. Signals from growth factor receptors activate PI3K resulting in Akt activation and finally activation of the centrally located downstream mTOR. It has been demonstrated that Akt, mTOR, and S6K1 are phosphorylated (activated) in most cancer types. These data suggest that activation of PI3K/Akt/mTOR is essential for proliferation and survival of malignancies and mTOR might therefore be a promising target in cancer treatment. [16][17]

Temsirolimus and the active metabolite sirolimus, when bound to an intracellular protein (FKBP-12), form a protein-drug complex inhibiting the activity of mTOR that controls cell division. Inhibition of mTOR activity resulted in a G1 growth arrest in treated tumor cells. When mTOR is inhibited, the downstream targets p70S6k and S6 ribosomal protein are not phosphorylated. Additionally, temsirolimus and sirolimus, are thought to retard tumor angiogenesis, induce apoptosis, reduce expression of hypoxia-inducible factor-1 alpha (HIF1-a) and sensitize cancer cells to apoptosis induction by DNA-damaging agents such as cisplatin. [18-20] In cell lines mTOR inhibitors are able to sensitize cancer cells to chemotherapy, radiation and overcome chemo- or endocrine therapy-resistance. [21-24] Combination therapy studies of mTOR inhibitors with either endocrine- or chemotherapy in metastatic cancer showed only moderate additional benefit. [13][25]

Bioanalysis

Temsirolimus and everolimus blood concentrations can be measured by validated liquid chromatography/tandem mass spectrometry combination procedures. [26] Simultaneous quantification of co-medication is possible. [27] The high costs of the mass spectrometry technique and the demanding technical knowledge is a disadvantage. An alternative is a validated ELISA platform, which has been applied in several studies. [28-30]

Table 1 Pharmacological features of everolimus and temsirolimus

Summary table		
Generic name	**Temsirolimus**	**Everolimus**
Commercial name(s)	CCI-779, Torisel®, CCI-779	SDZ-RAD, RAD001, Certican®, Afinitor®
Mechanism of action	Inhibition of PI3K/Akt/mTOR pathway resulting in modulation of cellular metabolism, growth, proliferation and angiogenesis	
Molecular weight	1030.30 g/mol	958.22 g/mol
Cell-cycle specificity	G1 phase	
Route of administration	Intravenously	Oral; low bioavailability (16%) high fat meal reduces absorption
Protein binding	~85%	75%
Metabolism	By CYP3A4 to active metabolite sirolimus	By CYP3A4 to main metabolite hydroxy-everolimus
Elimination	Primarily via feces (82%), via urine 4.6%	Metabolites via feces (>90%) and urine (>2%).
Terminal half-life	9-27 hrs	26-30 hrs
Main toxicities	Mucositis, thrombocytopenia, rash, asthenia, nausea, edema, anorexia, anemia, hyperglycemia, hyperlipemia, hypertriglyceridemia, elevated alkaline phosphatase, elevated serum creatinine, lymphopenia, hypophosphatemia	
Pharmacogenetics	To be investigated. Data for sirolimus, the main metabolite of temsirolimus, shows a correlation of CYP3A4*1/*1B polymorphism and sirolimus pharmacokinetics.	
Resistance	Activation of IGFR and Akt/PKB results in activation of both, PI3K pathway and anti-apoptotic signalling.	
Unique features	Ability to penetrate the brain-tumor barrier[69]; allergic reaction possible	Extended experience as immune-suppressant in drug-eluting stents
Main drug-drug and drug-complementary and alternative medication interactions	50% dose if concurrent strong inhibitors of CYP3A4*	Interaction with CYP3A4, 3A5 and 2C8 inducers /inhibitors* (e.g. rifampin leads to greater CL/F of everolimus by ~172%)

* Strong CYP3A4 inhibitors include nelfinavir, ritonavir, clarithromycin, itraconazole, ketokonazole; moderate CYP3A4 inhibitors include aprepitant, erythromycin, fluconazole, grapefruit juice, verapamil; CYP3A4 inducers include barbiturates, glucocorticoids, phenytoin, St. John's Wort; CYP3A4 substrate include dexamethasone, taxol, sunitinib, sorafenib, vincristine, irinotecan, docetaxel.

CL/F clearance of the absorbed drug fraction
CYP cytochrome P450
IGFR insulin-like growth factor receptor
mTOR mammalian target of rapamycin
PI3K phosphoinositide 3-kinase
PKB protein kinase B.

Pharmacokinetics absorption

Everolimus absorption is rapid. After ~30 minutes (range 0.5–1 hour) the maximum concentration (Cmax) of everolimus is reached (44.2 ± 13.3 µg/L) with area under the curve (AUC) of 219 ± 69 µg • h/L.31 Steady-state is reached within 7 days, with a median 3-fold accumulation of everolimus exposure. Absorption of everolimus is reduced by about 50% after a high fat meal. It is therefore recommended to take the drug consistently with or without food.[28] The overall absorption of everolimus, like that of sirolimus, is probably affected by the activity of P-glycoprotein (P-gp).[32-34] Liver impairment (Liver cirrhosis Child-Pugh B) did not alter absorption of everolimus.[35]

In rats the oral bioavailability of everolimus is low (16%), but higher than that of sirolimus (10%).[32] Steady-state Cmax (Cmaxss), steady-state trough concentration (Cminss) and area under the AUC showed a dose-proportional increase, and Cminss correlated well with the AUC of everolimus. There is a wide interindividual pharmacokinetic (PK) variability for AUC of 85.4% and intraindividual interoccasion variability of 40.8%, suggesting a role for therapeutic drug monitoring using Cminss.[36]

Protein binding

Protein binding of temsirolimus after intravenous injection is ~85% (FDA label). Sirolimus, the major active metabolite of temsirolimus, is approximately 40% bound to the lipoprotein fraction in blood over a sirolimus concentration range of 5 to 100 ng/mL. Increases in plasma lipoproteins may increase the sirolimus plasma concentrations. For patients with liver cirrhosis Child-Pugh grade A or B may require dose reduction by 33%. [37][38] At therapeutic concentrations of everolimus, more than 75% is partitioned into red blood cells and approximately 75% of the plasma fraction is protein bound. The protein binding of everolimus was not influenced by moderate hepatic impairment (liver cirrhosis vs healthy, 73.8 ± 3.6% vs 73.5 ± 2.4%). [35][36]

Metabolism

Temsirolimus and its primary metabolite, sirolimus, are metabolized by the cytochrome P450 3A 4 pathway (CYP3A4). Sirolimus appears 15 minutes after infusion of temsirolimus with a peak at 0.5 to 2.0 hours, followed by a monoexponential decrease. Exposure to sirolimus is typically higher than that of temsirolimus, with a mean AUC ratio (sirolimus/temsirolimus) of ~ 2.5 to 3.5 (Coefficient of variation [CV], 0.2% to 69%). Dose-related increases in the sum (sirolimus + temsirolimus) of AUCs during treatment (AUCsum) were significantly less than proportional. Mean terminal half-life for sirolimus ranged from 61 to 69 hours (CV, 7% to 60%). At doses higher than 34 mg/m², residual concentrations of sirolimus were detectable before the next infusion seven days later. However, this did not result in higher AUC of sirolimus after repeated cycles. [39]

Everolimus is metabolized mainly in the gut and liver by cytochrome P450 (CYP) 3A4, 3A5 and 2C8. [40] Everolimus and four main metabolites, hydroxy-, dihydroxy-, and demethyl-

everolimus and the ring-opened form of everolimus, were found in blood. Hydroxy-everolimus was the most important metabolite, with a dose normalized AUC of the first 24 hours (AUC24) nearly half that of the parent compound (16.0 ± 6.5 vs 35.4 ± 13.1 µg • h/L), followed by demethyl-everolimus (AUC24 10.7 ± 15.8 µg • h/L), dihydroxy-everolimus (AUC24 8.5 ± 5.7 µg • h/L) and ring-opened everolimus (AUC24 2.3 ± 2.1 µg • h/L). All metabolites appeared relatively soon after administration (Time to maximal concentration [tmax] 1.2–2.0 hours vs 1.5 hours for everolimus). The immunosuppressive or toxic activity of everolimus metabolites is unknown. [27][41]

Elimination

Half-life (t1/2) of standard dose of 25 mg weekly of temsirolimus is 13 hours with a total plasma clearance (CL) of 16 L/h. [42] Increasing doses of temsirolimus induce significant increase of clearance (equivalent to the clearance of the absorbed drug fraction [CL/F]) and a decrease of the mean t1/2 (CL/F: 19 to 51 L/h, CV 14% to 32%; t1/2: 22 hours following 34 mg/m² dose to 13 hours following 220 mg/m² dose, CV 7% to 29%). This suggests auto-induction of factors increasing clearance of the drug at higher than clinically used doses.[43] Multiple dosing of temsirolimus has no significant influence on the PK profile[39] Temsirolimus is excreted predominantly via the feces (78%) and to a minor extent via urine (5%). About 98% of everolimus is excreted in the bile as metabolites and only 2% of everolimus is eliminated via the urine. The half-life of everolimus applied at a dose of 70 mg weekly is approximately 26 hours. [44] For the standard dose of 10 mg everolimus daily in cancer patients no complete PK data are available yet. Extensive data in non cancer patients are available.[27] Compared with healthy subjects, patients with moderate hepatic impairment (liver cirrhosis Child-Pugh B) had significantly lower CL/F of everolimus, by 53% on average.

Drug and complementary and alternative medicine interactions

Potential PK drug-drug interactions for temsirolimus exist with agents that modulate CYP3A4 isozyme activity. Co-administration of 400 mg oral ketoconazole with 5 mg i.v. temsirolimus increased the mean AUC by 3.1-fold compared with temsirolimus alone. P450-inducing anticonvulsant agents (EIACs) decrease Cmax and AUC by 73% and 50%. [45] If a concomitant strong CYP3A4 inhibitor is necessary, a temsirolimus dose reduction to 12.5 mg weekly should be considered. [46] In vitro studies showed that temsirolimus and sirolimus inhibit the CYP2D6 isozyme (Ki = 1.5 and 5 µM, respectively), indicating potential for PK interaction with agents that are substrates of CYP2D6. However, a single i.v. dose of 25-mg temsirolimus did not alter the disposition of desipramine, widely used as a probe to study potential CYP2D6 drug interactions. [47]

Everolimus is a substrate of CYP3A4, 3A5, 2C8 and the efflux transporter P-gp. Everolimus is at the same time a moderate inhibitor of P-gp, a competitive inhibitor of CYP3A4 and a mixed inhibitor of CYP2D6 *in vitro*. [32][40] Drug interaction is most likely by drugs influencing activity of the above mentioned enzyme systems and transporters. Potent or moderately potent inhibitors of CYP3A4 like ketoconazole, erythromycin, or verapamil

cause a pharmacokinetic interaction resulting in increased Cmax and AUC of everolimus. Concomitant use of potent or moderately potent CYP3A4 inhibitors (e.g., aprepitant, clarithromycin, diltiazem, erythromycin, fluconazole, grapefruit, itraconazole, ketoconazole, nefazodone, telithromycin, verapamil, voriconazole and most anti-HIV medication should be avoided.

Potent inducers of CYP3A4, like rifampin, pharmacokinetically interact with everolimus, leading to decreased Cmax and AUC. [31] Concomitant use of potent CYP3A4 inducers (e.g., carbamazepine, dexamethasone, phenobarbital, phenytoin, rifabutin, rifampin) should be avoided. If concomitant use of a potent CYP3A4 inducer cannot be avoided, an increase in everolimus dosage (from 10 mg daily up to 20 mg daily, titrated in 5-mg increments) should be considered based on pharmacokinetic considerations; however, no clinical data on this dosage adjustment in this patient population are currently available. If the potent CYP3A4 inducer is discontinued, the dosage of everolimus should be reduced to the usual recommended dosage. Neither statins like atorvastin or pravastatin nor anti-cancer drugs like letrozole or gefitinib cause significant alteration of drug exposure. [30][48] There is no specific data available about interaction between complementary alternative medicine and mTOR inhibitors.

Alterations with disease or age

Efficacy and dosing of temsirolimus is independent of age. Increased incidence of thrombocytopenia has been noted in patients with mild hepatic impairment treated with temsirolimus. [49] Moderate or severe hepatic impairment have not been studied in the treatment of temsirolimus. Concurrent hemodialysis did not show any influence on temsirolimus and sirolimus PK excluding the need of temsirolimus dose adjustments for renal impairment. [50] Interestingly, higher doses of temsirolimus (weekly 175mg for 3 weeks followed by 75mg weekly) have been well tolerated in a phase III study of mantle cell lymphoma. [51]

Everolimus PK characteristics did not differ with age, sex and weight in adults. [36] The dosage of everolimus should be reduced by half in patients with significant hepatic impairment. [35] Only 2% of everolimus is eliminated in the urine, therefore renal impairment is not expected to influence drug exposure. No dosage adjustment of everolimus is recommended in patients with renal impairment.

Pharmacogenetics

Boni et al. found in a population PK study that the pharmacogenomic profiling of identified peripheral blood mononuclear cells (PBMCs) altered the expression of ribonucleic acid transcript levels that correlate with exposure. [52] However, sirolimus pharmacogenetic studies from renal organ transplant show an association between the CYP3A4*1/*1B polymorphism and sirolimus PKs as well as lower dose-corrected trough levels in CYP3A4*1B/*1B than in CYP3A4*1/*1 patients. [53] This was, however, not confirmed by other studies.[54] None of the CYP3A4, CYP3A5, and ABCB1 polymorphisms has been associated with sirolimus toxicity or efficacy. [53][55]

Drug resistance

Rapamycin induces the Akt activation, an oncogenic kinase, in some models. [56][57] Insulin-like growth factor I (IGF-I) and insulin-dependent induction of the PI3K/Akt pathway leads to feedback inhibition of signalling due to mTOR/S6K-mediated phosphorylation. Rapamycin-induced Akt activation has been attributed to loss of this negative-feedback loop. The effect of rapamycin on Akt may vary with drug dose, with lower doses leading to an increase in Akt activation and higher doses to diminishing Akt activity. [58][59]

Sirolimus inhibits only mTOR1 and not mTOR2, whereas the latter is responsible for Akt/PKB activation via a positive feedback loop. Activation of IGFR and Akt/PKB results in activation of both the PI3 kinase pathway and anti-apoptotic signalling. [60][61] To overcome this problem dual inhibition of PI3K and mTORC1/2 signalling by NVP-BEZ235 as a new therapeutic strategy for acute myeloid leukemia has been investigated.[62] In addition, other strategies to downregulate mTOR signalling, such as the use of the antidiabetic drug metformin, an activator of AMPK, are being pursued in clinical trials. [63]

Pharmacodynamics

For mTOR, the two best studied targets are S6K1 and 4E-BP1. Preclinically, rapamycin and its analogs inhibit phosphorylation of 4E-BP1 and S6K1 in tumor, skin and PBMCs. [64][65] Time- and dose-dependent inhibition of S6K1 was demonstrated in PBMCs. In preclinical models, a correlation with antitumor effect of rapamycin and prolonged (≥ 7 days) PBMC-derived S6K1 activity has been observed. For everolimus, preclinical simulations suggest that the administration regimen has a greater influence on S6K1 activity in the tumor than PBMCs, with daily dosing exerting greater activity than weekly doses, [66] sustained S6K inhibition occurring with ≥ 20-mg everolimus weekly and ≥ 5 mg daily. [44] These findings highlight that although PBMC S6K1 activity is often measured as a PD marker, it is not a perfect readout of target inhibition in the tumor. [67]

A phase I study of everolimus in solid tumors, pretreatment and at steady-state tumor and skin biopsies were evaluated showing a mTOR signaling inhibition at all doselevels and schedules tested (between 5 mg daily, and 70 mg weekly). [68] Dose- and schedule-dependent inhibition of mTOR was observed with near complete inhibition of p-S6 and p-eIF4G at 10 mg/d and ≥50 mg/wk. This study demonstrates that inhibition of mTOR signaling may be dependent on dose and schedule, and downstream targets may not always be inhibited concordantly. Downstream effects of mTOR inhibition in rapamycin-sensitive versus rapamycin-resistant tumors elucidated rapamycin's mechanism of action. Potential pharmacodynamic (PD) markers of response being examined are p-4EBP1, p-PRAS40 (Thr246), p-Akt and cyclin D1 levels. In a recent review it has been stated that it is unlikely that any single marker will sufficiently separate responders from non-responders and it has been suggested to evaluate a panel of rapamycin effectors for PD monitoring. [67]
Another option is the use of serial biopsies of the tumor, but it is an inconvenient way to determine early signs of response. [67] Molecular imaging with tracers that assess metabolic and proliferative function ([18F]fluorodeoxyglucose and [18F]fluorothymidine uptake) has shown promise in preclinical models. [67]

Patient instructions and recommendations for supportive care

Oral ulcerations (i.e., mouth ulcers, stomatitis, oral mucositis) are very common in mTOR inhibition. Topical therapy is recommended; however, alcohol- or peroxidecontaining mouthwashes should be avoided. Myelosuppression is the second most common toxicity and requires monitoring of serial blood counts. Hyperglycemia and dyslipidemia can worsen, regular blood tests are warranted and the use of antidiabetic and hypertensive medication to optimize blood glucoses and blood pressure is recommended. The use of mTOR inhibitors may cause drug induced pneumonitis, which usually respond well to steroids and withdrawal of the mTOR inhibitor. In case of dyspnea during treatment other causes should be excluded. Immunosuppressive activities of mTOR inhibitors are rare, but infections should be treated according to standard of care. For herpes lesions topic and systemic treatments with antiviral drugs is recommended. H1 blocker should be given approximately 30 minutes before each weekly temsirolimus infusion as prophylaxis against an allergic reaction.

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Chapter 5

Phase I study of temsirolimus in combination with nelfinavir in patients with solid tumors

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Submitted for publication

ABSTRACT

Purpose

Temsirolimus is an inhibitor of the mammalian target of rapamycin (mTOR) with proven anti-cancer activity. Nelfinavir, a human immunodeficiency virus protease inhibitor with Akt inhibiting activity, might provide a strategy to circumvent drug resistance to temsirolimus. This phase I trial was conducted to determine the safety, tolerability, pharmacokinetics, and pharmacodynamics of combined treatment with temsirolimus and nelfinavir.

Patients and Methods

Eighteen adult patients with advanced solid malignancies were treated in dose escalation cohorts of 3-6 patients. Intravenous temsirolimus was given once weekly and oral nelfinavir was taken daily. Temsirolimus was dosed at 10 or 15 mg, and nelfinavir at 1000 mg or 1500 mg, divided into two daily doses. Pharmacokinetics of each drug and its active metabolites were determined both in a run-in phase where each drug was taken as a single agent, and when combined. Pharmacodynamic studies both before and during treatment, in peripheral blood mononuclear cells (PBMCs) and in tumor material of mTOR and PI3kinase signalling were performed.

Results

The recommended doses were determined to be temsirolimus 10 mg and nelfinavir 1000 mg. Dose-limiting toxicities were mucositis and nausea. One patient died during treatment due to fatal lung embolism, most likely not related to treatment. When temsirolimus was combined with nelfinavir, an increase of the sirolimus area under the curve (AUC) was seen ($p = 0.0003$), probably due to the inhibitory effect of nelfinavir on CYP3A4. Three patients had stable disease up to 16 months, and median progression free survival was 71 days. Pre-existing high levels of mTOR in PBMCs and higher levels of sirolimus were predictive for response to treatment.

Conclusion

Nelfinavir 1000 mg/day and temsirolimus 10mg/week can be safely combined. DLTs were mucositis and nausea.

INTRODUCTION

The mammalian target of rapamycin (mTOR) is a highly conserved serine-threonine kinase that functions as a central controller of cell growth, metabolism, and aging. mTOR is activated in response to nutrients, growth factors, and cellular energy. Dysregulated mTOR signalling has been implicated in several diseases including cancer [1]. Aberrant activity of the PI3K/Akt pathway plays an important part in mTOR activation, as has been demonstrated in several cancer types [2]. These data suggest that activation of PI3K/Akt/mTOR is essential for proliferation and survival of malignancies, and mTOR might therefore be a promising target in cancer treatment [3-5]. Several mTOR inhibitors (rapamycin analogues) have been developed, including temsirolimus (CCI-779 / Torisel®), which is active in renal cell cancer and mantle cell lymphoma [6][7]. Temsirolimus (TEM) and its equally potent active metabolite, sirolimus (SIR), are both metabolized by the cytochrome P450 3A4 (CYP3A4). Enzymatic hydrolysis is responsible for the conversion of TEM into SIR and is independent of CYP3A4 [8]. Exposure to SIR is typically higher than exposure to TEM due to the longer half-life of the former (2.7 fold) [9]. TEM and SIR, when bound to the intracellular FK506 tacrolimus-binding protein 12 (FKBP-12), form a protein–drug complex inhibiting the activity of mTOR. The disruption of mTOR signalling suppresses the activation of proteins (eg. p70S6 kinase [p70S6K] and elF4E) that regulate progression through the cell cycle and angiogenesis [6][10].

Although rapamycin and its analogues have antineoplastic activity in several experimental cancer models, and are clinically active in renal cell cancer [6][11] and neuroendocrine tumors [12], most cancer types are resistant. A potential mechanism of drug resistance is loss of a feedback loop inhibiting Akt after exposure of tumor cells to mTOR inhibitors, leading to activation of Akt. Akt is a crucial molecule in the PI3K pathway involved in carcinogenesis and cancer progression [4]. Dual inhibition of Akt and mTOR may therefore be an interesting option to counteract mTOR-inhibition-induced Akt activation and other downstream molecules in the PI3K signalling pathway [5][13]. Nelfinavir (NFV), a protease inhibitor used in HIV treatment, is a strong CYP3A4 inhibitor which has Akt inhibiting activity, resulting in downregulation of PI3K signaling [14][15].

Pharmacodynamic investigations demonstrated the predictive value of phospho-p70S6K^{Thr389} in peripheral blood mononuclear cells (PBMCs) for mTOR inhibition and activity of phospho-Akt^{Ser473} in PBMCs for Akt inhibition [16][17]. Other pharmacodynamic studies have been performed on tumor tissue using phospho-S6 ribosomal protein (phospho-S6RP$^{Ser235/236}$) [18].

This first–in-human, phase I, non-randomized, single-center, dose escalation study was designed to determine the maximum-tolerated dose (MTD), safety profile, pharmacokinetic (PK) parameters, pharmacodynamic activity, and preliminary antitumor activity of intravenous TEM in combination with NFV in patients with solid tumors.

Patients and Methods

Eligibility Criteria

Patients with solid malignancies progressive on standard therapy or for whom no adequate therapy was available, were eligible. Additional eligibility criteria included age > 18 years; an Eastern Cooperative Oncology Group performance status of 2 or less; adequate hematopoietic, hepatic, and renal function. Patients were excluded for concomitant significant systemic disorders, pregnancy and concomitant use of strong CYP3A4 inhibitors or inducers.[19] All patients provided informed consent. The study was approved by the Institutional Review Board and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Study Treatments

TEM was administered intravenously as a weekly 30 minute infusion in 250 ml NaCl 0.9%, after 2 mg of clemastine premedication to prevent potential allergic reactions. NFV was supplied as 250 mg tablets for oral administration twice daily. In order to investigate potential pharmacokinetic interaction, the treatment schedule was: day 1 NFV single dose, day 4 TEM single dose, day 11 start of continuous combination therapy of weekly TEM and daily NFV (Figure 1). Three weeks of the combined treatment was defined as one cycle. The first dose level was: TEM 10 mg, which is 40% of the standard weekly approved dose in renal cell carcinoma (25 mg) [6][20], in combination with NFV 750 mg BID, 60% of the dose commonly used in HIV patients. The rationale for these dose adaptations for TEM and NFV in the first dose cohort was the CYP3A4 inhibiting activity of NFV leading to potential toxic levels of both TEM and SIR. Previously it was shown that co-administration of ketoconazol, another CYP3A4 inhibitor, increased AUCsum (sum of TEM plus SIR AUCs) 2.3-fold compared with TEM alone[21]. In case of dose limiting toxicity (DLT) at dose level 1, the dose of NFV was reduced to 500 mg BID (dose level -1) with the option to increase in a further cohort TEM to 15 mg (dose level –1A). In the absence of DLTs at dose level 1, the dose of NFV was escalated to 1250 mg BID in combination with TEM 10 mg (dose level 2). In the next cohort the dose of TEM was escalated to 15 mg in combination with NFV 1250 mg (dose level 3) with a further increase of TEM to 20 mg for dose level 4. No intra-patient dose escalations were allowed. At least three patients per dose level were recruited and expanded to a maximum of six if one out of three patients experienced DLT. In case patients were not evaluable to measure DLT (stop of treatment within the first 6 weeks without DLT), additional patients were added to that dose cohort. Dose escalation to the next dose level was permitted if no DLT occurred in any of three or in ≤ 1 of six patients. In case of DLT in one or more out of three, or in two or more out of six patients, that dose level was declared intolerable and no further dose escalation occurred. The immediately preceding dose level was declared the MTD. DLT was defined as: neutropenia CTC grade 4 lasting more than five days or neutropenia CTC grade 3 with fever, CTC grade ≥ 4 platelets or grade 3 platelets with bleeding, vomiting CTC grade ≥ 2 or any other toxicity CTC grade ≥ 3, despite best supportive care in the first treatment cycle. Patients continued on therapy until disease progression, treatment-related toxicities, or withdrawal of consent.

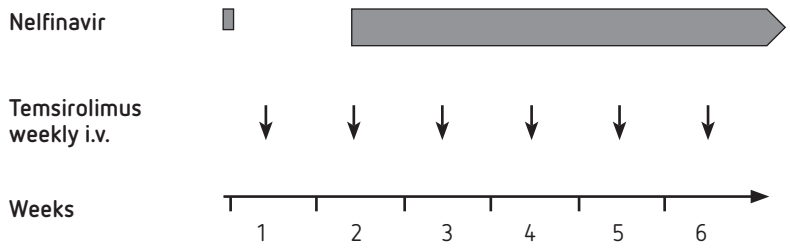

Figure 1 Treatment schedule, nelfinavir given only once on day one, from on day 11 twice daily.

Pharmacokinetic Assessments

Blood samples for PK profiles of TEM, its active metabolite SIR and NFV were obtained in each patient both as single agent after the first administration (day 1 for NFV and day 4 for TEM/SIR) and after combination of TEM and NFV (day 11) at time points 0, 1, 2, 3, 4, 8, and 24 hours after TEM and 0, 1, 2, 3, 4 and 8 hours after NFV. In addition, TEM, SIR and NFV trough levels at day 18, 25 and 32 were collected. NFV plasma concentrations and TEM and SIR whole blood concentrations were determined using liquid chromatography coupled to tandem-mass spectrometry (LC-MS/MS) (for more details see the Supplementary Appendix 1). The mean and coefficient of variation (CV) of the following PK parameters were reported: the maximum observed plasma concentration (C_{max}), time to reach C_{max} (t_{max}) and area under the plasma concentration-time curve between 0 and the last quantified time point (t=8 and 24 hours for NFV and TEM/SIR, respectively).

Pharmacodynamic Analyses

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood collected before start of treatment and at day 18 and day 25 as described in supplementary Appendix 1. Total protein was then extracted from the PBMC pellet and stored at -80°C until analyzed. Phospho-specific antibodies were used to determine the levels of phospho-p70S6K^{Thr389} and phospho-Akt^{Ser473} by Western blotting [23][24]. In patients who gave optional consent, additional biomarker evaluation was performed in tumor biopsies collected before and during treatment. Inhibition of mTOR signaling was evaluated by changes in phosphorylation of S6RP$^{Ser235/236}$ (Supplementary Appendix 1).

Evaluation of Clinical Activity

Disease status was assessed before start of study treatment, after 9 weeks of therapy, and every 9 weeks thereafter until withdrawal from the study, using the Response Evaluation Criteria in Solid Tumors (RECIST) 1.0 Guidelines [25].

Statistical analyses

PK parameters of TEM, SIR and NFV were calculated using non-compartmental methods. PK parameters obtained as single agent after the first administration or after combination of TEM and NFV were rank-transformed and compared using non-parametric repeated measures ANOVA with correction for dose level. PK parameters are presented as median (interquartile range, IQR). Differences between responders and non-responders were tested using a Mann-Whitney or Fisher exact test for continuous and categorical variables, respectively. Relationships between PK and PD parameters and between PK and toxicity parameters were analysed using Spearman correlation coefficients (rho). All statistical analyses were carried out using R (Version 2.10, http://www.r-project.org/). p values < 0.05 were considered statistically significant.

Results

Patient characteristics

Eighteen patients were enrolled at a single center in The Netherlands between June 2009 and April 2011. Table 1 shows the characteristics of these patients, who were treated on three dose levels. Sixteen patients (89%) had received previous chemotherapy (2 or more lines). Two patients had had no prior systemic treatment. They were diagnosed with metastatic gallbladder cancer, for which no standard systemic treatment but best supportive care existed in The Netherlands at the time of study entry.

Treatment characteristics

Seven patients were treated at dose level 1: weekly TEM 10 mg/NFV 750 mg BID. Two patients discontinued treatment during the first cycle, one patient withdrew consent and one withdrew for grade 3 toxicity. Therefore dose level 1 had 7 participating patients. Six patients were treated at dose level -1: weekly TEM 10 mg/NFV 500 mg BID and five patients at dose level -1A: weekly TEM 15 mg/NFV 500 mg BID. Three weeks of the combined treatment was defined as one cycle. Table 2 shows treatment durations and dose reductions. Participating patients received a median number of 8 weekly TEM treatments, and 9 (50%) patients completed 3 or more cycles. Median time on treatment for the overall population was 67 days (range, 20 to 487 days).

Table 1 Baseline Patient Characteristics

Characteristic		Median	Range	No
Total number				18
Age (years)		52	24-74	
Body Surface Area (average in m^2)		1.84	1.62-2.17	
Sex	Male			9
	Female			9
Ethnicity	Caucasian			18
Primary malignancy	Pancreas			3
	Glioblastoma multiforme			3
	Sarcoma			3
	Esophageal			3
	Galbladder			2
	Colon			1
	RCC			1
	Ovarian			1
	Bladder			1
Prior anticancer therapy	Chemotherapy			15
	Radiotherapy			6
	Surgery			16
	Targeted therapy			6
WHO performance status	0			9
	1			7
	2			2
Site of metastatic disease	Liver			6
	Bone			1
	Lung			6

RCC Renal-cell carcinoma
WHO World Heath Organization

Table 2 Treatment administration of the combination of temsirolimus and nelfinavir by dose level

	Dose level	1 N=7	-1 N=6	-1A N=5	Total
					18
Nelfinavir	Daily dose	1500 mg	1000 mg	1000 mg	
	Dose reduction	4	0	0	4
Temsirolimus	Dose /week	10 mg	10 mg	15 mg	
	Dose reduction	0	1	4	5
No. of iv. TEM given in weeks					
Median		9	7	11	8
Range		2-32	5-9	2-55	2-55
No. of weeks of NFV treatment					
Median		10	9	12	10
Range		3-33	6-10	3-61	3-61
Treatment duration in days					
Median		72	61	87	67
Range		20-231	44-73	24-490	24-490

TEM temsirolimus
NFV nelfinavir
SD standard deviation

Table 3 Treatment-related adverse events occurring in 3* or more treated patients

Event	Dose level						Totaal, n(%)	
	1500 mg NFV/day, 10m g TEM/week (n = 7)		1000 mg NFV/day, 10 mg TEM/week (n = 6)		1000 mg NFV/day, 15 mg TEM/week (n = 5)			
	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4
Oral aphthous ulcer/ mucositis	5	1	2	0	2	1	9 (50)	2 (11)
Infection	3	1	0	0	0	1	3 (17)	2 (11)
Nausea/vomiting	3	1	2	0	0	1	5 (28)	2 (11)
Fatigue	3	0	2	0	4	0	9 (50)	0
Rash	3	0	3	0	1	0	7 (39)	0
Diarrhea	2	0	3	0	2	0	7 (39)	0
Hematology								
Thrombopenia	3	0	3	0	3	0	9 (50)	0
Hyperglycemia	4	0	3	0	1	0	8 (44)	0
Anemia	4	0	1	0	2	0	7 (39)	0
Leukopenia	1	0	2	0	1	2	4 (22)	2 (11)
Clinical chemistry								
Hyperlipidemia	3	0	4	0	1	1	8 (39)	1 (6)
Hypokalemia*	0	1	0	0	0	1	0	2 (11)
Others	6	0	8	0	1	0	15	0
DLT		2		0		2		4
Total AEs							94	12

*Grade 3 blood potassium abnormalities occurred in two patients and was therefore included in the table.

NFV nelfinavir
DLT dose limiting toxicity.

Safety and Tolerability

Three dose levels were evaluated for DLT. During treatment on dose level 1 five CTC grade 3/4 AEs and 2 DLTs occurred (one grade 3 nausea and one grade 3 mucositis). When patients stopped NFV the toxicities diminished. These toxicities led to a dose reduction of NFV from 750 mg to 500 mg BID. Therefore, the next dose level (-1) started at 10 mg TEM and 500 mg NFV BID resulting in acceptable toxicity (no CTC grade 3/4 AEs). Due to the high rate of toxicity in the first dose level it was agreed to expand the cohort by another 3 patients before dose escalation, which was associated again with acceptable toxicity. At dose level -1A (TEM 15 mg/week, NFV 500 mg BID) one DLT occurred in the first cohort of 3 patients and another one in the expanded cohort defining this dose level as intolerable. The MTD was thus determined to be TEM 10 mg/week, NFV 500 mg BID.

All patients experienced drug related AEs. Most of the drug related AEs (94 of 106) were mild in intensity (grade 1or 2) (see table 3.). The most frequently reported drug related AEs were oral aphthous ulcers/mucositis (50%), fatigue (50%), rash (39%) and diarrhea (39%). Most common grade 1-2 blood test abnormalities were thrombocytopenia (50%), hyperglycemia (44%), anemia (44%) and hyperlipidemia (39%). Less frequently reported drug related AEs were nausea/vomiting (28%), infections (17%), anorexia (17%) and leukocytopenia (22%). Eleven grade 3 AEs experienced by eight patients were considered to be drug related (table 3). Two patients had a CTC grade 3 hypokalemia, which resolved with adequate supplementation. One patient died during treatment on dose level 1, due to fatal lung embolism, which was considered to be most likely not related to treatment. One patient developed grade 4 hyperlipidemia, which increased from pre-existing grade 2. Drug related toxicity caused dose reductions of NFV in 4 patients on dose level 1 (TEM 10 mg/NFV 750 mg BID). One patient on dose level -1 (TEM 10 mg/NFV 500 mg BID) had a grade 2 headache 3 days a week which occurred in a TEM treatment-related pattern until dose reduction of TEM. Four patients on dose level -1A (TEM 15 mg/NFV 500 mg BID) needed dose reduction of TEM. All reported infections were possibly drug related, i.e. pneumonia, cholangitis with liver abscess (in a cholangiocarcinoma patient), tooth-root infection and fungal infection of the feet.

Pharmacokinetics

Pharmacokinetic data were available for all 18 patients (table 4), except for the NFV data of the first 4 patients in dose level 1. There was a wide interpatient variation of the AUC0-8 and Cmax for single agent NFV at all three dose levels, up to 4-fold (Coefficient of variation [CV] 42-65% and 46-53%, respectively). There was no significant difference of PK both for NFV with or without TEM, and for TEM with or without NFV, which indicates the absence of a drug-drug interaction between TEM and NFV. In contrast, Tmax and AUC0-24 of SIR increased when NFV was co-administered. The mean Tmax increased up to 14-fold and the mean AUC0-24 up to 2.4-fold. Trough levels were measured for TEM and SIR (data not shown). TEM levels were in most cases undetectable 168 hours after administration of TEM. Mean SIR trough levels at day 18, 25 and 32 were 13.7 (standard deviation [StD] 8.4), 21.5 (StD 13.2) and 22.5 (StD 14.8) ng/mL, respectively, for all dose levels combined.

Table 4

Median pharmacokinetic parameters (interquartile range) of nelfinavir and temsirolimus and its active metabolite sirolimus as single agent and in combination. The p value shows the significance of drug-drug interaction by measurement of the difference between single agent treatments PK data of all 3 dose levels and the PK data of the combination therapy.

Drug interaction	Dose level*	Patients with values (*N*)	T_{max} hr	C_{max} µg/mL	P value	AUC# µg·h/mL	P value
Nefinavir without temsirolimus (day 1)	1	3	3 (1)	1.73 (1.10)		8.0 (5.4)	
	-1	6	2 (0.75)	2.46 (1.01)		11.0 (8.7)	
	-1A	5	3 (2)	1.72 (0.32)		8.7 (4.4)	
Nelfinavir with temsirolimus (day 11)	1	3	3 (0)	1.82 (0.12)	0.479	8.9 (1.3)	0.876
	-1	6	3 (0.75)	2.56 (2.78)		6.5 (10.9)	
	-1A	5	3 (2)	3.07 (1.92)		18.5 (3.1)	
				C_{max} ng/mL		AUC# ng·h/mL	
Temsirolims without nelfinavir (day 4)	1	7	1 (0)	165 (12)		1161 (229)	
	-1	6	1 (0)	133 (55)		730 (516)	
	-1A	5	1 (0)	132 (31)		1008 (182)	
Temsirolimus with nelfinavir (day 11)	1	7	1 (0)	163 (15)	0.439	1181 (324)	0.746
	-1	6	1 (0)	162 (42)		1054 (176)	
	-1A	5	1 (0)	108 (5)		801 (156)	
Sirolimus without nefinavir (day 4)	1	7	2 (1)	30 (37)		467 (792)	
	-1	6	1.5 (1)	30 (19)		368 (299)	
	-1A	5	1 (0)	30 (26)		413 (363)	
Sirolimus with nelfinavir (day 11)	1	7	24 (8)	49 (16)	0.025	1005 (507)	0.0003
	-1	6	6 (18)	37 (11)		758 (202)	
	-1A	5	24 (16)	33 (3)		678 (72)	

AUC for NFV is calculated as AUC0-8, AUC of TEM and SIR are calculated as AUC0-24

* Dose level 1 TEM 10 mg weekly, NFV 750 mg BID,
-1 TEM 10 mg weekly, NFV 500 mg BID,
-1A TEM 15 mg weekly, NFV 500 mg BID

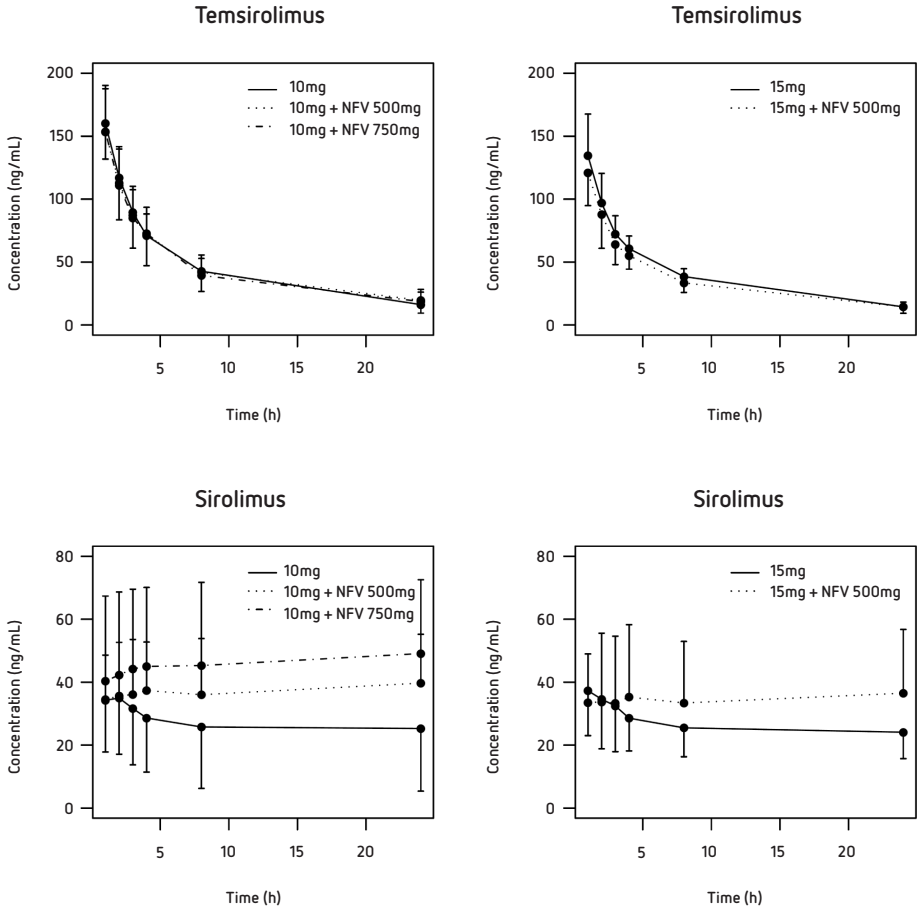

Figure 2 Pharmacokinetics of temsirolimus and sirolimus with and without nelfinavir

Black dots with error bars represent median drug levels with standard deviation.

A Blood concentrations of temsirolimus after intravenous dose of 10 mg (solid line) and

B 15mg temsirolimus with nelfinavir 500 mg BID (dotted line) and nelfinavir 750 mg BID (dash-dotted line).

C Blood concentrations of sirolimus after intravenous dose of 10 mg temsirolimus (solid line) and

D 15mg temsirolimus with nelfinavir 500 mg BID (dotted line) and nelfinavir 750 mg BID (dash-dotted line).

Tumor Response

Three patients (17%) had prolonged stable disease (SD). SD was seen in one patient with osteosarcoma (progression free survival [PFS] 123 days), in one patient with glioblastoma multiforme (GBM) (PFS 232 days) and in one patient with platinum-resistant ovarian-cancer (PFS 487 days). Three patients had no radiological evaluation: two patients withdrew consent and one patient died of pulmonary-embolism before evaluation. The majority (15 patients) had either radiological or clinical progression within the first 70 days of treatment.

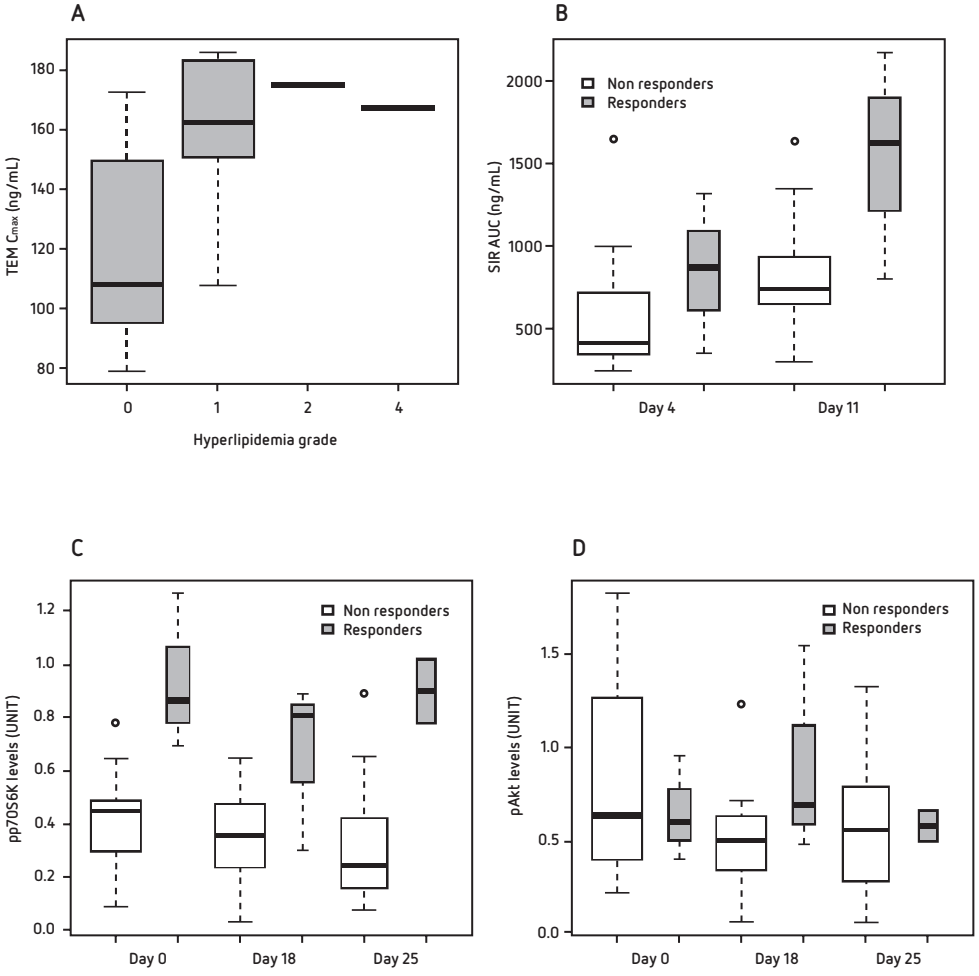

Figure 3

Pharmacokinetic and pharmacodynamic parameters of responders (n = 3) and non-responders (n= 15).

- **A** Correlation between grade of hyperlipidemia (grade by CTCAE) and TEM C_{max} at day 11;
- **B** Correlation between of SIR $AUC_{0\text{-}24}$ in ng-h/ml on day 4 and day 11;
- **C** Pharmacodynamic analysis of phospho-p70S6K^{Thr389} in PBMCs measured before treatment, at day 18 and day 25;
- **D** Pharmacodynamic analysis of phospho-AktSer473 in PBMCs measured before treatment, at day 18 and day 25.

Densitometric quantification of the Western blotting images was performed using ImageJ software. The levels of phospho-p70S6K^{Thr389} and phospho-Akt^{Ser473} were both normalized by the content of β-actin (used to control for sample-to-sample differences in protein concentration) and expressed in arbitrary units (UNIT).

Pharmacodynamics

Molecular response to combination of TEM and NFV was evaluated in PBMCs of all patients and in tumor biopsies of three patients. Reduced phospho-p70S6K^{Thr389} and phospho-Akt^{Ser473} levels were a surrogate marker of mTOR and Akt inhibition respectively. PBMC numbers decreased about twofold after long term treatment with TEM and NFV (data not shown).

Pharmacodynamic data were correlated with the effect of treatment and toxicity (Figure 3 and 4). For this analysis effect of treatment was divided in responders (n=3) and non-responders (n=15). Responders in this study were defined as patients with prolonged stable disease (stable disease for ≥4 months). Non-responders were defined as patients with either clinically or radiologically progressive disease. The presence of high activity levels of phosphorylated p70S6K in PBMCs at day 0 and 25 was predictive for prolonged stable disease (p = 0.0059 and 0.0440 respectively) (Figure 3). The change of phospho-Akt^{Ser473} level in PBMCs between day 0 and 18 was also significantly different between responders and non-responders (p = 0.032). Reduction in phospho-S6RP$^{Ser235/236}$ was profound after treatment, particularly in the biopsy of the patient with ovarian carcinoma who experienced the most prolonged period of stable disease (Figure 4A), and indicated sustained inhibition of mTOR signaling. In a patient with esophageal cancer the phospho-S6RP$^{Ser235/236}$ level in a skin metastasis was also remarkably lower than before treatment (Figure 4B).

There was no significant correlation between NFV PK data and Akt activity in PBMCs (for NFV AUC and C_{max} p = 0.923 and 0.443 respectively), but a strong positive correlation existed between NFV AUC0-8 at D11 and absolute levels of phospho-p70S6K^{Thr389} at day 25 (p = 0.001).

Discussion

This study explored the combination of the intravenous mTOR inhibitor temsirolimus and the oral Akt inhibitor nelfinavir. Treatment at dose level –1 (TEM 10 mg weekly and NFV 500 mg BID) was well tolerated and showed an acceptable toxicity profile in patients with solid tumors. Higher dose levels showed grade 3 nausea and mucositis as unacceptable toxicity with two DLTs at dose level 1 (TEM 10 mg/NFV 750 mg BID) and also two DLTs at dose level -1A (TEM 15 mg/NFV 500 mg BID). There were more non-hematological toxicities compared with the results of single-agent temsirolimus at the same dose [9]. Grade 3 and 4 toxicities occurring after the first treatment cycle were mucositis, nausea/vomiting, infections, hyperlipidemia and hypokalemia.

We found an increase of SIR exposure (AUC0-24) after co-administration of NFV, due to CYP3A4 inhibition by NFV. The decreased metabolism of SIR can be explained by CYP3A4 inhibition as previously demonstrated with ketoconazol, another CYP3A4 inhibitor [21][26]. There was no significant increase of TEM AUC_{0-24}, even though TEM is also metabolized by CYP3A4. This might be explained by the ongoing enzymatic hydrolysis of TEM to SIR [8]. The increase in the T_{max} of SIR when combined with NFV is thought to be due to the

phospho-S6RP $^{Ser235/236}$ in skin metastasis collected

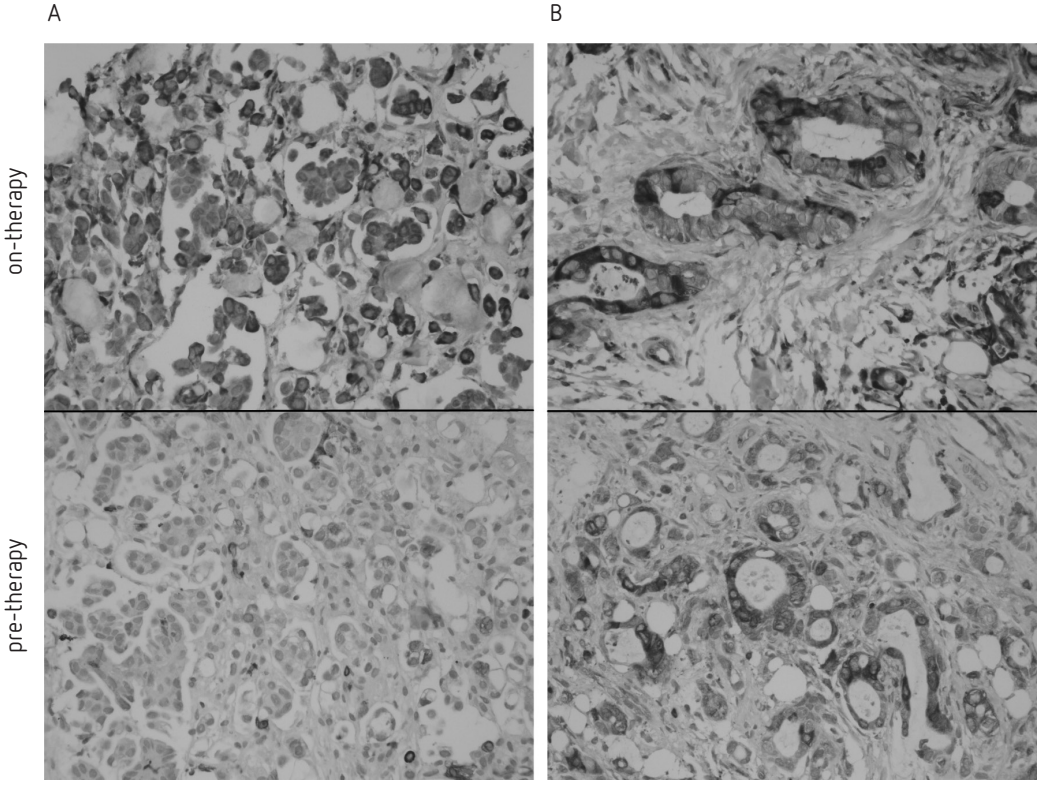

Figure 4

mTOR pathway status changes in response to temsirolimus treatment in combination with nelfinavir. Changes in phospho-S6 ribosomal protein $^{Ser235/236}$ (pS6RP$^{Ser235/23}{}_{6}$) were determined in biopsies from skin metastasis collected before treatment and during treatment, on day 25 of treatment in a patient with ovarian carcinoma **(A)** and on day 25 in a patient with esophageal carcinoma **(B)** (200x magnification). There is a clear decrease of mTOR activity, marked by decrease stainging of pS6RP$^{Ser235/236,}$ between pre-therapy and on-therapy. This decease is more pronounced in in colom **A** , than in colom **B**.

ongoing increase of SIR levels most likely due to ongoing hydrolysis of TEM to SIR and the increased elimination half-life of SIR (Figure 2). Levels of NFV showed a wide interpatient variation (more than 4 fold NFV AUC_{0-8}), as demonstrated before in other patient cohorts [27][28]. This variation does not correlate with the variation in SIR levels when both drugs are combined (for SIR AUC_{0-24} and C_{max} p = 0.98 and p = 0.88 respectively). Due to toxicity we were not able to increase the dose of NFV, most likely due to high SIR concentrations. Previously, a much higher MTD (3125 mg) was found in a single agent Phase I study of NFV in cancer patients [29]. This finding suggests that toxicities of TEM and SIR rather than NFV toxicity are the main dose limiting factors in this combination study. The DLT is therefore probably not a direct effect of NFV, but the result of the altered metabolism of TEM to SIR.

None of the treated patients in this study had an objective response to treatment. In cancer treatment objective responses to mTOR inhibitors are uncommon. Clinical benefit with prolonged SD is seen in about one third of patients in large randomized trials and seen in three unselected patients in this study [6][11]. All patients with SD had dose reductions during their treatment due to toxicity, but tolerated TEM 10 mg/NFV 500 mg BID. The particularly prolonged SD in a patient with platinum-resistant ovarian cancer may be a confirmation of preclinical data, which show the potential of mTOR inhibition to delay tumor progression in a mouse model of ovarian cancer [30].

The pharmacodynamic analysis yielded several interesting results. When phospho-p70S6K^{Thr389}, a predictor of mTOR inhibition, was correlated with PK data, it showed SIR and NFV levels to be positively and significantly correlated. This finding confirms the dose dependent influence of SIR and NFV on inhibition of mTOR.

This study investigated only a small group of patients. The patients with prolonged stable disease versus patients without any clinical benefit were studied in a separate analysis to generate potential predictive markers for future studies. A trend was observed of higher SIR levels for responders vs non-responders at day 11 (p = 0.0760 for SIR C_{max} and AUC_{0-24}). Preexisting levels of mTOR activation (measured by active p70S6K in PBMCs) were significantly different between responders and non-responders (p = 0.0059), which remained significant on day 25 (p = 0.040), with responders having higher values (Figure 3C). It is unclear why the levels of mTOR activation in non-tumor cells (PBMCs in this study) would be predictive for the response of the tumor. We speculate that inherited mutations of the host induce tumors with a certain dominant driver of cancer proliferation with subsequently a higher chance to respond to inhibition of that specific driver, as seen before in certain breast-cancer patients [31]. Active Akt levels in PBMCs were also different between responders and non-responders with a significant change in phospho-Akt^{Ser473} between day 0 and 18 (p = 0.0324) (Figure 3D). While active Akt levels in responders increased on day 18, it decreased in non-responders. It has been shown before that mTOR inhibition results in an increase of Akt activity. Our observation about the difference between responders and non-responders raises questions, such as: Does increased Akt activity due to mTOR inhibition imply that the right target was attacked? Is the increased Akt activity in PBMCs evidence against the additional effect of NFV as Akt-inhibitor to

TEM? We hope that ongoing studies with dual mTOR and Akt inhibition will elucidate these issues.

We conclude that TEM and SIR trough levels (C_{min}) are predictive for measured PK data. Our study showed significant positive correlations between TEM AUC_{0-24} at D11 and trough levels of TEM at day 25 and 32 (p = 0.005 and 0.018 respectively). C_{max} of TEM also correlated with its trough level at day 25 (p = 0.005), and the same was seen for SIR at day 18 (for SIR AUC_{0-24} and C_{max} p= 0.003 and 0.002 respectively). More interestingly, there was a positive correlation between SIR trough levels at day 18 and phospho-Akt^{Ser473} levels at day 25 (p = 0.037) in PBMCs. These results strongly suggest to tailor treatment in a future study by using trough levels during treatment with mTOR inhibitors. Another way to prospectively tailor treatment is the use of surrogate markers. In our study we found that the presence and the grade of hyperlipidaemia was significantly correlated with C_{max} of TEM and SIR (p = 0.007 and 0.004 respectively) (Figure 3A). Rash was a less useful surrogate marker for PK (correlation with TEM AUC_{0-24} at day 11 p= 0.048).

In summary, combination of IV TEM 10 mg weekly and PO NFV 500 mg BID is well tolerated and has some clinical activity in heavily pre-treated patients. Especially the promising response in a platinum-resistant ovarian cancer patient deserves prospective investigations. Further studies are warranted to investigate the potentially increased activity of TEM and NFV in GBM patients due to the fact that TEM is able to cross the blood brain barrier and has shown promising anti-tumor activity in GBM [5, 26]. Other prospective studies are needed to investigate combination treatment of mTOR inhibition and Akt inhibition with drugs such as metformine, a widely used antidiabetic drug which inhibits cancer cell viability through the inhibition of mTOR [32][33]. Several new drugs with dual inihibiton of mTOR and Akt/PI3K are currently under investigation in clinical studies [34].

Supplementary Appendix 1

Pharmacokinetic Methods

TEM, SIR and the internal standard Ascomycin (ASC), were analyzed using LC-MS/MS in the positive ionisation mode on a Thermo Scientific (Waltham, USA) Surveyor LC coupled to a Thermo Scientific Quantum Access MS. To 100 µl of patients EDTA whole blood 500 µl acetonitril/methanol 50:50 (v%/v%) containing 15 µg/l ASC as internal standard was added to precipitate proteins. Samples were vortexed, stored at -20°C for 10 minutes, vortexed again and centrifuged. Of the supernatant 8 µl was injected onto a Thermo Scientific Hypersil C18 (50 x 2.1 mm, 3 µm) column. A ternary gradient was applied using deionized water (A), methanol (B) and 2% formic acid/0.004% sodium formate in water (C). C was set at 5% throughout the run using a stepwise gradient. The flow was 500 µl/min and column-oven temperature was 60°C. TEM, SIR and ASC were measured as sodium adducts using the mass transitions 1052.6/409.3, 936.6/409.3 and 814.5/604.4, respectively. The method was validated over a range of 1 – 500 µg/L following FDA guidelines.[35] The accuracies ranged from 93.3% to 107.8% and the precisions were less than 14.7%.

NFV and the internal standard D5-saquinavir (D5-SQV) were analyzed using a LC-MS/MS method validated according to FDA guidelines.[35][36] In short, to 100 µL of plasma 200 µL of a protein precipitation solution containing a mixture of methanol, acetronitril and D5-SQV was added. Chromatographic separation was performed on a reversed phase C18 column (150 x 2.0 mm, particle size 5 µm) with a stepwise gradient using an acetate buffer (pH 5) and methanol. The flow rate was 0.25 mL/min. A triple quadrupole mass spectrometer operating in the positive ionization mode was used for measuring NFV and D5-SQV (mass transitions 568/330 and 676/575, respectively). The method was validated over a range of 0.1 to 20 ug/mL. The accuracies ranged from 88.5% to 102.2% and the precisions were less than 9.5%.

Pharmacodynamic Methods

PBMCs isolation

PBMCs were isolated from whole blood by Ficoll–Hypaque density gradient centrifugation. Briefly, fresh blood was mixed with equal volume of RPMI-1640. Diluted blood was gently layered on 15 ml of Ficoll-Paque Plus (Amersham Biosciences, Piscataway, NJ) in a 50 ml conical tube and centrifuged at 1400 g at 24 °C for 20 min with no brake. Cells were collected, washed with cold PBS and subsequently lysed (M-PER Mammalian protein extraction reagent, Pierce, Rockford, IL).

Western Blotting Analysis

PBMCs lysates were resolved by electrophoresis on denaturing SDS polyacrylamide gels (SDS-PAGE), transferred to polyvinylidene difluoride (Millipore Corp., Bedford, MA), and probed with the following primary antibodies:, anti-phospho-p70S6K(Thr389), anti-phosho-Akt (Ser473) (both from Cell Signaling Technology Inc., Beverly, MA); and anti-ß-actin (Santa Cruz Biotechnology; Santa Cruz, CA). Proteins were revealed using an IRDye

680 goat anti-rabbit secondary antibody. Images were acquired with the Odyssey infrared imaging system and analyzed by the software program as specified in the Odyssey software manual. Densitometric quantification was performed using ImageJ software.

Immunohistochemistry

Biopsies were fixed and paraffin embedded according to routine procedures. For immunohistochemistry, four-micron sections were deparaffinized, endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide for 15 minutes at room temperature, and antigen retrieval was performed for 10 minutes at 100°C in 10 mM sodium citrate buffer, pH 6. After subsequent washing at room temperature and rehydration in PBS, sections were blocked with 10% normal goat serum in PBS for 30 minutes and subsequently incubated with antibodies against phospho-S6 ribosomal protein Ser236/235 (phosho-S6RP$^{Ser235/236}$) (1:200; Cell Signaling Technology; USA) overnight at 4°C. After that slides were incubated with the appropriate HRP-conjugated secondary antibodies and DAB staining was used to visualize peroxidase activity. Slides were photographed with a microscope equipped with a digital camera (Leica CTR500, Leica Microsystems, Wetzlar, Germany). 200x magnification.

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Acknowledgements: This study was made possible thanks to research grant and drug supply by Wyeth Pharmaceuticals (now Pfizer). The grant is on the name of the first author.

Chapter 6

Phase I and pharmacokinetic study of capecitabine and the oral mTOR inhibitor everolimus in patients with advanced solid malignancies

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Invest New Drugs Published online: 02 Aug 2011

ABSTRACT

Background

Everolimus is an oral mTORinhibitor. Preclinical data show synergistic effects of mTOR inhibition in combination with 5-fluorouracil-based anticancer therapy. The combination of everolimus with capecitabine seems therefore an attractive new, orally available, treatment regimen.

Patients and methods

Safety, preliminary efficacy and pharmacokinetics of everolimus in combination with capecitabine were investigated in patients with advanced solid malignancies. Patients were treated with fixed dose everolimus 10 mg/day continuously, plus capecitabine bid for 14 days in three-weekly cycles. Dose escalation of capecitabine proceeded according to the standard 3×3 phase I design in four predefined dose levels (500–1,000 mg/m^2 bid).

Results

In total, 18 patients were enrolled. Median (range) treatment duration with everolimus was 70 days (21–414). Capecitabine 1,000 mg/m^2 bid combined with 10 mg/day everolimus was declared the maximum tolerated dose, at which level one patient developed dose-limiting toxicity (stomatitis grade 3). Drug-related adverse events were mostly grade ≤2 and included mainly fatigue (56%), stomatitis (50%), and handfoot syndrome (33%). Partial response was documented in three patients, and four had stable disease. There was no pharmacokinetic interaction between everolimus and capecitabine.

Conclusion

Everolimus 10 mg/day continuously combined with capecitabine 1,000 mg/m^2 bid for 14 days every 3 weeks is a patient-convenient, safe and tolerable oral treatment regimen. This is the first study to demonstrate feasibility of this combination at doses with proven single agent efficacy in a number of tumors. Prolonged clinical benefit was observed in an encouraging 39% of patients with advanced solid malignancies.

INTRODUCTION

The PI3K/Akt pathway is an important intracellular signalling pathway that is often dysregulated in multiple types of cancers. Signal transduction of activated PI3K/Akt is transmitted through several downstream pathways, including the mammalian target of rapamycin (mTOR) [1][2]. Everolimus, an oral mTOR inhibitor, has demonstrated antitumor properties including inhibition of cell proliferation, cell survival and angiogenesis in preclinical studies [3–11]. Interestingly, in cancer cell lines synergistic anticancer effects of mTOR inhibition were observed when used in combination with 5-fluorouracil (5-FU) [12–15]. Recently, everolimus has been investigated as single agent in phase I–III clinical trials in patients with advanced renal cell carcinoma [16][17], metastatic pancreatic cancer [18], advanced pancreatic neuroendocrine tumors [19], metastatic breast cancer [20], advanced non-small cell lung cancer [21], and in patients with various advanced solid tumors [22–24]. These trials showed that treatment with everolimus continuously at 10 mg per day was well tolerated and showed biological activity with an acceptable side effect profile, consisting of mainly stomatitis and fatigue. Overall, the preclinical and clinical data suggest that everolimus may be more efficacious when used in combination with other anticancer drugs. Indeed, everolimus has been combined in phase I–II trials with paclitaxel [25][26], gemcitabine [27], gefitinib [28], bevacizumab [29] and letrozole [30], and several other early clinical trials using combinations of everolimus with cytotoxic agents are currently ongoing. Capecitabine is an oral pre-prodrug of 5-FU. Recently, capecitabine combined with everolimus has been tested in 15 heavily pretreated Korean patients with advanced gastric cancer [31]. The combination of capecitabine plus everolimus seems especially attractive due to its oral availability and easy use in an outpatient setting. This patient-friendly approach, the preclinical synergistic effects of 5-FU-based anticancer therapy and mTOR inhibition, plus the available clinical experience of mTOR inhibitors in patients with cancer served as the rationale to initiate this phase I trial of everolimus in combination with capecitabine. The purpose of this study was to determine the safety, tolerability and the pharmacokinetic interaction of capecitabine and everolimus in patients with advanced solid malignancies.

Materials and methods

Patient population

Eligible patients were aged ≥18 years with histological or cytological confirmed solid malignancies refractory to standard therapies, or for whom no standard treatment existed. Other eligibility criteria included WHO performance status ≤2, estimated life expectancy of ≥3 months, adequate bone marrow (white blood cell count ≥3.0×109/L, platelets ≥100×109/L) and adequate hepatic and renal function (serum bilirubin ≤1.5 × upper limit of normal (ULN), ALAT/ASAT ≤2.5 × ULN or in case of liver metastases ≤5 × ULN and serum creatinine ≤150 µmol/L). Patients were ineligible if they were known with alcoholism, drug addiction and/or psychotic disorders that were not suitable for adequate follow-up. Women who were pregnant or lactating, or able to conceive but unwilling to practice effective anticonception were also excluded. All patients gave written informed consent. The study was approved

Table 1 Patient Characteristics

Characteristic		Median	Range	No	%
Total number				18	
Gender	Male			14	78
	Female			4	22
Race	White patients			18	100
Age (years)		61	21–71		
Body Surface Area (average in m^2)		1.9	1.6–2.2		
WHO performance status	0			7	39
	1			10	56
	2			1	6
Primary tumor	Pancreas			7	39
	Major duodenal papilla			2	11
	Esophagus			2	11
	Gallbladder			2	11
	Brain			1	6
	Osteosarcoma			1	6
	Hepatocellular carcinoma			1	6
	Unknown			2	11
Prior anticancer therapy	Surgery			10	56
	Radiotherapy			4	22
	Chemotherapy			10	56
Prior chemotherapy regimens	1			5	28
	2			4	22
	3			1	6

WHO World Health Organization

Table 2 Treatment administration of the combination of everolimus and capecitabine by dose level

	Dose level				
	1	2	3	4	All
Daily dose of everolimus	10 mg	10 mg	10 mg	10 mg	10 mg
Dose of capecitabine	500 mg/m^2 bid	650 mg/m^2 bid	800 mg/m^2 bid	1000 mg/m^2 bid	500 – 1000 mg/m^2 bid
Evaluable patients (*N*)	4	5	3	6	18
No. of treatment cycles					
Median	2.5	9	2	3	3
Range	1 – 15	1 – 15	2 – 3	3 – 19	1 – 19
No. of treatment days with everolimus					
Mean ± SD	108 ± 126	192 ± 141	50 ± 20	168 ± 153	141 ± 131
Median	61	202	50	78	70
Range	21 – 290	29 – 329	30 – 70	65 – 414	21 – 414
DLT	None	None	None	1 patient	1 patient

bid twice daily
DLT dose-limiting toxicity
SD standard deviation

by the Medical Ethics Committee of the participating institutions and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Study design and treatment

This was a phase I, open-label, multi-center, dose escalation study to assess the safety, dose-limiting toxicity (DLT), maximum-tolerated dose (MTD) and the pharmacokinetic interaction of the combination of everolimus and capecitabine. The study was conducted at the Amsterdam Medical Center and the Netherlands Cancer Institute (the Netherlands). Everolimus was administered continuously twice daily (bid) at a fixed total oral dose of 10 mg (5 mg bid). The first 7 days of treatment patients were treated with single agent everolimus to reach steady state concentrations. Treatment with capecitabine started on day 8 and was given twice daily for 14 days every 3 weeks. Capecitabine was dose-escalated according to four predefined dose levels: 500, 650, 800 and 1,000 mg/m^2 capecitabine bid.

At least three patients per dose level were recruited and expanded to six if one of three patients experienced DLT. Dose escalation to the next dose level was permitted if no DLT occurred in any of three or in ≤1 of six patients. In case of DLT in one or more out of three, or in two or more out of six patients, that dose level was declared intolerable and no further dose escalation occurred. The immediately preceding dose level was declared the MTD. No intra-patient dose escalations were allowed. DLT was defined as any of the following events related to study treatment and occurring during the first treatment cycle: neutropenia CTC grade 4 lasting more than 5 days, CTC grade ≥4 platelets or grade 3 platelets with bleeding, vomiting CTC grade ≥2 or any other toxicity CTC grade ≥3 (excluding alopecia), despite best supportive care. Toxicity was graded using the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 and was assessed every treatment cycle. In case of measurable disease, tumor measurements were performed at baseline and every three cycles and were evaluated in accordance with the Response Evaluation Criteria in Solid Tumors (RECIST 1.0) [32].

Pharmacokinetic analysis

To determine the pharmacokinetic interaction between everolimus and capecitabine plus metabolites, patients received (only for pharmacokinetic purposes) one single morning administration of capecitabine 7 days prior to start of treatment (day –7), at the dose level that the patient would receive at start of treatment. Plasma samples for capecitabine were obtained on day –7 (without everolimus) and on day 8 (with everolimus at steady state), both at predose and at 0.5, 1, 1.5, 2, 3, 4, 6 and 8 h after capecitabine intake. Vice versa, blood samples for everolimus were drawn on day 7 (without capecitabine) and on day 8 (with capecitabine), both at predose and at 0.5, 1, 1.5, 2, 3, 4, 6, 9 and 12 h after administration of everolimus. In addition, trough concentrations (immediately before the morning dose) of everolimus were determined on days 1, 4, 7, 8, 15, 22 and 29 to assess the formation and continuation of steady state blood concentrations. Everolimus was determined in whole blood by highperformance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) after protein precipitation with acetonitrile using the deuterated stable

isotope everolimus-d_4 as internal standard. Capecitabine and the metabolites 5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy- 5- fluorouridine (5'-dFUR), 5-fluorouracil (5-FU) and 5-fluorodihydrouracil (FUH_2) were determined in plasma by a validated HPLC-MS/MS method as described previously [33]. The pharmacokinetic parameters were calculated using PK Solutions 2.0 (Summit Research Services, Montrose, CO 81401; www.summitpk.com) and included the area under the concentration-time curve (AUC), maximum concentration (C_{max}), time to C_{max} (t_{max}), and in addition for everolimus trough concentrations at steady state (C_o^{ss}).

Statistical analysis

Descriptive statistics were used for evaluation of the safety, efficacy and pharmacokinetic parameters using SPSS statistics version 17.0.

Results

In total, 18 patients with advanced solid tumors were enrolled between February 2008 and June 2010. Patient characteristics are listed in Table 1. All patients received at least one course of treatment and were evaluable for toxicity assessments (Table 2). Overall, a total of 114 treatment cycles were given, with a median (range) of 3 (1–19) cycles per patient. Median (range) treatment duration with everolimus was 70 (21–414) days (Table 2). Four patients temporarily interrupted treatment with everolimus: in three cases due to adverse events and in one due to fever. Following treatment interruption, two patients received a 50% dose reduction of everolimus and the others continued treatment at full dose everolimus. Dose reductions for capecitabine were applied in nine patients, mostly due to adverse events.

MTD and DLT

No DLT was observed up to 800 mg/m² of capecitabine bid, and none of the first three patients treated at the 1,000 mg/ m² level developed DLT. Since capecitabine 1,000 mg/m² bid was the highest predefined dose level and no DLT had occurred thus far, it was decided to include an additional three patients to ensure the tolerability of this level. In one of these additional patients, a man with advanced hepatocellular carcinoma, severe angioneurotic edema occurred 10 days after start of treatment with everolimus (i.e. 3 days after start of capecitabine), which could however be excluded as being drug-related. Nonetheless, treatment with both capecitabine and everolimus was discontinued. Edema quickly resolved, and after 5 days of treatment interruption, treatment was resumed and the first cycle completed with a 50% and 33% reduced dose of everolimus and capecitabine, respectively. Subsequently, the patient developed mucositis grade 3 and thrombocytopenia grade 2 for which start of the second cycle with capecitabine had to be delayed by more than 4 weeks. This toxicity was considered intolerable and dose-limiting. Since none of the other five patients treated at the 1,000 mg/m² level developed DLT, everolimus 5 mg bid continuously combined with capecitabine 1,000mg/m² bid for 14 days every 3 weeks was declared the MTD.

Table 3

Possibly, probably or definitively treatment related grade 1-2 and grade 3-4 adverse events reported in 2* or more patients

Dose level	1		2		3		4		Total, n(%)	
No. of patients	N = 4		N = 5		N = 3		N = 6		N = 18 (100)	
CTC grade	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4	Grade 1-2	Grade 3-4
Non-Hematology										
Fatigue	1	2	1		1	1	4		7 (39)	3 (17)
Stomatitis	1		2		1		5		9 (50)	
Hand-foot syndrome			3				1	2	4 (22)	2 (11)
Nausea					2	1	1		3 (17)	1 (6)
Diarrhea	1				1		1		3 (17)	
Mucositis			1		1			1	2 (11)	1 (6)
Anorexia			1		1				2 (11)	
Taste loss							2		2 (11)	
Neuropathy							2		2 (11)	
Constipation							2		2 (11)	
Skin rash			1				1		2 (11)	
Hematology										
Hemoglobin	3		5		3		4		15 (83)	
Platelets	2	1	2		2		3		9 (50)	1 (6)
Leukocytes	1		2		2		2		7 (39)	
Clinical chemistry										
GGT	1	2	4		1		2	1	8 (44)	3 (17)
ASAT	2		3		2		3		10 (56)	
ALAT	2		4				1	1	7 (39)	1 (6)
Hypertriglyceridemia	1		3	1	1		2	1	7 (39)	2 (11)
AP		2	1		1		3		5 (28)	2 (11)
Hypokalemia	2				2		1		5 (28)	
Hyponatremia	2		1		1		1		5 (28)	
Hypercholesteremia	1		2				1		4 (22)	
Hyperkalemia	2						1		3 (17)	
Bilirubin	1	2							1 (6)	2 (11)
Hypercalcemia*								1		1 (6)

Note: Numbers represent number of patients

* Grade 4 hypercalcemia occurred in one patient and was therefore included in the table.

GGT gamma-glutamyltransferase
ASAT aspartate aminotransferase
ALAT alanine aminotransferase
AP alkaline phosphatase

Safety

Table 3 lists the treatment-related CTC grade 1–2 and grade 3–4 adverse events per dose level. The most frequently reported clinical toxicities of any grade included fatigue (56%), stomatitis (50%), hand-foot syndrome (33%) and nausea (22%). Other clinical toxicities included diarrhea and mucositis (both 17%), and anorexia, taste loss, constipation, skin rash and neuropathy (all 11%). Clinical toxicities were never severe (≤ grade 2), with the exception of fatigue in three patients, hand-foot syndrome in two, and nausea in one. The six patients that developed hand-foot syndrome were on average treated with 12.2±5.6 cycles, while the average onset of hand-foot syndrome was after 4.6±2.3 cycles of treatment. With 20–25% dose reductions of capecitabine, five patients were able to continue treatment safely, and one patient went off study due to disease progression. Decreased hemoglobin was the most frequently reported drug-related hematological adverse event (83%), followed by a decrease in platelets (56%) and leukocytes (39%). Except for one patient in dose level 1 with grade 3 thrombocytopenia, hematological toxicity remained always ≤grade 2. Elevation of gamma-glutamyltransferase (GGT) of any grade was the most frequently reported biochemical toxicity (61%), but appeared almost never clinically relevant (grade≤2). Only two patients presented with GGT grade 3, and one patient with GGT grade 4, though her GGT-level was already grade 3 at baseline. Elevated levels of ASAT (56%), ALAT (44%) and alkaline phosphatise (39%) were mostly of grade ≤2, except for one patient in whom ALAT grade 3 occurred, and in two patients alkaline phosphatase grade 3. Hypertriglyceridemia and hypercholesteremia occurred in 50% and 22% of the patients, respectively. Hypercalcemia was the second grade 4 toxicity that occurred in this study, which developed after the 3rd cycle of treatment in a patient treated in dose level 4. Due to clinical progression, the patient went off study and was given pamidronate, however a possible relationship with study medication could not be excluded.

Pharmacokinetics of everolimus and capecitabine

Blood samples for pharmacokinetic analyses of everolimus and capecitabine plus metabolites were obtained from 17 to 14 patients, respectively. Pharmacokinetic parameters are provided in Table 4. Everolimus showed a rapid absorption with a median (range) time to reach maximum blood concentrations of 1 (0.5–4) hour. Steady state for everolimus was achieved by day 4 of treatment (Fig. 1a). Steady state was stable and remained steady at least up to day 29 (the last analyzed trough sample in this study) with an average (±SD) concentration of C_o^{ss}=18 (±8.5) ng/mL. This demonstrates that no induction or inhibition of metabolism of everolimus occurred. The mean half-life for everolimus was $t_{1/2}$=15 (±8.2) hours. Furthermore, there was a linear relationship between the AUC of everolimus within a 12 h dosing interval (AUC_{0-12}) and the C_o^{ss} concentration (r^2=0.67). No effect was observed of capecitabine on the pharmacokinetics of everolimus (Figs. 1b and 2a). The AUC_{0-12} of everolimus at steady state alone and with capecitabine was 302±97 h*ng/mL and 290±114 h*ng/mL, respectively. The inter-patient variability of the exposure to everolimus expressed as the coefficient of variation (%CV) was 36%.

Capecitabine was rapidly absorbed and the median time to reach C_{max} was 1 (0.5–2) hour. Plasma concentrations for capecitabine metabolites peaked within 1–2 h after

absorption. Since the dose of capecitabine very poorly correlated with the AUCs of capecitabine and its main metabolites 5'-dFCR, 5'-dFUR, 5-FU and FUH_2 (r2=0.064, 0.063, 0.063, 0.13 and 0.14, respectively), the pharmacokinetic parameters were not dose-corrected (Fig. 1c). Everolimus did not interact with the pharmacokinetics of capecitabine. The AUC of capecitabine alone and with everolimus was 5.1± 3.5 h*mg/L and 4.6±2.3 h*mg/L, respectively. Furthermore, C_{max} and tmax of capecitabine did not differ between capecitabine alone or with everolimus. Likewise, no differences in the AUC, Cmax and tmax for capecitabine metabolites were observed with concomitantly everolimus (Table 4 and Fig. 2b–f). The inter-patient variabilities in the AUC for capecitabine, 5'-dFCR, 5'-dFUR, 5-FU and FUH_2 were %CV=60%, 37%, 32%, 71% and 57%, respectively.

Table 4 Pharmacokinetic parameters of everolimus, capecitabine and capecitabine metabolites

Drug interaction	Patients with values (*N*)	T_{max} (hr)	C_{max} (ng/mL)	AUC (hr*ng/mL)
Everolimus without capecitabine	15	1.0 (0.5 – 4.0)	50 ± 23	302 ± 97
Everolimus with capecitabine	17	1.0 (0.5 – 4.0)	52 ± 21	290 ± 114
Capecitabine without everolimus	12	0.5 (0.5 – 2.0)	5627 ± 5815	5104 ± 3479
Capecitabine with everolimus	11	1.0 (0.5 – 2.0)	3864 ± 3247	4575 ± 2285
dFCR without everolimus	14	1.0 (0.5 – 2.0)	6972 ± 3069	11994 ± 5150
dFCR with everolimus	13	1.5 (0.5 – 4.0)	6019 ± 2343	11513 ± 3458
dFUR without everolimus	14	1.0 (0.5 – 2.0)	6739 ± 3438	9773 ± 3113
dFUR with everolimus	13	1.5 (0.5 – 4.0)	5357 ± 3048	9209 ± 3070
5-FU without everolimus	7	0.5 (0.5 – 4.0)	203 ± 230	268 ± 219
5-FU with everolimus	6	1.0 (0.5 – 4.0)	157 ± 120	264 ± 170
FUH_2 without everolimus	11	2.0 (1.0 – 4.0)	817 ± 301	2275 ± 730
FUH_2 with everolimus	10	2.0 (1.0 – 4.0)	782 ± 482	2805 ± 1976

t_{max} values are median (range), the other parameters are mean ± standard deviations.

C_{max}	maximum concentration	dFCR	5'-deoxy-5-fluorocytidine
T_{max}	time to C_{max}	dFUR	5'-deoxy-5-fluorouridine
AUC	Area under the concentration-time curve	5-FU	5-fluorouracil
hr	hour	FUH_2	5-fluorodihydrouracil
ng/mL	nanogram per millilitre		

Efficacy

Overall, 14 patients were evaluable for response by RECIST 1.0: three patients had a confirmed partial response, four had stable disease and seven had progression of disease. Four patients could not be evaluated for response, but had clinical progression of disease, and therefore withdrew early.

Discussion

This study explored the safety, pharmacokinetics and preliminary efficacy of the combination of capecitabine with the oral mTOR inhibitor everolimus in patients with advanced solid malignancies. Treatment with everolimus combined with capecitabine was generally well tolerated and showed an acceptable toxicity profile. In total, one DLT (mucositis grade 3) was observed, which occurred in one out of six patients treated at the highest dose level. In this study, we did not reach the maximum of two or more DLTs out of six treated patients in any dose level. The study protocol was not amended though with an additional higher dose level, given the fact that dose reductions of capecitabine were indicated in 50% of all patients during treatment. This was mostly due to hand-foot syndrome, for which the dose of capecitabine was reduced by 25% after on average 6 cycles of treatment. Therefore, our recommendation for further phase II trials with this combination is everolimus 10 mg daily continuously combined with 1,000 mg/m2 capecitabine twice daily for 14 days in 3-weekly cycles.

The most commonly reported treatment-related clinical side effects were fatigue, stomatitis and hand-foot syndrome. Hand-foot syndrome can be attributed to capecitabine, since this has not been observed before in single agent everolimus trials. This well-known side effect of capecitabine resulted in only modest dose reductions of capecitabine in our patients. Although stomatitis is a common adverse event of both capecitabine and everolimus as single agent as well, this overlapping toxicity always remained mild to moderate in severity in this study and was not dose-limiting. Moreover, the frequency of stomatitis in this study was similar compared to studies with single agent everolimus, which suggests that there is no, or only a marginal additive toxic effect of capecitabine.

The frequency of fatigue was similar to that of single agent studies with everolimus as well [21–24]. Other adverse events included, diarrhea, anorexia, taste loss, neuropathy and skin rash, but remained non-severe. While in this study the oral treatment regimen of everolimus combined with capecitabine appeared safe and feasible, it is of note that a previous phase I study in which the intravenous mTOR inhibitor temsirolimus was combined with infusional 5-FU and leucovorin, stomatitis/mucositis occurred at all dose-levels and was dose-limiting. Moreover, this combination also resulted in treatment-related fatal bowel perforation in two patients [34]. Similarly, another phase I study reported that everolimus 20 mg per week combined with the antimetabolite gemcitabine 600 mg/m^2 on days 1, 8 and 15 in 4-weekly cycles was not tolerated due to severe myelosuppression [27]. However, no such unexplained severe toxicities were observed in our study, which has important implications for further studies. Similarly to what we report in this trial, both previous studies did not show a pharmacokinetic interaction between the mTOR inhibitor and the antimetabolite.

Figure 1

Figure 1A
Mean (standard deviation) C_{trough} (C_0^{ss}) concentrations of everolimus in whole blood assessed at days 1, 4, 7, 8, 15, 22 and 29 from start of treatment at a dose 5 mg twice daily.

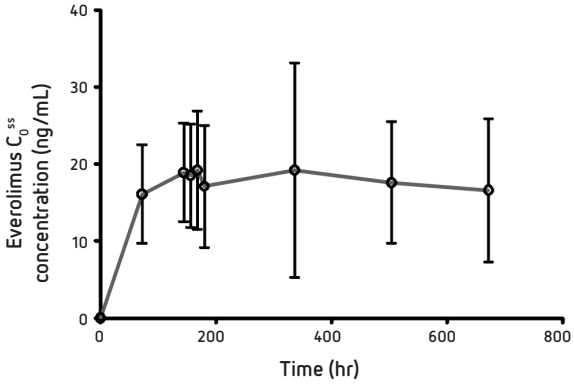


Figure 1B
Mean (standard deviation) concentrations of everolimus in whole blood during a 12 hour interval at a dose of 5 mg bid at steady state alone (red circles, solid line) and with (blue triangles, dotted line) capecitabine.

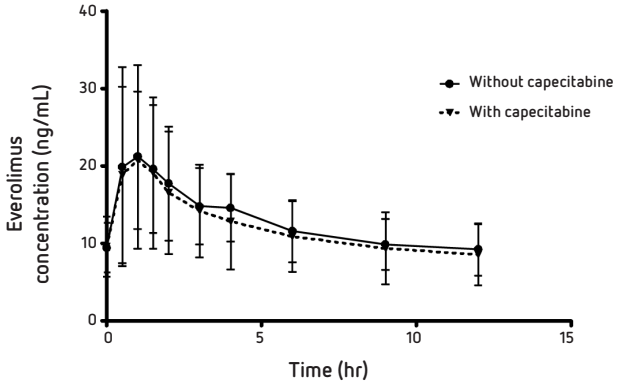
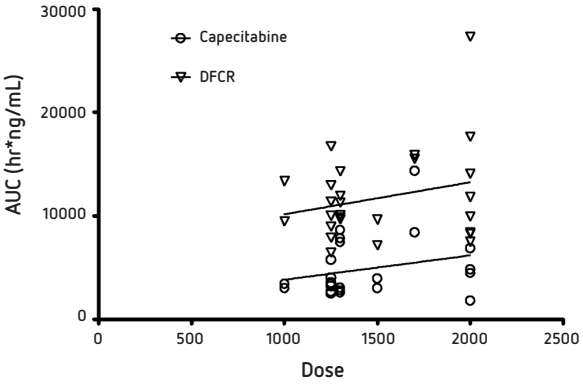


Figure 1C
Scatter plot of the administered dose of capecitabine versus the observed area under the plasma-concentration time curve (AUC) of capecitabine and dFCR. (Not shown for other capecitabine metabolites for the sake of clarity).



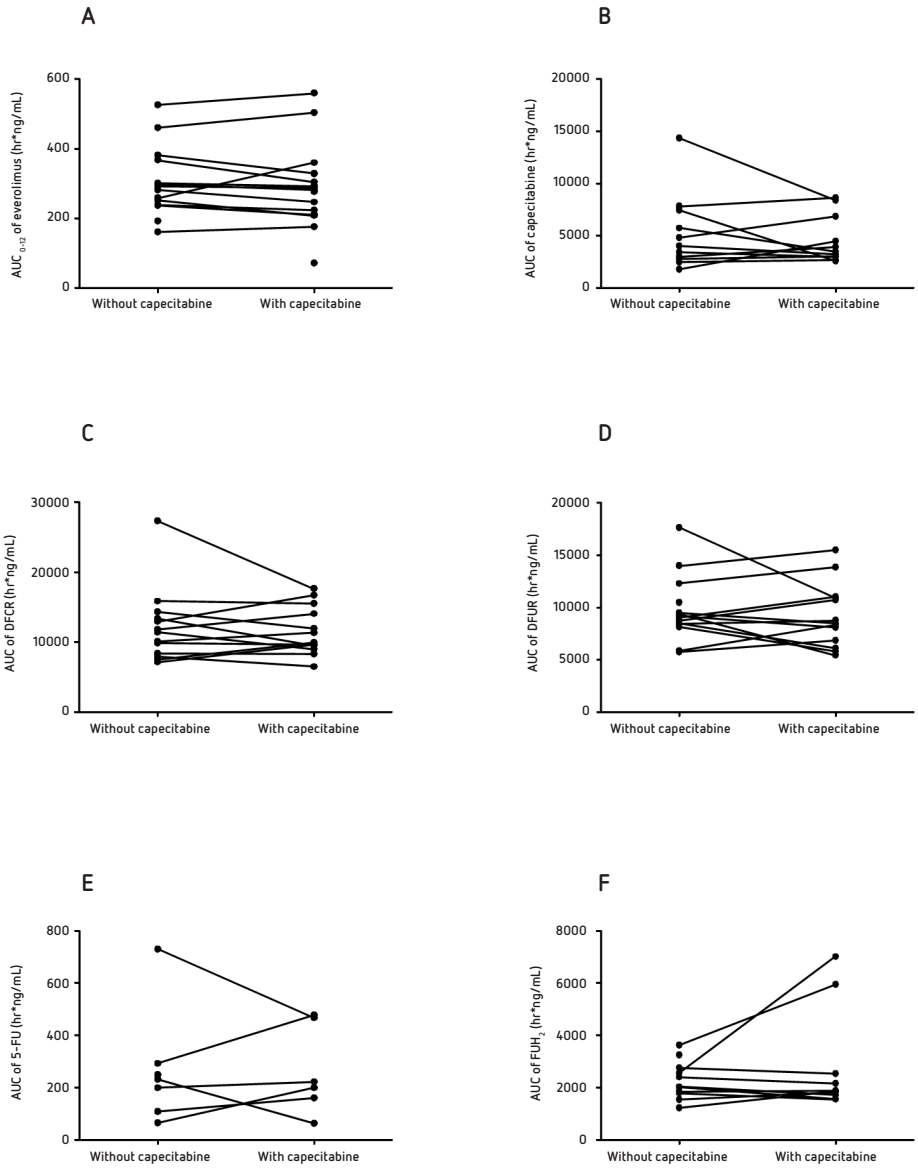


Figure 2

Individual patient area under the concentration-time curve (AUC) of

A everolimus in whole blood without and with capecitabine

B capecitabine in plasma without and with everolimus

C dFCR in plasma without and with everolimus

D dFUR in plasma without and with everolimus

E 5-FU in plasma without and with everolimus

F FUH_2 in plasma without and with everolimus.

This suggests that drug interactions at a pharmacodynamic level, or schedule differences might explain the observed variations in severity of overlapping toxicities when different mTOR inhibitors and cytotoxic antimetabolites are combined.

Lim et al. recently published their trial of everolimus combined with capecitabine in Korean patients with advanced gastric cancer [31]. In contrast to their findings of a rather low maximum-tolerated dose (capecitabine 650 mg/m²), our study is the first to demonstrate the feasibility of this combination at doses with proven single agent efficacy in a number of tumors. This surprisingly large difference (650 mg/m² versus 1,000 mg/m² capecitabine bid) between the studies might possibly be caused by the gastrectomy in more than half of the patients in the Korean trial already had undergone. It is known that gastrectomy results in a higher systemic exposure and higher C_{max} to capecitabine, possibly affecting tolerability [35].

The pharmacokinetic profile of everolimus assessed in this study showed a comparable absorption, systemic exposure, and trough concentration as in other white and Japanese patient cohorts treated with 10 mg everolimus per day [22][23][25]. The absorption was relatively fast with a median time to C_{max} of 1 h, and steady state was reached within 4 days of treatment. Likewise, capecitabine was rapidly absorbed, and the AUC, C_{max} and time to C_{max} were in line with previously reported data [36].

Since this was a phase I study, efficacy was not a primary endpoint; nonetheless, 14 patients were evaluable for response. In seven patients a clinical response, including prolonged disease stabilization was achieved. The three patients with a partial response (two patients with pancreatic cancer) and half of patients with stable disease had not received any prior chemotherapy at study entry. But because of the minimal survival advantage of gemcitabine in pancreatic cancer, first line treatment with experimental anticancer therapy is considered a reasonable alternative in this group of patients. Obviously, the relative contribution of everolimus to this clinical benefit in these patients is difficult to determine. However, previous studies with single agent everolimus rarely showed better responses than prolonged disease stabilization.

In conclusion, we showed that everolimus twice daily in a total dose of 10 mg/day (5 mg bid) continuously combined with capecitabine 1,000 mg/m² for 14 days every 3 weeks is a safe and tolerable oral treatment regimen, and achieved prolonged clinical benefit in a significant number of patients. Toxicities were generally mild to moderate severe and were well manageable. No unexplained severe toxicities were reported, and no pharmacokinetic interaction between everolimus and capecitabine was observed. Therefore, the results obtained in this study provided for us a solid basis for our ongoing phase II trial of everolimus and capecitabine in pancreatic cancer patients (Trial identifier NTC01079702. Moreover, the interesting balance between efficacy and burden on the patient of this treatment combination is attractive to investigate in other tumor types as well.

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Chapter 7

Phase II study of the efficacy capecitabine and the oral mTOR inhibitor everolimus in patients with advanced pancreatic cancer

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Submitted for publication

ABSTRACT

Background

Capecitabine has activity in pancreatic cancer. The combination of 5-fluouracil-and mTOR inhibitor based anticancer therapy is attractive because of preclinical evidence of synergy between the two drugs.

Patients and methods

Patients with unresectable or metastatic adenocarcinoma of the pancreas, World Health Organization (WHO) performance status 0-2 and adequate hepatic and renal function were eligible. All patients were treated with the combination of capecitabine 1000 mg/m^2 BID day 1-14 and everolimus 10 mg daily (5mg bid) of a 21-day schedule. Tumor assessment was performed with computed tomography every 3 cycles. Primary endpoint was response rate according to RECIST 1.1. Secondary endpoints were time to treatment failure, overall survival and one year survival rate.

Results

In total, 25 patients were enrolled. Median (range) treatment duration with everolimus was 72 days (16-217). Principal grade 3/4 toxicities were hyperglycemia (36%), hand-foot syndrome (12%), diarrhea (8%), and stomatitis (4%). Prominent grade 1/2 toxicities were stomatitis (60%), fatigue (44%). Among 19 evaluable patients, one (4%) had a partial response and ten (40%) had stable disease at least for a median of 2.7 months resulting in a clinical benefit rate of 44%. Median overall survival was not yet reached at time of analysis yet.

Conclusion

The combination of capecitabine day 1-14 and daily everolimus combination schedule is a moderately active second line treatment for advanced pancreatic cancer.

INTRODUCTION

The PI3K/Akt pathway is an important intracellular signalling pathway that is often dysregulated in cancer.[1] Signal transduction of activated PI3K/Akt is transmitted through several downstream targets, including the mammalian target of rapamycin (mTOR) [2][3]. Everolimus, an oral mTOR inhibitor, has demonstrated antitumor properties including inhibition of cell proliferation, cell survival and angiogenesis, and showed additive as well as synergistic effects when combined with other anticancer agents [4-12]. This synergistic antiproliferative effect was confirmed when used in combination with 5-FU [13-15]. Recently, single agent everolimus has been investigated in phase I – III clinical trials in patients with advanced renal cell carcinoma [16][17], metastatic pancreatic cancer [18], metastatic breast cancer [19], advanced non-small cell lung cancer [20] and in patients with various advanced solid tumors [21-23]. These trials demonstrated that treatment with everolimus continuously at 10 mg daily was well tolerated and showed biological activity with an acceptable toxicity profile, consisting mainly of stomatitis and fatigue. Clinical trials investigating everolimus in combination with other anticancer drugs have been performed or are ongoing.[24-29] In short, everolimus may be more efficacious when used in combination with other anticancer drugs. An important class of anticancer agents that are widely used in the treatment of cancer are the antimetabolites, which include the fluoropyrimidines 5-fluorouracil (5-FU) and its oral pre-prodrug capecitabine.[30] Recently we demonstrated the safety and feasibility of the combination of capecitabine 1000 mg/m² BID day 1-14 and everolimus 10 mg daily in a 21-day schedule.[31] In that phase I study we found unexpectedly good responses in patients with pancreatic cancer, namely 2 out of 3 patients had a partial remission (PR). Moreover, this combination is attractive due to its oral availability and easy use in an outpatient setting. These findings served as the rationale for this phase II trial of everolimus combined with capecitabine in pancreatic cancer patients. The purpose of this study was to determine the efficacy of capecitabine plus the oral mTOR inhibitor everolimus in patients with advanced pancreatic cancer.

Materials and methods

Patient population

Eligible patients were aged ≥ 18 years, with histologically or cytologically confirmed advanced pancreatic cancer, and measurable lesions according to Response Evaluation Criteria in Solid Tumors (RECIST). Patients who had prior chemotherapy in the adjuvant setting or for metastatic disease were eligible. Other eligibility criteria included WHO performance status ≤ 2, estimated life expectancy of ≥ 3 months, adequate bone marrow (white blood cell count ≥ 3.0 x 109 /L, platelets ≥ 100 x 109 /L) and adequate hepatic and renal function (serum bilirubin ≤ 1.5 x upper limit of normal (ULN), ALAT/ASAT ≤ 2.5 x ULN or in case of liver metastases ≤ 5 x ULN and serum creatinine ≤ 150 µmol/L). Patients were ineligible if they were known with alcoholism, drug addiction and/or psychotic disorders that were not suitable for adequate follow-up. Women who were pregnant or lactating were also excluded. All patients gave written informed consent. The study was approved by the

Medical Ethics Committee of the Academic Medical Center Amsterdam, and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. The trial was registered online (ClinicalTrials.gov identifier: NCT01079702).

Study design and treatment

This was a phase II, open-label, single-center study to assess the antitumor activity and safety of the combination of everolimus and capecitabine. The primary endpoint was response rate. The study was conducted at the Amsterdam Medical Center, The Netherlands. Everolimus was administered continuously twice daily (bid) at a fixed total oral dose of 10 mg (5 mg bid). The first seven days of treatment patients were treated with single agent everolimus to reach steady state concentrations. Treatment with capecitabine 1000 mg/m^2 started on day 8 and was given twice daily for 14 days in a three weekly cycle. Toxicity was graded using the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) version 3.0 [32] and was assessed every treatment cycle. Tumor measurements were performed at baseline and every three cycles, and responses were evaluated in accordance with the RECIST 1.0 [33]. Patients continued on therapy until disease progression, treatment-related toxicities, or withdrawal of consent. All patients were followed for survival.

Statistical analysis

For sample size calculations the two-stage design according to Gehan for estimating the response rate was used. In the first stage, 14 patients were entered. If no responses were observed in the first stage, then the trial would be terminated because this event (0/14) has a probability < 0.05 if the true response rate is 0.20. We choose for an estimate with approximately a 10% standard error, with an accrual of 11 patients in the second stage. For the evaluation of the safety, efficacy parameters descriptive statistics were applied using SPSS statistics version 16.0. Intention to treat analysis was used.

Results

In total, 25 patients with advanced pancreatic cancer were enrolled between June 2010 and August 2011. Patient characteristics are listed in table 1. All patients had metastatic disease, and none had locally advanced disease. All patients received at least one course of treatment and were evaluable for toxicity assessments (table 2). Overall, a total of 82 treatment cycles was given, with a median (range) of 3.3 (1 – 9) cycles per patient. Median (range) treatment duration with everolimus was 72 (16-217) days (table 2). Eleven patients temporarily interrupted treatment with everolimus due to adverse events. Following treatment interruption, six patients received a 50% dose reduction of everolimus and the others continued treatment at full dose everolimus. Due to adverse events, treatment with capecitabine was interrupted in 14 patients resulting in dose reductions for capecitabine in 12 patients.

Table 1 Patient Characteristics

Characteristic		Median	Range	No	%
No. of patients				25	
Gender	Male			14	56
	Female			11	44
Race	Caucasian			24	96
	Asian			1	4
Age (years)		61	37 - 77		
Body Surface Area (m^2)		1.82	1.4 - 2.13		
WHO performance status	0			12	48
	1			10	40
	2			3	12
Histology				25	100
Adenocarcinoma				24	96
Acinar-cell-carcinoma				1	4
Prior anticancer therapy	Surgery			10	40
	Radiotherapy			2	8
	Chemotherapy			14	56
	adjuvant			7	28
	palliative			7	28
Prior chemotherapy regimens	0			10	40
	1			14	56
	2			1	4

WHO World Health Organization

Table 2 Treatment administration of the combination of everolimus and capecitabine

Evaluable patients (N=25)	All
No. of treatment cycles,	
Median	3.3
Range	1-9
No. of treatment days with everolimus,	
Mean ± SD	87 ± 49
Median	72
Range	16 - 217
Daily dose of everolimus	10 mg
No. of patients (%)	
undergoing dose reduction due to toxicity	6 (24)
undergoing temporary treatment disruption due to toxicity	11 (44)
Dose of capecitabine	1000 mg/m^2 BID
No. of patients (%)	
undergoing dose reduction due to toxicity	12 (48)
undergoing temporary treatment disruption due to toxicity	14 (56)

bid twice daily
SD standard deviation

Table 3

Possibly, probably or definitively treatment related grade 1-2 and grade 3-4 adverse events reported in 2 or more patients.

Dose level	Total, n(%)	
No. of patients	N = 25 (100)	
CTC grade	Grade 1-2	Grade 3-4
Non-Hematology		
Stomatitis*	15 (60)	1 (4)
Fatigue	11 (44)	
Hand-foot syndrome	7 (28)	3 (12)
Diarrhea	8 (32)	2 (8)
Nausea	8 (32)	
Skin rash	6 (24)	1 (4)
Anorexia	6 (24)	
Vomiting	4 (16)	1 (4)
Neuropathy	4 (16)	
Constipation	3 (12)	
Ankle edema	3 (12)	
Epistaxis	3 (12)	
Itching	2 (8)	
Hematology		
Hemoglobin	21 (84)	
Platelets	12 (48)	2 (8)
Leukocytes	7 (28)	
Clinical chemistry		
Hyperglycemia	14 (56)	9 (36)
AP	10 (40)	1 (4)
ASAT	7 (28)	
ALAT	6 (24)	
Hypokalemia	5 (20)	3 (12)
Hyponatremia	5 (20)	
Hypertriglyceridemia	4 (16)	
Bilirubin	3 (12)	1 (4)
Hypercholesteremia	2 (8)	

* Stomatitis including aphteus ulcaeration and mucositis of the mouth.

GGT gamma-glutamyltransferase
ASAT aspartate aminotransferase
ALAT alanine aminotransferase
AP alkaline phosphatase

Safety

Table 3 lists the treatment-related CTC grade 1 – 2 and grade 3 – 4 adverse events. The most frequently reported clinical toxicities of any grade included stomatitis (64%), fatigue (44%), hand-foot syndrome (40%) and diarrhea (40%). Other clinical toxicities included nausea (32%), skin rash (28%), anorexia (24%), vomiting (20%), neuropathy (16%), constipation, ankle edema and epistaxis (all 12%). Severe clinical toxicities were not frequent. Grade 3-4 hand-foot syndrome was observed in three patients, diarrhea in two, stomatitis, skin rash and vomiting in one. Decreased hemoglobin was the most frequently reported drug-related hematological adverse event (84%). In addition, a decrease in platelets and leukocytes occurred in 48% and 28% of the patients, respectively. Only two patients developed grade 3 hematological toxicity (thrombocytopenia). Hyperglycemia of any grade was the most frequently reported biochemical toxicity (92%), resulting in clinical relevance for nine patients (grade 3). Elevated levels of alkaline phosphatase (44%), ASAT (28%) and ALAT (24%) were mostly of grade ≤ 2, except for one patient in whom alkaline phosphatase grade 3 occurred. Hypokalemia and hyponatremia became apparent in 32% and 20% of the patients, respectively, of which grade 3/4 hypokalemia was reported in 3 patients. Hypertriglyceridemia and hypercholesteremia not exceeding grade 2 was seen in 16% and 8% of the patients, respectively. Four patients presented with hyperbilirubinemia of any grade, of which grade 3 was reported in one patient.

Antitumor activity

Overall 19 patients were evaluable for response by RECIST 1.0. Fourteen of these 19 underwent study treatment after failure of prior systemic treatment. Five patients received study medication as first line treatment. One patient had a confirmed partial response and was part of the first stage of this phase II study. Ten patients had stable disease and 8 had progression of disease. Of these ten patients with stable disease, 7 were treated with first-line therapy and three as second-line chemotherapy. Stable disease lasted for a median of 145 days, including 7 patients still on treatment. Six patients could not be evaluated for response, of whom 5 had clinical progression of disease, with early withdrawal and one patient has not yet had a radiological evaluation. Median progression free survival (PFS) was 82 days (range 23-230 days). The median PFS was 89 and 79 days for first- and second-line chemotherapy, respectively. The second endpoints median overall survival and 1 year survival rate were not available at time of study analysis, but will follow as soon as sufficient events have occurred. The median follow up was 155 days (range 66-374 days). Figure 2 shows the Kaplan-Meier curve for all patients in an intent-to-treat analysis.

Table 4

Response rates for the entire cohort (N = 25) and per protocol radiological response evaluable patients (N = 19). Two patients had no tumor response evaluation yet at time of analysis. Four patients had clinical progressive disease and did not have a radiological tumor response evaluation.

Type of response	No. of patients	Entire cohort (%)	Response evaluable (%)
PR	1	4	5
SD	10	40	53
Clinical PD	5	20	
PD	8	32	42
No evaluation yet	1	4	
Clinical benefit PR + SD	11	44	58

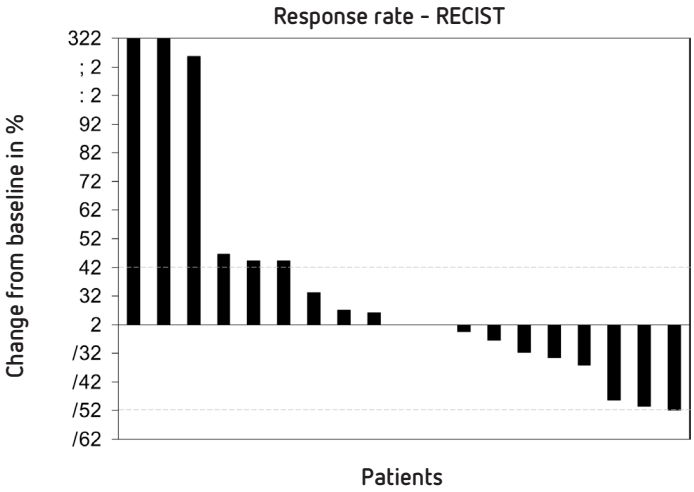

Figure 1

Best confirmed change from baseline in sum of longest diameters of target lesion size (%), by RECIST.

Waterfall plot of maximal change from baseline (pre-therapy) in sum of longest diameters of target lesion size (%), by RECIST for N = 19. Two patients had a change of 133 % and 253 % from baseline which was cut-off to 100% change from baseline for illustrational reasons. Five patients were still ongoing with the study treatment at time of the analysis, of whom two had no radiological evaluation of response yet. One patient stopped due to toxicity and had no radiological evaluation of response. Five patients stopped treatment due to clinical progressive disease before they had radiological response evaluation. The grey dotted lines represent the threshold for either progressive disease (PD, upper line, 20% or more increase from baseline), or partial remission (PR, lower line, 30 or more % decrease from baseline).

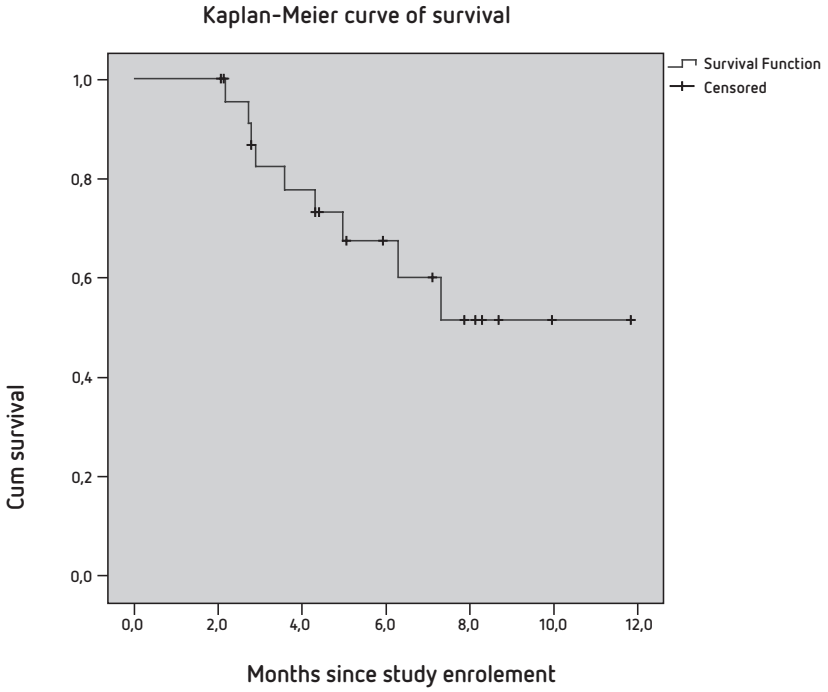

Figure 2 Kaplan-Meier curve of survival

Discussion

This phase II study explored the combination of capecitabine with the oral mTOR inhibitor everolimus in patients with advanced pancreatic cancer. Treatment with everolimus combined with capecitabine was generally well tolerated.

The most commonly reported treatment-related clinical side effects were stomatitis, fatigue and hand-foot syndrome. Although stomatitis is a common adverse event of both capecitabine and everolimus as single agent, this overlapping toxicity always remained mild to moderate in severity in this study and led to dose reduction in only one patient. We assume at most marginally additive toxicity of capecitabine to everolimus because the frequency of stomatitis in this study was similar to that in studies with single agent everolimus[34]. Hand-foot syndrome can be solely attributed to capecitabine, since this has not been observed before in single agent everolimus trials. This well-known side effect of capecitabine resulted in dose reductions of capecitabine by 25% and 50% in eight and four patients, respectively, which is comparable with previous studies of capecitabine monotherapy. [35]

The frequency of fatigue was increased compared with single agent studies of either agent[16][34][36], suggesting an additive toxic effect of the combination. An alternative explanation for the increased incidence of fatigue might be disease related. Patients with

pancreatic cancer have a high probability to develop disease related fatigue.[37] Other adverse events included diarrhea, anorexia, taste loss, neuropathy and skin rash, but remained non-severe. The incidence and frequency of toxicity confirmed the data from our previous phase I study. [31]

In contrast to our findings, two prior studies of combination treatment of mTOR inhibitors and 5FU [38] or gemcitabine [26] did not show acceptable safety and feasibility due to dose limiting stomatitis and hematological toxicities. However, no such severe toxicities were observed in either our phase I or this phase II studies. These findings provide a feasible treatment schedule for further studies.

To explain the different findings of toxicity between the combination study by Punt et al. and our study, we compared the regimens and the patient population. The majority of study participants (21 out of 28) were diagnosed with a cancer type that would be treated as standard of care with first line platinum based chemotherapy. The observed increased myelosupression might therefore be explained by this pretreatment.

Eleven (44%) of the 19 for response evaluable patients had some clinical benefit, including prolonged disease stabilization. The only patient with a partial response had received prior chemotherapy at study entry. Obviously, the relative contribution of everolimus to the clinical benefit in these patients is difficult to determine. Comparable clinical response rates (50%) were seen in the first study of capecitabine (1250 mg/m² bid) in 41 evaluable patients with advanced pancreatic cancer failing first line treatment.[39] In another study in the same setting by Bodoky et al., clinical benefit was seen in 32% of the patients.[40]

Chemonaive patients were more likely to achieve clinical benefit (64 vs. 29%), although treatment duration was similar in both groups (first line 73 days and second line 70 days, respectively).

There is no standard second line treatment for advanced pancreatic cancer, [41] but 5FU-based regimens are occasionally employed. [42] In view of the disappointing treatment results even in first line, enrolment in clinical trials could arguably be considered for all irresectable or metastatic pancreatic cancer patients. [41] The majority of the patients treated in this study progressed after at least one line of palliative treatment for advanced pancreatic cancer (56%), but a substantial minority was chemotherapy naïve.

Stratification of patients with higher mTOR activity in their pancreatic cancer might be a selection factor to increase the likelihood of response to treatment with mTOR inhibitors, as hypothesized before in pancreatic neuroendocrine tumors.[43] A retrospective analysis of the tumor material of patients with pancreatic cancer who were treated with mTOR inhibitors at our institute is ongoing. A positive correlation with a higher likelihood to respond to mTOR inhibitors would build the basis for a prospective study of mTOR inhibition with or without combination chemotherapy.

In conclusion, we showed that everolimus twice daily in a total dose of 10 mg/day (5 mg bid) continuously combined with capecitabine 1000 mg/m² for 14 days every three weeks

is a safe and efficacious oral treatment regimen, and achieved prolonged albeit limited clinical benefit in a significant number of patients (44%). Toxicities were generally mild to moderately and were well manageable. No unexplained severe toxicities were reported. Therefore, the results obtained in this study provide a basis for future phase III trials with the combination of everolimus and capecitabine versus capecitabine alone as a second line treatment after failure of gemcitabine based treatment in advanced pancreatic cancer, assuming better methods can be defined to select patients with the highest response likelihood.

FUNDING

This work was supported by the Academic Medical Center Amsterdam. Everolimus was supplied free of charge by Novartis.

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Chapter 8

Conclusions and perspectives

Systemic cancer treatment has changed tremendously over the last decade, both in targets and in practical execution, as well as in efficacy and side-effects. Most pronounced changes have been the shift from treatment choice based on the origin of the tumor to tumor biology and mutation specific treatment choices, from cytotoxic to cytostatic treatment, and the move from short intravenous infusions to chronic oral treatment. Insights into the cause of each form of cancer led to the development of drugs targeting molecular drivers of these cancers, or targeted therapy as it is now known.

In the era of classical chemotherapy the strategy was to 'hit early, hit hard', which translated into intermittent short-term use of cytotoxic drugs to either eradicate or reduce the tumor burden by killing fast replicating cells. To maximize efficacy of classical cytotoxic drugs, the maximum tolerated dose was determined in Phase I studies based on the assumption that there is a relationship between the dose and the likelihood of response. The dose-response relationship is usually non-linear, implying no further increase of response likelihood abovea certain dose-level. Body-surface-area is usually used for dosing in the individual patient. Alternative dosing methods have been investigated over the last decade to minimize the wide interpatient variation of drug exposure for classical chemotherapy.

In the era of targeted therapies in cancer treatment, the prolonged use of orally given single agent treatments is more and more common with the aim of blocking certain biological pathways. This has dramatically changed the aim of cancer treatment, as most targeted therapies are thought to have mainly cytostatic properties. Some forms of cancer that used to have very limited options for life extending treatment, let alone cure, became chronic diseases in some patients. Examples are renal-cell cancer (RCC), Gastrointestinal stromal tumor (GIST), chronic myelogenous leukemia (CML), hepato-cellular carcinoma HCC) and certain forms of lung cancer.

Pathway blocking by targeted therapies is most often reversible. Consequently targeted therapies are given continuously. Treatment with oral drugs usually is characterized by wide interpatient variation in drug exposure. It may result in enzyme inhibition or induction of metabolism and absorption systems in the individual over time. This variation is often wider for oral drugs given continuously than for intermittently applied intravenously given drugs.

The use of a fixed dose is based on the observation that BSA based dosing is not necessarily more precise than flat-fixed dosing. For oral targeted therapies the need for dose individualization has been extensively been advocated, in order to minimize interindividual variation of drug exposure. High interindividual variation of drug exposure increases the likelihood of toxicity and underdosing. This is of particular concern for orally given drugs, where absorption is an additional factor influencing the interindividual variation in drug exposure. Controlling systemic exposure based on dose individualization should maximize treatment efficacy, coinciding with manageable effects, thus resulting in an optimal balance between efficacy and adverse effects.

In the first part of this thesis the factors predicting toxicity and response of anti-cancer tyrosine kinase inhibitors (TKIs) are investigated in collaboration with an international team of experts in this field. Imatinib and sunitinib were studied to get closer to an answer to the question "how to get the right dose for each individual patient".

The other big question is "do I aim at the right target?" New potential targets of anti-cancer treatment are frequently discovered. Targets are mutated drivers of proliferation. Each individual cancer in each individual has a unique variation of drivers and mutations within the primary cancer, and even more in their metastases as mutation continues. One of the most common drivers in cancer proliferation is mTOR. Inhibitors of mTOR have proven efficacy for cancer types like RCC and neuro-endocrine tumors (NET). To improve the efficacy of mTOR inihibitors combination with other anti-cancer treatments is a current topic of great importance. The inhibition of only one target of cancer proliferation is often insufficient to induce response to treatment. In most cancers there are more than one mutations in proliferation mechanisms. Consequently combination therapy with inhibition of other targets may increase response to treatment.

In this thesis several combination therapies are investigated clinically with the aim to select the optimal dose and schedule of the combinations and to explore its pharmacological effects. Translational research was used to select patients with a higher probability to respond to treatment. Here peripheral blood mononuclear cells (PBMCs) and where possible tumor tissues have been investigated to predict if the projected target is inhibited during treatment. To unravel potential drug interactions, blood drug levels of treated patients were analyzed for each drug separately and in combination. The dose considered feasible for use as a combination was tested for anti-tumor activity in a Phase II study.

In this thesis the wide interpatient variation of ten commonly clinically used TKIs is described (chapter 2) based on published data. Until predictive factors to individualize dosing are found and implemented, surrogate markers of drug exposure and, more importantly, of response may guide clinicians in the use of TKIs. In table (page 20) an overview of these TKIs is given for clinicians with information on drug-drug interaction along with the influence of food on drug absorption. The importance of compliance, particularly for the reviewed orally used drugs, is emphasized.

A retrospective study of imatinib, used to treat CML patients, confirmed the clinical observation that toxicity decreases over time due to reduced exposure and that dose escalations in case of treatment failure in individuals are feasible, even after dose limiting toxicity at start of treatment (chapter 3). This phenomenon is rarely seen in classical chemotherapy and underlines the different dose response relationship between these two treatment modalities. Interestingly the possibility to increase the dose is predictable using genetic analysis of single nucleotide polymorphisms (SNPs). Prior studies of SNPs significantly associated with toxicity have been confirmed especially for a SNP in the gene for CYP3A5.

For sunitinib an interim analysis of a prospective study (Appendix) found the association between the ABC drug transporter gene ABCG2 and the likelihood of the development of nausea and fatigue. Another observation is the association of the gene encoding for the metabolic enzyme CYP3A5 and the chance to develop diarrhea, hand-foot syndrome and rash. Hypertension, a common side-effect of sunitinib appear to be more pronounced when in patients selected genetic markers were present encoding for one of the vascular endothelial growth factors. Trough levels of sunitinib seem to predict the likelihood of development of inflammation of the mouth, also a common side-effect of sunitinib. Clearance of midazolam, which is a probe for determination of the activity of the drug metabolism enzyme CYP3A4 involved in the metabolic conversion of sunitinib, was predictive for the blood levels of the active metabolite of sunitinib (N-desethylsunitinib) at start of treatment. These preliminary observations appear to point into the direction of significant correlations between pharmacogenetic parameters and exposure and toxicity of sunitinib. However, additional and more thorough investigations are currently in progress including population pharmacokinetic and pharmacodynamic analysis also incorporating haplotype analysis to further analyse the data obtained in this study.

In the second part of this thesis the properties of the mTOR inhibitors everolimus and temsirolimus are reviewed (chapter 5). Clinical trials investigating the feasibility and anti-tumor activity of combination treatment with mTOR inhibition as one of the component are reported. Temsirolimus, an intravenously applied mTOR inhibitor, may show drug resistance due to drug induced Akt activation, bypassing mTOR inhibition. In a Phase I study, Akt inhibition by nelfinavir, a potent Akt inhibitor, was feasible in combination with temsirolimus (chapter 6). Even though the recommended dose of temsirolimus was less than the standard dose for RCC, serum levels of the active metabolite sirolimus did show adequate levels comparable to single agent in RCC, suggesting adequate exposure. When temsirolimus was combined with nelfinavir an increase of sirolimus AUC was seen ($p = 0.0003$). In fact, the increased active sirolimus levels are an expected consequence of the inhibited metabolism of temsirolimus by the strong CYP3A4 inhibitior nelfinavir. When correlated with outcome, higher levels of SIR were predictive for response to treatment. Pharmacodynamic investigations of the PBMCs showed that pre-existing high levels of mTOR are predictive for response to treatment. Hyperlipidaemia is a surrogate marker for blood levels of temsirolimus and sirolimus.

Synergistic anticancer effects of mTOR inhibition and 5-fluorouracil (5-FU) have been observed in cancer cell lines. The observed high toxicity in a study of temsirolimus and 5FU led to a different approach, i.e. the administration of the oral formulations of both drugs. Surprisingly we found the combination of everolimus and capecitabine for the first time feasible in a Phase I study, even in active single agent doses (chapter 7). Drug interactions were not seen and in PK studies an expected wide inter-patient variation of drug levels of each of the drugs was found. The documented feasibility of this combination therapy generates options for clinical trials in many other cancer types. Hopefully this combination will increase the anti-tumor activity of capecitabine alone. Interestingly, a similar phase I study of this combination in a Korean gastric cancer cohort recommended capecitabine at a 33% lower dose. This difference might be explained by the genetic ethnic

differences of drug disposition and probably prior gastrectomies in the Korean gastric cancer cohort. Recently it has been found that after total or partial gastrectomy absorption of capecitabine is faster, with higher plasma concentrations and a higher systemic exposure to capecitabine compared to patients with an intact stomach.[1]

Finally, the recommended doses of everolimus and capecitabine showed promising efficacy in a phase II study of advanced pancreatic cancer patients, even after gemcitabine pretreatment (chapter 8).

The data presented in this thesis may have consequences for future studies. Clinicians prescribing TKIs should be aware of factors involved in drug disposition. The need to dose-adjust, not only because of toxicity, as is common practice nowadays, but with subsequent dose escalations in an attempt to increase treatment efficacy requires confirmation in prospective studies. A dosing method based on the presence or absence of toxicity (toxicity-adjusted dosing [TAD]) is proposed. TAD is a practical and easy to use tool derived from the theory that drug induced toxicity is correlated with outcome. To confirm the latter theory two steps should be determined: (1) establishment of the correlation between toxicity and response to treatment, (2) prospective studies to compare TAD vs fixed dosing in regards to outcome.

Another dosing method is based on the genetic fingerprint of the genes involved in drug disposition (pharmacogenetics). The pharmacogenetic data of imatinib described in this thesis demand a confirmation in a prospective study. A study proposal comparing flat-fixed dosing imatinib with dose-prediction, based on PG, may help to determine whether underdosing in an early stage of the disease can be prevented. This is of great importance due to the fact that imatinib is the first TKI that may cure CML, in particular when treated early with adequate doses. Complicating factors of this promising approach are prognostic CML mutations, but also co-morbidity and co-medication, which might have a strong effect on drug disposition.

Wide interpatient variation has been confirmed for most TKIs, but insufficient data are published on variation within an individual (intrapatient variation). Factors like the influence of different food styles, fasting and different sleep patterns including sleep deprivation on PK are easy to investigate in small groups of patients. These studies would increase the awareness of clinicians about factors affecting systemic exposure and possibly about the importance of how en when patients should take oral anti-cancer medication to optimize efficacy and minimize toxicity.

As shown for imatinib previously and for sunitinib in this thesis, association studies of e.g. trough levels and response-rate for all TKIs currently in routine clinical use, or in ongoing phase II and III trials are warranted. Consequently, in case of positive predictive associations, prospective studies comparing flat-fixed dosing with dosing based on Therapeutic drug-monitoring (TDM) would be of interest to perform.

In regards to the mTOR inhibition results, several promising studies deserve to be carried out:

1 Phase II study of temsirolimus and nelfinavir for platinum resistant ovarian cancer, on the basis of the remarkably long term response of one ovarian cancer patient and some promising preclinical data [2]
2 Phase II study of temsirolimus and nelfinavir in AIDS-related lymphomas, due to the known activity of nelfinavir in HIV patients as an antiretroviral drug and the preclinical evidence of efficacy of mTOR inhibition in lymphomas[3][4]

3 Phase II study of capecitabine BSA dosed and everolimus fixed dose versus both drugs TDM dosed as second line treatment for pancreatic cancer after failure of gemcitabine based regimens. This proposal is based on the observation of a wide interpatient variation of serum drug levels of both drugs and the promising drug activity in pancreatic cancer.

In conclusion, in this thesis new dosing methods for TKIs are reviewed and clinical data of two TKIs are given showing the feasibility of implementation of PK and PG in the Netherlands as well as the feasibility of international cooperation. Furthermore new promising therapy combination regimens have been found, forming the basis for subsequent efficacy studies. Pharmacodynamic analysis, an important tool to select patients for more effective future studies, was successfully implemented as a second endpoint in some of these studies. Future studies of novel targeted anti-cancer agents are not only dependent on their activity but also on the choice of the 'right' dose, which underscores the need for smarter phase I studies using PD markers and intrapatient dose escalations.

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Appendix

Predictive value of drug elimination gene polymorphisms on clearance and dose adjustment of sunitinib in patients with cancer *(interim analysis)*

Analysis in progress.

Financial disclosure: An unrestricted research grant was provided by Pfizer.

ABSTRACT

Background

Cancer patients treated with sunitinib show a wide inter-patient variability in drug exposure. This partly explains the variation in toxicity related to sunitinib treatment. The primairy aim of this study was to investigate whether the pharmacogenetic characteristics and clearance of phenotyping probes of the patient could be related to the pharmacokinetics (PK) and toxicity of sunitinib determined at continuous daily dosing.

Patients and Methods

A prospective multi-center study was performed in cancer patients treated with single agent sunitinib. Pharmacogenetic analysis of eight target single nucleotide polymorphisms (SNPs) in 5 genes of patients DNA were correlated with toxicity. Trough concentrations at continuous daily dosing of sunitinib and its active metabolite N-desethyl-sunitinib were correlated to midazolam metabolic ratio (CYP3A probe), hepatic ^{99m}Tc-MIBI scans (ABCB1 probe) and pharmacogenetics.

Results

All of the 52 patients included were evaluable for pharmacogenetic and toxicity analysis. Forty-six patients were evaluable for analysis of pharmacokinetics and phenotype probe study at continuous daily dosing. There was a correlation between sunitinib trough levels and the midazolam metabolic ratio ($r = 0.62$, $p = 0.006$). Pharmacogenetic markers such as CYP3A5 (rs776746), ABCG2 (rs2231142) and VEGF (rs3025039) were correlated with an increase risk of development of toxicity.

Conclusions

These preliminary observations appear to point into the direction of significant correlations between pharmacogenetic parameters and exposure and toxicity of sunitinib. However, additional and more thorough investigations are currently in progress including population PK-pharmacodynamic (PD) analysis also incorporating haplotype analysis to further analyse the data obtained in this study.

INTRODUCTION

Sunitinib malate (Sutent; Pfizer Pharmaceuticals Group, New York) is an oral multi-targeted tyrosine kinase inhibitor with antitumor and antiangiogenic activities. It is approved for first-line treatment of metastatic renal-cell cancer (mRCC) and imatinib-resistant metastatic gastrointestinal stromal tumors (GIST) [1][2]. A recent Phase III study showed survival benefit for patients with pancreatic-neuroendocrine tumors (p-NET) treated with sunitinib [3].

Treatment schedules for mRCC and GIST consist of a flat-fixed daily dose of 50 mg (4 weeks on 2 weeks off) and for p-NET a flat-fixed daily dose of 37.5 mg continuously. Dose reductions due to toxicity are frequently applied (31% for P-NET, 32% for RCC, 11% for GIST) [1][2][4]. This may be the result of wide inter-patient variability in sunitinib drug exposure due to differences in activity of drug metabolizing enzymes. There are many genetic and non-genetic factors influencing drug exposure and metabolism. Pharmacogenetics are increasingly used to identify genetic predisposing factors for both drug efficacy and adverse drug reactions [5]. For sunitinib, polymorphisms of metabolizing enzymes, efflux transporters, and drug targets have been found to predict sunitinib related toxicity [6]. This method may help to optimize drug treatment in individual patients. The activity of sunitinib metabolism in the individual patient may be measured by applying so-called phenotype tests. Midazolam metabolic ratio is correlated with cytochrome P450 enzyme 3A4 (CYP3A4) activity [7][8]. Sunitinib is primarily metabolized by CYP3A4, resulting in the formation of its active metabolite SU012662, which is further metabolized by CYP3A4. If midazolam metabolic ratio is correlated to sunitinib clearance, midazolam metabolic ratio may be used as a predictor of sunitinib exposure and possible also sunitinib induced toxicity, assuming a direct relationship between drug exposure and this toxicity. ABCB1 is an adenosine triphosphate binding cassette (ABC) transporter, involved in hepatic clearance of many classes of anticancer drugs, including sunitinib. Hepatobiliary clearance of technetium-99m (^{99m}Tc)- 2-methoxy isobutyl isonitrile (MIBI) is markedly reduced in the presence of ABCB1 inhibitors in humans. Thus it is likely that ABCB1 is the principle mediator of ^{99m}Tc-MIBI elimination. Hepatic ^{99m}Tc-MIBI scans may provide an indicator of ABCB1 activity [9].

The aim of this interim analysis is to investigate whether clearance of these phenotype probes are related to sunitinib trough levels. Furthermore sunitinib trough levels were related to adverse events.

Patients and Methods

Between February 2009 and September 2011, in four centers in Australia and the Netherlands, patients with solid tumors treated with single agent sunitinib were asked to participate in this study. Patients were eligible at any course during treatment. All dose levels of sunitinib were allowed in the study. Additional eligibility criteria were age at least 18 years; Eastern Cooperative Oncology Group (ECOG) performance status of 2 or less;

adequate hematopoietic, hepatic, and renal functions. Patients were excluded for any significant systemic disorders and pregnancy. All patients provided informed consent according to federal and institutional guidelines. The trial was conducted in accordance with current Good Clinical Practice.

Study design

This was a prospective study of patients treated with sunitinib. Patients were asked to undergo four study tests at day 1 of a new treatment cycle and in the fourth week of continuous treatment. Study tests consisted of a blood DNA analysis, pharmacokinetic sampling of sunitinib, midazolam metabolic ratio test and a 99mTc-MIBI liver scan. Sunitinib toxicity was evaluated during treatment by National Cancer Institute Common Toxicity Criteria for Adverse Events (CTCAE) version 3.0 for up to 3 months after the first day of study participation. Demographic and clinical data of patients were reported on case record forms designed for data collection in this study. All samples for pharmacokinetics on sunitinib and midazolam, and the performed ^{99m}Tc-MIBI lever scans 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

Eight candidate polymorphisms previously found as significant predictors of sunitinib induced toxicity in 5 genes involved in pharmacokinetics and pharmacodynamics of sunitinib were selected [6]. The polymorphisms were in de genes of ABCB1, ABCG2, CYP3A5, vascular endothelial growth factor (VEGF) and its receptor VEGFR (Table 5). The analysis of all samples took place in one laboratory. All samples were analysed in the same through put.

Pharmacokinetic sampling of sunitinib

Pharmacokinetic blood withdrawal was performed on day 1 and at repeated contiuous dosing of sunitinib treatment and stored at - 80°C at the participating center. Drug concentrations were measured after a minimum of 21 days of treatment. Limited blood sampling regimen was used with accurately timed blood samples taken pre-dose and at t=4, t=8 and t=24 h after sunitinib administration. All pharmacokinetic measurements of sunitinib were undertaken at the department of Pharmacology of the Erasmus Medical centre. Plasma concentrations of sunitinib were determined by a high-performance liquid chromatography mass spectrometry method [10]. Trough levels were corrected for dose-adjustment, using the assumption that dose adjustment of sunitinib results in equally adjusted exposure to sunitinib and its active metabolite

Midazolam metabolic ratio test

Midazolam at a dose of 75 µg was administered before sunitinib administration to determine CYP3A activity [7]. The 30 minute post-administration 1-OH- midazolam/midazolam ratio was determined in a single plasma sample using an LC-MS/MS method, after enzymatic hydrolysis of the glucuronic conjugates, as described previously [11][12]. Midazolam metabolic ratio was related to sunitinib trough levels.

Hepatic technetium labeled sestamibi (^{99m}Tc-MIBI) clearance

Within 2 days before commencement of treatment and after repeated daily dosing pharmacokinetic measurements, hepatic elimination of 99mTc-MIBI was determined and interpreted as an indicator of ABCB1 (P-glycoprotein, MDR1) activity. An elimination rate constant (kH) for 99mTc-MIBI was used and correlated with sunitinib trough levels [9]. Hepatic ^{99m}Tc-MIBI clearance (kH*liver volume) was used for comparison with drug clearance with liver volume estimated from body surface area as described previously [13][14].

Definition of Toxicity

All adverse events were graded at the participating centres. Clinical symptoms and haematological abnormalities were considered drug induced, such as: fatigue, mucosal inflammation, hand-foot syndrome, thrombocytopenia, neutropenia and any other adverse events higher than grade 2. The considerations if an adverse event is related to sunitinib treatment and recommendations on dose adjustments were not given but up to investigators discretion.

Statistical Design and Data Analysis

All data were analyzed using SPSS 17.0. To compare phenotype tests (midazolam metabolic ratio and hepatic ^{99m}Tc-MIBI scans) with dose-adjusted sunitinib trough levels, linear regression model was used. For the correlation between sunitinib trough levels and toxicity the chi-squared statistic test was used.

Results

Patients and sunitinib dosage

A total of 52 patients were enrolled at four centres in Australia and The Netherlands between February 2009 and September 2011. Forty-six of all participating patients underwent pharmacokinetic measurements and ^{99m}Tc-MIBI scans of the liver in the fourth week of treatment and were therefore evaluable. Patient's demographics and disease characteristics at study enrolment are summarized in Table 1.

Table 1 Patient Characteristics

Characteristic		Median	Range	No	%
Age (years)		61	29 - 81		
Gender	Male			33	64
	Female			19	36
Body Surface Area (m^2)		2.08	1.26 - 2.5		
Ethnicity	Caucasian			48	92
	Asian			3	6
	Hispanic			1	2
Type of malignancy	Renal cell cancer			43	83
	Pancreatic neuro endocrine tumor			7	13
	Gastrointestinal stromal tumor			2	4
Previous medical antitumoral treatments	Yes			9	17
	No			43	83
ECOG performance status	0			29	56
	1			21	40
	2			2	4
Starting dose of sunitinib at enrolment	25 mg			5	10
	37.5 mg			15	29
	50 mg			32	61
Dose of sunitinib at continuous dosing	25 mg			6	11
	37.5 mg			14	27
	50 mg			26	50
	Patients not participating 2nd test			6	11

Baseline chemistry and hematology	Median	Range
Creatinine, µM	101	45-147
Ttotal bilirubin, µM	7	3-31
Albumine, gram/L	42	26-50
ALT, units/L	27	9-239
AST, units/L	28	14-134
Hemoglobin, mmol/L	7.7	5.2-10.6
Leukocytes, $\times 10^9$/L	6.3	2.9-11.5
Thrombocytes, $\times 10^9$/L	276	108-587

ECOG Eastern Cooperative Oncology Groupn

Table 2 Distribution of sunitinib doses and dose adjustments (N=52)

Dose of sunitinib and doseadjustments	No	%
Dose at start of sunitinib treatment		
25 mg	3	6
37.5 mg	6	11
50 mg	43	83
Dose changes of sunitinib during treatment		
increase	2	4
no change	22	42
decrease	28	64

Toxicities

Seventeen (37%) patients of 46 suffered from grade 3 toxicity and 24 patients underwent dose reduction (n=24) at any time during treatment with sunitinib (Table 2). At the time of determination of the pharmacokinetics six patients suffered from grade 3 sunitinib related toxicity. Of all the 52 patients 21 (40%) suffered from grade 3 toxicity with the need for dose reduction in 28 patients (53%). Within the first 3 months of treatment with sunitinib 31 patients (60%) had grade 1/2 toxicity and 21 patients (40%) had more that grade 2 toxicity (Table 3).
Hand-foot syndrome was the most frequently reported grade 3 toxicity, occurring in 6 patients. Forty-one (89.1%) patients suffered from any grade fatigue in the first year of treatment with sunitinib.

Table 3 Toxicity seen within the first 3 months of treatment with sunitinib

	Total, n (%)	
No. of patients	*N* = 52 (100)	
CTC grade	**Grade 1 – 2**	**Grade 3 – 4**
Non-Hematological		
Fatigue	37 (71)	4 (8)
Stomatitis*	32 (62)	2 (4)
Diarrhea	29 (56)	2 (4)
Nausea	22 (42)	4 (8)
Hand-foot syndrome	19 (37)	6 (12)
Hypertension	20 (39)	3 (6)
Anorexia	20 (39)	1 (2)
Taste alterations	19 (37)	
Skin rash	19 (37)	
Hypothyroidism	7 (14)	
Any	31 (60)	21 (40)
Hematological		
Platelets	25 (48)	
Leukocytes	25 (48)	

* Stomatitis including aphteus ulcaeration and mucositis of the mouth.

Sunitinib systemic exposure and toxicity

Of the 46 patients undergoing pharmacokinetic investigations, 15 patients already had undergone a dose reduction due to toxicity by the time of pharmacokinetic blood drawing. The mean level of sunitinib plus *N*-desethylsunitinib, adjusted for dose reduction was

88µg/mL (Table 4). The mean increase of the absolute sunitinib trough level at day 1 and at steady state was from 14 to 49, respectively (standard deviation [SD] 19). Trough levels at steady state were positively correlated with the occurrence of stomatitis (p=0.013) and rash (p=0.001). This difference between the trough levels measured on day 1 and after repeated continuous dosing confirmed the observation for stomatitis (p=0.009) and rash (p=0.003). However, a population pharmacokinetic model needs to be developed for more detailed analysis of PK and PK-PD relationships, which is currently being performed.

Table 3 Toxicity seen within the first 3 months of treatment with sunitinib

	Total, n (%)	
No. of patients	*N* = 52 (100)	
CTC grade	**Grade 1 – 2**	**Grade 3 – 4**
Non-Hematological		
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Hand-foot syndrome	19 (37)	6 (12)
Hypertension	20 (39)	3 (6)
Anorexia	20 (39)	1 (2)
Taste alterations	19 (37)	
Skin rash	19 (37)	
Hypothyroidism	7 (14)	
Any	31 (60)	21 (40)
Hematological		
Platelets	25 (48)	
Leukocytes	25 (48)	

* Stomatitis including aphteus ulcaeration and mucositis of the mouth.

Sunitinib exposure and drug elimination phenotype

Midazolam metabolic ratio is significantly correlated with *N*-desethylsunitinib levels 24 hours after first intake of sunitinib in the first course of treatment (n=18, r=0.62, p=0.006). There is a trend towards significance in the correlation between midazolam metabolic ratio and *N*-desethylsunitinib levels at steady state (n=15, r=0.45, p=0.09), however, sample size is small (figure 1). We found no correlation between hepatic clearance of ^{99m}Tc-MIBI and trough levels of sunitinib or its active metabolite in this interim analysis (data not shown).

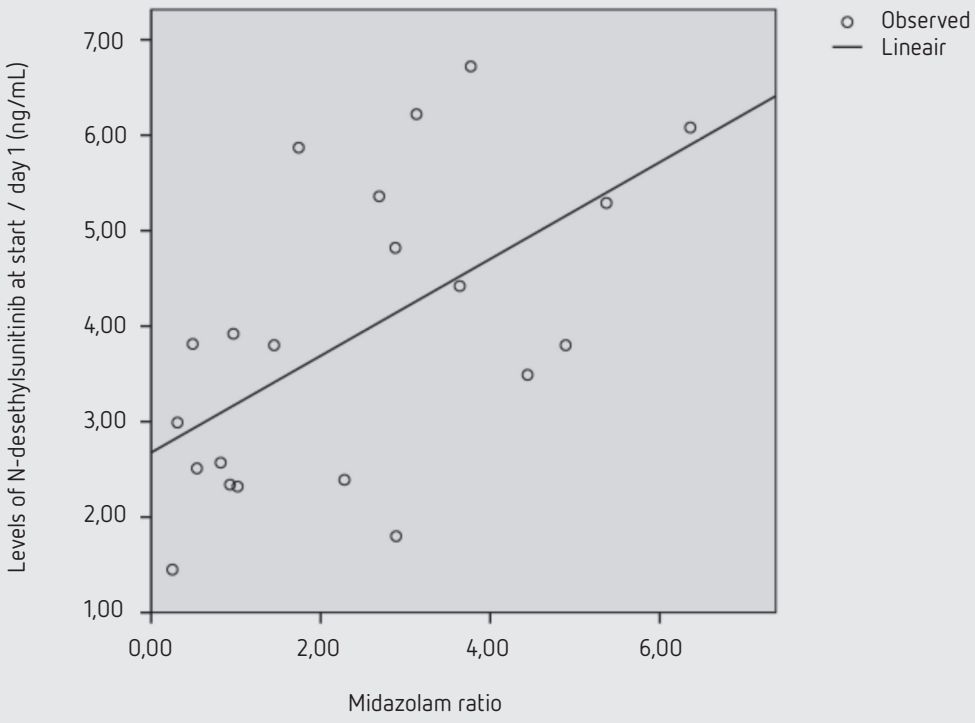

Figure 1
Correlation between midazolam metabolic ratio and dose adjusted level of N-desethyl sunitinib (ng/mL) at start/day 1 (n=21, r=0.58, p=0.006)

Table 4 Pharmacokinetic and elimination phenotype tests

	n	Day1 mean±SD (min-max)	n	Steady-state mean±SD (min-max)
Pharmacokinetic measurements				
Sunitinib trough level (C_{min}) (ng/mL)	51	14.1±4.9 (6.5-28.9)	46	49.8±20.9 (9.6-113)
N-desethyl-sunitinib (ng/mL)	51	4.2±1.7 (1.5-9.8)	46	25.6±11.8 (1.6-57.8)
Sum (ng/mL)	51	18.3±5.7 (9.7-33.7)	46	75.3±29.8 (11.2-147.8)
Phenotype measurements				
1-OH- midazolam/midazolam ratios	48	2.73±2.18 (0.2-9.9)	42	2.76±2.0 (0.2-7.9)
^{99m}Tc-MIBI scan described as distribution of elimination constant (kH) min^{-1} x1000	22	14.15±7.9 (4.16-35.6)	20	11.79±5.2 (4.5-27.6)

Table 5 Genotype analysis of 52 patients

Gene and SNP	Genotype	N
ABCB1		
rs1128503	1236C>T	
	CC	16
	CT	26
	TT	10
rs203582	2677G>/T	
	GG	42
	GT	1
	TT	3
rs1045642	3435C>T	
	CC	14
	CT	28
	TT	10
ABCG2		
rs2231142	421C>A	
	CC	1
	CA	14
	AA	37
rs2231137	34G>A	
	GG	0
	GA	1
	AA	51
CYP3A5		
rs776746	6986A>G	
	AA	2
	AG	6
	GG	44
VEGF		
rs3025039	936C>T	
	CC	31
	CT	21
	TT	0
VEGFR		
rs2305948	G>A	
	GG	39
	GA	13
	AA	0

Sunitinib exposure and genotype

Through levels of sunitinib, its metabolite *N*-desethylsunitinib and their sum at steady state was correlated with the eight SNPs of the 5 candidate genes. There was no significant correlation found. However, more detailed analysis including haplotype analysis needs to be performed.

Sunitinib toxicity and genotype

The toxicity of sunitinib of 52 patients within the first 3 months of treatment, as described in Table 3, was correlated with the eight SNPs and showed significant correlations, without correction for multiple comparisons.

The genetic factor of the drug transporter ABCG2 (rs2231142) was correlated with fatigue (p=0.026) and nausea (p=0.028). The drug metabolism enzyme CYP3A5 (rs776746) was correlated with diarrea, hand/foot syndrome and rash (p= 0.016, 0.015, and 0.068 respectively). Hypertension was correlated (p=0.028) with a SNP encoding for VEGF (rs3025039). However, more detailed analysis including haplotype analysis needs to be performed.

Preliminary Discussion

Considering sunitinib is mainly metabolized by CYP3A4 to its active metabolite SU12668, and eliminated in the liver by ABCB1 transporter, substrate probe tests as a marker of CYP3A4 and ABCB1, might predict serum levels of sunitinib and its active metabolite. In our study we correlated midazolam metabolic ratio and hepatic ^{99m}Tc-MIBI scans with sunitinib trough levels and its active metabolite, to test whether clearance of these well known substrate drugs for CYP3A4 and ABCB1 activity could be used as a predictor of exposure and toxicity in patients treated with sunitinib. In this interim analysis, midazolam metabolic ratio at start of treatment correlated with exposure to N-desethylsunitinib in the first week of treatment with sunitinib. In contrast, no significant relationship was found between hepatic ^{99m}Tc-MIBI scans and trough levels of sunitinib.

Currently, the data are used to establish a population PK model in order to optimally determine relationships between sunitinib exposure and toxicity as well as between sunitinib exposure and midazolam metabolic ratio and sestamibi clearance.

Preliminary analysis of single nucleotide polymorphisms in genes coding for the metabolizing enzymes of sunitinib did not reveal significant relationships between trough levels of sunitinib and presence of identified SNPs. Also, this analysis should be expanded incorporating the population PK model and a thorough haplotype analysis.

Several significant correlations were found between identified SNPs and toxicity parameters. The most frequently observed toxicity fatigue, but also nausea, appeared to be related to genotypic variation in ABCG2. The presence of this genotype (rs2231142) was associated with the likelihood to develop toxicity greater than grade 2 [6]. CYP3A5 is involved in the metabolism of sunitinib [15]. The SNP rs776746 in CYP3A5 seems to be correlated to the development of toxicities of sunitinib [6][16][17], which our data seem to

confirm as patients with this SNP were more prone to develop several sunitinib induced toxicities, such as diarrea, hand/foot syndrome and rash. We also found that a specific SNP of the gene encoding for VEGF (rs3025039) was associated with the presence of hypertension, a well known toxicity of sunitinib. Although hypertension appears to be one of the most common adverse effects of VEGF inhibitors, the pathophysiological mechanisms underlying the increase in blood pressure in response to VEGF pathway inhibitors have yet to be fully elucidated [18].

Our current research is focussed on completion of the dataset of this study, on assessment of a population PK model, on modelling PK to PD and on extensive SNP and haplotype analysis in relation to sunitinib exposure and toxicities.

ACKNOWLEDGMENTS

This study was made possible thanks to an unrestricted research grant by Pfizer.

The following investigators contribute to this study:
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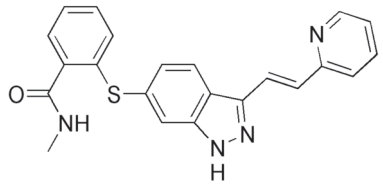
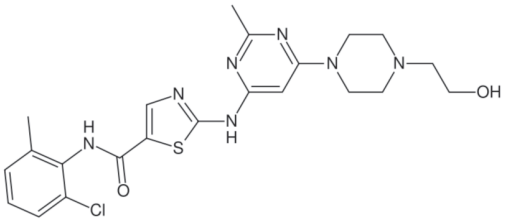
Chemical structures of investigated molecules in this thesis

Part 1

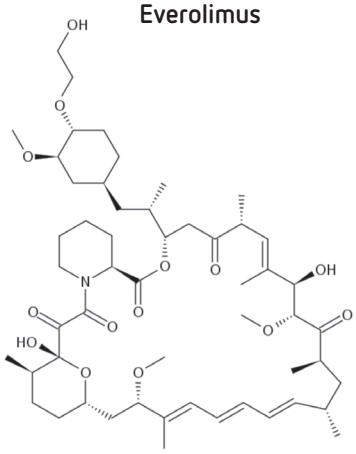
Axitinib



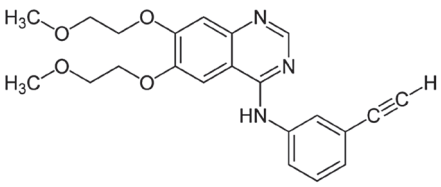
Dasatinib



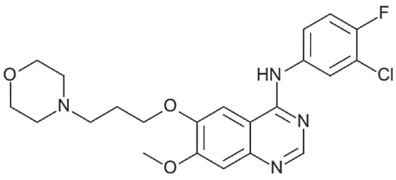
Everolimus



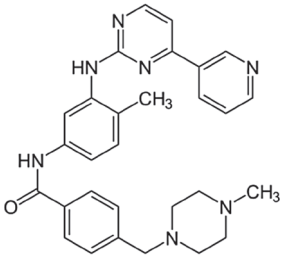
Erlotinib



Gefitinib



Imatinib



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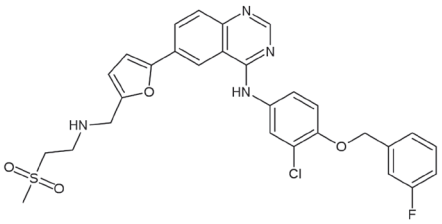
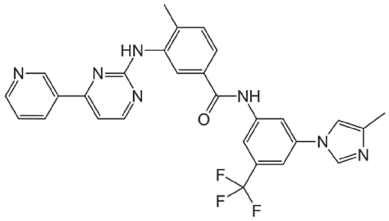
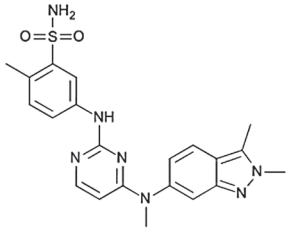


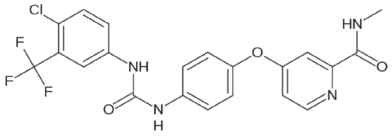
Nilotinib



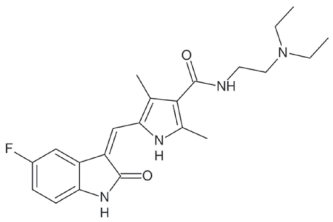
Pazopanib



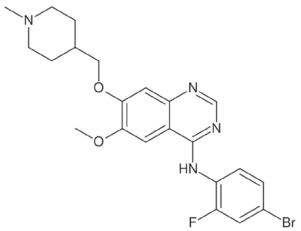
Sorafenib



Sunitinib



Vandetanib



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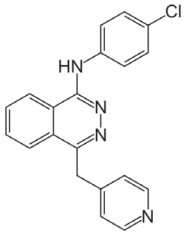
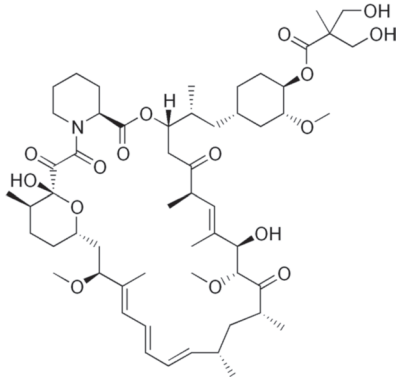
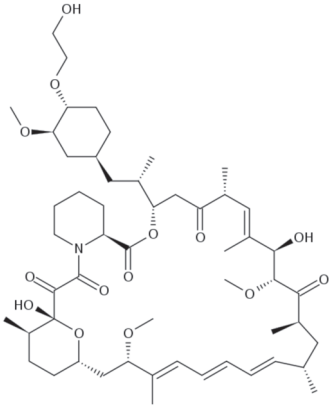


Part 2

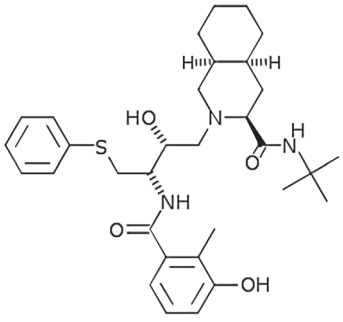
Temsirolimus



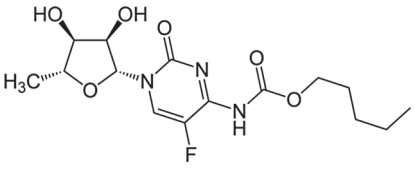
Everolimus



Nelfinavir



Capecitabine



Summary

Personalized medicine is an emerging practice of medicine that uses an individual's genetic profile to guide decisions made in regard to the prevention, diagnosis, and treatment of disease. In cancer care, personalized treatment is tailored to the genes and proteins in an individual's tumor. This approach could improve survival rates, avoid unnecessary treatment, and reduce health care costs. The potential of personalized medicine is limited by the growing but as of yet incomplete understanding of the genetic variation of an individual patient and his individual tumor. The goal of this thesis was to increase the understanding of the genetic variation in the use of several molecular targeted agents, which form the basis of a new approach in cancer treatment in recent years.

Pharmacognetics of small molecules

Chapter 2 gives an overview of molecular targeted therapies, also called tyrosine kinase inhibitors (TKI) or small molecules. Currently these agents are mainly used at a fixed dose, ignoring the possible need for dose individualization. Fixed dosing may indeed result in suboptimal treatment or excessive toxicity considering the high inter-individual variability in the pharmacokinetics (PK) of these therapies. The PK, toxicity and efficacy of ten commonly used molecular targeted anti-cancer therapies were reviewed in order to optimize their prescription. A wide interpatient variability in the pharmacokinetics of these small molecules was demonstrated. Moreover, associations between certain toxicities and treatment efficacy have also been demonstrated for some agents, such as erlotinib and skin rash, that may be used as a surrogate marker. Other biomarkers intended to substitute for clinical endpoints have been described for some TKIs and may be useful for dose individualization. Promising alternatives to fixed dosing were explored, such as therapeutic drug monitoring, genotype and phenotype adjusted dosing, and toxicity-adjusted dosing.

In **Chapter 3** the genetic profile of 77 patients with chronic myeloid leukemia (CML) was investigated to predict toxicity and dose adjustments of imatinib, a small molecule with long term responses in CML treatment. A validation of a previously found genetic marker of drug metabolism (CYP3A5) was seen, and was predictive for the tolerability of a higher dose of imatinib. This marker might help to overcome pharmacokinetic resistance to imatinib for patients who tolerate a higher dose of imatinib. But also several novel relationships between metabolizing enzymes and drug transporters and tolerability of imatinib were found, e.g. ABCC12, a member of the multi-resistance protein subfamily. Imatinib-induced non-hematological toxicity was highly correlated with genes in CYP2B6 and a glutathione transferase gene (GSTM5). Hematological toxicity was associated with multiple SNPs including those in CYP8B1, and a number of ABC transporters.

For sunitinib, another small molecule used in the treatment of cancer such as renal-cell cancer, factors involved in drug disposition of sunitinib were investigated. In the **appendix** the preliminary pharmacogenetic and phenotype results of these investigations

in 52 patients are described. The ABC drug transporter gene ABCG2 seem to predict nausea and fatigue, and the gene of the metabolism enzyme CYP3A5 appreat to predict for the chance to develop diarrhea, hand-foot syndrome and rash. Hypertension, a common side-effect of sunitinib, was more pronounced when patients had the presence of a certain genetic marker encoding for one of the vascular endothelial growth factors. Analysis in progress shows that trough levels of sunitinib predicted the likelihood of development of inflammation of the mouth, another common side-effect of sunitinib. The clearance of midazolam, which is a probe for the activity of the drug metabolism enzyme CYP3A4 involved in the conversion of sunitinib, was predictive for the blood levels of the active metabolite of sunitinib (N-desethylsunitinib). Additional and more thorough investigations are currently in progress including population PK-pharmacodynamic (PD) analysis also incorporating haplotype analysis to further analyse the data obtained in this study.

mTOR inhibition in cancer treatment

The mammalian target of rapamycin (mTOR) was first discovered in lichen on the Easter Island (Rapa Nui), and it is an intracellular protein responsible for cell-proliferation. Inhibitors of mTOR have the potential to inhibit the proliferation of certain cancer cells, which initiated the use of mTOR inhibitors in the treatment of cancer, such as renal cell cancer.

A concise drug review of mTOR inhibitors from a clinical pharmacology perspective is summarized in **chapter 4**. This review provides pharmacokinetic and dynamic properties of the commonly used mTOR inhibitor drugs everolimus and temsirolimus. Additionally information on the clinical use, mechanism of action, bioanalysis, drug-drug interaction, alterations with disease or age, pharmacogenetics and drug resistance is supplied. This overview should assist the treating medical oncologist to adjust mTOR inhibitor treatment to the individual patient circumstances.

The ability of mTOR inhibitors to activate Akt, a protein leading to cell proliferation, counteracts its antiproliferative activity. Combined treatment with both mTOR inhibitors and Akt inhibitors may overcome this issue and thus increase therapeutic efficay. Nelfinavir, a protease inhibitor used in the treatment of the human immunodeficiency virus, is a strong Akt inhibitor. **Chapter 5** shows the results of a dose finding study of combination treatment with the mTOR inhibitor temsirolimus and the Akt inhibitor nelfinavir in 18 patients with advanced solid tumors. It was feasible and safe to combine both drugs at a dose level of nelfinavir 1000 mg/day and temsirolimus 10mg/week. Dose limiting toxicities were mucositis and nausea. The combination increased the blood levels of sirolimus, the active metabolite of temsirolimus, compared with single agent use of temsirolimus. This was most likely due to the fact that nelfinavir is also a strong CYP3A4 inhibitor. Translational investigations were performed to identify potential predictive markers of increased response. These investigations showed that patients who had an increased mTOR activity before start of treatment had an increased chance to have clinical treatment benefit. Patients who developed dyslipidemia had higher blood levels of temsirolimus and sirolimus. The potential use of dyslipidemia as a surrogate marker for drug exposure needs to be validated in prospective studies.

Another promising candidate drug to improve efficacy of mTOR inhibition is 5-fluorouracil-based anticancer therapy, due to preclinical data showing synergistic effects of this combination. A dose finding study in 18 patients with advanced solid tumors treated with everolimus in combination with the 5FU prodrug capecitabine, both orally available, is presented in **chapter 6**. Everolimus 10 mg/day continuously combined with capecitabine 1,000 mg/m^2 bid for 14 days every 3 weeks was a fully outpatient, convenient, safe and tolerable oral treatment regimen. There was no drug-drug interaction seen in pharmacokinetic studies. Prolonged clinical benefit was observed in an encouraging 39% of patients with advanced solid malignancies. Two of the three patients with a partial response to treatment had pancreatic cancer. This is the first study to demonstrate feasibility of this combination at doses with proven single agent efficacy in a number of tumors.

The observation of partial response in two pancreatic cancer patients led to a study of the efficacy of everolimus and capecitabine in a cohort of advanced pancreatic patients. **Chapter 7** describes the results of this phase II study. The majority of the 25 treated patients failed prior systemic treatment. Median (range) treatment duration with everolimus was 72 days (16-217). Principal grade 3/4 toxicities were hyperglycemia (36%), hand-foot syndrome (12%), diarrhea (8%), and stomatitis (4%). Prominent grade 1/2 toxicities were stomatitis (60%) and fatigue (44%). Among 19 patients evaluable for response, one (4%) had a partial response and ten (40%) had stable disease for a median of at least 78 days, resulting in a clinical benefit rate of 44%.

In conclusion, personalised medicine is a major novel goal of cancer treatment. The investigations described in this thesis present new ways to tailor anti-cancer treatment.

Nederlandse samenvatting

Geïndividualiseerde geneeskunde is een opkomende praktijk in de geneeskunde. Met hulp van het genetisch profiel en inzicht in variabiliteit in blootstelling (farmacokinetiek; PK) van een individu kunnen beslissingen met betrekking tot de preventie, diagnose en behandeling van de ziekte verbeterd worden. In de kankerzorg wordt een geïndividualiseerde behandeling afgestemd op de genen en eiwitten van de tumor van een individu en de genoemde PK variabiliteit. Deze aanpak zou tot een verbeterde overleving kunnen leiden, onnodige behandeling kunnen voorkomen en tot vermindering van kosten in de gezondheidszorg kunnen leiden. Het potentieel van geïndividualiseerde geneeskunde wordt beperkt door het weliswaar groeiende, maar vooralsnog onvolledige inzicht in de genetische variatie van de tumor een individuele patiënt. Het doel van dit proefschrift is om het inzicht te geven in de genetische factoren die een rol spelen bij de dosis-werkingsrelatie van enkele moleculair gerichte antikanker medicijnen. Deze moleculair gerichte medicijnen, ook wel 'small molecules' genoemd, vormen de basis voor een nieuwe aanpak in de behandeling van kanker de afgelopen jaren.

Farmacogenetica van 'small molecules'

Hoofdstuk 2 geeft een overzicht van de moleculair gerichte antikanker therapieën ook wel tyrosine kinase remmers genoemd. Op dit moment worden deze middelen vooral gebruikt in een standaard of gefixeerde dosis waarbij het inzicht ontbreekt om de dosis te individualiseren. Een standaard dosering kan leiden tot een suboptimale behandeling of het ontstaan van overmatige bijwerkingen. Dit is het gevolg van de grote verschillen in de PK van deze therapieën tussen individuele patiënten. De PK, de bijwerkingen en de werkzaamheid van tien veel gebruikte moleculaire gerichte antikanker therapieën werden beoordeeld met het doel het gebruik ervan te optimaliseren. Een grote variatie in de PK van deze 'small molecules' werd aangetoond. Bovendien is ook de samenhang tussen bepaalde bijwerkingen en de werkzaamheid van de behandeling van sommige medicijnen aangetoond, zoals erlotinib en huiduitslag. Zo wordt huiduitslag als een voorspeller gezien van werkzaamheid. Andere voorspellers (markers) bedoeld als alternatieve voor het meten van de effectiviteit (het zogenaamde klinische eindpunt) zijn beschreven voor sommige 'small molecules' en kunnen nuttig zijn voor de dosisindividualisering. Veelbelovende alternatieven voor het gebruik van standaarddoseringen werden verkend, zoals metingen van de concentratie van middelen in bloed, en het aanpassen van de dosering op basis van erfelijke eigenschappen (genotype), uitingen daarvan (fenotype) en bijwerkingen.

In **hoofdstuk 3** worden de erfelijke eigenschappen (het genetisch profiel) van 77 patiënten met chronische myeloïde leukemie (CML) onderzocht. Dit om de bijwerkingen en de dosisaanpassingen te voorspellen van de behandeling met imatinib, een tyrosine kinase remmer die een hoge kans geeft op een langdurige respons voor patiënten met CML. De eerder gevonden genetische marker voor de transformatie van geneesmiddelen in het lichaam

(CYP3A5) werd bevestigd. Deze marker was voorspellend voor in welke mate een patiënt een hogere dosis van imatinib kan verdragen. De marker kan patiënten, die een hogere dosis imatinib verdragen, mogelijk helpen om de ontwikkeling van een zogenaamde farmacokinetische resistentie tegen imatinib tegen te gaan. In dit onderzoek werden ook een aantal nieuwe relaties tussen enzymen en vervoermiddelen van geneesmiddelen in het lichaam vastgesteld, almede ook een beter inzicht in de verdraagbaarheid van imatinib, bijvoorbeeld ABCC12, een lid van de groep van multi drug-resistentie eiwitten. Door imatinib veroorzaakte bijwerkingen waren gerelateerd aan de variatie van het gen CYP2B6 en aan een glutathion transferase gen (GSTM5). Bepaalde bijwerkingen konden ook in verband worden gebracht met meerdere genen waaronder die van CYP8B1, en een aantal ABC-drug transporters.

Voor sunitinib, een ander 'small molecule' gebruikt bij de behandeling van onder andere niercelkanker, werden factoren onderzocht die betrokken zijn bij de graad van blootstelling aan sunitinib. In het **appendix** worden de voorlopige resultaten van premature farmacogenetische en fenotypische onderzoeken van 52 patiënten beschreven. Het gen voor de ABC-transporter ABCG2 voorspelde het ontstaan van misselijkheid en vermoeidheid. Het gen van het stofwisselings enzym CYP3A5 voorspelde de kans om diarree, hand-voet-syndroom en huiduitslag te ontwikkelen. Hoge bloeddruk, een vaak voorkomende bijwerking van sunitinib, was meer uitgesproken bij patiënten bij wie een bepaalde genetische eigenschap aanwezig was die codeert voor een van de vasculaire endotheliale groeifactoren. Concentraties van sunitinib in het bloed voorspelden de kans op het ontwikkelen van ontsteking van de mondholte, ook een regelmatig voorkomende bijwerking van sunitinib. De activiteit van het enzym CYP3A4, dat sunitinib in de lever afbreekt, kon afgeleid worden uit de snelheid van de afbraak van een lage dosis van een ander middel, midazolam. Uitslag van de midazolam test was voorspellend voor de concentratie van het afbraakproduct van sunitinib (N-desethylsunitinib) bij de start van sunitinib behandeling. Aanvullend genetisch onderzoek is op dit moment in ontwikkeling. Ook de halplotype analyse (genetische patronen) die in deze studie werden verkregen worden op het ogenblik diepgaand onderzocht.

mTOR remming bij de behandeling van kanker

Het 'mammalian target of rapamycine' (mTOR) is een eiwit dat voor het eerst werd ontdekt in cellen van een bepaald soort korstmos dat voorkomt op het Paaseiland (Paaseiland wordt door de lokale bevolking Rapa Nui genoemd). Het bleek snel dat dit eiwit een verantwoordelijk rol heeft voor de celdeling ook in menselijke cellen. In sommige kankersoorten zorgt dit eiwit voor een overmatige celdeling. Remmers van mTOR hebben daarom het potentieel om ook de celdeling in bepaalde kankercellen te remmen. Deze bevinding heeft ertoe geleid dat mTOR-remmers bij de behandeling van kanker worden gebruikt. Een beknopt overzicht van de klinische farmacologie van mTOR-remmers wordt beschreven in **hoofdstuk 4**. In een overzicht worden de farmacokinetische en dynamische eigenschappen van mTOR-remmers everolimus en temsirolimus behandeld. Daarnaast worden er gegevens gepresenteerd over klinisch gebruik, werkings¬mechanisme, bioanalyse, interactie tussen geneesmiddelen, eventuele dosis-aanpassingen bij ziekte of hoge leef-

tijd, farmacogenetica en resistentiemechanismen tegen deze geneesmiddelen. Dit overzicht kan mogelijk de behandelend arts van behulp zijn om de behandeling met mTOR-remmers aan te passen aan de individuele behoefte van de patiënt. Omdat mTOR-remmers slechts een beperkt vermogen hebben om tumoren te verkleinen, is men, in een poging om de effectiviteit van behandeling met mTOR te vergroten, begonnen om de behandeling met mTOR-remmers te combineren met andere antikanker therapieën. mTOR-remmers zijn in staat om de activiteit van Akt, een eiwit dat een belangrijke rol speelt in de intracellulaire signaaloverdracht in kankercellen, te laten toenemen. Dit kan leiden tot een ongewenste toename van celdeling van kankercellen. In het licht van deze bevinding is verder onderzoek naar de combinatie van de mTOR-remmers met Akt-remmers wenselijk. Nelfinavir, een protease-remmer dat wordt gebruikt bij de behandeling van het humaan immunodeficiëntie virus (HIV), is ook een sterke Akt-remmer. **Hoofdstuk 5** toont de resultaten van een 'dose-finding' studie van de combinatie van de mTOR-remmer temsirolimus en de Akt-remmer nelfinavir bij 18 patiënten met verschillende vormen van kanker. Het was haalbaar en veilig om beide geneesmiddelen te combineren in een dosering van nelfinavir 1000 mg/dag en temsirolimus 10 mg/week. Dosis-limiterende bijwerkingen waren slijmvliesontstekingen in de mond en misselijkheid. De combinatie met nelfinavir en temsirolimus leidde ertoe dat de concentratie in het bloed van het actieve afbraakprodukt sirolimus toenam in vergelijking met het van gebruik van temsirolimus alleen. Dit was waarschijnlijk te wijten aan het feit dat nelfinavir ook een sterke CYP3A4-remmer is, waardoor de afbraak van sirolimus geremd werd. Aanvullend translationeel onderzoek werd verricht om potentiële markers te identificeren, die een gunstig effect van deze combinatie behandeling kunnen voorspellen. Dit onderzoek toonde aan dat patiënten die een verhoogde activiteit van mTOR hadden voor aanvang van de behandeling, een verhoogde kans hadden op klinisch voordeel van de behandeling. Patiënten die verhoogde bloedvetten (dyslipidemie) kregen door de medicijnen hadden hogere bloedconcentraties van temsirolimus en sirolimus. Het gebruik van dyslipidemie als marker voor blootstelling aan het geneesmiddel dient nog te worden bevestigd in prospectieve studies.

Een andere veelbelovende groep antikanker medicijnen zijn fluoropyrimidines. Deze groep van anti-kanker medicijnen kunnen de effectiviteit van mTOR remmers doen toenemen. Dit inzicht is gebaseerd op laboratorium onderzoek waaruit blijkt dat de combinatie van deze middelen tot een versterkte activiteit van beide middelen (synergisme) leidt. Een 'dose-finding' studie bij 18 patiënten met verschillende soorten kanker die behandeld werden met everolimus in combinatie met capecitabine (een op fluoropyrimidines gebaseerde vorm van chemotherapie), beide in tabletvorm beschikbaar, wordt gepresenteerd in **hoofdstuk 6**. Everolimus 10 mg/dag continu in combinatie met capecitabine 1000 mg/m^2 twee keer per dag gedurende 14 dagen, in een cyclus van 3 weken, bleek een patiëntvriendelijke, veilige en qua bijwerkingen aanvaardbare orale behandeling te zijn. In farmacokinetische studies werd vastgesteld dat deze middelen elkaars afbraak niet beïnvloeden Langdurig klinisch voordeel werd waargenomen bij een bemoedigende 39% van de patiënten. Twee van de drie patiënten met een forse afname van kanker op de behandeling hadden alvleesklierkanker. Ten opzichte van een eerdere Japanse studie is dit de eerste studie die de haalbaarheid van deze combinatie aantoont in een dosering die ook als monotherapie een bewezen werkzaamheid heeft in een aantal tumoren.

De waarneming van anti-kanker activiteit bij de genoemde twee alvleesklierkanker-patiënten, heeft tot de beslissing geleid om de werkzaamheid van everolimus en capecitabine verder te onderzoeken in een grotere groep van patiënten met alvleesklierkanker. **Hoofdstuk 7** illustreert de resultaten van deze fase II studie. Het merendeel van de 25 behandelde patiënten had toename van ziekte op eerdere systemische behandeling. De mediane duur van de behandeling met everolimus was 72 dagen (minimaal 16 en maximaal 217dagen). De belangrijkste ernstige bijwerkingen waren verhoging van het suikergehalte (36%), pijnlijke handen en voeten (hand-voet syndroom) (12%), diarree (8%) en ontstekingen van de mondholte (4%). Ook minder ernstige bijwerkingen waren ontstekingen van de mondholte (60%) en ver¬moeidheid (44%). Een patiënt had een duidelijke afname van kanker (4% partiële respons) en tien (40%) hadden een stabiele ziekte gedurende mediaan 2,7 maanden, resulterend in een klinisch voordeel van 44% voor de hele groep van patiënten.

Samenvattend is individualisatie van de behandeling van kanker een belangrijk nieuw doel in de geneeskunde. Het onderzoek beschreven in dit proefschrift presenteert nieuwe markers voor deze op maat toegesneden antikanker behandeling.

Acknowledgment

A few years ago I embarked on a wondrous journey. It started with a working trip to Australia and ends here with this book, the result of my PhD research. On this journey, that lead me to new horizons and insights and across all the highs and lows that seem to be part of a getting a PhD, I have received tremendous support and encouragement from many, many people. Without their help, this book would have been impossibly hard to finish, and without their commitment, dedication and indeed love, my journey would have been so much less inspiring. I thank all these generous people from the bottom of my heart for everything they have given. Without discounting anybody, there are some people I would like to mention specifically.

First, my appreciation goes towards the many patients who voluntarily participated in all the clinical trials performed for this PhD. For certain studies, many generously gave their time and energy for the research purposes only, knowing there would be no personal benefit. For this I am greatly thankful. I would like to thank my supervisors Prof. Howard Gurney, Prof. Jan Schellens, Prof. Dick Richel and Anneke Westermann for their continual guidance and support during the long process of research and writing.

Dear Howard, pragmatic approach and your optimism, with the focus on seeking for solutions to difficult situations, not only helped me in the darker days, but also changed the way I now perceive and handle complications. You gave me the feeling that you had faith in me to manage whatever new situation I got into. Your faith inspired me to develop creativity. With your support I was able to learn, grow and develop skills I never thought I had. I admire your helicopter-view in the confusing and rapidly changing field of medical oncology. Your visionary ideas about the future of cancer care have motivated me in many ways. Your warm welcome, including that of your family and your team at the hospital, was a gift I still cherish. I have great memories of our time in Australia.

Dear Jan, without your confidence in this “long distance” project (to be a supervisor of a PhD student more than 16.000 km away in a totally different time zone) this book would have never become reality. Your clear guidance was of great help in the perplexing world of pharmacokinetics and genomics. It still is a mystery to me how you are able to achieve so many things in your live and still remain calm and patient with all my shortcomings. Your clear expectations kept me focused and helped me persist with my thesis, also at night, after a long day at the hospital, or in the weekends. Thank you for your constructive and critical comments on the articles.

Dear Dick, even though you joined this PhD project in a later phase, you are the true origin of this whole project. You approved my application to be a fellow in medical oncology despite my clear statement, I was not at all interested in doing research (and at that time I was convinced I wasn’t). That unreserved acceptance of my personality helped me change

my attitude and as a result I participated in a few small research projects without angst. With my participation in the Flims workshop my doubts about running clinical trials evaporated. Subsequently, you supported my plan to leave for a research position in Sydney, Australia. I still remember your words "I wish I would have done that when I was your age". And I am so happy you made it possible for me to do it at my age.

The first two and a half years I spent on this thesis I was employed at the department of Medical Oncology at the Westmead Hospital in Australia (since November 2011 called Crown Princess Mary Cancer Center). Patricia Quarles van Ufford kindly recommended me for that position in Sydney.

My first Sydney desk was in the office of the clinical nurse consultants Eucharia, Leanne, Dianne and Louise. You ladies are an asset to any oncology team. Louise, I still regard you as my first and only personal nurse. You made my life so much easier. The continual support of Jodie, Sue-Ellen, Vicky and all the others at the of the clinical trial unit was much appreciated. Many thanks to my always warm, kind and highly professional colleague medical oncology consultants Rick Kefford, Nicholas Wilken (thanks for letting us use your home during our first weeks in scorching Sydney), Mark Wong, Rina Hui, Kylie Passage, Paul Harnett and the great admin support by Jenny Butler, Kylie and many more. Thanks to the many registrars who worked with me, especially Bo Gao and Phuoung Dinh and Shang Yeap. Bavanthi Balakrishnar took over the trial coordination. Even though we never met, you were of great help. Heleen Grootsjans, the visiting student from the University of Groningen, helped to get the CLEARSUN study up and running. Thanks also to the team of the Pharmacy at Westmead especially Deirdre D'Sauza and Sonia Cuan.

Many credits to the Hematology team at Westmead, particularly Angela Bailey, research nurse, and the always cooperative consultants David Gottlieb, Mark Herzberg, Ian Kerridge and Sally Greenaway.

Val Gebski always had an open ear and a big smile for me. I received lots of inspiration from Rosemary Ballain (Translational research), Chris Liddle's team from Clinical Pharmacology, Caroline Samer and Vijay Suppiah. Laboratory- and logistical support was generously provided by Greg Kaplan, Kumar Savrabh and Linda Frost at the Westmead Millenium Institute. I am very thankful to David Farlow and Brad Camden of the department of Nuclear Medicine at Westmead Hospital for their support. And last but not least: I still miss the true "family-style" outpatient clinic each Thursday with Sandra Turner and Meg Hughes (and her delicious cakes).

Special appreciation to the colleagues at the collaborating hospitals in several cities throughout Australia including: David Goldstein and his research nurse Cristie Norris at the Prince of Wales Hospital in Sydney. Thony Schwarer and Jenny Muirhead at The Alfred Hospital in Melbourne. Jeff Szer at the Royal Melbourne and his team, especially Rosemary Hoyt. John Seymour at the Peter MacCallum Cancer Centre and his team. Timothy Hughes and Deb White at The Royal Adelaide Hospital. Desmond Yip at the Canberra Hospital. Tony Mills at the Princess Alexandra Hospital in Brisbane. Kerry Taylor and Jenny Larson

at the Mater Hospital in Brisbane. Special thanks to Rachel Koelemeyer, who is doing great work as the clinical trials manager of the Australian Leukemia & Lymphoma Group located at the Peter MacCallum Cancer Centre in Melbourne.

Not only do I want to thanks my colleagues in Australia, but also to the many good friends we made during our stay. They made it so easy for us to love the time we had in Sydney (and Melbourne of course).

The second part of this thesis was finalized in the Netherlands at the Academic Medical Center of the University of Amsterdam.

Daarom voor mijn fijne collega's nu in het Nederlands. Natuurlijk ga ik beginnen met Anneke Westermann. Zij heeft mij vanaf mijn eerste dag dat ik in Nederland woon en werk gesteund, niet zonder constructieve kritiek natuurlijk. Daarom was ik ook heel blij dat zij mijn co-promotor werd. Van alle collega's heeft Mariëtte Weterman ontzettend veel tijd en inspanning geleverd voor dit promotie onderzoek. Fijn dat je er bent, enthousiast, kritisch, altijd duidelijk maar ook gul in het geven van een "hug". Mijn dank is ook groot voor de stille krachten die zoveel werk verzetten zoals Eva van Daalen, Lyda ter Hofstede, Arnaud Stevens en Marga Boerrigter. Om alles soepel te laten verlopen op de poli en dagbehandeling maar ook op F6 heeft een hele ploeg aan medewerkers geholpen aan de balie, aan bed en in de planning. Zonder iedereen apart te noemen bedank ik allen hiervoor. Wees verzekerd dat ik jullie hulp nooit voor vanzelfsprekend heb genomen.

Terwijl ik druk met dit manuscript bezig was hebben mijn collega's Hanneke Wilmink, Sander Dalhuisen en Anneke Westermann extreem collegiaal mijn taken overgenomen als dit nodig was. Dat was uitermate genereus en hartelijk van jullie. Hanneke, ik ben onder de indruk van de rust en de lichtheid van je handelen. Het lijkt als of jij alles zo uit jouw mouw schudt. Het afronden van de klinische studies was zeker niet zo snel gelukt als niet ook onze arts-assisten meegeholpen hadden. Hiervoor mijn dank aan Dirkje Sommijer, Hans van Thienen, Annet van Zweeden, Annete Coumou, Serge Dohmen, Noortje Schoenmaker, Nadia Haj Mohammad, Diederik ten Oever en Susan Vrijaldenhoven. Ook dank voor de fijne samenwerking met de apotheek, in bijzonder met Marleen Kemper en Jan Toersche. Met verbazing en bewondering kijk ik naar het werk van Koos Zwinderman, die mij ook onder tijdsdruk altijd geduldig heeft gesteund met al het statistische werk. Ik ben ook bijzonder dankbaar voor de bereidwilligheid van Roel Bennink van de afdeling Nucleaire Geneeskunde in het AMC om altijd met enthousiasme zich in te zetten voor wetenschappelijk onderzoek.

Zeer erkentelijk ben ik ook jegens mijn collega's in samenwerkende instituten, zoals het Slotervaart Ziekenhuis in Amsterdam, waar ik snel en adequaat geholpen werd door Wiete Kromdijk, Alwin Huitema, Maarten Deenen en Jos Beijnen. Ook dank aan Jolanda Slijkermans voor de altijd vriendelijke en snelle acties om de geografische afstanden tussen Jan Schellens en mij moeiteloos te overbruggen.

Het andere invloedrijke instituut is het Erasmus Medisch Centrum in Rotterdam waarmee ik vanuit Sydney al de eerste contacten had gelegd. Er is een vruchtbare samenwerking ontstaan met de inzet van Ron Matthijssen, Karel Eechoute, Jaap Verweij en Jacqueline Kloth en velen meer.

Heel fijn dat mijn goede vrienden Myra en Christiaan mijn paranimfen zijn. Van hen en van heel veel andere lieve vrienden heb ik tijdens mijn promotiejaren heel veel steun, advies en aanmoediging gekregen: dankjewel Remco, Ben, Kees, Eddo, Elisabeth, Peter, Marc, Tim, Bert en Truus en alle anderen, die mij altijd met een goede raad, opmonterende woorden en interesse in het onderzoek hielpen door te zetten.

Nicht zuletzt mein Dank an meine Familie. Eure Geduld mit mir, um wieder nicht zu Geburtstagen, Weihnachten und anderen Familienfesten kommen konnten war sehr nett von Euch. Lieber Josef und liebe Ursula, Eure Fürsorge und Liebe hat mir das Fundament gegeben um genug Energie und Durchsetzungsvermögen zu entwickeln diese Arbeit erfolgreich abzuschliessen, neben meiner Arbeit als Arzt in der Klinik. Schön das Ihr in Australien ward um ein Teil unseres Lebens dort mit zu bekommen. Danke auch an meine Geschwister und deren Familien, für Eure Nachsicht und die Ausdauer mit mir Kontakt zu halten.

Maar niet in woorden te vatten is mijn dank aan mijn echtgenoot Maarten, die met eindeloos geduld en liefde mij in dit proces begeleid (en verdragen) heeft. Zonder jou was dit boekje nooit ontstaan.

Curriculum Vitae

Curriculum Vitae (Nederlands)

Heinz-Josef Klümpen werd geboren op 5 juni 1969 te Sevelen (Duitsland). In 1988 behaalde hij het eindexamen aan het Friedrich Spee Gymnasium te Geldern (Duitsland). Zijn vervangende dienstplicht vervulde hij in het bacteriologische laboratorium van het St. Clemens ziekenhuis te Geldern. De In 1990 begon hij de studie geneeskunde aan de Heinrich-Heine Universiteit te Düsseldorf. Zijn artsenexamen legde hij in 1996 met succes af. Tijdens zijn studie volgde hij coschappen neurologie zowel in New-York aan het Mount-Sinai Hospital en in London aan het National Hospital for Neurology and Neurosurgery. Een extra-curriculaire klinische studie op het gebied van Neuro-AIDS deed hij aan de afdeling neurologie van de universiteits kliniek Düsseldorf. Het pad van de medische oncologie sloeg hij in nadat hij in 1997 als basisarts in het Antoni van Leeuwenhoek Ziekenhuis te Amsterdam begon te werken. Van 1998 tot 2005 volgde hij de opleiding tot internist-oncoloog aan de Universiteit van Utrecht en Universiteit van Amsterdam. In 2006 ontving hij van de Universiteit van Sydney (Australië) een beurs als Clinical Fellow Medical Oncology. In Sydney, waar hij tot 2008 verbleef als medisch oncoloog, zette hij onder de supervisie van prof. H. Gurney verschillende klinische studies op. In deze periode werd het in dit proefschrift beschreven onderzoek uitgevoerd met als Nederlandse promotor prof. dr. J.H.M. Schellens. Vanaf 2008 werd het tweede deel van dit onderzoek voortgezet aan het Academisch Medisch Centrum te Amsterdam met prof. dr. D.J. Richel als tweede promotor en dr. A.M. Westermann als co-promotor.

Curriculum Vitae (English)

Heinz-Josef Klümpen was born on June 5th, 1969 in Sevelen (Germany). In 1988 he completed his final exam at the Friedrich Spee Gymnasium in Geldern (Germany). His community service was fulfilled at the bacteriological laboratory of the St. Clement hospital Geldern. From 1990 to 1996 he studied medicine at the Heinrich-Heine University in Düsseldorf. During his studies he took two neurology internships, one at the Mount Sinai Hospital in New York, the other at the National Hospital for Neurology and Neurosurgery in London. Extra-curricular clinical studies, in the field of Neuro-AIDS at the department of neurology of the University of Düsseldorf, were the first experiences in the field of clinical research. His interest in medical oncology was raised when in 1997 he worded as an MD at the Antoni van Leeuwenhoek Hospital (specialized in cancer care) in Amsterdam. This prompted him to undergo training for internal medicine and medical oncology, which he did starting in 1998, at both the University of Utrecht and the University of Amsterdam. In 2005, after successfully completing his training, the University of Sydney (Australia) offered him a position as Medical Oncologist. There he initiated clinical trials under the supervision of Prof. H. Gurney. This research, which was carried out under the Dutch supervision of Prof. JHM Schellens, eventually lead to this PhD thesis. From 2008 until 2011, the second part of the research for this thesis was continued at the Academic Medical Centre in Amsterdam under co-supervision of Prof. Dr. D.J. Richel and with Dr. A.M. Westermann as a co-promotor.

List of publications

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