Pharmacokinetics and pharmacogenetics of tacrolimus in renal transplant patients

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Pharmacokinetics and
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Pharmacokinetics and pharmacogenetics of tacrolimus in renal transplant patients

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ter verkrijging van de graad van doctor
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Chapter 1

Outline and aim of the thesis

Outline and aim of the thesis

A number of enzymes in humans are capable of biotransforming lipid-soluble xenobiotics in such a way as to render them more water soluble. There are two types of enzyme reactions: phase I and phase II type reactions. Phase I type reactions are involved in hydrolysis, oxidation and reduction reactions, whereas phase II type reactions have been referred to as conjugative reactions which include glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione (mercapturic acid synthesis), or with amino acids (e.g., glycine, taurine and glutamic acid). Although it is well known that not all conjugation reactions are secondary phase II reactions since many endobiotics (e.g. bilirubin, steroids, retinoids, and bile acids) and xenobiotics (e.g. acetaminophen, morphine, and oxazepam) are conjugated without prior conversion to products generated from oxidative, reductive or hydrolytic enzymes. Conjugative biotransformation results in an increase in the hydrophilicity of a compound which promotes its excretion. The balance between the cytochrome P450 and conjugative drug biotransforming enzymes is responsible for either the detoxification or the accumulation of toxic metabolites in the body. The calcineurin inhibitor tacrolimus has highly variable pharmacokinetic characteristics and therefore close therapeutic drug monitoring is required especially to prevent subtherapeutic and toxic blood levels. Sub-therapeutic drug levels increase the risk of rejection significantly while toxic drug levels cause serious side effects, as described in CHAPTER 2. Additionally, this CHAPTER includes a complete review of the pharmacokinetics and pharmacogenetics of tacrolimus. Although several commercial DNA isolation kits are available to extract deoxyribonucleic acid (DNA) from whole blood samples, less information is available regarding the extraction efficiency and the quality of the isolated DNA. CHAPTER 3 presents a study that compares three commercially available DNA isolation kits regarding their ability to extract DNA from whole blood samples as well as the DNA quality.

In CHAPTER 4 a locked nucleotide acid (LNA) on the polymorphic position in the sensor probe is introduced. By using a LNA on the polymorphic position, a better discrimination between the wild type allele and the variant allele is possible. The influence of a LNA in a FRET probe and its consequence on the melting curves is described in CHAPTER 4. Due to the introduction of real-time PCR technology it is possible to detect single nucleotide polymorphisms (SNPs) faster, with less contamination risk and less hands on time than previously used PCR methods, like PCR restriction fragment length polymorphism (RFLP) and PCR amplification refractory mutation system (ARMS). CHAPTER 5 reports new real-time PCR fluorescence resonance energy transfer (FRET) assays for SNPs which are compared with PCR RFLP and PCR ARMS assays. CHAPTER 6 evaluates 24 limited sampling strategies for tacrolimus that have been published by other research groups in an independent renal transplant recipient group. This comprehensive study demonstrates that a limited sampling strategy using two (C_2 , C_4) or three (C_0 , C_2 , C_4) sample time points are a better indication for the tacrolimus

exposure than the trough (C_0) levels alone. In most transplant centres monitoring of the tacrolimus blood concentration is performed by C_0 concentrations after the morning tacrolimus administration. However, conflicting evidence is present regarding the usefulness of these morning tacrolimus C_0 concentrations. Knowing this, several studies recently developed a limited sampling strategy for tacrolimus using stepwise multiple regression analysis or Bayesian fitting to predict the AUC_{0-12} as accurate as possible, since the AUC_{0-12} is considered to be the gold standard of measuring the total tacrolimus exposure. Most of these studies that developed a limited sampling strategy for tacrolimus used no independent transplant patient population to validate their limited sampling strategies. Several studies reported an association between the ABCB1 polymorphisms C1236T, G2677T/A and C3435T or ABCB1 haplotypes and the tacrolimus C_0 levels or the pharmacokinetic parameters of tacrolimus in different transplantation patients, while other studies found no correlation between the ABCB1 polymorphisms and the tacrolimus blood levels. In CHAPTER 7 and 8 the influence of CYP3A and ABCB1 polymorphisms is studied in a Caucasian and a Chinese renal transplant recipient group while CHAPTER 9 examines the presence of the CYP3A7*1C variant allele in Chinese and Caucasian renal transplant recipients and investigates the association between the CYP3A7*1C variant allele and the pharmacokinetic tacrolimus parameters. Previously, it was demonstrated that UGT2B7 is the most important member of the UGT family involved in the tacrolimus glucuronidation although the influence of the recently identified UGT2B7 polymorphisms on the tacrolimus blood levels was never reported. CHAPTER 10 reports the contribution of three UGT2B7 polymorphisms on the tacrolimus blood levels in a Chinese renal transplant recipient group. The nuclear receptor pregnane X receptor (PXR) activates transcription as a response to a diversity of natural and synthetic compounds. Recently, it was demonstrated that eight SNPs present in the untranslated and intron regions of the PXR gene showed a significant difference or trend in the erythromycin breath test, in the CYP450 3A located in the human hepatocytes or intestines and in the ABCB1 expression in the intestines after induction with rifampicin. The role of PXR polymorphisms in correlation with a limited sampling strategy for tacrolimus is examined in a Chinese renal transplant recipient group as described in CHAPTER 11. Since it is also known that corticosteroids can induce the CYP3A enzymes, these PXR polymorphisms may also play a role in the tacrolimus metabolism, especially in the early transplant period when transplant patients receive high daily doses of corticosteroids. In CHAPTER 12 the influence of corticosteroids on the contribution of the PXR polymorphism demonstrates that the role of CYP3A enzymes may be significantly influenced by the combination of an induced PXR gene and the presence of PXR polymorphisms. The corticosteroids activate the PXR gene which as a result induce the CYP3A enzymes that lead to lower tacrolimus blood levels. CHAPTER 13 presents some general conclusions and comments on the obtained results. Future perspectives and recommendations on the different pharmacokinetic and pharmacogenetic

approaches used nowadays and possibly in the (near) future and its consequences for transplantation patients in daily practice are also discussed.

This thesis includes three important aims which have been studied:

- I A comparison and validation of limited sampling strategies for tacrolimus previously published, which may provide a better indication of the tacrolimus exposure in different transplant patients (CHAPTER 6).
- II The introduction of real-time PCR FRET technology that makes genotyping of several SNPs present in different genes possible in a shorter analytical time, with less hands on time, and with less contamination risk compared to the conventional PCR methods (CHAPTER 3, 4 and 5).
- III Knowledge on the presence of SNPs in the phase I and phase II enzymes, in transporters and nuclear receptors in the tacrolimus metabolism may contribute to decrease the high inter-individual pharmacokinetic variation in the tacrolimus blood levels of Caucasian and Chinese renal transplant patients (CHAPTER 7, 8, 9, 10, 11 and 12).

CHAPTER 2

Pharmacokinetics and pharmacogenomics of tacrolimus; a review

Submitted in an adapted form

1 Introduction

Tacrolimus (FK506) is a 23-membered macrolide lactone ($C_{44}H_{69}NO_{12}$; for molecular structure see Figure 2.1) which is isolated from the fermentation broth of *Streptomyces tsukubaensis*^{1,2}. Additionally, tacrolimus like cyclosporin is an immunosuppressive agent belonging to the calcineurin inhibitor group that has emerged as a valuable therapeutic alternative to cyclosporine following solid organ transplantation³. It is highly effective at preventing rejection in heart⁴⁻⁷, small bowel^{8,9}, pancreas¹⁰⁻¹⁴, bone marrow¹⁰⁻¹⁴, lung¹⁵⁻¹⁹, liver²⁰⁻²⁴, and kidney²⁵⁻²⁸ recipients and for the therapy of autoimmune diseases^{29,30}.

Figure 2.1 Structure of tacrolimus.

Although there are structural differences, cyclosporin and tacrolimus share a similar cellular mechanism of action, though tacrolimus, is 10 to 100 times more potent at the molecular level³¹. After entry into the cell, both agents bind to their respective cytosolic immunophilins: cyclosporine to cyclophilin and tacrolimus to the FK506-binding proteins FKBP-12 and FKBP-52, a component of the glucocorticoid receptor complex. Immunophilins are a family of highly conserved proteins that likely participate in protein folding. The drug-immunophilin complex binds to and inhibits the activity of the enzyme calcineurin, a calcium/calmodulin-dependent protein phosphatase that is expressed in all mammalian tissues. As a result, the complex interrupts the calcium-dependent signal transduction pathway in T-cells. Inhibition of calcineurin by cyclosporin or tacrolimus leads to interference with translocation to the nucleus of various nuclear factors involved in the transcription of cytokine genes, such as the cytosolic subunit of the nuclear factor of activated T-cells (NF-ATc). It also antagonizes the interaction of the transcription factor, cyclic adenosine monophosphate (cAMP)-response element binding protein (CREB), with its putative deoxyribonucleic acid (DNA) binding site, which in turn inhibits

cAMP-directed transcriptional events. As a result of calcineurin inhibition, the transcription of early T-cell activation genes is suppressed, affecting the production of interleukin-2 (IL-2) and many other cytokines, such as interleukin-3 (IL-3), interferon- γ (INF- γ), and tumor necrosis factor- α (TNF- α). Figure 2.2 illustrates a simplified model of the intracellular mode of action of tacrolimus and cyclosporine.

Figure 2.2 Intracellular mode of action of tacrolimus and cyclosporine. Adapted from LC Paul. Mechanistic differences of cornerstone immunosuppressants. ISBN 1-898729-15-8.

2 Absorption, distribution, metabolism and elimination

2.1 Absorption

Tacrolimus, orally administrated, has a large variability in the rate of absorption and absolute bioavailability which implies that the mean bioavailability is approximately 25% but can range from 5% to 93%^{32,33}. In general oral doses of tacrolimus should be 3 to 4 times higher than intravenous doses to achieve comparable drug exposure after oral and intravenous administration. A reduced bioavailability has been reported in patients awaiting renal transplantation³⁴, in small bowel recipients with open stomas³⁵, in African American and non-Caucasian individuals³⁶⁻⁴⁰, in diabetic patients^{41,42}, and following administration of food with a moderate fat content⁴³⁻⁴⁵. However, the bioavailability is

similar for paediatric and adult transplant patients⁴². In most patients tacrolimus is absorbed rapidly with peak plasma/blood concentrations obtained in 0.5-1 hour³² however a lag time of 0 to 2 hours has also been reported in some liver transplant recipients³⁷ or an extended lag time or secondary peaks^{34,46,47}.

The shape of the plasma concentration-time profile in some patients (sharp peaks), and higher dose-normalised maximum blood concentration at lower doses is seen in some patients who received different doses, are suggestive of the involvement of a zero order/saturable process in the absorption of tacrolimus³⁸. The poor aqueous solubility of tacrolimus and alterations in gut motility in transplant patients may be partially responsible for poor and erratic drug uptake. Presystemic metabolism of tacrolimus by gastrointestinal cytochrome P450 (CYP) 3A iso-enzymes and removal by P-glycoprotein transport is extensive⁴⁸⁻⁵⁰. P-glycoprotein lowers the intracellular concentration of tacrolimus by pumping absorbed drug back out into the intestinal lumen. P-glycoprotein may also regulate access of tacrolimus to CYP3A enzymes and prevents these enzymes from being overwhelmed by high drug concentrations in the intestine^{51,52}. As tacrolimus is repeatedly transported out of the intestinal mucosa cells and then passively reabsorbed, this continuous repeated exposure should lead to more efficient metabolism^{52,53}. P-glycoprotein belongs to a family of adenosine triphosphate-binding cassette transporters with considerable overlap in substrate specificity. Bile is not essential for tacrolimus absorption⁵⁴⁻⁵⁶. Simultaneous administration of enteral feeds does not appear to interfere with tacrolimus bioavailability. Contrasting studies appeared regarding the role of ABCB1 polymorphisms in the tacrolimus metabolism process. A number of studies⁵⁷⁻⁶⁵ found a significant association between the ABCB1 polymorphism C3435T or the ABCB1 haplotype consisting of the polymorphisms C1236T, G2677T/A and C3435T and tacrolimus C_0 concentrations or pharmacokinetic parameters, while other studies⁶⁶⁻⁷⁶ found no significant correlation between these parameters. A large diversity of transplant recipient populations were used to examine this ABCB1 genotype tacrolimus blood concentration correlation which may explain the difference in the results observed. Especially, when the role of these ABCB1 polymorphisms is minor compared to the CYP3A polymorphisms.

2.2 Distribution

Transplant recipients have significantly higher blood tacrolimus concentrations (mean 15 times; range 4 to 114 times) than the corresponding plasma concentrations due to the extensive binding of tacrolimus to the red blood cells^{46,54,77-81}. Venkataramanan *et al.*⁵⁴ reported that the maximum amount of tacrolimus bound to red blood cells ($B_{\max}$) is $418 \pm 258 \mu\text{g/l}$ with an apparent dissociation constant (K_D) of $3.8 \pm 4.7 \mu\text{g/l}$ in transplant patients while $B_{\max}$ is $1127 \mu\text{g/l}$ and K_D of $13.5 \mu\text{g/l}$ in healthy volunteers. The diffusion of tacrolimus from erythrocytes is slow in comparison with the transit time of blood through an organ, but tacrolimus is readily released from the erythrocytes⁸²⁻⁸⁴, and the binding of tacrolimus to erythrocytes may in part protect it from hepatic metabolism⁸⁵. An

intracellular protein in erythrocytes, with a molecular weight range (14 to 15 kDa) corresponding to FKBP⁸², or a molecular weight of 11 to 12 kDa⁸⁶ appears to be primarily responsible for binding tacrolimus. Tacrolimus does not bind to haemoglobin. When the tacrolimus concentration in whole blood increases, the uptake of tacrolimus by erythrocytes is saturated, resulting in a lower blood *versus* plasma ratio^{81,82,84,86}. Approximately 99% of tacrolimus in plasma is bound, with uptake not saturable at physiological drug concentrations⁸⁷⁻⁸⁹. Additionally, tacrolimus is principally associated with α_1 -acid glycoprotein, lipoproteins, globulins and albumin. Partitioning of tacrolimus between erythrocytes and plasma *ex vivo* is dependent on the haematocrit, tacrolimus concentration, temperature of the sample and plasma protein concentration^{87,90-92}. Tacrolimus passes through the placenta and into the breast milk^{78,93,94}. In one study⁹⁴, 21 female liver transplant recipients treated with tacrolimus before and throughout gestation, the mean tacrolimus concentrations ($\mu\text{g/l}$) on the day of delivery were 4.3 in the placenta *versus* 1.5, 0.7 and 0.5 in the maternal, cord and child plasma, and 0.6 in the first breast milk specimens. In paediatric liver recipients, a volume of distribution up to 1.8 times higher than in adult recipients has been reported⁹⁵. Possible reasons for this include increased membrane permeability of red blood cells and reduced amounts and affinity of plasma binding proteins in infants, enhancing drug entry into some compartments⁹⁶.

2.3 Metabolism

Tacrolimus undergoes O-demethylation, hydroxylation and/or oxidative metabolic reactions, predominantly by CYP3A4 and CYP3A5 in the liver and intestinal wall, with <0.5% of the parent drug appearing unchanged in the urine or faeces^{88,90,93,97-108}. Several metabolites are the product of a two-step reaction: oxidation by cytochrome P450 3A enzymes destabilising the macrolide ring and followed by its rearrangement^{92,102,105,109}. Schöler *et al.*¹⁰⁹ detected seven different isomers of 13-O-demethyl-tacrolimus by using two-dimensional homo- and heteronuclear magnetic resonance experiments. Figure 2.3 illustrates the major metabolic pathways of tacrolimus. Several other metabolites have been isolated, the structures of which have only partially been identified using mass spectrometry³². The CYP3A subfamily composed of four genes, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, and three pseudogenes CYP3AP1, CYP3AP2 and CYP3AP3, consists of 231 kb and is located on chromosome 7q21-q22.1⁵⁶. The first human CYP3A to be identified was CYP3A4, which was independently cloned in two separate laboratories in 1986^{110,111}. Another CYP3A member CYP3A5 was subsequently purified from liver¹¹² and the corresponding complementary DNA (cDNA) was cloned in two separate laboratories^{113,114}. A third CYP3A family member, CYP3A7, which is expressed at high levels in fetal liver, was also identified^{112,115} and cloned by Komori *et al.*¹¹⁶. More recently cDNA clones for a fourth CYP3A member, CYP3A43, have been isolated from the liver¹¹⁷⁻¹¹⁹.

Figure 2.3 The reactions involved in the tacrolimus metabolism are cytochrome P450 3A-mediated hydroxylation, demethylation and oxidation. The proposed metabolite structures and metabolic pathways are based on Iwasaki *et al.*^{87,92}. The metabolites are classified as first and second generation metabolites. First generation metabolites are those directly derived from tacrolimus and are changed in one position. Metabolic changes in certain positions such as 13-O-demethyltacrolimus, lead to secondary non-enzymatic rearrangement of the macrolide ring resulting in several isomers. This figure is derived from an illustration of a publication of Christians *et al.*²⁸⁸ and used in this thesis with permission of the publisher.

2.4 Elimination

The metabolites of tacrolimus are for more than 95% eliminated by the biliary route⁹⁷. Urinary excretion accounts for, on average, 2.4% of tacrolimus elimination. Biliary

obstruction is reported to increase the concentration of tacrolimus metabolites in the blood^{120,121}.

3 Pharmacokinetics

3.1 Genetic factors affecting the pharmacokinetics

3.1.1 *Cytochrome P450 (CYP450) 3A4*

The CYP3A4 gene is encoded by a 27 kb sequence on chromosome 7q21.3-q22.1 and spans 13 exons^{117,122,123}. CYP3A4 consists of 502 amino acids with a molecular weight of 57 kDa¹²⁴. CYP3A4 accounts for approximately 30% of the total cytochrome P450 activity in the liver and 70% of the cytochrome P450 activity in the small intestines^{125,126}. Since so much of the human cytochrome P450 activity is due to CYP3A4, it comes as no surprise that CYP3A4 performs the bulk of oxidative drug metabolism in humans. Therefore, the list of medicines that are metabolized by CYP3A4 is still growing and CYP3A4 may be involved in the metabolism of about 50% of all clinically used drugs. CYP3A4 is responsible for the hydroxylation, demethylation, and dealkylation of several endogenous and exogenous compounds. Due to the multiple enzymatic sites on the CYP3A4 molecule, CYP3A4 is able to affect so many compounds through multiple enzymatic steps. CYP3A4 is also an important enzyme in the metabolism of endogenous steroids, cholesterol and lipids through hydroxylation. Children and young adults seem to have more CYP3A4 function than do adults and this enzyme may be more active in women than in men, but studies of age and gender differences with regard to CYP3A4 have yielded conflicting results¹²⁷. CYP3A4 has a vast amount of metabolic activity, and it is often thought of as a high capacity/low affinity enzyme as compared with the other cytochrome P450 iso-enzymes, particularly CYP2D6, CYP2C9 and CYP2C19 which are low capacity/high affinity enzymes. CYP3A4 may function as “sink” where many drugs go to be metabolized at relatively high levels. At low levels, the low capacity/high affinity enzymes perform more of the enzymatic activity. If CYP3A4 is inhibited, the drugs may spill over to the other enzymes, but because these enzymes are low capacity, serum levels of the drugs may increase significantly and lead to toxicity¹²⁸. Drugs with evidence of effective competitive or non-competitive CYP3A4 inhibition include the azole anti-fungals itraconazole¹²⁹ and ketoconazole¹³⁰, diltiazem¹³¹, the macrolide antibiotics clarithromycin, erythromycin¹³², and troleandomycin¹³³, norfluoxetine, nefazodone¹²⁹, the quinolone antibiotics ciprofloxacin and norfloxacin¹³⁴, quinupristin/dalfopristin¹³⁵, and the protease inhibitors indinavir and ritonavir¹³⁶. Ketoconazole and ritonavir seem to be the most potent of all CYP3A4 inhibitors. A moderate CYP3A4 inhibitor is a chemical (or chemicals) in grapefruit and grapefruit juice^{137,138}. The action of this chemical (or these chemicals) is probably through inhibition of the gut wall CYP3A4, although there is some evidence that the

effect may be due to P-glycoprotein¹³⁹. However, regardless of the mechanism, when a second drug is ingested after grapefruit juice has been drunk, serum levels of the second drug increase if it is a substrate of CYP3A4 or P-glycoprotein. The strongest known inducer of CYP3A4 is carbamazepine and its mechanism is described by Levy *et al.*¹⁴⁰. Carbamazepine induces CYP3A4 and the drug is also metabolised by CYP3A4 however, carbamazepine induces not only CYP3A4 but also phase II conjugation enzymes¹⁴¹. Other anti-epileptics such as phenytoin, phenobarbital and primidone¹⁴² also induce CYP3A4. Additionally, there is evidence that rifampicin and rifabutin¹⁴³, nevirapine and efavirenz^{144,145}, troglitazone¹⁴⁶, dexamethasone and prednisone¹⁴⁷ all induce CYP3A4. In addition, St. John's wort is a popular herbal product used to treat depressions and thought to be implicated in drug interactions involving CYP3A4^{148,149} and/or P-glycoprotein¹⁵⁰. Markowitz *et al.*¹⁵¹ found that a 14-day course of St. John's wort administration significantly induced the activity of CYP3A4 as measured by changes in the pharmacokinetics of alprazolam. This suggested that long-term administration of St. John's wort may result in diminished clinical effectiveness or increased dosage requirement for all CYP3A4 substrates. Use of St John's wort has led to decreased cyclosporine levels, ultimately causing transplantation rejection¹⁵²⁻¹⁵⁴. St John's wort has also been shown to decrease digoxin levels¹⁵⁵. Given these reports, St John's wort may affect numerous other drugs and make them less effective. Unlike other cytochrome P450 iso-enzymes CYP3A4 is capable of being stimulated. Stimulation is similar to induction in that enhanced CYP3A4 activity occurs. It differs since it happens immediately while induction is delayed by 1 to 3 weeks. Stimulation is termed homotropic if performed by a substrate of CYP3A4 and heterotropic if performed by other effectors or drugs¹⁵⁶. Although more than 30 CYP3A4 variants have been reported¹⁵⁷, most CYP3A4 variants are SNPs with low allelic frequencies and many of these SNPs are population specific¹⁵⁷. In addition, the clinical implementation of all CYP3A4 polymorphisms of in drug metabolism have not been determined¹¹⁹. Rebbeck *et al.* described the most common variant in the 5'-untranslated region (UTR) of CYP3A4, CYP3A4*1B (A-392G)¹⁵⁸. The frequency of CYP3A4*1B is highly variable in different racial populations with an allele frequency of 0% in Chinese, Taiwanese and Japanese¹⁵⁹⁻¹⁶², 4-10% (Caucasians)¹⁶³⁻¹⁶⁶, 9-10% (Hispanics)^{161,162} and 48-80% (African-Americans)^{161,162,165,167,168}.

3.1.2 Cytochrome P450 (CYP450) 3A5

The CYP3A5 gene has 13 exons encoding 502 amino acids^{113,169}. CYP3A5 has been reported to be expressed at higher levels than CYP3A4 in extra hepatic tissue, such as in the small intestine¹⁷⁰, colon¹⁷¹, lung¹⁷², oesophagus¹⁷³, kidney^{174,175}, adrenal gland¹⁷⁶, anterior pituitary¹⁷⁷, breast¹⁷⁸, prostate¹⁷⁹ and polymorphonuclear leukocytes¹⁸⁰. The majority of compounds that are substrates for CYP3A4 are also metabolized by CYP3A5, usually with a higher catalytic efficiency. Rendic provided a detailed list of CYP3A4 and CYP3A5 substrates¹⁸¹. The induction mechanism for CYP3A4 and CYP3A5 is similar and it is therefore unlikely that drug interactions involving enzyme

inducers such as rifampicin will be affected by the CYP3A5 genotype. However, the interactions involving CYP3A enzyme inhibition are probably more common than those involving induction. There is increasing evidence that CYP3A4 and CYP3A5 show differences in their response to enzyme inhibitors and it is therefore possible that CYP3A5 expression may be a determinant of individual susceptibility to drug interactions involving inhibition. In a detailed study regarding the differences between CYP3A4 and CYP3A5 with respect to inhibition, differences in the extent of inhibition by ketoconazole and diltiazem were confirmed and there was also less inhibition of CYP3A5 than CYP3A4 by erythromycin and nicardipine¹⁸². Regarding the mechanism-based inhibitors, it was suggested that the lower level of inhibition of CYP3A5 was due to a decreased ability to form the metabolites associated with metabolite-intermediate complex rather than an inability to form such a complex. The CYP3A5*3 variant allele designated for the presence of a genomic A6986G transition within intron 3 is the most common and functionally important variant across all ethnic populations studied¹⁸³, while additional rare polymorphisms that affect CYP3A5 activity in individuals expressing the enzyme have also been identified¹⁸⁴. The non-coding CYP3A5 A6986G polymorphism creates a cryptic consensus splice site in the pre-mRNA, resulting in the incorporation of 131 bp of intron 3 sequence (named exon 3B and inserted between exon 3 and exon 4) in the mature mRNA, and the production of improperly spliced mRNA containing exon 3B (splice variant 1) and a small amount of properly spliced mRNA (wt-mRNA)^{157,185}. The insertion causes a frameshift and a predicted premature termination codon, so that the encoded protein is truncated at amino acid residue 102 with loss of enzyme activity. According to the cytochrome P450 nomenclature (see website cytochrome P450 iso-enzyme nomenclature: <http://www.cypalleles.ki.se/>) any allele positive for 6986A is designated CYP3A5*1 while any allele positive for 6986G is designated CYP3A5*3. The CYP3A5*1 allele has a frequency of 5-7% in European Caucasians compared with approximately 40% among Africans and African Americans, and 25% in various Asian ethnic groups^{163,184-193}. Additionally, Xie *et al.*¹⁸³ performed a meta-analysis on the ethnic distribution of CYP3A5 alleles and genotypes.

A large number of studies demonstrated the influence of the CYP3A5 genotype on the dose requirement and clearance of the widely used immunosuppressants tacrolimus^{58,60,61,64-67,69-71,74-76,194-205}, cyclosporine^{42,66,67,203,206-213} and sirolimus^{74,214-216} in organ transplant patients. A number of studies found the CYP3A5*3 polymorphism a good predictor of the tacrolimus dose requirement and the tacrolimus plasma levels^{58,60,61,64-67,69-71,74-76,194-203,205}. Additionally, two studies^{67,201} determined using stepwise multiple regression analysis that the CYP3A5 genotype can explain approximately 35% of the variation observed in the tacrolimus concentrations of the transplant patients. The influence of CYP3A5 genotypes on the cyclosporin metabolism has also been examined in a number of studies^{66,67,203,206-213,217}. Most of these studies^{66,203,206-208} found no significantly increased rate of drug clearance in patients carrying a CYP3A5*1 allele, although some other studies observed a significant decrease in dose adjusted plasma trough levels^{67,210-213,217}. Although the reason is not

clear why the CYP3A5 genotype is less predictive for cyclosporin than for tacrolimus pharmacokinetics a few possible explanations can be given. The most evident explanation is that tacrolimus might be a better substrate for CYP3A5 compared to CYP3A4 than is cyclosporin. Kamden *et al.*²¹⁸ demonstrated that the turnover percentage for CYP3A5 with tacrolimus is approximately 60% higher than that for CYP3A4. This could explain the apparent higher requirement for tacrolimus in patients carrying a CYP3A5*1 allele. The published data on the cyclosporin metabolism is limited and is based on the CYP3A isoforms expressed in HepG2 cells using a vaccinia virus system¹¹³. In this study the ability of CYP3A5 to form the M17 metabolite was approximately 60% of that of CYP3A4, and no formation of the other oxidation metabolites M1 and M21 could be detected by CYP3A5. Another *in vitro* study²¹⁹ demonstrated that CYP3A5 may contribute to the formation of primary and secondary metabolites of cyclosporine, particularly in kidneys carrying the wild type CYP3A5*1 allele. Another possible explanation for the discrepancy between the effect of CYP3A5 genotype on the metabolism of the two immunosuppressants could be related to differences in the extent of the first-pass metabolism. Despite a twofold difference observed in a recent study²⁰¹ between patients homozygous for the CYP3A5*1 allele and those homozygous for CYP3A5*3 variant allele, other factors like gender, age, body mass index (BMI), time since transplantation and the use of steroids also may contribute to the wide intersubject variability in dose requirement^{67,201,220,221}. Although at the moment the clinical relevance of the association between tacrolimus pharmacokinetics and CYP3A5 remains unclear, it is possible that CYP3A5 genotyping could form part of a genetic test to predict tacrolimus dose requirement. A recent study²⁰² proposed after examining 19 volunteers between two dialysis periods a twofold higher tacrolimus dose requirement for patients carrying at least one CYP3A5*1 compared to patients carrying a CYP3A5*3 variant allele.

3.1.3 Cytochrome P450 (CYP450) 3A7

The CYP3A7 gene is encoded by a 17.1 kb sequence on the human chromosome 7q22.1 and spans 13 exons²²². CYP3A7 accounts for between 30 and 50% of the total cytochrome P450 activity in fetal liver²²³, although it is now apparent that it is also expressed in adult liver. In adult livers expressing high levels of CYP3A7, it has been suggested that this isoform may contribute up to 20% of the total CYP3A¹⁷⁶. CYP3A7 is also expressed in adult extrahepatic tissues, including intestine, endometrium, adrenal gland and prostate^{176,224,225}. In the endometrium, expression of CYP3A7 has been demonstrated to occur at high levels during the proliferative phase of the menstrual cycle²²⁶. Expression patterns in adult intestine seem to parallel those in adult liver²²⁴ but it is still unclear whether expression in other tissues is subject to the same inter-individual variability. The majority of the CYP3A7 substrates are similar to those for CYP3A4 and CYP3A5 except that the $V_{\max}$ values tend to be lower and K_m higher in most cases. However, CYP3A7 shows a higher catalytic activity for the retinoic acid isomers all-*trans* retinoic acid, 13-*cis* retinoic acid and 9-*cis* retinoic acid compared with

both CYP3A4 and CYP3A5^{227,228}. This may indicate a role for CYP3A7 in certain key developmental processes in view of the central role of retinoic acid in regulating some pathways and the need to maintain very specific local concentrations of this compound during the development²²⁹. In addition, CYP3A7 shows a high activity in the 16 α -hydroxylation of estrone and dehydroepiandrosterone^{115,184}. This may be of considerable relevance to both normal physiological processes and in relation to cancer susceptibility in view of the fact that CYP3A7 has now been shown to be expressed in several different steroidresponsive adult tissues. A study on the induction of CYP3A7 in fetal hepatocytes found induction by dexamethasone but not by rifampicin and suggested that induction was not mediated by the pregnane X receptor (PXR) but by the glucocorticoid receptor²³⁰. The CYP3A7*1C variant allele was reported at a frequency of 3% in Caucasians, 6% in African American and 0% in Chinese^{185,224}; Op den Buijsch *et al.* Submitted 2007. While the CYP3A7*1B and CYP3A7*1D variant alleles have allele frequencies of 1% in Caucasians and the CYP3A7*1E variant allele has been determined in African Americans at a frequency of 8%^{185,224}. The cause of CYP3A7 expression in the adult liver appears to be the G291T substitution which results in the creation of a binding site for the hepatocyte transcription factor hepatocyte nuclear factor-3 (HNF-3), whereas the A232C change destroys an HNF-3 binding site and creates a putative octamer motif identical to that found in the upstream sequence of CYP3A4²³¹.

3.1.4 Uridine 5'-diphosphate glucuronosyltransferase (UGT) 2B7

The analysis of tacrolimus metabolites in humans has provided evidence not only for CYP3A catalysed metabolism, but also for the formation of glucuronides²³². Strassburg *et al.*²³³ examined the glucuronidation activities of different uridine 5'-diphosphate glucuronosyltransferase (UGT) subfamilies for both tacrolimus and cyclosporine and found that UGT2B7 showed the highest glucuronidation activity in the human gastrointestinal tract. The UGT family, a group of proteins responsible for the glucuronidation of several endogene and exogene compounds in humans, is mainly present in the liver. However, UGTs are also found throughout the gastrointestinal tract, where they are an integral part of prehepatic first-pass metabolism. In addition, UGTs also work in the kidneys, brain, placenta and in several other locations in the human body. The major function of glucuronidation is to increase the polarity of the target compounds, a process which facilitates their detoxification and excretion. However, glucuronidation can also result in compounds which are biologically active or demonstrate increased toxicity. After glucuronidation, intestinal bacterial β -glucuronidases break down glucuronidation products and release the unconjugated drugs via enterohepatic recirculation. This "recycling" system slowly clears conjugated compounds and releases glucuronides for re-use²³⁴⁻²³⁸. Metabolites of tacrolimus have been isolated from human plasma, bile and urine and have been generated in human liver microsomes^{98,120,239,240}. Up to 15 metabolites (demethyl-, demethylhydroxy-, didemethyl-, didemethylhydroxy-, and hydroxytacrolimus) have been identified in blood

samples obtained from liver and renal transplant recipients, with the demethyl (approximately 3% of the area under the concentrationtime curve (AUC) of tacrolimus) and the demethylhydroxy (approximately 10% of the AUC of tacrolimus) compounds being the most prevalent¹²¹. Tacrolimus is predominantly metabolised into 13-O-demethyltacrolimus in liver and blood^{98,120,239,240}. This metabolite was found to be approximately one-tenth as active as tacrolimus^{87,92}. Based on the blood concentration data, tacrolimus is considered a low clearance drug with clearance equivalent to 3% of the liver blood flow⁹⁷. Figure 2.4 illustrates an overview of the tacrolimus metabolism.

Figure 2.4 Proposed interactions between tacrolimus metabolism and active efflux in the small intestine mucosa. Two potential cooperative mechanisms between cytochrome P450 (CYP) enzymes and active efflux transporters have been proposed. P-glycoprotein regulates the access of drugs to CYP enzymes and prevents CYP enzymes from being overwhelmed by the high drug concentrations in the intestine^{51,52,369}. With the drug being repeatedly transported out of the mucosa cells and being reabsorbed, repeated exposure leads to more efficient metabolism. The metabolites are better substrates of another active transporter than the parent, thus avoiding inhibition of metabolite efflux by the high concentrations of the parent drug³⁷⁰. ABC = ATP binding cassette transporter other than P-glycoprotein; met = tacrolimus metabolite; P-gp = P-glycoprotein; tac = tacrolimus.

3.1.5 Pregnane X receptor (PXR)

The pregnane X receptor (PXR)²⁴¹ also known as SXR²⁴², hPAR²⁴³ or NR (nuclear receptor) 1I2 has been identified as a transcriptional regulator of CYP3A4^{241,243} and ABCB1^{244,245}. PXR binds as a heterodimer with the 9-*cis* retinoic acid receptor (RXR; NR2B) to previously characterized xenobiotic response elements in CYP3A gene promoters, and importantly, PXR is activated by the spectrum of chemicals that are known to induce CYP3A gene expression^{246,247}. Single nucleotide polymorphisms (SNPs) in the PXR gene can possibly influence the PXR activity and thereby the CYP3A4 and ABCB1 expression.

3.1.6 Constitutive androstane receptor (CAR)

The constitutive androstane receptor (CAR) encoded by NR1H3 is a member of the orphan nuclear receptor superfamily that plays a major role in the control of drug metabolism and disposition²⁴⁸. CAR binds to the DNA of target genes as a heterodimer with the retinoid X receptor (RXR α) and regulates gene transcription²⁴⁹⁻²⁵¹. CAR is predominantly expressed in the liver and intestine^{252,253}. CAR regulates transcription of the genes encoding drug/steroid metabolizing enzymes as well as other physiologically important enzymes. Moreover, CAR transactivates several hepatic cytochrome P450s and other enzymes involved in drug metabolism including UGT1A1²⁵⁴, CYP2B6²⁵⁵, CYP3A4²⁵¹, CYP2C9^{251,255} and CYP2C19²⁵⁶. Therefore, polymorphisms inducing changes in the function or expression of the NR1H3 are thought to be potential sources for variation in cytochrome P450 expression or drug metabolism in humans.

3.1.7 Glucocorticoid receptor (GR)

The glucocorticoid receptor (GR), encoded by NR3C1, is a member of the nuclear hormone receptor of transcription factors. In the cytosol, GRs are associated with heat-shock and other proteins, and the binding of glucocorticoid leads to their nuclear translocation and positive or negative regulation of various genes^{257,258}. The GR causes anti-inflammatory effects through transcriptional activation of glucocorticoid induced leucine zipper genes or transcriptional suppression of genes of inflammatory cytokines induced by NF-kappaB or AP-1²⁵⁹⁻²⁶¹. Therefore, it is possible that the altered transcriptional activity of GR associated with polymorphisms of NR3C1 might affect the expression levels of target genes including CYP450 enzymes. Furthermore the GR regulates the expression of many drug metabolising enzymes. For instance, it is reported that GR activates the transcription of drug metabolizing enzymes CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP3A4 and CYP3A5 through GREs in the promoter/enhancer regions of these genes or induction of both pregnane X receptor and constitutive androstane receptor^{251,256,262-265}. Individual differences of CYP450 enzymes are thought to result from differences in their expression levels and/or activities²⁶⁵.

3.2 Non-genetic factors affecting the pharmacokinetics

3.2.1 Sex

Fitzsimmons *et al.*³⁶ observed no sexspecific effects on the pharmacokinetic profile of tacrolimus after examining the pharmacokinetic data collected from 127 adult (49 female and 78 male) who participated in three phase I studies and five multicentre, phase II clinical trails and 343 transplant recipients (149 female and 194 male) who participated in two phase III clinical trails. Healthy volunteers, pre and posttransplant renal recipients and bone marrow recipients demonstrated no sex differences in pharmacokinetics. However, Kuypers *et al.*²²⁰ recently reported that female renal transplant recipients reached overall higher C_{max} levels compared to their male counterparts (mean 26.1 $\pm$ 9.1

$\mu\text{g/l}$ versus $22.5 \pm 8.8 \mu\text{g/l}$; $P = 0.04$, respectively) and six months after grafting, the tacrolimus AUC_{0-12} was significantly higher in female renal transplant recipients ($148.7 \pm 35.2 \mu\text{g} \times \text{hr/l}$ versus $132.9 \pm 32.5 \mu\text{g} \times \text{hr/l}$; $P = 0.003$)

3.2.2 Age

Numerous studies already demonstrated that paediatric transplant recipients require two to fourfold higher tacrolimus doses than adults to maintain similar trough concentrations^{95,266-267}. The higher tacrolimus doses required in pediatric patients have been attributed to differences in cytochrome P450 3A although differences in bowel length, hepatic blood flow and P-glycoprotein expression also need to be considered²⁷². The CYP3A4 content in hepatic tissue is extremely low in the foetus but increases rapidly after birth to 120% of adult levels after the age of one year. In addition, CYP3A5 is expressed in nearly 50% of all infant livers, but could be found in only 29% of adult livers²⁷³. However, it is unclear whether intestinal CYP3A expression changes in parallel with hepatic CYP3A expression during human maturation. Moreover, it is also not known whether P-glycoprotein expression changes with age. In addition Kuypers *et al.*²²⁰ found that six months after grafting the tacrolimus AUC_{0-12} increased with younger age ($R^2 = -0.20$; $P = 0.007$). Tacrolimus trough levels were lower with increasing age ($R^2 = -0.13$; $P = 0.05$ day 7 and $R^2 = -0.20$; $P = 0.005$ at six months).

3.2.3 Race

African-American transplant patients require higher tacrolimus dosages (mg/kg) than Asians (Chinese or Japanese) and Caucasians^{36-40,274,275}. Moreover, bioavailability was significantly reduced among Black patients (9.9% versus 19%)³⁶. After oral tacrolimus administration, tacrolimus C_{max} were significantly lower in the African-American than in the White transplant patients. The differences between ethnic groups may result from racial differences in intestinal CYP3A or P-glycoprotein activity^{40,275}.

3.2.4 Haematocrit and albumin concentrations

Undre *et al.*²⁷⁶ found, after examining 303 renal transplant recipients, a correlation between the relative clearance and both haematocrit ($r = 0.81$) and albumin ($r = 0.74$) concentration over the first 12 weeks posttransplantation. After examining blood samples from 33 patients with autoimmune diseases and recipients of bone marrow and liver transplant undergoing cyclosporine immunosuppressive therapy, Kuzuya *et al.*²⁷⁷ found, after adding tacrolimus to each blood sample at a final concentration of 7.5 $\mu\text{g/l}$ and 15 $\mu\text{g/l}$, that haematocrit interferes with the MEIA II for tacrolimus. Furthermore that the magnitude of the interference is clinically significant, especially during the early posttransplantation period, because the haematocrit fluctuates widely and the target concentration of tacrolimus is variable. Additionally, Kuzuya *et al.*²⁷⁷ even proposed that beyond the range from 30% to 40% of haematocrit values, caution should be exercised in interpreting results as one may need to compensate for the levels of tacrolimus.

Recently Brown *et al.*²⁷⁸ demonstrated, after examining 1156 blood samples obtained from 121 paediatric recipients of renal, liver and bone marrow grafts or hepatocyte or pancreatic islet cell implants, that patients with a low packed cell volume and plasma albumin are likely to show artificially high tacrolimus concentration when measured by MEIA. This increased risk of underimmunosuppression must be considered should doses be reduced to lower these seemingly high tacrolimus concentrations. More recently Cheung *et al.*²⁰¹ also reported, after using stepwise multiple regression analysis, that the haematocrit levels may explain 35% of the variability in the tacrolimus blood concentrations. Knowing that in this study Cheung *et al.*²⁰¹ used blood samples obtained from a wide spread period after transplantation namely (133-4982 days) it is likely that this percentage will be much higher in a transplant recipient group just after transplantation. These results are in line with the strong binding of tacrolimus to red blood cells and serum albumin. Haematocrit and α_1 -acid glycoprotein concentrations are generally lower in renal transplant patients immediately post surgery and increase significantly when the patients recovers²⁷⁹. Ghoshal *et al.*²⁸⁰ demonstrated a correlation between IMx II and LC MS/MS of respectively $r = 0.612$ and 0.829 for tacrolimus concentrations above and below 9 ng/ml which may imply that influence of a low haematocrit fraction is minor for tacrolimus concentration above 9 ng/ml. Additionally, Ghosal *et al.*²⁸⁰ suggested that tacrolimus concentrations below 9 ng/ml measured by MEIA II are questionable and should be interpreted with caution.

3.2.5 Time after transplant action

Several studies among patients who underwent a different organ transplantation have reported a decrease in the dosage of tacrolimus required to maintain similar trough concentrations with increasing time posttransplant^{42,276,281,282}. A generally considered cause for the decrease in the tacrolimus dosage is a decrease in the tacrolimus clearance with time, although an increased bioavailability also has to be considered as a feasible explanation. Pou *et al.*²⁸¹ found, in 50 adult liver transplant recipients, significant reductions in the dose-normalised trough concentration of tacrolimus after the first and third month of treatment. Additionally, Undre *et al.*²⁷⁶ demonstrated in 303 renal transplant recipients after two years that the decline in the dose-normalised trough concentration was 61.1% and the tacrolimus dosage was reduced by 50%. Potential explanations for this are a reduction in the corticosteroid dosage and the increased haematocrit and albumin concentrations.

3.2.6 Corticosteroid dosage

The concomitant use of corticosteroids, which may induce CYP3A iso-enzymes, also has the potential to influence the tacrolimus elimination although contradictory studies appeared. Undre *et al.*²⁷⁶ found, after examining 303 renal transplant recipients, a significant correlation between the relative clearance and the mean oral corticosteroid dosage ($r = 0.94$) during months 2-12 posttransplant which indicates that corticosteroids

increase the tacrolimus metabolism. Other studies demonstrated, or at least suggested the opposite effect^{283,284}, while researchers examining bone marrow transplant patients could not demonstrate any influence²⁸⁵.

3.2.7 Population

A higher tacrolimus clearance was found for adult renal transplant recipients compared to liver transplant recipients and healthy volunteers^{286,287}. The presence of low haematocrit and albumin concentrations and differences in corticosteroid dosage may be partly responsible for this observations^{287,288}. A 38% reduction in the AUC₀₋₁₂ associated with tacrolimus was reported in diabetics compared to non-diabetic patients pre-transplant⁴¹. This may be due to disturbed gastrointestinal motility in patients with diabetes mellitus.

3.2.8 Hepatic dysfunction

Several studies already reported that a poor liver function can decrease tacrolimus clearance up to 67% and increase the elimination half-life with a threefold^{283,286,289-292}. Cold ischaemia time and reperfusion injury to a transplanted liver may also alter the clearance of tacrolimus. However, tacrolimus clearance has been reported to be similar between healthy volunteers and patients with mild hepatic impairment²⁸⁹. Transplant patient who are hepatitis C positive require a significantly lower mean dosage of tacrolimus than hepatic C negative patients to obtain the same trough concentrations^{268,293,294}. Horina *et al.*²⁹⁵ suggested that replication of the hepatitis virus in liver cells alters the cytochrome P450 system which results in reduced tacrolimus metabolism.

3.2.9 Renal function

No significant correlations have been reported between serum creatinine concentrations and the clearance of tacrolimus ($r = 0.36$)³⁴. Patients with severe renal dysfunction (serum creatinine concentrations of 344 - 1061 $\mu\text{mol/l}$) and patients on dialysis prior to renal transplantation have similar tacrolimus clearance to healthy volunteers.

3.2.10 Donor liver characteristics

The characteristics of the donor organ may influence the pharmacokinetics of tacrolimus in liver transplant recipients²⁹⁶. It is demonstrated in 118 liver transplant recipients that the mean dosage requirements were lower in patients who received a liver from donors of an older age group (≥ 65 years) than from donors of a younger age group (10-25 years), possibly as a result of decreased metabolic capacity in older livers. Additionally, several studies have reported that transplant patients who received a partial liver from a living donor require significantly lower tacrolimus doses than transplant patients who received a whole liver from a deceased donor to achieve similar tacrolimus trough

concentrations in the early posttransplant period²⁹⁷⁻²⁹⁹. Two studies performed in transplant patients of related living-donor liver transplant (LDLT), the tacrolimus dose needed to reach a target blood concentration correlated with the ratio of graft weight to recipient standard liver volume which indicating an increased metabolic capacity with increase graft size^{300,301}.

3.2.11 Impact of organ donor genotype

CYP3A is expressed in the enterocyte and the liver, while the P-gp barrier to drug absorption probably only acts at the levels of the intestine. Liver transplantation offers a fascinating opportunity to study the impact of different genotypes in the intestine and liver with metabolism of drugs by the transplanted liver. A further layer of complexity is added by the functional regeneration of hepatic grafts with time which may lead to a progressively increasing role in drug metabolism over time^{302,303}.

3.2.12 Administration of food

The influence of food on the oral absorption of tacrolimus appears to be dependent on its fat content and relative time of administration. Previous research indicated that coadministration of low fat food had minimal effect on the extent of absorption, but delayed the time to reach C_{max} ³⁰⁴. A significant reduction in both the rate and extent of tacrolimus absorption (bioavailability reduced 27%) was observed after concurrent administration of moderate fat food⁴³. Another study examined the effect of meal-timing on the tacrolimus absorption and found that the tacrolimus absorption in the fasting state had a significantly greater relative bioavailability than all other treatments⁴⁴. Moreover, the absorption of tacrolimus was also significantly prolonged after a meal. Van Duijnhoven *et al.*³⁰⁵ recently examined in 27 renal transplant recipients that conversion to non-fasting ingestion of tacrolimus, without dosage adjustments did not significantly change the tacrolimus trough concentrations in stable patients.

4 Drug-drug interactions

After oral administration, there are several factors involved in absorption of a drug which all can be the target of drug interactions: delivery to the intestine (pH, gastric emptying and food), absorption from the intestinal lumen (dissolution, lipophilicity, stability active uptake), intestinal metabolism (phase I and phase II metabolism), active intestinal drug efflux pumps, and subsequent hepatic first pass extraction^{51,306}. Previously, Christians *et al.*²⁸⁸ summarizes many relevant drug interactions, the information on which the drug interactions are based (*in vitro* / animal studies, controlled clinical trials and clinical observations), their proposed mechanisms and their clinical effects. Recently, Wilkinson³⁰⁷ described in a review article about the influence of both inducers and inhibitors on felodipine blood levels (a blood pressure lowering drug). Similar reports

have also been written tacrolimus interactions³⁰⁸⁻³¹¹. This emphasized that to decrease the variability in the tacrolimus exposure among transplant patients multiple factors like pharmacokinetics (C_0 , abbreviated AUC_{0-12} or complete 12 hour AUC), genetics (CYP3A, ABCB1, UGT2B7 and PXR polymorphisms, the possible influence of co-medication and the influence of other compounds that the patient eventually use like St John's wort or grapefruit juice.

5 Therapeutic drug monitoring

Tacrolimus has a narrow therapeutic index and highly variable pharmacokinetic characteristics. Close monitoring of the tacrolimus concentration is required to achieve an optimal efficiency and thus minimizing the risk of subtherapeutic or toxic blood concentrations. Efficacy and side effects of tacrolimus are highly correlated with the area under the curve (AUC_{0-12})³¹². The most exact way to monitor the total tacrolimus exposure is by creating 12 hour pharmacokinetic profiles, which implicates that the tacrolimus concentration should be measured at at least 6 different time points. The AUC_{0-12} can then be calculated according to the trapezoidal rule using the tacrolimus concentrations measured at different time points. Since recording a complete 12 hour pharmacokinetic profile for every patient is not feasible in clinical practice, traditionally many transplant centres use tacrolimus trough (C_0) concentrations to estimate the tacrolimus exposure. Although tacrolimus C_0 concentrations are generally considered to be a good indication of the total systemic drug exposure^{312,313}, its usefulness in differentiating graft rejection episodes from nephrotoxicity has been questioned³¹⁴⁻³¹⁷. Recently, the correlation between individual tacrolimus concentrations and AUC_{0-12} has been studied in kidney^{220,318-323}, liver³²⁴, heart^{325,326} and lung³²⁷ transplant recipients. In these studies a poor association was found between the tacrolimus C_0 concentrations and the AUC_{0-12} while tacrolimus concentrations measured at other time points showed much better correlations with the AUC_{0-12} . Additionally, strategies have been developed that consisted of a limited number of sampling time points within a short time post dose, the so called limited sampling strategies. Several two and three time point sampling strategies showed a high correlation with the AUC_{0-12} in the published studies and were able to predict the AUC_{0-12} more accurately than the C_0 concentration alone^{318,320,321,323,325,327}. Moreover, Op den Buijsch *et al.*³²⁸ recently evaluated 24 different limited sampling strategies for tacrolimus that already have been published in a different renal transplant recipient group. They found that the limited sampling strategies that used two (C_2 and C_4) or three (C_0 , C_2 and C_4) sample time points demonstrated the best results in their own renal transplant group. Based on the half-life of tacrolimus which is approximately ten hours, it is necessary to wait at least 36 hours (3.3 half-lives) to reach a steady state tacrolimus concentration after initiation of therapy or after a change in the administration regime of tacrolimus. Ideally, after starting the infusion, blood concentrations should be monitored on day 2 or 3 on average 3 to 7 times weekly

during the first few weeks after transplantation, and less frequently thereafter. Special circumstances such as changes in liver function, presence of adverse effects or use of drugs that may alter tacrolimus kinetics may warrant more frequent monitoring³²⁹.

6 Analytical methods used to determine the tacrolimus blood concentration

Tacrolimus concentrations in biological fluids have been measured using a number of methods. However, the development of a simple, specific and sensitive assay method for measuring tacrolimus in biological fluids is limited by the low absorptivity of tacrolimus, the low concentrations of tacrolimus in plasma or blood and the presence of several other drugs in the blood samples of patients, which potentially interfere with the analysis of tacrolimus. The currently available assays can be categorized as enzyme immunoassays, chromatographic/mass spectrometric assays, radioreceptor assay and a bioassay.

6.1 Enzyme immunoassays

Tamura *et al.*³³⁰ reported in 1987 the first method for quantification of tacrolimus in plasma using an enzyme-linked immunosorbent assay (ELISA) method following a solid phase extraction procedure to separate tacrolimus from other components in the sample. The clinical application of this assay was first reported in 1990⁵⁵ and a modification of this method has been used to measure tacrolimus in whole blood^{77,317}. A number of drugs (Table 2.1) used to treat transplant patients do not appear to cross-react with the antibody used in the ELISA procedure^{77,331,332}. A semi-automated technique, based on the principle of microparticle enzyme immunoassay (MEIA) method for the IMx analyser developed by Abbott that measures the concentrations of tacrolimus in whole blood has been reported³³³. The immunoassay MEIA I assay was found to have insufficient sensitivity to monitor patients with tacrolimus concentrations less than 5.0 ng/ml and is also not completely specific to tacrolimus with a cross reactivity of 56.2% and 5.4% for respectively 15-O-demethyltacrolimus and 13-O-demethyltacrolimus³³⁴. Therefore, the development of a MEIA II method with a suggested lower analytical range (1.5 ng/ml) was released³³⁵.

Table 2.1 Drugs which do not appear to cross-react with the antibody used in enzyme-linked immunosorbent assay (ELISA)^{77,87,92,331,332}.

Amikacin	Netilmicin
Amphotericin B	Nifedipine
Azathioprine	Paracetamol (acetaminophen)
Carbamazepine	Phenobarbital (phenobarbitone)
Cyclosporin	Phenytoin
Digitoxin	Prednisolone
Digoxin	Primadone
Disopyramide	Procainamide
Erythromycin	Quinidine
Ethosuximide	Salicylates
Flecainide	Theophylline
Fluconazole	Tobramycin
Gentamycin	Valproic acid
Lidocaine (Lignocaine)	Vancomycin
Methylprednisolone	

6.2 Chromatographic / mass spectrometric methods

An LC tandem mass spectrometric (LC MS/MS) method for measuring tacrolimus and its metabolites in patients' blood, bile and urine samples is also available^{120,239,240}. This method involves solid-phase extraction of the biological samples and the use of LC to separate various components, followed by the use of a mass spectrometer as a detector. Although the LC MS/MS assay is highly specific due to its capability of quantifying tacrolimus and its major metabolites and its sensitivity with a detection limit of 0.2 ng/ml, the lack of routine availability of this instrumentation in transplant centres, and the difficulty in analysing large volumes of samples on a regular basis, the use of this technique is limited to pharmacokinetic and metabolism studies at the present time.

6.3 Radioreceptor assay

The radioreceptor assay uses tritiated dihydrotacrolimus for competition with tacrolimus extracted from the blood sample to bind partially purified preparation of FK binding protein (FKBP)³³⁶. The radioreceptor assay is simple to perform, requires a small volume of blood, can provide a rapid turn-around time and the results of this assay correlate well with the whole blood ELISA assay ($r = 0.97$). However, consistently higher tacrolimus concentrations are estimated by this assay in comparison to the ELISA, indicating that the assay is non-specific. It is not clear whether the affinity of a molecule towards FKBP is related to the immunosuppressive activity of that molecule. Any further development of the radio receptor assay dependent on establishing a relationship between the factors mentioned above.

6.4 Bioassay

Zeevi *et al.*³³⁷ reported a biological assay based on inhibition of the alloantigen-driven proliferation of a clone of allo-reactive T-cells. Although in this bioassay the metabolites with activity are being measured as tacrolimus in a biological specimen, the limitations of this procedure are the slow turn around time (> 72 hours) and the inability to directly assay whole blood samples.

7 Comparison of the analytical methods

Venkataramanan *et al.*³² reviewed several studies that compared two or more analytical methods for measuring tacrolimus. The Protac ELISA, the MEIA and the LC tandem MS method are currently used clinically to measure the blood concentrations of tacrolimus. Whereas the ELISA method generally tends to have a higher coefficient of variation than the MEIA method, the MEIA method lacks the sensitivity required for routine clinical use. The tacrolimus blood concentrations measured by MEIA have been reported to correlate well with those of the ELISA method^{317,338,339} since both methods are non-specific as they also measure some of the tacrolimus metabolites. A method which uses LC prior to ELISA, MEIA or a mass spectrometric method is specific for the parent tacrolimus molecule. Other methods seem to measure additional tacrolimus-related components in blood or plasma, owing to the non-specificity of the monoclonal antibody used. Larger discrepancies between different methods are observed in blood samples obtained from patients with an impaired liver function, indicating the accumulation of some metabolites of tacrolimus which cross-react with the antibody used in the assay procedure.

8 Toxicity

Generally occurring adverse effects associated with tacrolimus include nephrotoxicity, neurotoxicity, diabetogenesis, gastrointestinal disturbances, hypertension, infections and malignant complications^{3,340,341}. Adverse reactions of tacrolimus tend to occur the most frequently in the first few months after transplantation and decrease with time possibly in line with reductions in tacrolimus concentration^{341,342}. At higher tacrolimus concentrations nephrotoxicity, neurotoxicity, diabetogenesis, gastrointestinal disturbances and infections occur more frequently or are more severe^{340,341,343-347}.

8.1 Nephrotoxicity

The use of tacrolimus can be limited by nephrotoxicity which occurs in approximately 50% of the patients treated with this drug^{340,341} although it is not always easy to attribute

renal failure directly to tacrolimus toxicity since transplant patients often also receive other nephrotoxic drugs and may have pre-existing or ongoing kidney diseases (e.g. diabetic patients).

8.2 Neurotoxicity

Minor neurological adverse effects include insomnia, mild tremors, headache, photophobia, nightmares and hyperaesthesia^{340,341}. Clinical studies point out that tremors occurred in 35 - 56% of the transplant patients receiving tacrolimus while headaches occurred in 20 - 64%, insomnia in 24 - 32% and paraesthesia in 14 - 40%^{341,342}. Higher blood concentrations of tacrolimus are associated with less occurring severe neurological events like seizures, akinetic mutism, expressive aphasia, coma and delirium. Liver transplant recipients requiring a transplantation due to hepatitis B or C have an increased risk of severe tacrolimus-induced neurotoxicity^{344,348,349}.

8.3 Diabetogenic effects

One of the most serious adverse effects of tacrolimus is posttransplant diabetes and this is most likely exacerbated by concomitant administration of corticosteroids^{347,350}. Three major randomised, multicentre trials^{340,341} demonstrated that the incidence of diabetes in the first year after renal or liver transplantation ranged from 8% to 20%. Bonomini *et al.*³⁵¹ reported, after examining 245 renal transplant patients treated with triple therapy (tacrolimus, corticosteroids and azathioprine), an 4% incidence of new onset type I diabetes, while among the 246 patients receiving a dual therapy (tacrolimus and corticosteroids) the incidence of new onset type I diabetes was 5.6% after a follow up period of three months. Posttransplant diabetes is reversible in some patients^{340,341}. Risk factors for the development of posttransplant diabetes include, race, high tacrolimus trough concentrations and a high corticosteroid dosage³⁴¹.

8.4 Hypertension

Hypertension occurred in up to 50% of the liver and renal transplant patients treated with tacrolimus in several clinical studies^{340,341} although it is difficult to determine with certainty an association between tacrolimus therapy and hypertension^{352,353}. In transplant patients who were previously normotensive, hypertension may be caused by the excessive intravascular volume, intrinsic renal damage or increased vasomotor tone³⁵². Moreover, corticosteroids may also lead to excess water retention, while liver failure prior to transplantation causing low peripheral resistance can mask pre-existing blood pressure problems³⁵³.

8.5 Gastrointestinal disturbances

Several major clinical studies in renal and liver transplant recipients indicated that gastrointestinal disturbances like diarrhoea, nausea and constipation with frequencies of respectively 22 - 72%, 17 - 46% and 31 - 35% were common in patients treated with tacrolimus^{340,341}.

8.6 Infectious complications and malignancies

It is known that all immunosuppressive drugs increase the risk of infection and malignancy, especially lymphoma. In large clinical studies similar infection rates were found in the tacrolimus and cyclosporine treated groups in renal and liver transplant patients. Within the first year after transplantation approximately 75% of the patients had one or more infections^{341,344,354}. According to two studies^{341,354} malignancies (excluding lymphoma or lymphoproliferative disease) developed in 1% of the adult renal transplant patients taking tacrolimus and the rate of lymphomas was approximately 1.5%^{341,354}.

8.7 Other adverse effects

During the tacrolimus therapy mild hyperkalaemia, associated with low or low-normal renin and aldosterone concentrations occur regularly^{352,355-357} and is not always correctable by dosage adjustment and may even occur at low blood tacrolimus concentrations³⁴⁶. Furthermore, hypomagnesaemia has also been reported during tacrolimus treatment in renal transplant recipients and appears to be related to impairment of normal magnesium conserving mechanisms³⁵⁸.

8.8 Paediatric patients

The acceptability profile of tacrolimus in children is generally similar to that in adults^{3,347,359-361}. However, children appear to be at an increased risk of, to the Epstein-Barr virus related, posttransplant lymphoproliferative disorders^{22,347,362-367}. Although some data suggest that paediatric patients may be at a particular risk of developing tacrolimus associated diabetes^{368,369}, further study is required to confirm these findings.

9 Concluding remarks

Tacrolimus is an immunosuppressive drug with a large inter- and intra-individual variation in its pharmacokinetics, with variable rates and extents of absorption, variable extents of blood protein binding and variable rates of elimination. It is incompletely bioavailable after oral administration, is bound extensively to red blood cells (the binding being saturable), is primarily eliminated by hepatic metabolism and has a narrow therapeutic index. Furthermore, tacrolimus is predominantly metabolised by

cytochrome P450 (CYP450) 3A iso-enzymes into 13-O-demethyltacrolimus which has a neglectable immunosuppressive activity. Previously, several studies have indicated that the CYP3A5 A6986G polymorphism is the most relevant polymorphism present in the CYP450 3A iso-enzyme family with a significant impact on both the tacrolimus exposure and the daily tacrolimus dose. In order to decrease the variability in the tacrolimus exposure of transplant recipients it is also recommendable to look for possible drug interactions and possible interactions with other CYP450 inducers or inhibitors like St John's wort or grapefruit juice.

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Chapter 3

Comparison of DNA isolation kits to extract DNA from whole blood samples

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Abstract

Background and aim

Genomic deoxyribonucleic acid (DNA) from white blood cells (WBCs) is widely used for polymerase chain reaction (PCR). Although kits for DNA isolation are in common use, there is scarce information about their performance and the PCR quality of the genomic DNA obtained. Hence, three different kits, QIAamp blood mini kit, High Pure PCR Template Preparation Kit and the Puregene DNA isolation kit were evaluated on these aspects.

Materials and methods

Genomic DNA was isolated from whole blood samples with WBC counts ranging from 0.5 to 20×10^9 WBC/l. The WBC count was used to calculate the amount of genomic DNA. The actual amount of genomic DNA isolated, was determined spectrophotometrically. The DNA extraction efficiency was obtained by dividing the actual amount of DNA by the calculated amount yielded. PCR quality was analysed by measuring Cycle threshold (C_t) values with a quantitative real-time PCR β -globin assay.

Results

The extraction efficiency of the three DNA isolation kits was 20% to 40%. Spectrophotometrically determined DNA concentrations correlated inversely with C_t values, regardless of the DNA isolation kit applied, whereas the strongest correlation was obtained for the Puregene DNA isolation kit. WBC counts also correlated inversely with C_t values and here the strongest correlation was found for the QIAamp blood mini kit.

Conclusion

The overall performance of the DNA isolation kits was quite comparable (DNA recoveries of 20 - 40%), albeit the QIAamp blood mini kit yielded the most reproducible extraction efficiencies and lowest C_t values within every WBC count category.

Introduction

White blood cells (WBCs) are easily obtained and therefore a common source of genomic deoxyribonucleic acid (DNA), which is the starting material for a variety of molecular biological techniques. One of the most important applications is the use of genomic DNA in polymerase chain reaction (PCR), for either polymorphism analysis or quantitative analysis with fluorescent real-time PCR. In the latter, the number of genome copies present at the start of the PCR is related to the Cycle threshold (C_t) values^{1,2}. DNA isolation kits are commonly used for the isolation of genomic DNA from WBCs. However, it is not known whether the performance of DNA isolation kits is influenced by the number of WBCs present. Neither, has been investigated whether the PCR quality of genomic DNA depends on the DNA isolation kit used. To answer these questions three different DNA isolation kits are tested. The QIAamp blood mini kit (Qiagen, Leusden, the Netherlands) and the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) rely on the preparation of a chaotropic mixture, in which DNA binds to a column and is subsequently eluted. The principle of DNA isolation with the Puregene DNA isolation kit (Puregene, Landgraaf, the Netherlands) is based on a salting out method. The WBC count was used as the gold standard to calculate the exact amount of genomic DNA in a given volume. The diploid genome of one WBC equals the amount of 6.6 pg DNA². The calculated amount of DNA is set as the 100% score. The actual amount of genomic DNA isolated was measured spectrophotometrically. Dividing the actual amount of genomic DNA isolated, by the calculated amount of genomic DNA, yields the extraction efficiency. The C_t value was used as a rough indicator for the PCR quality of genomic DNA, provided that an equal number of genome copies is present at the start of the PCR^{1,2}. Genomic DNA with the best PCR quality should result in the lowest C_t value. A quantitative real-time PCR β -globin assay was used in this study to compare the PCR quality of genomic DNA, obtained with the different DNA isolation kits^{2,3}.

Materials and methods

Sample collection

Thirty ethylene diamine tetra-acetic acid (EDTA) anticoagulated blood samples with known WBC counts were collected from anonymized patients referred to the haematology department for routine investigations. These samples were categorized in six different WBC categories each category consisting of five samples. The range of WBC counts was set at 0.5×10^9 WBC/l, being the lowest number of WBC counts that could be counted reliably. The highest level was set at 20×10^9 WBC/l, being twice the number of the upper limit of a normal WBC count. Four intermediate categories of 2.0, 5.0, 10 and 15×10^9 WBC/l were formed. Each sample was divided into three equal

aliquots of 300 μl whole blood. Until DNA isolation was performed all blood samples were stored at -20°C . WBC counts were performed on a Beckman Coulter GenS (Beckman Coulter Inc., Fullerton, CA, USA).

DNA isolation procedures

DNA isolation was performed with the QIAamp blood mini kit (Qiagen, Leusden, the Netherlands), the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH Mannheim, Germany) and the Puregene DNA isolation Kit (Biozyme, Landgraaf, the Netherlands), all according to the manufacturers' protocols. Isolated genomic DNA was quantified spectrophotometrically by measuring absorptions at 260, 280 and 320 nm ⁴⁻⁷ with the Ultrospec 2000 (Pharmacia Biotech, Roosendaal, the Netherlands). Absorption at 320 nm (A^{320}) gives information about the presence of e.g. phenolic substances, whereas the ratio of the absorptions at 260 nm *versus* 280 nm ($A^{260/280}$) provides information about impurities due to proteins or RNA.

Quantitative real-time PCR β -globin TaqMan[®] assay

A quantitative real-time PCR β -globin TaqMan[®] assay, described by Lo *et al.* for the ABI Prism 7700, was used to obtain C_t values². This quantitative real-time PCR TaqMan[®] assay was adapted for use on the LightCycler[®] (Roche Diagnostics by Almere, the Netherlands). The fluorescence signal from the liberated FAM reporter group was measured at the end of the extension step (72°C) in channel 1. The 2nd derivative method was used to calculate C_t values from the sigmoid real-time PCR curves. The PCR mixture contained 7.0 μl sterile water, 4.0 mM MgCl_2 , 2.0 μl LC Faststart DNA Master Hybridization Probes (Roche Diagnostics, GmbH, Mannheim, Germany), 0.2 μM of the dual labelled FAM/TAMRA TaqMan[®] probe BGFT13 (Eurogentec, Seraing, Belgium) and 0.70 μM of both primers BGLOBF13 and BGLOBR13 (Eurogentec, Seraing, Belgium). After adding 1.0 μl genomic DNA to the PCR mixture, the total volume was 20 μl . The PCR protocol included the following steps a) denaturation for 10 minutes at 95°C b) 45 cycles at 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 5 seconds. The temperature transition rates were programmed at $20^{\circ}\text{C}/\text{second}$ for all three temperatures. C_t values of thirty DNA samples, isolated with the same DNA isolation kit, were determined within one PCR run.

Statistical methods

Statistical analysis was performed using SPSS 10.0 for Windows (SPSS, Chicago, IL, USA). As initial distributions of the variables extraction efficiencies and C_t values were positively skewed. When appropriate, parametric tests were performed, otherwise non parametric statistics were used. To explore the influence of the WBC count (6 different levels from 0.5 to 20×10^9 WBC/l) on the extraction efficiency, the analysis was performed on the dataset where the outliers with extraction efficiency $< 1.3\%$ and

> 104% were omitted from the analysis (three for the Puregene method, two for the Roche method). As the data were thereafter readily normalized, univariate ANOVA was performed. The correlation between C_t values and DNA concentrations based on either WBC counts or UVspectrophotometry was determined with the non parametric Spearman rank correlation. The Spearman correlation coefficient (r_s) indicates the degree of association between either DNA concentrations and the C_t values ($r_{s(UV)}$) or WBC counts and C_t values ($r_{s(WBC)}$).

Results

Following the manufacturers' protocols, the end volume for the genomic DNA solution was 200 μ l for the Roche, 100 μ l for the Qiagen and the Puregene DNA isolation kit. DNA extraction efficiencies appeared to be similar for all three DNA isolation kits in all WBC count categories, except in the lowest category (Figure 3.1). In this category the chaotropic isolation methods (Qiagen and Roche) seemed to exceed the salting out based method (Puregene). Background A^{320} readings $\pm$ standard deviation (SD) showed different optical density (OD) values over the complete range for each DNA isolation kit, but were higher for the chaotropic isolation methods than for the salting out isolation method, with respectively 0.20 ± 0.12 and 0.08 ± 0.06 for the Roche and Qiagen, and 0.02 ± 0.02 for the Puregene DNA isolation kit. The background corrected $A^{260/280}$ ratio of genomic DNA of the WBC count categories of 5.0×10^9 WBC/l and higher, varied from 1.8 to 1.9. Only the two lowest WBC count categories had a different background corrected $A^{260/280}$ ratio. Genomic DNA, isolated with the chaotropic isolation methods, showed in this area lower ratios than genomic DNA, isolated with the salting out method, 1.7 and 1.8 for the Qiagen and Roche DNA isolation kits, respectively, and 2.0 for the Puregene DNA isolation kit (Table 3.1). To explore the influence of the WBC counts on the extraction efficiency, the univariate ANOVA analysis was performed including all WBC count categories for each DNA isolation kit. The results were respectively $F = 13.4$, $P < 0.0001$ for Qiagen, $F = 20.8$, $P < 0.0001$ for Roche and $F = 1.67$, $P = 0.186$ for Puregene. This indicates that the mean value of at least one WBC level was significantly different as compared to other WBC levels for the Qiagen and Roche DNA isolation kit. The correlations of different DNA isolation methods (taking all WBC count levels into account) with the WBC count were as follows: $r = -0.596$, $P = 0.001$ for Qiagen, $r = -0.782$, $P < 0.0001$ for Roche and $r = 0.153$, $P = 0.445$ for Puregene. We speculated that two factors might be attributable to these findings. The first one was large variation in the extraction efficiency results in the group with WBC count of 0.5×10^9 WBC/l. The second one might be that extraction efficiency is dependent on the WBC count. Therefore, the analysis was performed once more, thereby omitting WBC count group of 0.5×10^9 WBC/l. The results of univariate ANOVA were respectively $F = 1.33$, $P = 0.293$ for Qiagen, $F = 22.1$, $P < 0.0001$ for Roche and $F = 1.87$, $P = 0.162$ for Puregene. Only for the Roche DNA isolation kit at least one

WBC level was significantly different as compared to other WBC levels. The correlations of different DNA isolation methods (omitting WBC count level of 0.5×10^9 WBC/l) with the WBC count were as follows: $r = -0.398$, $P = 0.05$ for Qiagen, $r = -0.844$, $P < 0.0001$ for Roche and $r = 0.326$, $P = 0.138$ for Puregene. In the tenfold range of WBC counts, the extraction efficiency varies roughly from 20% to 40%. In the lowest WBC count category, the extraction efficiency is higher for the chaotropic isolation methods than for the salting out isolation method (Figure 3.1). The real-time PCR curves are shown in Figure 3.2.

Figure 3.1 White blood cell (WBC) counts *versus* extraction efficiency of (A) Qiagen, (B) Roche and (C) Puregene with a WBC range of ± 0.5 to $\pm 20 \times 10^9$ WBC/l. The WBC count category of 0.5×10^9 WBC/l is excluded before calculating the r_p and p values of all DNA isolation kits. The closed circles in Figure 3.1B are not included in the trend line.

Table 3.1

Figure 3.2 Real-time PCR amplification curves of three different DNA isolation kits (A) Qiagen, (B) Roche and (C) Puregene.

The median C_t values calculated from these curves seemed to be equal for the three DNA isolation kits in the lower three WBC count categories. Albeit that the median C_t value was somewhat lower for the Qiagen DNA isolation kit with the smallest C_t value interval ranges (Table 3.1). The median C_t values in the three higher WBC count categories seemed to be the lowest for the Qiagen DNA isolation kit, with also the narrowest C_t value interval ranges (Table 3.1). It was one and two C_t units lower than the median C_t values of the Puregene and the Roche DNA isolation kits, respectively. The variability of the C_t values within one WBC count category is lower for the

chaotropic isolation methods than for the salting out method (Table 3.1). For all the DNA isolation kits it was found that increased concentrations of genomic DNA was paralleled by decreased C_t values. Likewise, increased WBC counts were related with a decrease in C_t values. However, there seem to be subtle differences in the strength of these associations between the DNA isolation kits. This is apparent from the r_s values obtained with the non parametric Spearman rank correlation (Figure 3.3).

Figure 3.3 Log concentration DNA *versus* C_t values. The Spearman correlation coefficients (r_s) indicate the correlation between the log concentrations DNA in copies *versus* the C_t values of respectively (A) Qiagen, (B) Roche and (C) Puregene. The closed circles indicate the samples of which the DNA concentration is determined UV-spectrophotometrically ($r_{s(UV)}$). The open circles indicate the samples of which the DNA concentration is determined by counting white blood cells ($r_{s(WBC)}$).

The $r_{s(UV)}$ value for the association of the concentration of genomic DNA with the C_t value was the highest for the Puregene and the lowest for the Roche DNA isolation kit, 0.98 and 0.88, respectively. The $r_{s(WBC)}$ value for the association of the WBC counts with the C_t value was the highest for the Qiagen and the lowest for the Puregene DNA isolation kit, 0.97 and 0.71, respectively. The C_t values of the Qiagen DNA isolation kit correlated very well with the concentration of genomic DNA as well as with the WBC counts. Also, the C_t values of the Roche DNA isolation kit correlated well with the concentration of genomic DNA as well as with the WBC counts. In contrast, the C_t values of the Puregene DNA isolation kit correlated very well with the concentration of genomic DNA but relatively poorly with the WBC counts.

Discussion

WBC counts generate unambiguous data about the amount of genomic DNA present in a given volume of anticoagulated whole blood samples. Hence, the efficiency of a genomic DNA isolation with a DNA isolation kit can be determined unequivocally from the number of WBCs. In this study three different DNA isolation kits were compared, using samples containing low as well as high WBC counts, divided into six different WBC count categories. We showed that the different DNA isolation kits were comparable with regard to the total amount of genomic DNA that can be isolated. This is determined spectrophotometrically⁴⁻⁷. However, the background A^{320} readings are substantially higher in the chaotropic isolation methods than in the salting out method, this is especially prominent in the lowest WBC count category. OD readings for samples with low concentrations of genomic DNA, obtained with the chaotropic DNA isolation kits, might therefore overestimate the actual genomic DNA present. Only in the lowest WBC count category there is a marked difference in the calculated extraction efficiencies between the salting out and the chaotropic DNA isolation methods. The salting out method showed an extraction efficiency of about 40%, whereas the extraction efficiency of the chaotropic method was much higher. However, the high background in genomic DNA isolated with the chaotropic method might lead to an overestimation of the actual amount of genomic DNA present, and hence, to an exaggerated extraction efficiency. Turning to 2.0×10^9 WBC/l and higher, there is a remarkable constancy in the extraction efficiency. In a tenfold increase of WBC counts, there is a decrease from 40% to 20% in the extraction efficiency. This is observed for all the DNA isolation kits examined. It seems therefore that extraction efficiency depends on neither the method of DNA isolation nor the WBC count (Qiagen and Puregene), given that the WBC count is 2.0×10^9 WBC/l or higher. The PCR quality of the genomic DNA is evaluated by comparison of C_t values, obtained with a quantitative real-time PCR TaqMan[®] assay for the β -globin gene. Provided that the same copy number is present at the start of the PCR, genomic DNA with consistently the lowest C_t value, offers the best PCR quality. Although the C_t values are almost identical within a WBC

count category, the median C_t value of genomic DNA isolated with the Qiagen kit seems to be consistently lower than the C_t values obtained with genomic DNA isolated with the other DNA isolation kits. The Qiagen kit also yields genomic DNA with the narrowest C_t value interval ranges (Table 3.1). The largest C_t value interval ranges are observed with genomic DNA isolated with the salting out method. Most likely, this is related to the fact that three samples in three different WBC count categories yielded a very low extraction efficiency. In general, C_t values are inversely related to DNA concentrations. Because the DNA concentration in a whole blood sample is largely determined by the number of WBCs, one should also find an inverse relation between C_t values and WBC counts. Actually, the association found between C_t values and WBC counts for the chaotropic isolation methods is better than the association between C_t values and genomic DNA concentration, as measured with UV spectrophotometry. For the salting out method this association is the other way around and most likely, due to the very low extraction efficiency of three samples in three highest WBC count categories, which impairs the association of the C_t values with WBC counts, but not the association of C_t values with genomic DNA concentration, as measured with UVspectrophotometry. It implies that WBC counts could be used to predict the yield of genomic DNA if a chaotropic DNA isolation is used in addition of an UVspectrophotometric measurement. This would be worthwhile considering if samples with a low WBC count are used.

In conclusion, the use of WBC counts to calculate the amount of genomic DNA in a given volume allowed us to establish that the extraction efficiency ranges roughly from 20% to 40%, independent of the DNA isolation kit used and the number of WBCs present (Qiagen and Puregene). Using C_t values as a measure for the PCR quality of genomic DNA, it was found that the Qiagen blood mini kit seemed to yield the best quality of genomic DNA for PCR in the three highest WBC count categories.

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Chapter 4

Genotyping of the pregnane X receptor A11156C polymorphism with locked nucleic acid containing fluorogenic probes

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Abstract

Background and aim

The discriminative power of oligonucleotide probes to differentiate between two alleles of a biallelic gene locus is an important aspect in polymorphism analysis.

Materials and methods

This study investigates the enhancement of discriminative power in a fluorescence resonance energy transfer (FRET) assay by incorporation of a locked nucleic acid (LNA) at the polymorphic site applied on genotyping of the pregnane X receptor (PXR) A11156C polymorphism. Fifty individuals were genotyped for this PXR A11156C polymorphism. Polymerase chain reaction (PCR) products of the different PXR 11156 allelic variants were sequenced to validate the results of this FRET assay.

Results

It follows that incorporation of the LNA significantly improved the genotyping of the PXR A11156C polymorphism. Especially, heterozygotes could be discriminated clearly by showing two melting peaks, whereas the probes without a LNA were not able to separate melting peaks in the melting curve analysis.

Conclusion

A LNA within a sensor probe may improve the discriminating power of FRET assays significantly.

Introduction

The introduction of molecular technologies that combine the polymerase chain reaction (PCR) with the use of fluorescent hybridisation probes significantly reduced the risk of contamination, since no post PCR steps are involved. Although these techniques have been successfully used for the identification of a huge variety of single nucleotide polymorphisms (SNPs)^{1,2}, there are target sequences in which the discriminating power of deoxyribonucleic acid (DNA) probes is not sufficient to differentiate between the two alleles of a biallelic gene locus. The nuclear pregnane X receptor (PXR), (also known as SXR, PAR, PRR and NR1I2) is an important component of the body's adaptive defensive mechanism against toxic substances including drugs. Ligand activated PXR is involved in the transcriptional activation of multiple drug resistance gene 1 (MDR1) and cytochrome P450 3A4 (CYP3A4). Both are main regulators in the uptake and transformation of many orally prescribed medicines. Hence, knowledge with regard to known PXR polymorphisms, which influence the expression of MDR1 and/or CYP3A4, is therefore relevant to understand the interindividual responses towards drugs³⁻⁵. One of the putative relevant polymorphisms is PXR A1156C because it has been shown that the presence of the 1156C allele is associated with a decreased expression of MDR1 mRNA in intestinal villi cells derived from gut biopsies⁴. In order to determine the genotype of the PXR A1156C polymorphism we developed a real-time PCR fluorescence resonance energy transfer (FRET) assay. Although homozygotes could be discriminated unequivocally, separate melting peaks could not be obtained in putative heterozygote genotypes. To improve the discriminative power of the sensor probe two routes were followed. The first was to shorten the sensor probe and the second route was to modify the sensor probe with a locked nucleic acid (LNA) on the polymorphic position⁶⁻¹³. This last mentioned possibility is based on some recently published reports, which documented the high discriminative power of fluorogenic probes containing LNA to differentiate between matched and mismatched duplexes in several techniques, but not yet applied in FRET assays¹⁴⁻¹⁶. However, FRET assays have become a major technique in the field of polymorphism analysis. It is therefore worthwhile to examine the usefulness of LNA in FRET assays. The present study describes a real-time PCR FRET assay on the LightCycler in which three different sensor probes, of which one contains a LNA, are compared for their ability to differentiate between matched and mismatched duplexes.

Materials and methods

Ethylene diamine tetra-acetic acid (EDTA) whole blood samples of 50 anonymized patients referred for routine haematologic laboratory investigation were collected. The whole blood samples were stored at -20°C until DNA isolation, with a QIAamp blood mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturers'

instructions, was performed. To obtain the different allelic variants of the PXR A11156C polymorphism a real-time PCR FRET assay consisted of a primerset, an anchor probe and the sensor probe Sen [A] is used. The PCR primers were chosen to specifically target the human PXR gene. The primerset and the different fluorogenic probes used were designed and synthesised by TIB MOLBIOL (Berlin, Germany). The primers F22: 5'-CAA TCA gTT AAC ACA CCg gAg-'3 (sense; 81370-391) and R22: 5'-TTT TAT gTT CTT ACg CCg gAg T-'3 (anti-sense; 81766-745) were used to amplify a 397 bp fragment from the human PXR gene which covers the A11156C polymorphism in the 3'-untranslated region (3'-UTR) area (Genbank acces no: AF280107). Detection was carried out using the anchor probe 56Anchor: 5'-TTT Tgg gAA ATg TAg CCC Tgg gT-'3 (sense; 81553-575), which was labelled at the 5'-end with LCRed640 and phosphorylated at the 3'-end to block extension, and combined with one of the three sensor probes shown in Table 4.1.

Table 4.1 Melting curve analysis obtained by genotyping fifty individuals with three different sensor probes.

Name ^a	Sequence sensor probe ^b	Tm _[A] ^c (°C)	Tm _[C] ^d (°C)	ΔTm _{[A]/[C]} ^e (°C)
Sen [A]	Agg CAT TCC ACA CCT AAg AAC TA-F	62.4 ± 0.18	59.3	Nd
Sen [A]s	CAT TCC ACA CCT AAg AAC TA-F	57.2 ± 0.08	53.1	Nd
Sen [A]sL	CAT TCC ACA CCT AAg AAC TA-F	58.7 ± 0.08	51.9	7.5 ± 0.10

^a "s" is the short variant of the sensor probe; "L" short variant with a locked nucleic acid (LNA) on the polymorphic site; ^b Probe position in human PXR sequence AF280107 is sense between 81529-551 bp; the polymorphic nucleotide is underlined and expressed in bold; F is the position of the labelled fluorescein; ^c The mean melting temperature (Tm) ± standard deviation (SD) of 33 homozygote PXR 11156 [A]/[A]; ^d The melting temperature (Tm) of a homozygote PXR 11156 [C]/[C]; ^e The mean melting temperature difference (ΔTm) ± SD of 16 samples with the PXR 11156 [A]/[C] genotype; Nd is not discriminable.

PCR and melting curve analysis were performed on the LightCycler (Roche Diagnostics, Almere, the Netherlands). The PCR mixture contained 3.0 mM MgCl₂, 2.0 μl LC Faststart DNA Master Hybridization Probes Mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.50 μM of each primer, 0.20 μM of the anchor probe and 0.20 μM of the sensor probe. After adding 2.0 μl containing 50-100 ng genomic DNA to the PCR mixture the total volume was 20 μl. The PCR protocol included the following steps: a) denaturation for ten minutes at 95°C b) 45 cycles at 95°C for ten seconds; 55°C for ten seconds and 72°C for 16 seconds. After amplification was completed, a melting curve was recorded by cooling to 45°C at a ramp rate of 20°C/second, holding at 40°C for one minute and then heating slowly to 70°C at 0.2°C/second. The derivative melting curves are read in channel 2 of the LightCycler for the detection of the PXR fragment. The PCR products were sequenced according to a direct sequence procedure performed with the capillary sequencer ABI 3100[®] (Applied Biosystems, Fostercity, USA) using the Bridge version 1.1 sequence kit.

Results and discussion

Fifty individuals were genotyped for the PXR A11156C polymorphism by a real-time PCR FRET assay using the sensor probes illustrated in Table 4.1. Since sensor probe Sen [A] is complementary to the PXR 11156 [A] allele, melting of the PCR product homozygous for this 11156 [A] allele produced a melting peak at $62.4 \pm 0.18^\circ\text{C}$, whereas for a PCR product homozygous for the PXR 11156 [C] allele the melting peak was obtained at 59.3°C . In accordance with the melting temperatures obtained, 33 samples were genotyped as PXR 11156 [A]/[A] and one sample was genotyped as PXR 11156 [C]/[C]. A heterozygous sample contains both types of targets and should therefore generate both peaks. However, the real-time PCR FRET assay using the sensor probe Sen [A] produced for 16 samples one unspecific melting peak around $60.0 \pm 0.66^\circ\text{C}$ (see Figure 4.1A), which in our hands under no conditions could be resolved into two separated peaks. Most likely, these samples are heterozygous for the PXR A11156C polymorphism. Hence, the allele frequency for the 11156 [A] polymorphism is 84 % and for the 11156 [C] 16% which is in line with the other study⁴. The PCR products of three different PXR 11156 allelic variants were sequenced to confirm the results obtained by the real-time PCR FRET assays. The results provided by the sequence analysis matched exactly with the genotypes as classified by the melting peaks. The discriminating power of SNP detection by FRET probes is based on the difference in melting behaviour of mismatches *versus* perfect matches to the target probe. The greater the difference between the melting temperature (T_m) of the matched *versus* mismatched duplexes (ΔT_m), the better is the discriminating potential of the sensor probes in the FRET assay. This ΔT_m is dependent upon the length of the sensor probe, the type of mismatch and the neighbouring nucleotides. The longer the sensor probe, the smaller the effect of a single-base mismatch on overall duplex stability, hence the ΔT_m between match and mismatch duplexes decreases. Therefore to improve the melting curve analysis the sensor probe was shortened by three nucleotides which resulted in Sen [A]_s. The T_m of both homozygotes was lowered as to be expected. The difference between the T_m of both homozygotes was 4.1, which is 1°C more than obtained with Sen [A] (Table 4.1). However the melting curve analysis of heterozygotes did not yield two separate melting peaks, this is illustrated in Figure 4.1B. The differential stability of a match *versus* mismatch duplex is the main limitation of the use of FRET probes for detection of SNPs. Fluorogenic probes containing LNAs have shown strong affinity for their complementary targets. The main structural feature of these probes is the presence of an additional methylene bridge linking the 2'-hydroxyl group of a RNA monomer to the 4'-carbon of the ribose ring. The presence of this bridge "locks" the sugar ring in one fixed conformation (3'-endo), which is the conformation observed for the ribose ring in RNA or DNA hybrids. Based on NMR studies of LNA/DNA duplexes, it has been concluded that the main factor contributing to the extraordinary high stability of LNA containing duplexes is a local change of the phosphate backbone geometry that favours a much higher degree of base stacking⁶⁻¹³. Thus, the

Sen [A]s was modified with a LNA on the polymorphic site, resulting in Sen [A]sL. The melting curve analysis showed an increment in the temperature of the melting peaks of homozygotes 11156 [A]/[A]. This could be attributed to the improved binding of the LNA probe. The melting peak for the homozygote 11156 [C]/[C] seemed to be somewhat lowered. Figure 4.1C shows these melting curves. The heterozygotes now yielded two separated peaks for with a ΔT_m of 7.5°C (Table 4.1). From the present study it is obvious that by introducing a LNA on the polymorphic location in a sensor probe the discriminative power of a real-time PCR FRET assay may increase enormously. Therefore, we conclude that the incorporation of a LNA in the sensor probe of a FRET assay is an additional tool to improve further the analysis of SNPs.

Figure 4.1 Genotyping of the A11156C polymorphism of the PXR gene with allele specific fluorescent probes using (A) Sen [A], (B) Sen [A]s and (C) Sen [A]sL by derivative melting curve plots. Representative derivative melting curves are plotted for the sample homozygous for the (1) 11156 [C] allele; (2) a heterozygous sample; (3) a homozygous sample for the 11156 [A] allele; (4) a no template control.

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Chapter 5

Rapid genotyping of the organic anion transporter polypeptide 1B1 polymorphisms A388G and T521C with real-time polymerase chain reaction fluorescence resonance energy transfer assays

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Abstract

Background and aim

The organic anion transporting polypeptide 1B1 (OATP1B1) polymorphisms A388G and T521C of the solute carrier organic anion-transporter family member 1B1 gene (SLCO1B1), previously known as OATP-C, have potential impact on the drug metabolism.

Materials and methods

In order to set up a fast and consistent assay for these polymorphisms, we developed for both OATP1B1 polymorphisms rapid speed polymerase chain reaction (PCR) fluorescence resonance energy transfer (FRET) assays on the LightCycler.

Results

A locked nucleic acid (LNA) on the polymorphic location within the sensor probe was necessary to discriminate both alleles of the OATP1B1 T521C polymorphism. To confirm the reliability of both real-time PCR FRET assays, these new methods were validated by genotyping 120 samples using a PCR restriction fragment length polymorphism (RFLP) assay and an allele-specific PCR.

Conclusion

The results of the real-time PCR FRET assays were completely in line with the conventional PCR methods, indicating that the real-time PCR FRET assays are appropriate for clinical settings.

Introduction

The solute carrier organic anion-transporter (SLCO) family, also known as the organic anion-transporting polypeptides (OATP), represent a family of proteins responsible for the membrane transport of a large number of endogenous and xenobiotic compounds. Within this family, OATP1B1, OATP2B1 and OATP1B3 have now been established as the major OATPs located at the basolateral membrane of human hepatocytes. OATP1B1, also known as liver specific transporter-1 (LST-1), OATP-C or OATP2, is involved in the hepatocellular uptake of a variety of endogenous and foreign chemicals¹⁻⁴. Different groups have identified a number of single nucleotide polymorphisms (SNPs) in the SLCO1B1 gene^{5,6}. *In vitro* experiments showed that some OATP1B1 allelic variants exhibit a markedly reduced uptake of the OATP1B1 substrates estrone sulfate and estradiol 17 β -D-glucuronide^{5,7,8}. Several SNPs introduce amino acid changes in the OATP1B1 protein that result either in altered transport activity or affect the substrate specificity of this protein, as has for instance been shown for the substrates E₂17 β G and cholytaurine^{5,8,9}. More recently, several publications reported the OATP1B1*1b (388G), OATP1B1*5 (521C) and OATP1B1*15 (388G and 521C) variant alleles, which have relatively high allele frequencies in different ethnic populations and are associated with significantly altered pharmacokinetics of pravastatin, irinotecan and its active metabolite SN-38¹⁰⁻¹³. Until now these OATP1B1 alleles have been genotyped with either a PCR restriction fragment length polymorphism (RFLP) assay (A388G) or with an allele-specific PCR assay (T521C)^{5,6}. In order to perform large epidemiological studies or for routine clinical use, a rapid genotype method is preferred. Therefore, the present study describes real-time PCR fluorescence resonance energy transfer (FRET) assays, which are able to genotype these OATP1B1 polymorphisms in considerably less hands on time and with less contamination risk. The results are compared with both a PCR RFLP assay and with an allele-specific PCR assay.

Materials and methods

Study design

For the present study genomic deoxyribonucleic acid (DNA) was isolated from 120 anonymized samples originating from a healthy Caucasian population volunteers which consisted of 73 males and 47 females with a median age of 37 years (19-94 years). DNA was isolated from 200 μ l ethylene diamine tetra-acetic acid (EDTA) anti-coagulated blood with a High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) or a QIAamp blood mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturers' instructions. Genotyping for the OATP1B1 A388G and T521C polymorphisms was performed using real-time PCR FRET assays

on the LightCycler (Roche Diagnostics, Almere, the Netherlands). A PCR RFLP for the A388G polymorphism and an allele-specific PCR for the T521C polymorphism were used to confirm the results obtained by the real-time PCR FRET assays⁶. The digested and allele-specific PCR products were electrophoresed on a 3% MetaPhor[®] agarose gel containing 0.3 mg/l ethidium bromide at 75V for 1 hour and 30 minutes, respectively, and examined under ultraviolet illumination. FRET genotyping was also validated by sequencing a homozygote allelic variant sample, a heterozygote sample and a wild type sample of both OATP1B1 polymorphisms. The PCR products were sequenced according to a direct sequence procedure performed with the capillary sequencer ABI 3100[®] (Applied Biosystems, Fostercity, USA) using the Bridge version 1.1 sequence kit (Applied Biosystems, Fostercity, USA).

Genotyping for OATP1B1 T521C polymorphism

The primers F27: 5'-gTA gAC AAA ggg AAA gTg ATC ATA -'3 (sense; 80677-700) and R27: 5'-gTT AAA TTT gTA ATA gAA ATg C-'3 (anti-sense; 80936-915) were used to amplify a 260 bp part from the SLCO1B1 gene which covers the T521C polymorphism in exon 5 (Genbank acces no: AC022335). Detection was carried out using the anchor probe OATPCA27: 5'-TAA TAT gCT TCg Tgg AAT Agg ggA gAC TC-'3 (anti-sense; 80823-795) which was labelled at the 5'-end with LCRed 640 and phosphorylated at the 3'-end to block extension and the sensor probe OATPCP27L: 5'-Tgg ATA TAT gCg TTC ATg g-'3 (anti-sense; 80843-825) which is complementary to the OATP1B1 521C polymorphism. This 3'-fluorescein-labelled sensor probe, in which the polymorphic locked nucleic acid (LNA) is underlined, binds with a distance of one base 5' to the detection probe^{14,15}. The PCR mixture contained 5.9 µl sterile water; 3.0 mM MgCl₂; 2.0 µl LC Faststart DNA Master Hybridization Probes (Roche Diagnostics GmbH, Mannheim, Germany); 0.50 µM of each primer and 0.225 µM of both anchor and sensor probe (TIB MOLBIOL, Berlin, Germany). After adding 2.0 µl containing 50-100 ng genomic DNA to the PCR mixture the total volume is 20 µl. The PCR protocol included the following steps: a) denaturation for ten minutes at 95°C b) 55 cycles at 95°C for five seconds; 51°C for ten seconds and 72°C for 20 seconds. After amplification was completed, a melting curve was recorded by heating to 95°C (20°C/second), holding at 95°C for 20 seconds then cooling to 40°C (20°C/second), holding at 40°C for 20 seconds and then heating slowly to 85°C at 0.2°C/s.

Genotyping for OATP1B1 A388G polymorphism

The primers F28: 5'-CTC Agg TgA TgC TCT ATT gAg TgA -'3 (sense; 82564-587) and R28: 5'-ggA AAT TgA CAg AAA gTA CTC Tgg T-'3 (anti-sense; 82809-785) were used to amplify a 246 bp part from the SLCO1B1 gene which covers the A388G polymorphism in exon 4 (Genbank acces no: AC022335). Detection was carried out using the anchor probe OATPCA28: 5'-TAC CTg TAA CTg TAA gAA CAT CAC TgA ATT AAA CAT TTT gC-'3 (sense; 82664-704) which was labelled at the 5'-end

with LCRed640 and phosphorylated at the 3'-end to block extension and the sensor probe OATPCP28: 5'-TTT CTg ATg AAT IgA TAT TAg TTT CTT TAg A-3' (sense; 82632-662) which is complementary to the OATP1B1 388A polymorphism. This 3'-fluorescein-labelled sensor probe, in which the polymorphic nucleotide is underlined, binds with a distance of one base 5' to the detection probe. The PCR mixture contained 6.4 μ l sterile water; 3.0 mM MgCl₂; 2.0 μ l LC Faststart DNA Master Hybridization Probes, 0.50 μ M of each primer and 0.20 μ M of both anchor and sensor probe. After adding 2.0 μ l containing 50-100 ng genomic DNA to the PCR mixture the total volume is 20 μ l. The PCR protocol included the following steps: a) denaturation for ten minutes at 95°C b) 45 cycles at 95°C for ten seconds; 55°C for ten seconds and 72°C for ten seconds. After amplification was completed, a melting curve was recorded by heating to 95°C (20°C/second), holding at 95°C for 30 seconds, subsequently cooling to 60°C (0.5°C/second), holding at 60°C for 30 seconds and finally cooling to 45°C for 0 second (20°C/second) and then heating slowly to 75°C at 0.2°C/second. During the slow heating procedure of the melting curves, fluorescence was measured continuously to monitor the dissociation of the fluorophore-labelled detection probe. The fluorescence signals were then converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature *versus* temperature ($-d(F2/dT$ *versus* T).

Results

During the optimisation of the real-time PCR FRET assay for the OATP1B1 T521C polymorphism, several FRET designs have been tried. For the OATP1B1 T521C assay we used the same primers as Tirona *et al.* for their allele-specific PCR⁵. However, discrimination of the different allelic variants of the OATP1B1 T521C polymorphism was not possible using standard FRET probes. The modified FRET T521C assay applied a sensor probe in which the cytosine on the polymorphic location is substituted by an analogue locked nucleic acid (LNA). Due to their more rigid sugar moiety, LNA bases have a higher binding affinity to their complementary nucleic acids¹⁵. Hence, shorter sensor probes can be used which makes a better allelic discrimination possible¹⁴. Because the binding affinity of a well-designed LNA probe is remarkably decreased if hybrids contain one mismatched basepair (bp). For the OATP1B1 T521C polymorphism, the mean melting point $\pm$ standard deviation (SD) of the sensor probes was $52.2 \pm 0.29^\circ\text{C}$ ($n = 82$) when hybridized to the OATP1B1 521TT polymorphism and 59.1°C ($n = 3$) when hybridized to the OATP1B1 521CC polymorphism. The mean difference $\pm$ SD between two melting temperatures of 35 heterozygous samples of the OATP1B1 T521C polymorphism was $7.6 \pm 0.19^\circ\text{C}$ (Figure 5.1A).

Figure 5.1 Genotyping of the OATP1B1 A388G and T521C polymorphisms with allele specific fluorescent probes by derivative melting curve plots. The $-d(F2/dT)$ derivative melting curves are read in channel 2 for the detection of both OATP1B1 fragments.

A. The derivative melting curve is plotted for a sample homozygous for the 521T allele (1; T_m 52.1°C); a sample homozygous for the 521C allele (2; T_m 59.4°C) and a heterozygous sample (3; T_m 52.3°C and 60.0°C).

B. The derivative melting curve is plotted for a sample homozygous for the 388G allele (1; T_m 57.6°C); a sample homozygous for the 388A allele (2; T_m 60.0°C) and a heterozygous sample (3; T_m 57.9°C and 60.4°C). Melting analysis of a no template control was also performed for both assays (4).

Although we tried to optimise several FRET designs for the OATP1B1 A388G polymorphism, including the use of a LNA in the sensor probe, the discrimination between the melting peaks of heterozygous samples is still limited. Tirona *et al.* used primers for the OATP1B1 A388G assay which have a melting temperature (T_m) that is too low for application in this real-time PCR FRET assay⁵. Because this whole sequence area is very A/T rich, unusual long sensor and anchor probes were necessary to obtain a sufficient high probe T_m ¹⁶. For the OATP1B1 A388G polymorphism, the mean melting point $\pm$ SD of the sensor probes was $59.5 \pm 0.14^\circ\text{C}$ ($n = 43$) when hybridised to the OATP1B1 388AA genotype and $57.3 \pm 0.14^\circ\text{C}$ ($n = 16$) when hybridised to the OATP1B1 388GG genotype. This is close, hence 61 heterozygotes do not demonstrate two separated melting peaks. Yet, as is shown in Figure 5.1B, they can be discriminated from either wild type or variant homozygotes. In total, after genotyping 120 individuals in five different PCR runs for both polymorphisms, the frequency of the OATP1B1 521TT genotype is 68%, for the 521CT genotype 29% and for the OATP1B1 521CC genotype 3%. In addition, the frequency of the OATP1B1 388AA genotype is 36%, for the 388AG genotype 51% and for the OATP1B1 388GG genotype 13%. The allele frequencies of the volunteers examined is 17% (521C) and 39% (388G) which is in line with those published in other studies^{5,6,10}. To evaluate the reliability of the real-time PCR FRET assays on the LightCycler the 120 samples were also genotyped for the OATP1B1 A388G polymorphism with the PCR RFLP assay using TaqI (Roche Diagnostics GmbH, Basel, Switzerland) and for the T521C polymorphism using an allele-specific PCR⁶. The A388G substitution introduces a restriction enzyme cleavage site for TaqI. Accordingly,

after digestion with TaqI, the fragments of the 388A allele were 90 and 72 bp, whereas the amplicon of the 388G allele is digested into 90, 49 and 23 bp fragments. Individuals carrying both 388 alleles yield 90, 72, 49 and 23 bp fragments. An allele-specific PCR was used for the confirmation of the T521C polymorphism. Depending on the presence of a 521T or C allele a 142 bp fragment appears after electrophoresis of the PCR products. The PCR products and fragments obtained by allele-specific PCR and PCR RFLP were of the expected sizes and the genotypes determined by both methods matched completely with the genotypes determined with the real-time PCR FRET assays. Furthermore, sequence analysis confirmed the results obtained by both real-time PCR FRET assays, the PCR RFLP and the allele-specific PCR.

Highlights

- Recently, several studies found a correlation between OATP1B1 388G and OATP1B1 521C allelic variants and blood levels of several drugs and/or their metabolites. Individuals carrying one of both OATP1B1 521C alleles eventually in combination with OATP1B1 388G showed significantly higher blood levels.
- Nowadays, genotyping of these polymorphisms is still performed by an allele-specific PCR or a PCR RFLP method which are conventional genotyping methods that are time consuming and with a high contamination risk in contrast to the real-time PCR methods.
- In this study we describe two real-time PCR FRET assays on the LightCycler for genotyping the OATP1B1 A388G and T521C allelic variants. The results of both conventional PCR RFLP and allele-specific PCR methods are compared with the results of the real-time PCR FRET assays. The real-time PCR FRET assays are also validated by sequencing a homozygote allelic variant sample, a heterozygote sample and a wild type sample of both OATP1B1 polymorphisms.
- The locked nucleic acid (LNA) on the polymorphic location used in the sensor probe of the FRET assay makes discrimination of the OATP1B1 521C allelic variants possible. Therefore, implementation of a LNA on a polymorphic location in a sensor probe can contribute to applications for FRET probes in real-time PCR assays for polymorphism detection.
- Given the potential impact of functional OATP1B1 polymorphisms on drug metabolism, reliable and fast assays are clearly needed. Therefore, the overall goal of this study is to provide a fast and consistent genotyping method for the OATP1B1 A388G and T521C polymorphisms which can be used in epidemiological studies or routine clinical settings.

Outlook and conclusions

Since new polymorphisms are being discovered in a high speed, the need for fast and reliable genotyping methods becomes clear. Especially, polymorphisms in genes which have a significant effect on several drug blood levels and with a high allelic frequency, are of special interest. Recently, two functional OATP1B1 polymorphisms were discovered, which have apparently a cumulative effect on pravastatin blood levels. Genotyping 120 volunteers for these OATP1B1 polymorphisms with both real-time PCR FRET assays indicates complete concordance with the genotyping obtained by the PCR RFLP assay for OATP1B1 A388G and the allele-specific PCR assay for OATP1B1 T521C. Based on these results, we conclude that genotyping of the OATP1B1 A388G and T521C polymorphism with a real-time PCR FRET assay on the LightCycler is a very reliable, robust and reproducible technique.

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Chapter 6

Evaluation of limited sampling strategies for tacrolimus

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Abstract

Background and aim

In literature, a great diversity of limited sampling strategies (LSS) have been recommended for tacrolimus monitoring, although proper validation is limited. The question is if these LSS might be useful for AUC prediction of other patient populations.

Materials and methods

The performance of 24 of these LSS in 37 renal transplant patients with known AUC's has been evaluated and the results were also compared with the predictability of the trough concentrations, C_0 and C_{12} . Criterion was an absolute prediction error (APE%) that differs less than 15% from the complete AUC_{0-12} .

Results

Thirteen of the 18 (72%) LSS based on regression analysis were capable of predicting at least 90% of the 37 individual AUC_{0-12} within an APE of 15%. Predictions based on C_0 , C_{12} and Bayesian fitting were lower than 90%: 62% and 66.8%, respectively.

Conclusion

The present study indicates that implementation of LSS based on regression analysis could produce satisfactory predictions although careful evaluation is mandatory.

Introduction

The calcineurin inhibitor tacrolimus, used widely after organ transplantation, has a narrow therapeutic index and highly variable pharmacokinetic characteristics. Close monitoring of the drug concentration is required to achieve an optimal efficiency by minimizing the risk of subtherapeutic and toxic blood concentrations. Efficacy and side effects of tacrolimus are highly correlated with the area under the time tacrolimus concentration curve (AUC_{0-12})¹. Elevated tacrolimus concentrations may lead to severe side effects such as nephrotoxicity, neurotoxicity and hyperglycaemia²⁻⁴ while subtherapeutic tacrolimus concentrations increase the risk of transplant rejection enormously⁵⁻⁷. The most exact way to monitor the total tacrolimus exposure is by creating 12 hour pharmacokinetic profiles, which implicates that the tacrolimus concentration should be measured on at least six different time points. The AUC_{0-12} can then be calculated according to the trapezoidal rule using the tacrolimus concentrations measured at different time points. Since recording a complete 12 hour pharmacokinetic profile for every patient is not feasible in clinical practice, traditionally many transplant centres use tacrolimus trough (C_0) concentrations to estimate the tacrolimus exposure. Although tacrolimus C_0 concentrations are generally considered to be a good indication of the total systemic drug exposure^{1,8}, its usefulness in differentiating graft rejection episodes from nephrotoxicity has been questioned^{6,9-11}. Recently, the correlation between individual tacrolimus concentrations and AUC_{0-12} has been studied in kidney¹²⁻¹⁸, liver¹⁹, heart^{20,21} and lung²² transplant recipients. In these studies, a poor association was found between the tacrolimus C_0 concentrations and the AUC_{0-12} , while tacrolimus concentrations measured at other time points showed much better correlations with the AUC_{0-12} . Additionally, strategies have been developed that consisted of a limited number of sampling time points within a short time post dose, the so called limited sampling strategies. Several two and three time point sampling strategies showed a high correlation with the AUC_{0-12} in the published studies and were able to predict the AUC_{0-12} more accurately than the C_0 concentration alone^{12,15,16,18,20,22}. Based on the number of published studies regarding limited sampling strategies for tacrolimus, there seems to be a growing interest for non C_0 concentration measurements as an indicator of between patient variability and as a guide for dose adjustments. Most of these studies recommend different limited sampling strategies but these strategies have not been validated with a separate population. Ting *et al.*²³ recently reported that validation of the different limited sampling strategies with an independent transplant population is without doubt an absolute prerequisite. The question is if limited sampling strategies, described in literature could be used for the own population. Different limited sampling strategies have been selected for an evaluation in this study based on their predictive performances claimed in previous studies and their number of sample points (two or three) within a short time post dose (≤ 4 hours). Evaluation of these limited sampling strategies was performed by evaluating the predictive value of 24 different limited sampling strategies from literature and also

the trough concentrations C_0 and C_{12} , determined in our own population. Parameters used for the strategy comparison was the associated determination coefficient (R^2) between the $AUC_{\text{predicted}}$ (AUC_{pred}) from the limited sampling strategy and the actual 12 hour AUC (AUC_{actual}) calculated by the trapezoidal rule, the percentage of prediction error (PE%) and the percentage of absolute prediction error (APE%) was determined using our own well-characterised late posttransplant patient group of 37 patients.

Materials and methods

Patient populations

In total 37 Caucasian renal transplant recipients of whom in the past, for a clinical trial, a 12 hour time tacrolimus concentration curve was performed, were included in this study. The transplant recipients underwent a renal transplantation at least one year ago. Patients taking medication known to interact with tacrolimus, suffered from gastrointestinal or liver disease, pre-transplantation diabetes mellitus or other disorders that could have altered the absorption of tacrolimus were excluded for this study as is illustrated in Table 6.1. Prior to the blood sample collection, there was no tacrolimus dose change for at least one week. After overnight fasting the blood samples were collected immediately pre (C_0) and 0.5 ($C_{0.5}$), 1 (C_1), 2 (C_2), 3 (C_3), 4 (C_4), 5 (C_5), 7.5 ($C_{7.5}$) and 12 (C_{12}) hours after the morning tacrolimus administration. Patients were not allowed to take food until one hour after ingesting the tacrolimus dose and were advised to avoid grapefruit juice to prevent alterations in the tacrolimus metabolism. Demographic as well as clinical data were determined at the time of recording the 12 hour time tacrolimus concentration curve. Figure 6.1 shows the mean pharmacokinetic profile of the renal transplant recipients. The study was performed in accordance to the Declaration of Helsinki and its amendments. The protocol was approved by the local Medical Ethics Committee and written informed consent for participation in this study was obtained from all patients.

Determination of tacrolimus concentrations

The tacrolimus blood concentrations were determined in ethylene diamine tetra-acetic acid (EDTA) whole blood, using a method based on high pressure liquid chromatography (HPLC) tandem mass spectrometry (MS/MS). The assay is linear from 1 to 300 $\mu\text{g/l}$. Intra-assay precision and accuracy was 3.4%, 2.2%, 3.0% and 102%, 94% and 94 % respectively at 3.04, 6.23 and 13.0 $\mu\text{g/l}$ ($n = 6$). Inter-assay precision and accuracy were 8.2%, 5.2%, 4.6% and 102%, 94% and 93% ($n = 9$) respectively. Limit of quantification was 1.0 $\mu\text{g/l}$. The laboratory participates in an International Tacrolimus Proficiency Testing Scheme.

Table 6.1 Demographic characteristics of the two renal transplant recipients groups.

Demographic characteristics		Patients (n=37)
Gender (male/female)		24/13
Age (years, mean $\pm$ SD)		51.3 $\pm$ 10.9
Length (cm, mean $\pm$ SD)		174 $\pm$ 8.4
Weight (kg, mean $\pm$ SD)		77.4 $\pm$ 13.5
Body Mass Index (kg/m ² , mean $\pm$ SD)		25.6 $\pm$ 3.42
Primary kidney disease	Glomerulonephritis	1
	Chronic pyelonephritis	2
	IgA nephropathy	4
	Hypertensive nephropathy	7
	Diabetes Mellitus nephropathy	0
	Polycystic kidney disease	8
	Unknown	4
	Other	11
Transplantation number	First	30
	Second	6
	Third or more	1
Tacrolimus mono therapy		29
Tacrolimus dose (mg/kg/day, mean $\pm$ SD)		0.054 $\pm$ 0.029
C ₀ (ng/mL, mean $\pm$ SD)		6.59 $\pm$ 1.39
AUC ₀₋₁₂ (ng $\times$ hr/mL, mean $\pm$ SD)		122.5 $\pm$ 31.1
C _{max} (ng/mL, mean $\pm$ SD)		20.9 $\pm$ 6.5
T _{max} (hr, mean $\pm$ SD)		1.24 $\pm$ 0.43
Use of azothioprine, MMF ^a , rapamycine, steroids		3/4/0/0
Time since transplantation (days, mean, (range))		1542 (453–4128)
Haemoglobin (mmol/L, mean $\pm$ SD)		8.52 $\pm$ 0.83
Haematocrit fraction (mean $\pm$ SD)		0.41 $\pm$ 0.04
ALAT (U/L, mean $\pm$ SD)		24 $\pm$ 13
ASAT (U/L, mean $\pm$ SD)		17 $\pm$ 10
Serum albumin (g/L, mean $\pm$ SD,)		37.0 $\pm$ 3.84
Serum creatinine (μ mol/L, mean $\pm$ SD)		128 $\pm$ 29
Creatinine Clearance (Cockcroft – Gault; mL/min, mean $\pm$ SD)		58.4 $\pm$ 26.6

^a mycophenolate mofetil Figure 6.1 Mean ($\pm$ SD) whole blood concentrations of tacrolimus in 37 renal transplant recipients.

Pharmacokinetical and statistical analysis

The area under the time tacrolimus concentration curve (AUC_{0-12}) was calculated from the time *versus* tacrolimus concentration plot using the linear trapezoidal rule in MWPharm 3.50 (Mediware, Groningen, the Netherlands). The predicted AUC_{0-12} (AUC_{pred}), calculated with 24 limited sampling strategies, were validated by determining the predictive performance as described by Sheiner and Beal²⁴. The percentage of the prediction error (PE (%)) and the percentage of the absolute prediction error (APE (%)) are parameters often used for the validation in the studies that describe limited sampling strategies^{12,14-16,18,20,22}. In our opinion, a suitable limited sampling strategy for tacrolimus should consist of two or three time concentration points within a short time post dose (≤ 4 hour) and is able to predict at least 90% of AUC_{0-12} within an APE (%) of 15%. Given the high pharmacokinetic variability, an APE (%) of less than 15% was considered clinically acceptable^{16,25,26}. Finally, a strategy based on the tacrolimus C_0 and C_{12} concentrations is developed for our own renal transplant patient population and compared with the different limited sampling strategies that already have been published.

Prediction bias was measured by a percentage of the prediction error (PE (%)) using the following formula:

$$PE (\%) = 100 \times (AUC_{pred} - AUC_{actual})/AUC_{actual}$$

Prediction precision was measured by the percentage of the absolute prediction error (APE (%)) using the following formula:

$$APE (\%) = 100 \times |(AUC_{pred} - AUC_{actual})|/AUC_{actual}$$

The variance in the strength of association between the AUC_{pred} and the AUC_{actual} was reflected by the linear regression coefficient of multiple determination (R^2). All values are expressed as mean $\pm$ SD. All statistical analyses were performed with use of SPSS 12.0 software for windows (Chicago, IL, USA).

Results

Evaluation of predictive performances of the limited sampling strategies

Table 6.2 shows an overview of the studies describing the limited sampling strategies evaluated in the present study. The regression equations and the R^2 found by the investigators of the limited sampling strategies evaluated are summarised in Table 6.3.

Table 6.2 Overview of the characteristics of the transplant patients included in the studies that described limited sampling strategies.

Study	Transplanted Organ	Number of patients ^a	Number of AUC ₀₋₁₂ curves for validation (I ^b /NI ^c)	Analytical method ^d	Time since transplantation ^e	Inclusion criteria ^f
Wong <i>et al.</i> ¹⁶	Kidney	18	0/18	IMx II	2.5 years	1,2
Aumente Rubio <i>et al.</i> ²⁰	Heart	22	0/25	IMx	< 1 year	---
Pisitkun <i>et al.</i> ¹⁸	Kidney	15	0/15	IMx II	8.7 months	1,2,3
Armendariz <i>et al.</i> ¹²	Kidney	22	13/14	IMx	Unknown	---
Scholten <i>et al.</i> ¹⁵	Kidney	43	64/20	IMx	Differs ^g	2
Ragette <i>et al.</i> ²²	Lung	15	0/31	IMx	7.3 months	---

^a number of transplant patients used in the included study for both developing and validating the limited sampling strategies. ^b number of AUC₀₋₁₂ used for developing the limited sampling strategies described in this study. ^c number of independent (I) and dependent (NI) AUC₀₋₁₂ used in the study to validate the created limited sampling strategies. ^d the analytical method used to determine the whole blood tacrolimus concentration. ^e the mean time after transplantation. ^f the inclusion criteria used for the transplant patients in the different studies. 1. tacrolimus is administrated when patients were in the fasting state. 2. patients have been selected for using no interfering medication with tacrolimus. 3. patients have been selected with a normal liver function test. ^g Twenty-two pharmacokinetic profiles were obtained within two weeks after transplantation, 11 pharmacokinetic profiles were obtained between two and six weeks after transplantation and 51 pharmacokinetic profile were obtained between 6 and 52 weeks after transplantation.

Table 6.3 Overview limited sampling strategies and their reported correlation coefficient (R^2) with the complete tacrolimus AUC₀₋₁₂.

Equation	Time points	Regression equations	R^2	Ref
1.	C ₀	$14.550 + 16.387 \times C_0$	0.54	
2.	C ₁₂	$15.892 + 17.852 \times C_{12}$	0.79	
3. ^a	C ₀ , C ₂ , C ₄	$13.3 + 1.2 \times C_0 + 2.4 \times C_2 + 5.6 \times C_4$	0.93	16
4. ^a	C ₂ , C ₄	$16.2 + 2.4 \times C_2 + 5.9 \times C_4$	0.93	16
5. ^a	C ₀ , C ₂ , C ₄	$0.98 + 4.17 \times C_0 + 2.29 \times C_2 + 5.3 \times C_4$	0.97	20
6.	C ₀ , C ₄	$3.75 + 5.52 \times C_0 + 6.97 \times C_4$	0.95	20
7.	C ₀ , C ₁ , C ₂	$-5.496 + 7.189 \times C_0 + 2.357 \times C_1 + 2.131 \times C_2$	0.93	18
8. ^a	C ₀ , C ₁ , C ₄	$3.85 + 3.688 \times C_0 + 1.355 \times C_1 + 6.649 \times C_4$	0.97	18
9. ^a	C ₀ , C ₂ , C ₄	$-6.103 + 2.383 \times C_0 + 1.911 \times C_2 + 7.582 \times C_4$	0.97	18
10. ^a	C ₁ , C ₂ , C ₄	$1.304 + 0.465 \times C_1 + 1.636 \times C_2 + 8.256 \times C_4$	0.96	18
11.	C ₀ , C ₁	$9.345 + 8.408 \times C_0 + 3.23 \times C_1$	0.91	18
12. ^a	C ₀ , C ₄	$8.231 + 2.316 \times C_0 + 9.636 \times C_4$	0.95	18
13. ^a	C ₁ , C ₄	$13.114 + 0.873 \times C_1 + 9.291 \times C_4$	0.95	18
14. ^a	C ₂ , C ₄	$-0.192 + 1.888 \times C_2 + 8.783 \times C_4$	0.96	18
15. ^a	C ₀ , C ₁ , C ₄	$4.5 \times C_0 + 2 \times C_1 + 5.5 \times C_4$	0.97	18
16. ^a	C ₀ , C ₂ , C ₄	$5 \times C_0 + 2 \times C_2 + 5 \times C_4$	0.96	18
17.	C ₀ , C ₁ , C ₄	$8.90 + 4.0 \times C_0 + 1.77 \times C_1 + 5.47 \times C_4$	0.97	12
18.	C ₀ , C ₁ , C ₃	Bayesian estimation of the actual AUC ₀₋₁₂	0.97	15
19.	C ₀ , C ₂ , C ₃	Bayesian estimation of the actual AUC ₀₋₁₂	0.96	15
20.	C ₀ , C ₂ , C ₄	Bayesian estimation of the actual AUC ₀₋₁₂	0.97	15
21.	C ₀ , C ₂	Bayesian estimation of the actual AUC ₀₋₁₂	0.94	15
22.	C ₀ , C ₃	Bayesian estimation of the actual AUC ₀₋₁₂	0.96	15
23.	C ₀ , C ₄	Bayesian estimation of the actual AUC ₀₋₁₂	0.95	15
24. ^a	C ₀ , C ₂ , C ₄	$5.87 + 4.50 \times C_0 + 1.05 \times C_2 + 5.87 \times C_4$	0.98	22
25.	C ₀ , C ₄	$1.16 + 4.41 \times C_0 + 7.71 \times C_4$	0.96	22
26. ^a	C ₂ , C ₄	$24.36 + 0.97 \times C_2 + 7.94 \times C_4$	0.94	22

Limited sample strategies derived from the linear trapezoidal rule and the actual AUC₀₋₁₂. ^a Limited sampling strategies that are able to predict 90% of complete AUC₀₋₁₂ of the renal transplant recipients within the absolute prediction error (APE%) of 15%.

Table 6.4 describes the R^2 that represents the association between AUC_{pred} and AUC_{actual} and the calculated PE (%) and APE (%) of the 24 evaluated limited sampling strategies for our 37 pharmacokinetic profiles. Additionally, all but three of the LSS examined gave a better prediction of the complete AUC_{0-12} in comparison with trough concentrations C_0 and C_{12} (mean 62%). Predictivity of all six limited sampling strategies based on Bayesian fitting was < 90% (mean 66.8%).

Table 6.4 Evaluation of predictive performance of limited sampling strategies to estimate the complete AUC_{0-12} in the 37 renal transplant recipients.

Equation	Time points	R^2	Mean PE (%)	Mean APE (%)	$\pm 15\%^a$
23.	C_0, C_4^c	0.760	-14.9 ± 13.8 (-46.0-33.2)	17.9 ± 9.43 (1.12-46.0)	13 (35%)
22.	C_0, C_3^c	0.779	-11.5 ± 14.0 (-41.9-33.1)	15.7 ± 8.83 (2.0-41.9)	21 (57%)
1.	C_0	0.536	2.11 ± 14.8 (-27.1-24.4)	12.3 ± 8.22 (0.7-27.1)	22 (59%)
11.	C_0, C_1	0.703	6.58 ± 14.8 (-26.5-43.7)	12.6 ± 10.1 (0.1-43.7)	24 (65%)
2.	C_{12}	0.80	9.56 ± 11.6 (-12.7-29.9)	12.0 ± 8.97 (0.3-29.9)	24 (65%)
19.	C_0, C_2, C_3^c	0.502	-4.44 ± 17.4 (-45.3-50.6)	13.7 ± 11.4 (0.4-50.6)	25 (68%)
20.	C_0, C_2, C_4^c	0.537	-5.1 ± 16.3 (-43.1-50.3)	12.9 ± 10.4 (0.2-50.3)	28 (76%)
18.	C_0, C_1, C_3^c	0.525	9.95 ± 19.4 (-29.7-88.8)	13.1 ± 17.4 (0.4-88.8)	30 (81%)
25.	C_0, C_4	0.911	-7.83 ± 6.36 (-21.3-2.4)	8.08 ± 6.02 (0.1-21.3)	30 (81%)
7.	C_0, C_1, C_2	0.869	2.35 ± 9.96 (-17.2-27.3)	8.03 ± 6.22 (0.0-27.3)	31 (84%)
6.	C_0, C_4	0.896	-5.97 ± 6.71 (-20.1-4.7)	6.63 ± 6.04 (0.6-20.1)	31 (84%)
21.	C_0, C_2^b	0.802	-3.69 ± 10.2 (-19.6-18.6)	9.10 ± 5.67 (0.4-19.6)	31 (84%)
17.	C_0, C_1, C_4	0.943	5.91 ± 7.06 (-8.8-26.3)	7.02 ± 5.93 (0.2-26.3)	33 (89%)
15.	C_0, C_1, C_4^b	0.934	5.00 ± 7.28 (-9.8-25.8)	6.81 ± 5.57 (0.2-25.8)	34 (92%)
14.	C_2, C_4	0.964	2.28 ± 6.58 (-17.1-16.1)	5.45 ± 4.24 (0.7-17.1)	35 (95%)
24.	C_0, C_2, C_4	0.941	-4.81 ± 5.26 (-17.3-2.8)	5.32 ± 4.73 (0.1-17.3)	35 (95%)
13.	C_1, C_4	0.973	6.30 ± 4.84 (-5.9-17.8)	6.68 ± 4.28 (0.3-17.8)	36 (97%)
8.	C_0, C_1, C_4	0.967	3.37 ± 5.21 (-5.2-17.7)	4.87 ± 3.80 (0.2-17.7)	36 (97%)
9.	C_0, C_2, C_4	0.962	0.10 ± 6.37 (-16.7-14.7)	4.71 ± 4.22 (0.3-16.7)	36 (97%)
26.	C_2, C_4	0.959	3.38 ± 5.24 (-7.6-15.5)	5.20 ± 3.37 (0.0-15.5)	36 (97%)
10.	C_1, C_2, C_4	0.976	3.07 ± 5.40 (-14.9-13.2)	4.99 ± 3.64 (0.1-14.9)	37 (100%)
16.	C_0, C_2, C_4^b	0.953	-1.58 ± 5.29 (-14.9-10.1)	4.00 ± 3.75 (0.0-14.9)	37 (100%)
12.	C_0, C_4	0.930	3.55 ± 6.30 (-9.8-14.3)	6.29 ± 3.46 (0.1-14.3)	37 (100%)
4.	C_2, C_4	0.963	-1.66 ± 4.99 (-12.0-14.3)	4.13 ± 3.20 (0.2-14.3)	37 (100%)
5.	C_0, C_2, C_4	0.959	1.33 ± 5.24 (-11.8-14.0)	4.22 ± 3.32 (0.5-14.0)	37 (100%)
3.	C_0, C_2, C_4	0.965	-0.20 ± 4.79 (-10.4-13.7)	3.64 ± 3.06 (0.2-13.7)	37 (100%)

^a Number and percentage of calculated AUC_{0-12} with a prediction error within 15%; ^b Limited sample strategies derived from the linear trapezoidal rule and the actual AUC_{0-12} ; ^c Bayesian estimation of the actual AUC_{0-12}

Figure 6.2 An overview of the predictive performances of the limited sampling strategies published using 37 different pharmacokinetic profiles recorded in 37 different renal transplant recipients. The mean prediction error (PE%) < 15% is plotted *versus* the frequency of the limited sampling strategies examined.

Discussion

Our results indicate like several other studies^{12,14-16,18,20,22} that trough concentrations C_0 and C_{12} have a lower predictive value for the complete 12 hour AUC than most limited sampling strategies already published. Bayesian estimation of the AUC_{actual} using different two or three time point strategies is not able to predict the AUC_{actual} reliably in the renal transplant recipient group examined. The Bayesian estimation strategies showed for at least 16% of the complete AUC₀₋₁₂ an APE of more than 15%. Two or three time points in the early phase (≤ 4 hour) post dose seems not to be sufficient for a Bayesian estimation strategy to fit correctly most of the AUC₀₋₁₂ and thus predict the complete AUC₀₋₁₂ reliably. The differences in variability and shape between the curves of late posttransplant recipients combined with just two or three sample points may have caused the large differences found between the AUC_{pred} calculated according to the Bayesian estimation strategy and the complete AUC_{actual} . Ting *et al.*²³ recently suggests that limited sampling strategies should only be applied on transplant patient populations that are comparable with the transplant patient population that has been used to develop the limited sampling strategy. However, the renal transplant patient group examined in the present study is not exactly comparable with the transplant patient populations in which the equations for the limited sampling strategies are developed. For example: Aumente Rubio *et al.*²⁰ and Ragette *et al.*²² used respectively heart and lung transplant recipients to develop and validate their limited sampling strategies. Despite these limited sampling strategies being developed with the pharmacokinetic

profiles of patients who underwent a different kind of transplantation, equation 5, 24 and 26 are able to predict at least 90% of the AUC_{0-12} within an APE (%) of 15% which suggests that these limited sampling strategies are more robust than is expected by Ting *et al.*²³. In conclusion, the present study indicates that limited sampling strategies from literature based on regression analysis, could produce satisfactory predictions with own patients, although careful evaluation of its reliability is mandatory.

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Chapter 7

Influence of different allelic variants of the cytochrome 3A and adenosine triphosphate-binding cassette B1 gene on the tacrolimus pharmacokinetic profile of Chinese renal transplant recipients

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Abstract

Background and aim

Tacrolimus has a narrow therapeutic window and a wide interindividual variation in its pharmacokinetics. Cytochrome P450 3A (CYP3A) and the adenosine triphosphate-binding cassette B1 (ABCB1) gene encoding protein, multidrug resistance-1 (MDR1) or P-glycoprotein play an important role in the tacrolimus disposition. Therefore this study evaluates whether CYP3A and ABCB1 polymorphisms are associated with the area under the time tacrolimus concentration curve (AUC_{0-12}) calculated using a two time point sample strategy.

Materials and methods

The CYP3A and ABCB1 genotypes are determined by real-time polymerase chain reaction (PCR) fluorescence resonance energy transfer (FRET) assays in 103 Chinese renal transplant recipients and consequently related to their dose-normalized AUC_{0-12} ($dnAUC_{0-12}$).

Results

A significant allele-dependent effect (Kruskal Wallis; $P < 0.001$) is observed between the CYP3A5*3 polymorphism and the $dnAUC_{0-12}$. Multiple regression analysis showed that the CYP3A5*3 polymorphism is the most significant independent variable and explained 35% of the dose requirement variability in relation to tacrolimus use. Regarding the ABCB1 G2677T/A and C3435T polymorphisms, a trend is observed between the different genotypes and the $dnAUC_{0-12}$.

Conclusion

The CYP3A5*3 polymorphism may be an important factor in determining the dose requirement for tacrolimus and genotyping can help determine the initial daily dose needed by individual patients for adequate immunosuppression.

Introduction

The calcineurin inhibitor tacrolimus that is used worldwide for primary immunosuppression following renal transplantation has a narrow therapeutic index, which makes close therapeutic drug monitoring necessary to prevent both subtherapeutic blood levels as well as toxic blood levels. Subtherapeutic tacrolimus blood levels increase the risk of transplant rejection¹⁻³, while toxic tacrolimus blood levels may lead to severe side effects such as nephrotoxicity and neurotoxicity⁴⁻⁶. The high interindividual variability in tacrolimus pharmacokinetics complicates the realization of this narrow therapeutic index^{7,8}. Although tacrolimus trough (C_0) levels have been shown to provide a reliable indication of the total drug exposure⁹, their usefulness in differentiating graft rejection episodes from nephrotoxicity has been questioned^{2,10}. A more accurate estimation of the tacrolimus exposure by making 12 hour pharmacokinetic profiles may lead to a lower incidence of acute rejection like in the case of cyclosporine^{11,12}. Since obtaining complete 12 hour pharmacokinetic profiles for every renal transplant patient is not feasible in clinical practice, parameters have been examined that strongly correlate with the 12 hour pharmacokinetic profile. Recent studies showed higher correlations with the 12 hour pharmacokinetic profiles of tacrolimus when a limited sampling point sample strategy is used instead of C_0 levels alone¹³⁻¹⁷. Additionally, the two time point sample strategy used in the present study is able to calculate reliably the area under the time tacrolimus concentration curve or AUC_{0-12} in renal transplant patient groups^{15,17}. The cytochrome P450 (CYP) 3A iso-enzymes mainly represented by CYP3A4 and CYP3A5 have been identified as the major enzymes responsible for the disposition of tacrolimus¹⁸. Although a number of CYP3A4 polymorphisms have been identified (website Human Cytochrome P450 (CYP) Allele Nomenclature Committee¹⁰¹), these are rare and therefore do not contribute significantly to CYP3A4 expression variability. The single nucleotide polymorphism (SNP) CYP3A5 6986G or *3 displays a sequence variability in intron 3 that creates a cryptic splice site and encodes an aberrantly spliced mRNA with a premature stop codon, leading to the absence of protein expression. This CYP3A5*3 polymorphism occurred homozygously in 90% of the Caucasians, 73% of the Chinese and in 30% of the African-American population¹⁹⁻²¹. Although several studies²²⁻³¹ have described the association between the CYP3A5*3 polymorphism and tacrolimus C_0 levels, the present study examines the associations with the CYP3A polymorphisms using a more accurate approach to determine the tacrolimus exposure in renal transplant patients. Tacrolimus is also a substrate for the adenosine triphosphate-binding cassette B1 (ABCB1) gene encoding protein multidrug resistance-1 (MDR1) or P-glycoprotein. ABCB1 is an ATP-dependent efflux pump that contributes to the protection of the body from environmental toxins and drugs like tacrolimus by limiting their absorption from the gut lumen or increasing their biliary and urinary excretion³². Three partly linked polymorphisms in the ABCB1 gene located on exons 12, 21 and 26 have been studied widely and these polymorphisms account for the major haplotypes

encountered in Caucasians. Whereas two of these ABCB1 polymorphisms, C1236T and C3435T, result in silent mutations, the ABCB1 G2677T/A polymorphism in exon 21 is non-synonymous and results in an amino acid exchange (Ala893Ser/Thr). A number of studies already have reported that there seems to be no association between the ABCB1 polymorphisms and tacrolimus dose-normalized trough (dnC_0) levels^{25-27,30,31,33} or the dose-normalized area under the time tacrolimus concentration curve (dnAUC_{0-12})^{34,35}. However, some studies found a correlation between individual ABCB1 polymorphisms^{23,24,36} or the ABCB1 1236C-2677G-3435C haplotype³⁷ and a higher tacrolimus dose. The present study evaluates in Chinese renal transplant recipients the impact of CYP3A and ABCB1 polymorphisms on the dnAUC_{0-12} which is calculated according to a two time point sampling strategy. Furthermore, we described two new real-time polymerase chain reaction (PCR) fluorescence resonance energy transfer (FRET) assays to determine the genotype for the CYP3A5 A6986G and ABCB1 C1236T polymorphism.

Patients and methods

Study population

A total of 103 Chinese renal transplant recipients who received tacrolimus as part of the immunosuppressive therapy and had regular follow-up in Queen Elizabeth Hospital or Tuen Mun Hospital in Hong Kong were included in this retrospective study. There was no change in the daily tacrolimus dose for at least two weeks. Patients were advised to avoid St. John's wort and grapefruit juice which may affect the tacrolimus concentration. None of the patients included were taking medication known to have interaction with tacrolimus, such as calcium channel blockers, anti-epileptics, anti-mycotics and macrolide antibiotics. Additionally, patients who suffered from gastrointestinal disease, liver disease or other disorders that may alter the absorption of tacrolimus were excluded. Apart from tacrolimus and steroids, these patients were normally put on azathioprine, however some patients preferred to use mycophenolic acid on advice of their private physician. The dosage of azathioprine was 1.5 mg/kg/day while the dosage of mycophenolic acid was 0.5 gram twice daily. The initial tacrolimus dosage, administered twice daily, was 0.3 mg/kg/day for all patients. The daily tacrolimus dose was then adjusted according to the AUC_{0-12} value, which was kept at around 100-150 ng×hr/ml in the first 3 months. After three months the target AUC_{0-12} value was decreased to around 80-100 ng×hr/ml for long term maintenance. These AUC_{0-12} values were based on our previous pilot study¹⁵ and the experiences obtained in our transplantation centers. The steroid regimen for the first month was 30 mg/day of oral prednisolone, progressively tapered by 2.5 mg every two weeks until a daily maintenance dose of 5 mg. Demographic as well as clinical data were collected at the time of the tacrolimus blood sample collection.

Tacrolimus concentration determination

Tacrolimus blood concentrations were determined 2 (C2) and 4 (C4) hours after the morning tacrolimus administration in ethylene diamine tetra-acetic acid (EDTA) whole blood using a semi-automated microparticle enzyme immunoassay (MEIA) on an Imx II clinical analyser (Abbott Laboratories, Abbott Park, IL, USA). The Imx II does not cross-react enormously with the most important tacrolimus metabolite produced by CYP3A5, 13-O-demethyl tacrolimus, which has a minor immunosuppressive effect. Furthermore, a few metabolites show significant cross reactivity with the antibody but were present at low concentrations compared to tacrolimus²⁷. The two tacrolimus blood concentrations determined were used to calculate the AUC₀₋₁₂ according to the equation based strategy as described by our group¹⁵: $AUC_{0-12} = 16.2 + 2.4 \times C2 + 5.9 \times C4$. The dnAUC₀₋₁₂ was calculated by dividing the AUC₀₋₁₂ by the corresponding 24 hour dose on a milligrams per kilogram basis.

Ethics

The study was performed in accordance to the Declaration of Helsinki and its amendments. The protocol was approved by the Medical Ethics Committee of the Queen Elizabeth Hospital in Hong Kong and written informed consent for participation in this study was obtained from all patients.

DNA isolation

Genomic DNA was extracted from 103 Chinese renal transplant recipients by using 200 µl EDTA anti-coagulated blood for isolation with a QIAamp blood mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturers' instructions.

Genotyping of CYP3A and ABCB1 gene polymorphisms

Real-time PCR FRET assays were used for genotyping the CYP3A4 A-392G, CYP3A1 G-44A, ABCB1 G2677T/A and C3435T polymorphisms with the same primers and probes as described in the original publications³⁸⁻⁴¹. The real-time PCR FRET assays for the CYP3A5 A6986G and ABCB1 C1236T polymorphisms were designed, optimised and validated in our laboratory. The presence of the expected polymorphisms was confirmed by sequencing the different allelic variants of CYP3A5 and ABCB1 according to a direct sequence procedure on a capillary sequencer ABI Prism 3100® using the Bridge version 1.1 sequence kit (both products from Applied Biosystems, Fostercity, USA). Genotyping for the CYP3A5 A6986G polymorphism was performed using the primers F16: 5'-TTT gCC TCT TTg TAC TTC TTC ATC-'3 (sense; 172835-858) and R16: 5'- Tag TTg TAC gAC ACA Cag CAA CC-'3 (anti-sense; 173335-313) to amplify a 501 bp part from the CYP3A5 gene which covers the A6986G polymorphism in intron 3 (Genbank acces no: AF280107). Detection was carried out using the anchor probe CYP316R640: 5'-CCC TgT TTg gAC CAC ATT ACC CTT-'3 (sense; 173214-237) which

was labelled at the 5' end with LCRed 640 and phosphorylated at the 3'- end to block extension and the sensor probe CYP3A5A16: 5'-gAg CTC TTT TgT CTT TCA ATA TCT CT-'3 (sense; 173187-212) which is complementary to the CYP3A5 6986A or *1 allele with the polymorphic nucleotide indicated underlined. This 3'-fluorescein labelled anchor probe binds with a distance of one base 5' to the detection probe. The PCR mixture contained 3.3 µl sterile water; 2.0 mmol/l MgCl₂; 1.0 µl LC Faststart DNA Master Hybridisation Probes (Roche Diagnostics GmbH, Mannheim, Germany), 0.60 µmol/l of each primer, and 0.20 µmol/l of both anchor and sensor probe (TIB MOLBIOL, Berlin, Germany). After adding 1.0 µl containing 50-100 ng genomic DNA to the PCR mixture, the total volume is 10 µl. The PCR protocol included the following steps: a) denaturation for ten minutes at 95 °C b) 45 cycles at 95°C for ten seconds; 55°C for ten seconds and 72°C for 20 seconds (all 20°C/second). After amplification was completed, a melting curve was recorded by cooling to 45°C (20°C/second) holding at 45°C for two minutes and then heating slowly to 75°C at 0.1°C/second. Moreover, genotyping for the ABCB1 C1236T polymorphism is performed using the primers F35: 5'-gTT CCT ATA TCC TgT gTC TgT gAA T-'3 (sense; 208-32) and R35: 5'-AgT CTA gCT CgC ATg ggT CAT C-'3 (anti-sense; 448-27) to amplify a 241 bp part from the ABCB1 gene which covers the C1236T polymorphism in exon 12 (Genbank access no: M29432). Detection of the ABCB1 C1236T polymorphism was carried out using the anchor probe A35: 5'-gCC ACC gTC TgC CCA CTC TgC AC-3 (anti-sense; 343-321) which is labelled at the 3'-end with fluorescence and binds with a distance of one base 5' to the sensor probe. This 5' LCRed640 labelled sensor probe P35: TTC Agg TTC AgA CCC TTC AAg (anti-sense; 319-299) which is phosphorylated at the 3' end to block extension from the anchor probe and is complementary to the ABCB1 1236T polymorphism which is indicated underlined. The PCR mixture for the ABCB1 C1236T polymorphism contained: 2.95 µl sterile water; 2.0 mmol/l MgCl₂; 1.0 µl LC Faststart DNA Master Hybridization Probes; (Roche Diagnostics GmbH, Mannheim, Germany) 0.5 µM of each primer and 0.225 µmol/l of both sensor and anchor probe (TIB MOLBIOL, Berlin, Germany). After adding 1.0 µl containing 50-100 ng genomic DNA to the PCR mixture, the total volume was 10 µl. The PCR protocol included the following steps: denaturation for 10 minutes at 95°C; 45 cycles at 95°C for 5 seconds, 55°C for 10 seconds and 72°C for 20 seconds (all 20°C/second). After amplification was completed, a melting curve was recorded by heating to 95°C (20°C/second) holding at 95°C for 20 seconds; subsequently cooling to 40°C (20°C/second) holding at 40°C for 20 seconds and then heating slowly to 85°C at 0.2°C/second. All PCR and melting curve analysis were performed on the LightCycler (Roche Diagnostics, Almere, the Netherlands).

Statistical analysis

Statistical analysis of the data was performed with use of SPSS 12.0 software for windows (Chicago, IL, USA). To examine the population homogeneity of the patients, the genotype frequencies of the CYP3A and ABCB1 polymorphisms were tested against

Hardy-Weinberg equilibrium by the Pearson's goodness-of-fit test. For analysis of the dnAUC_{0-12} ($\text{ng} \times \text{hr/ml}$ per mg/kg) and the daily tacrolimus dose (mg/kg/day), two groups were compared using the non parametric Mann-Whitney test while the non parametric Kruskal Wallis test was used to compare several groups. Stepwise multiple regression analyses was used to calculate the relative contribution of several genetic and non-genetic factors on the variability in the daily tacrolimus dose requirement. The genetic factors examined are the CYP3A5*3 polymorphism and three ABCB1 polymorphisms while the non-genetic factors examined are gender, age, serum creatinine concentration, time since transplantation and steroid dosing. P values less than 0.05 were considered statistically significant. All values are expressed as median and range unless stated otherwise.

Results

Demographic characteristics of patients

Table 7.1 illustrates the characteristics of the 103 Chinese renal transplant patients that fulfilled the inclusion criteria and were enrolled in our study.

Real-time PCR FRET assays for CYP3A5 A6986G and ABCB1 C1236T genotyping

The melting point of the sensor probe covering the CYP3A5 A6986G polymorphism was 57°C when hybridised to the CYP3A5 6986G/G or *3/*3 genotype and 62°C when hybridised to the CYP3A5 6986A/A or *1/*1 genotype. A heterozygous sample contained both type of targets and thus generated both peaks (Figure 7.1A). In addition, for the ABCB1 C1236T polymorphism, the melting point of the sensor probe was 56.5°C when hybridised to the ABCB1 1236CC genotype and 62°C when hybridised to the ABCB1 1236TT genotype. A heterozygote sample for the ABCB1 C1236T polymorphism showed both melting peaks (Figure 7.1B). The presence of the expected polymorphisms was confirmed by sequencing these different allelic variants of CYP3A5 and ABCB1 gene. The resulting characterised heterozygote samples were used as controls in each run performed.

Table 7.1 Demographic characteristics of the renal transplant recipients

Demographic characteristics	
Gender (male/female)	58/45
Age (years, mean ± SD, (range))	43.1 ± 11.1 (21-77)
Weight (kg, mean ± SD, (range))	62.5 ± 11.4 (37.4-87.8)
Primary kidney disease	
Glomerulonephritis	57
Chronic pyelonephritis	1
Diabetic nephropathy	10
Hypertensive nephropathy	9
Polycystic kidney disease	2
Obstructive Uropathy	3
Unknown	18
Other	3
Transplantation number	
First	97
Second	6
Tacrolimus dose (mg/kg body weight/day, mean ± SD, (range))	0.067 ± 0.032 (0.01-0.18)
Tacrolimus conc. 2 hour post dose (ng/ml, mean ± SD, (range))	13.5 ± 4.30 (5.7-29.4)
Tacrolimus conc. 4 hour post dose (ng/ml, mean ± SD, (range))	8.7 ± 2.60 (2.3-16.4)
AUC ₀₋₁₂ (ng × hr/ml, mean, (range))	100.3 (47.8-183.5)
DnAUC ₀₋₁₂ (ng × hr/ml per mg/kg body weight, mean, (range))	1940 (300-11125)
Use of azathiopurine/mycophenolate mofetil	78/25
Current steroid dose (mg, mean ± SD, (range))	6.98 ± 2.37 (4-25)
Time since transplantation (days, mean, (range))	977 (133-4982)
Haemoglobin (mmol/l, mean ± SD, (range))	12.8 ± 2.0 (8.5-19)
Haematocrit fraction (mean ± SD, (range))	0.38 ± 0.058 (0.25-0.57)
ALAT (U/l, mean ± SD, (range))	22 ± 18 (8-147)
Serum albumin (g/l, mean ± SD, (range))	43 ± 3.2 (34-50)
Serum creatinine (µmol/l, mean ± SD, (range))	156 ± 76 (62-462)

Figure 7.1 Genotyping of CYP3A5 A6986G and ABCB1 C1236T with allele specific fluorescent probes by derivative melting curve plots. The derivative melting curves are read in channel 2 for the detection of the CYP3A5 and the ABCB1 fragments.

A. The derivative melting curve is plotted for a sample homozygous for the CYP3A5 6986G or *3 allele (1; Tm 57°C); a sample homozygous for the CYP3A5 6986A or *1 allele (2; Tm 62°C) and a heterozygous sample (3; Tm 57°C and 62°C). B. The derivative melting curve is plotted for a sample homozygous for the ABCB1 1236C allele (1; Tm 56.5°C); a sample homozygous for the ABCB1 1236T allele (2; Tm 62°C) and a heterozygous sample (3; Tm 56.5°C and 62°C). Melting analysis of a no template control was also performed for both assays (4).

The allele distribution of the different CYP3A and ABCB1 polymorphisms

Table 7.2 shows both the different genotypes and the allele frequencies of the CYP3A and ABCB1 polymorphisms. In our population, we found no homozygous or heterozygous variant allele for the CYP3A4*1B polymorphism, which confirmed its very low frequency in the Chinese population. The allele frequencies found for the CYP3A5, CYP3AP1 and ABCB1 polymorphisms are in line with the literature^{20,22,25}. The distribution of all polymorphisms examined is not significantly different from that predicted with the Hardy-Weinberg equilibrium (each $P > 0.05$). The genetic linkage of CYP3AP1 G-44A with CYP3A5 A6986G as already described by previous studies^{42,43}, was also confirmed in our study.

Table 7.2 Allelic distribution of CYP3A, CYP3AP1 and ABCB1 variants in 103 Chinese renal transplant recipients

SNP	wt/wt	wt/m	m/m	Allele frequency (%)		
CYP3A4				A	G	
A-392G	103 (100%)	0 (0%)	0 (0%)	100	0	
CYP3A5				A	G	
A6986G	10 (10%)	38 (37%)	55 (53%)	28	72	
CYP3AP1				G	A	
G-44A	9 (9%)	40 (39%)	54 (52%)	28	72	
ABCB1, exon 12				C	T	
C1236T	8 (8%)	52 (50%)	43 (42%)	33	67	
ABCB1, exon 21		GT: 32 (31%)	TT: 19 (19%)	G	T	A
G2677T/A	26 (25%)	GA: 16 (15%)	TA: 10 (10%)	48	39	13
ABCB1, exon 26				C	T	
C3435T	46 (45%)	43 (42%)	14 (13%)	66	34	

Effect of CYP3A and ABCB1 polymorphisms on tacrolimus dose requirements and pharmacokinetic profiles

Table 7.3 shows a significant decrease in the dnAUC_{0-12} which strongly depends on whether the patients were carrier of none, one or two CYP3A5*1 alleles (2143, 1228 and 920 $\text{ng} \times \text{hr}/\text{ml}$ per mg/kg ; Kruskal Wallis, $P < 0.001$), respectively. Consequently, the daily tacrolimus dose was 80% higher in homozygous carriers of a CYP3A5*1 allele compared to homozygous carriers of the CYP3A5*3 allele (0.09 $\text{mg}/\text{kg}/\text{day}$ *versus* 0.05 $\text{mg}/\text{kg}/\text{day}$; Kruskal Wallis, $P < 0.001$). This allele-dependent effect of the CYP3A5*3 polymorphism is also illustrated in Figure 7.2. Additionally, a significant correlation is found between the allelic variants of the CYP3AP1 G-44A polymorphism and both the dnAUC_{0-12} and the daily tacrolimus dose. Likely, this is caused by a very high linkage ($> 98\%$) between CYP3A5*3 and CYP3AP1 -44A (only two patients differed in genotype) in our study.

Figure 7.2 Influence of the CYP3A5 A6986G genotype on the pharmacological parameters recorded in 103 renal transplant recipients. A. The boxplot of the dose-normalized (dn)AUC₀₋₁₂ (ng×hr/ml per mg/kg body weight) clustered according to CYP3A5 A6986G genotype. B. The boxplot of the distribution of the daily tacrolimus dose (mg/kg body weight) clustered according to CYP3A5 A6986G genotype. *P* values for the pairwise comparisons of each genotype are given. Open circles indicate an outlier value at more than 1.5 boxlengths above the box, while asterisks indicate an extreme value at more than 3 boxlengths above the box.

Regarding the ABCB1 polymorphisms, we examined both the individual ABCB1 polymorphisms and the ABCB1 haplotypes present in the renal transplant patients. Table 7.3 shows the genetic effect of the three individual ABCB1 polymorphisms on both the dnAUC₀₋₁₂ and the daily tacrolimus dose. Individuals carrying the 2677TT or 3435TT genotype showed no significant difference in the dnAUC₀₋₁₂ compared to individuals carrying the 2677GG or 3435CC genotype. However, as is shown in Table 7.3, individuals carrying the 2677TT or 3435TT genotype required a significantly higher daily tacrolimus dose than individuals carrying the 2677GG or 3435CC genotype (0.066 mg/kg/day *versus* 0.053 mg/kg/day and 0.073 mg/kg/day *versus* 0.051 mg/kg/day; Mann-Whitney, *P* = 0.033 and *P* = 0.044), respectively. In contrast to ABCB1 G2677T/A and C3435T, the allelic variants of the ABCB1 C1236T polymorphism showed no significant difference on the dnAUC₀₋₁₂ and the daily tacrolimus dose. Additionally, we analysed the correlation between the ABCB1 haplotypes and the dnAUC₀₋₁₂ as well as the daily tacrolimus dose.

Table 7.3 Influence of CYP3A, CYP3AP1 and ABCB1 genotypes on the daily tacrolimus dose and the dose-normalized (Dn)AUC₀₋₁₂.

Genotype	Allelic status (n)	Dose (mg/kg/day)	DnAUC ₀₋₁₂ (ng×hr/ml per mg/kg)
CYP3A5	*1/*1 (10)	0.090 (0.05-0.18) ^a	920 (300-1981) ^a
A6986G	*1/*3 (38)	0.070 (0.04-0.15) ^a	1228 (589-2266) ^a
	*3/*3 (55)	0.050 (0.01-0.10) ^a	2143 (827-11125) ^a
CYP3AP1	G/G (9)	0.090 (0.05-0.18) ^a	929 (300-1981) ^a
G-44A	A/G (40)	0.070 (0.04-0.15) ^a	1298 (589-2265) ^a
	A/A (54)	0.050 (0.01-0.10) ^a	2156 (827-11125) ^a
ABCB1	C/C (8)	0.060 (0.03-0.15)	1484 (875-5166)
C1236T	C/T (52)	0.060 (0.01-0.18)	1494 (300-11125)
	T/T (43)	0.063 (0.01-0.15)	1651 (589-7579)
ABCB1	G/G (26)	0.053 (0.03-0.09) ^b	1887 (891-6166)
G2677T/A	G/T (32)	0.070 (0.01-0.18)	1365 (300-7579)
	G/A (16)	0.050 (0.01-0.15)	1864 (676-11125)
	T/A (10)	0.056 (0.05-0.13)	1765 (805-2870)
	T/T (19)	0.066 (0.02-0.15) ^b	1473 (589-4532)
ABCB1	C/C (46)	0.052 (0.01-0.15) ^b	1882 (676-11125)
C3435T	C/T (43)	0.063 (0.03-0.18)	1466 (300-6166)
	T/T (14)	0.073 (0.02-0.15) ^b	1332 (589-4532)
CYP3A5 – ABCB1	*3/*3 – GG (17)	0.048 (0.03-0.08)	2097 (1056-6166)
A6986G – G2677T/A	*3/*3 – GA/GT/TA (26)	0.050 (0.01-0.10)	2369 (827-11125)
	*3/*3 – TT (12)	0.063 (0.02-0.09)	1694 (1237-4532)
	*1/*3 – GG (8)	0.068 (0.04-0.09)	1346 (913-2168)
	*1/*3 – GA/GT/TA (24)	0.073 (0.05-0.15) ^c	1271 (691-1973) ^c
	*1/*3 – TT (6)	0.104 (0.05-0.15) ^c	788 (589-2266) ^c
	*1/*1 – GG (1)	0.0787	891
	*1/*1 – GA/GT/TA (8)	0.110 (0.05-0.18) ^c	878 (300-1981) ^c
	*1/*1 – TT (1)	0.0800	1106 [*]
CYP3A5 – ABCB1	*3/*3 – CC (28)	0.046 (0.01-0.08)	2120 (1056-11125)
A6986G – C3435T	*3/*3 – CT (21)	0.059 (0.03-0.10)	2179 (827-6166)
	*3/*3 – TT (6)	0.055 (0.02-0.09)	1845 (1322-4532)
	*1/*3 – CC (15)	0.069 (0.04-0.15) ^c	1106 (870-2168) ^c
	*1/*3 – CT (16)	0.073 (0.05-0.13) ^c	1271 (691-1882) ^c
	*1/*3 – TT (7)	0.098 (0.05-0.15) ^c	841 (589-2266) ^c
	*1/*1 – CC (3)	0.090 (0.08-0.13) ^c	891 (676-950) ^c
	*1/*1 – CT (6)	0.11 (0.05-0.18) ^c	977 (300-1981) ^c
	*1/*1 – TT (1)	0.800	1106

Values are indicated as median (range), ^a $P < 0.001$ (Kruskal Wallis); ^b $P < 0.05$ (Mann-Whitney), ^c $P < 0.05$ (Bonferroni), when the designated CYP3A5 - ABCB1 genotype combination is compared to the reference genotype combination, which is CYP3A5 - ABCB1 G2677T/A *3/*3 – GG and CYP3A5-ABCB1 C3435T *3/*3 – CC, respectively.

Table 7.4 illustrates that in contrast to the two individual ABCB1 polymorphisms, no significant difference or trend was observed between the ABCB1 haplotypes and the dnAUC₀₋₁₂ as well as the daily tacrolimus dose. Moreover, using stepwise multiple regression analysis to examine the relative contribution of the non-genetic and genetic factors to the daily tacrolimus dose variability, the CYP3A5*3 polymorphism appears to be the most significant independent variable when considering the daily tacrolimus dose

as a dependent variable. The CYP3A5*3 polymorphism can explain 35.3% ($P < 0.001$) of the variation in the daily tacrolimus dose observed in the renal transplant recipient population. Furthermore, gender (9.9%; $P < 0.001$) and the ABCB1 C3435T polymorphism (3.7%; $P < 0.009$) have a significant contribution in the variation of the daily tacrolimus dose. The two ABCB1 polymorphisms C1236T and G2677T/A, ABCB1 haplotypes and the non-genetic factors: age, serum creatinine concentration, time since transplantation and steroid dosing have no significant contribution on the variation of the daily tacrolimus dose.

Table 7.4 ABCB1 haplotypes in correlation to tacrolimus dose and dose-normalized (Dn)AUC₀₋₁₂

C1236T	G2677T/A	C3435T	n	Frequency (%)	Dose (mg/kg/day)	DnAUC ₀₋₁₂ (ng×hr/ml per mg/kg)
CC	GG	CC	4	3.9	0.064 (0.03-0.08)	1484 (1301-5166)
CC	GA	CC	4	3.9	0.054 (0.03-0.15)	1862 (875-3948)
CT	GG	CC	11	10.7	0.070 (0.03–0.09)	1073 (891-4002)
CT	GG	CT	2	1.9	0.034	4519
CT	GT	CC	2	1.9	0.067	1650
CT	GT	CT	15	14.6	0.070 (0.03–0.18)	1227 (300-4702)
CT	GT	TT	2	1.9	0.056	2156
CT	GA	CC	11	10.7	0.050 (0.01-0.13)	1846 (676-11125)
CT	TA	CC	1	1.0	0.050	1918
CT	TA	CT	8	7.8	0.056 (0.05-0.13)	1765 (805-2870)
TT	GG	CC	9	8.7	0.050 (0.03-0.08)	1919 (1438-3717)
TT	GT	CC	2	1.9	0.021	4814
TT	GT	CT	11	10.7	0.080 (0.05-0.10)	1325 (837-2309)
TT	GA	CC	1	1.0	0.039	2933
TT	TA	CC	1	1.0	0.072	1106
TT	TT	CT	7	6.7	0.063 (0.05-0.08)	1685 (1237-2626)
TT	TT	TT	12	11.7	0.075 (0.02-0.15)	1277 (589-4532)

Figure 7.3 suggests that a combined analysis of the CYP3A5*3 and ABCB1 G2677T/A or ABCB1 C3435T polymorphism may be helpful to identify those individuals that require a strong aberrant daily tacrolimus dose, although the contribution of the individual ABCB1 polymorphisms on the daily tacrolimus dose variation is not significant or minor compared to the CYP3A5*3 polymorphism.

Figure 7.3 The boxplot of the distribution of the daily tacrolimus dose (mg/kg) clustered according to the combination of CYP3A5 A6986G and ABCB1 G2677T/A genotypes. B. The boxplot of the distribution of the daily tacrolimus dose (mg/kg body weight) clustered according to the combination of CYP3A5 A6986G and ABCB1 C3435T genotypes. A P value is given for comparison of several groups of both CYP3A5 and ABCB1 genotype combinations. Open circles indicate an outlier value at more than 1.5 boxlengths below/above the box, while asterisks indicate an extreme value at more than 3 box lengths above the box.

Discussion

Although most centres still use C_0 levels for monitoring tacrolimus there are conflicting data about the correlation with the systemic tacrolimus exposure. Several studies reported a reasonable squared correlation between C_0 levels and AUC_{0-12} of tacrolimus⁴⁴⁻⁴⁶, while other studies found very poor correlations^{13-16,47,48}. Recently, Scholten *et al.*¹⁷ reported a reduction of the 95% prediction interval by 50%, when a two point sampling strategy was used consisting of a C_0 level and a second sample obtained between two and four hour post dose. Moreover, Scholten *et al.*¹⁷ evaluated the predictive performance of the two time point sample strategy used in the present study and confirmed its suitability. It is known that several non-genetic factors can modulate the post-operative CYP3A phenotype, especially in the early period after transplantation⁴⁹. Therefore, it is important to demonstrate with close drug monitoring of tacrolimus that steady state blood concentrations have been achieved in order to examine the genotype-phenotype associations more accurately. Therapeutic drug monitoring of tacrolimus can be performed accurately using complete AUC_{0-12} or an AUC_{0-12} calculated by a limited sampling strategy. In the present study a limited sampling strategy is used to calculate the AUC_{0-12} in 103 Chinese renal transplant recipients. To the best of our knowledge, two recent studies^{34,35} previously examined, in Japanese renal transplant recipients, the association between the CYP3A5*3 and the ABCB1 C3435T polymorphism and the pharmacokinetic parameters; (dn) C_0 ,

(dn)AUC₀₋₁₂, (dn)C_{max}, T_{max} and t_{1/2}. Although several studies found a significant effect of CYP3A5*3 polymorphism^{22-24,26-31,35} on tacrolimus levels or dosages, the number homozygous carriers of the CYP3A5*1 polymorphism was limited in these studies. Recently, Zhang *et al.*²⁵ correlated the different genotypes of the CYP3A5*3 and ABCB1 C3435T polymorphism of 118 Chinese renal transplant recipients with their corresponding C₀ levels and daily tacrolimus dosages respectively, 1 week, 1 month and 3 months after transplantation. Our findings showed in line with the results of Zhang *et al.*²⁵ a strong significant genetic effect between the CYP3A5*3 polymorphism and both the dnAUC₀₋₁₂ and the daily tacrolimus dose. After a mean time of 2.5 years post transplantation, carriers of the CYP3A5*3 polymorphism still require a remarkable lower daily tacrolimus dose compared to carriers of the CYP3A5*1 polymorphism. An estimation of the contribution of the different CYP3A and ABCB1 polymorphisms in the variation of the daily tacrolimus dose is performed by Haufroid *et al.*²⁷. They found that the CYP3A5*3 polymorphism is with a relative contribution of 38% the most significant independent variable when considering the daily tacrolimus dose as dependent variable, which is in line with the findings observed in the present study. The significant contribution of the gender in the variation of the daily tacrolimus dose is also found by Kuypers *et al.*¹⁴ although they examined the influence of several non-genetic variables within the first year after transplantation. However, no significant contribution is observed for the time since transplantation in the variation of the daily tacrolimus dose in contrast to the findings observed by Haufroid *et al.*²⁷. Despite the ABCB1 G2677T/A and C3435T polymorphisms showed a significant genetic effect after correlating these ABCB1 polymorphisms with the daily tacrolimus dose, no significant contribution is found using multiple regression analysis for the two individual ABCB1 polymorphisms or ABCB1 haplotype. Our findings seem therefore in contrast to several other studies which reported that carriers of the 3435CC allele^{23,24,36} and 1236C-2677G-3435C or 2677GG-3435CC haplotype required a higher daily tacrolimus dose³⁷. However, the significant genetic effect for ABCB1 polymorphisms or haplotypes found in these studies is like in our study not as pronounced as it is for the CYP3A5*3 polymorphism. The minor influence of the ABCB1 polymorphisms compared to the CYP3A5*3 polymorphism on the tacrolimus pharmacokinetics combined with the differences regarding pharmacokinetic parameter used (C₀ or AUC₀₋₁₂), the genetic (single SNP *versus* haplotype) analyses that were performed, the number of transplant patients included, and the difference in inclusion criteria for the transplant recipients may explain the differences in genotype-phenotype associations found by these studies. Also after combining the CYP3A5*3 polymorphism with the ABCB1 G2677T/A or C3435T polymorphism, the extra part of the variation in dnAUC₀₋₁₂ and daily tacrolimus dose that can be clarified above the CYP3A5*3 polymorphism is marginal.

Highlights

- The calcineurin inhibitor tacrolimus, an immunosuppressant used in transplantation therapy, has unpredictable drug levels and therefore requires close therapeutic drug monitoring in order to minimize the risk of under- or overimmunosuppression.
- Tacrolimus is metabolized by the cytochrome P450 3A (CYP3A) iso-enzymes, which is quantitatively the most important metabolic route of tacrolimus. Additionally, tacrolimus is also a substrate for the adenosine triphosphate-binding cassette B1 (ABCB1) gene encoding protein multidrug resistance-1 (MDR1) or P-glycoprotein.
- The present study used a two time point sampling strategy to calculate the area under the time tacrolimus concentration curve (AUC_{0-12}) and correlated these dose-normalized AUC_{0-12} with the corresponding CYP3A and ABCB1 genotypes in 103 Chinese renal transplant recipients.
- Several single nucleotide polymorphisms (SNPs) recently identified in CYP3A and ABCB1 gene may elucidate the interindividual differences in the tacrolimus pharmacokinetics.
- The CYP3A5*3 allele, which results in the absence of the functional CYP3A5 protein, has been strongly associated with a higher $dnAUC_{0-12}$ and consequently a lower daily tacrolimus dose. The different genotypes of the ABCB1 G2677T/A and C3435T SNPs showed a weaker association with the $dnAUC_{0-12}$ as well as the daily tacrolimus dose.
- Multiple regression analysis demonstrates that the CYP3A5*3 allele is the most important independent variable among several genetic and non-genetic variables examined in the renal transplant recipients.
- Knowing the impact of the CYP3A5*3 allele on the tacrolimus disposition, genotyping prior transplantation may help to assess the initial daily dose needed by individual patients for adequate immunosuppression.

Outlook and conclusion

Tacrolimus has a narrow therapeutic index which requires close monitoring of the drug concentration in order to achieve an optimal efficiency and minimizing the risks of acute rejection and drug overdose. The polymorphisms present in CYP3A and ABCB1 can explain their variability in expression and activity and thus clarify the interindividual differences in the tacrolimus pharmacokinetics. The majority of studies that examined the CYP3A and ABCB1 genotype-phenotype association were based on tacrolimus C_0 levels, despite the fact that an AUC_{0-12} is the best way to determine the tacrolimus exposure. To the best of our knowledge only two studies reported an association between the CYP3A5*3 polymorphism and both the different pharmacokinetic parameters of tacrolimus and the daily tacrolimus dose. Furthermore, several studies

reported an association between CYP3A5*3 polymorphism and the tacrolimus C_0 levels obtained within the early period after transplantation. However, non-genetic parameters have then also a large impact on the tacrolimus concentration in this period. The present study calculated an AUC_{0-12} according to a two time point sampling strategy for 103 Chinese renal transplant patients with a wide time interval starting on at least three months posttransplantation. Our results clearly point out that the CYP3A5*3 polymorphism is strongly allele-dependent associated with both the $dnAUC_{0-12}$ and the daily tacrolimus dose in renal transplant recipients. A weaker significant association is found for the ABCB1 G2677T/A and ABCB1 C3435T polymorphisms. Multiple regression analysis demonstrates that the CYP3A5*3 polymorphism is the most important independent variable among several genetic and non-genetic variables examined in the renal transplant recipients. Therefore, CYP3A5 genotyping prior transplantation may contribute to determine the initial tacrolimus dose required for individual patients.

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Chapter 8

Tacrolimus pharmacokinetics and pharmacogenetics: influence of adenosine triphosphate-binding cassette B1 (ABCB1) and cytochrome (CYP) 3A polymorphisms

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Abstract

Background and aim

Tacrolimus, an immunosuppressant used after organ transplantation, has a narrow therapeutic range and its pharmacokinetic variability complicates its daily dose assessment. P-glycoprotein (P-gp), encoded by the adenosine triphosphate-binding cassette B1 (ABCB1) and the cytochrome (CYP) 3A4 and 3A5 enzymes appear to play a role in the tacrolimus metabolism.

Materials and methods

In the present study, two different renal transplant recipients groups are used to examine the influence of ABCB1 and CYP3A polymorphisms on the daily tacrolimus dose and several pharmacokinetic parameters. In total 63 Caucasian renal transplant recipients divided into 26 early and 37 late posttransplant recipients were genotyped for ABCB1 and CYP3A polymorphisms. The pharmacokinetic parameters of tacrolimus were determined for all renal transplant recipients and correlated with their corresponding genotypes.

Results

A significant difference in allele frequencies of the CYP3A4*1B ($P = 0.028$) and CYP3A5*1 ($P = 0.022$) alleles was observed between the early and late posttransplant recipient group. Significant higher dose-normalized trough levels (dnC_0), dose-normalized area under the curve (dnAUC_{0-12}), and dose-normalized maximum concentration (dnC_{max}) were observed for carriers of the CYP3A5*3 variant allele in the two renal transplant patient groups. Except for the daily tacrolimus dose ($P = 0.025$) no significant differences were observed for carriers of the CYP3A4*1B variant allele. Neither the individual ABCB1 polymorphisms nor the ABCB1 haplotypes were associated with any pharmacokinetic parameter.

Conclusion

We noticed that a complete pharmacokinetic profile is more frequently requested for renal transplant recipients carrying a CYP3A5*1 allele suggesting that these patients have more difficulties in achieving and maintaining tacrolimus concentrations compared to homozygous carriers of the CYP3A5*3 variant allele. Additionally, patients carrying a CYP3A5*1 allele require a twofold higher tacrolimus dose compared to homozygous carriers of the CYP3A5*3 variant allele to maintain the target dnAUC_{0-12} . Therefore, genotyping for the CYP3A5*3 variant allele can contribute to a better and more individualized immunosuppressive therapy in transplant patients.

Introduction

The immunosuppressant tacrolimus is used worldwide in transplant patients although its narrow therapeutic window and high pharmacokinetic variability complicate the establishment of a dosage regime^{1,2}. Therefore, close therapeutic drug monitoring for tacrolimus is required to prevent the risk of subtherapeutic or toxic blood concentrations. Subtherapeutic tacrolimus blood concentrations increase the risk of transplant rejection enormously³⁻⁵, while high tacrolimus blood concentrations may lead to severe side effects such as nephrotoxicity, neurotoxicity and hyperglycemia⁶⁻⁸. Tacrolimus is like numerous other clinically used drugs, a substrate for P-glycoprotein (P-gp) and cytochrome P450 3A (CYP3A) enzymes. P-gp is encoded by the adenosine triphosphate (ATP)-binding cassette B1 (ABC B1) gene and co-expressed with the CYP3A enzymes in the liver and intestines. CYP3A4 and CYP3A5 are the most important members of the CYP3A subfamily^{9,10}. The differences in expression levels and activity of ABC B1 and CYP3A may explain the interindividual variations in the tacrolimus pharmacokinetics. P-gp is an ATP-dependent membranous transporter that contributes to the protection of the body from environmental toxins and drugs like tacrolimus by limiting their absorption from the intestine and promoting the efflux into bile and urine¹¹. Three partly linked polymorphisms in the ABC B1 gene located on exons 12, 21 and 26 have been studied extensively. Two of these ABC B1 polymorphisms, C1236T and C3435T, result in silent mutations while the ABC B1 G2677T/A polymorphism in exon 21 is non-synonymous and results in an amino acid exchange (Ala893Ser/Thr). A number of studies found no association between the ABC B1 genotypes and tacrolimus trough (C_0) concentrations¹²⁻¹⁷ or the pharmacokinetic parameters: area under the time tacrolimus concentration curve (AUC_{0-12}), the maximum concentration (C_{max}), the time that the maximum concentration is reached (T_{max}) and the half-life time ($t_{1/2}$)^{18,19}. However, some other studies found an association between the tacrolimus C_0 concentrations, the daily tacrolimus dose and individual ABC B1 polymorphisms^{20,21} or the ABC B1 haplotypes²². Homozygotes for the CYP3A4*1B and CYP3A5*3 variant allele carry at position -392 an A and at position 6986 a G, respectively. Moreover, homozygotes for the CYP3A4*1B variant allele show an altered CYP3A4 enzyme activity while homozygotes for the CYP3A5*3 variant allele show no CYP3A5 enzyme activity. A number of studies already demonstrated the impact of the CYP3A5*3 variant allele on tacrolimus C_0 concentrations^{12-17,23-25}, the pharmacokinetic parameters: AUC_{0-12} , C_{max} , T_{max} , $t_{1/2}$ ^{18,19} and the daily tacrolimus dose in different transplant patient groups. However, the influence of CYP3A4*1B and ABC B1 C1236T variant alleles on these pharmacokinetic parameters of tacrolimus has never been examined. In the present study, a complete 12 hour pharmacokinetic profile is recorded for all transplant patients and the influence of several clinical and genetic parameters is determined on the variation in pharmacokinetic parameters of tacrolimus in an early and a late posttransplant recipient group. Additionally, the association will be examined between the CYP3A4 A-392G, CYP3A5 A6986G, CYP3A1 G-44A

polymorphisms and the three ABCB1 polymorphisms C1236T, G2677T/A, C3435T on the pharmacokinetic parameters in the same renal transplant recipient groups.

Patients and methods

Study population

In total 63 Caucasian renal transplant recipients of whom a complete 12 hour time tacrolimus concentration curve was performed were divided over two different groups. Table 8.1 illustrates that most pharmacokinetic profiles of the patients included in group I were recorded within 6 weeks after transplantation, while group II included patients that underwent a renal transplantation at least 1 year ago. Moreover, in group I eight patients used calcium channel blockers which are known to interact with tacrolimus, whereas patients included in group II used no medication known to interfere with tacrolimus. In addition, patients that suffer from gastrointestinal, liver disease or other disorders that may alter the absorption of tacrolimus were disqualified for inclusion in both groups. Prior to the blood sample collection, there had been no tacrolimus dose change for at least three days in the two groups. After overnight fasting the blood samples were collected immediately pre (C_0) and 0.5 ($C_{0.5}$), 1 (C_1), 2 (C_2), 3 (C_3), 4 (C_4), 5 (C_5), 7.5 ($C_{7.5}$) and 12 (C_{12}) hours after the morning tacrolimus administration. Patients were not allowed to take food until 1 hour after ingesting the tacrolimus dose and were advised to avoid grapefruit juice after transplantation to prevent alterations in the tacrolimus metabolism. Demographic as well as clinical data were determined at the time of recording the 12 hour time tacrolimus concentration curve.

Ethics

The study was performed in accordance to the Declaration of Helsinki and its amendments. The protocol was approved by the local Medical Ethics Committee and written informed consent for participation in this study was obtained from all patients.

Tacrolimus concentration determinations

The tacrolimus blood concentrations were determined in ethylene diamine tetra-acetic acid (EDTA) whole blood, using a microparticle enzyme immunoassay with a monoclonal antibody (IMx II assay; Abbott Laboratories, Abbott Park, IL, USA) for group I and a method based on high pressure liquid chromatography (HPLC) tandem mass spectrometry (MS/MS) for group II. The laboratories in which the tacrolimus concentrations have been determined participate in the International Tacrolimus Proficiency Testing Scheme.

Table 8.1 Demographic characteristics of the two renal transplant recipients groups.

Demographic characteristics	Group I	Group II
Gender (male/female)	18/8	24/13
Age (years, mean $\pm$ SD)	43.0 $\pm$ 13.2	51.3 $\pm$ 10.9
Body Mass Index (kg/m ² , mean $\pm$ SD)	23.3 $\pm$ 4.40	25.6 $\pm$ 3.42
Primary kidney disease		
Glomerulonephritis	4	1
Chronic pyelonephritis	2	2
IgA nephropathy	3	4
Hypertensive nephropathy	4	7
Diabetes Mellitus nephropathy	4	0
Polycystic kidney disease	1	8
Unknown	1	4
Other	7	11
Transplantation number		
First	20	30
Second	5	6
Third or more	1	1
Tacrolimus mono therapy	1	29
Tacrolimus dose (mg/kg/day, mean $\pm$ SD)	0.39 $\pm$ 0.231	0.054 $\pm$ 0.029
C ₀ (ng/ml, mean $\pm$ SD)	16.8 $\pm$ 5.83	6.59 $\pm$ 1.39
AUC ₀₋₁₂ (ng $\times$ hr/ml, mean $\pm$ SD)	305.0 $\pm$ 96.8	122.5 $\pm$ 31.1
C _{max} (ng/ml, mean $\pm$ SD)	56.3 $\pm$ 21.3	20.9 $\pm$ 6.5
T _{max} (hr, mean $\pm$ SD)	1.46 $\pm$ 1.33	1.24 $\pm$ 0.43
Use of azothiopurine, MMF ^a , rapamycine, steroids	11/3/4/26	3/4/0/0
Current steroid dose (mg, dose, no. patients)		
0 mg/day	3	37
5 mg/day	2	0
8 mg/day	2	0
10 mg/day	10	0
15 mg/day	4	0
20 mg/day	3	0
> 20 mg/day	2	0
Time since transplantation (days, median, (range))	16 (3-74)	1465 (453-4128)
Haemoglobin (mmol/l, mean $\pm$ SD; ref. ♂ 8.2-11.0, ♀ 7.3-9.7)	5.43 $\pm$ 1.08	8.52 $\pm$ 0.83
Haematocrit (mean $\pm$ SD; ref. ♂ 0.41-0.52, ♀ 0.36-0.48)	0.25 $\pm$ 0.07	0.41 $\pm$ 0.04
ALAT (U/l, mean $\pm$ SD; ref. ♂ < 45, ♀ < 35)	34 $\pm$ 34	24 $\pm$ 13
Serum albumin (g/l, mean $\pm$ SD; ref. 34-45)	30.7 $\pm$ 3.86	37.0 $\pm$ 3.84
Serum creatinine (μ mol/l, mean $\pm$ SD; ref. ♂ 71-110, ♀ 53-97)	331 $\pm$ 293	128 $\pm$ 29
Creatinine clearance ^b (ml/min, mean $\pm$ SD; ref. 90-140)	37.5 $\pm$ 28.4	58.0 $\pm$ 26.6

ref. are the reference values applied in the clinical chemistry and haematology laboratory of the University Hospital Maastricht. ♂ male, ♀ female, ^a MMF is mycophenolate mofetil, ^b The creatinine clearance is determined with the Cockcroft-Gault equation.

The tacrolimus C₀ concentration and the peak tacrolimus blood concentration (C_{max}) during the assessed time interval were determined directly from the time *versus* tacrolimus blood concentration data. Additionally, the area under the time tacrolimus concentration curve (AUC₀₋₁₂) was calculated from the time *versus* tacrolimus concentration plot using the linear trapezoidal rule in MWPharm 3.50 (Mediware, Groningen, the Netherlands). Dose-normalized (Dn)C₀, dose-normalized (dn)AUC₀₋₁₂

and dose-normalized ($\text{dn}C_{\max}$) were calculated by dividing the C_0 , AUC_{0-12} and $C_{\max}$, respectively, by the corresponding morning dose on a milligram per kilogram basis.

DNA isolation

Genomic DNA isolation was performed on 63 renal transplant recipients by using 200 μl EDTA anti-coagulated blood for isolation with a QIAamp blood mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturers' instructions.

Genotyping of ABCB1 and CYP3A gene polymorphisms

Real-time polymerase chain reaction (PCR) fluorescence resonance energy transfer (FRET) assays were used for genotyping, ABCB1 G2677T/A, ABCB1 C3435T, CYP3A4 A-392G and CYP3A5 G-44A using the same primers and probes compared to the original publications²⁶⁻²⁹. Regarding the ABCB1 C1236T and CYP3A5 A6986G polymorphisms real-time PCR FRET assays were used as we described earlier³⁰. For each polymorphism, heterozygote samples were sequenced according to a direct sequence procedure on a capillary sequencer ABI Prism 3100® using the Bridge version 1.1 sequence kit (both products from Applied Biosystems, Fostercity, USA) and used in every real-time PCR FRET assay run as control sample.

Statistical analysis

Statistical analysis of the data was performed with use of the statistical software SPSS 11.0 for windows (Chicago, IL, USA). Correlations between pharmacokinetic parameters and clinical variables were evaluated using stepwise multiple regression analysis. To examine the population homogeneity of the patients, the genotype frequencies of the ABCB1 and CYP3A polymorphisms were tested against Hardy-Weinberg equilibrium by the Pearson's goodness-of-fit test³¹. Allele frequencies of the early and late posttransplant recipient group were compared using the χ^2 test. For analysis of the daily tacrolimus dose (mg/kg/day), $\text{dn}C_0$ (ng/ml per mg/kg), dnAUC_{0-12} ($\text{ng}\times\text{hr/ml per mg/kg}$), and $\text{dn}C_{\max}$ (ng/ml per mg/kg), groups were compared using non parametric statistical tests. To compare two groups we used the Mann-Whitney test, and to compare several groups the Kruskal Wallis test. P values less than 0.05 were considered statistically significant. All values are expressed as median and range unless stated otherwise.

Results

Influence of clinical and genetic parameters on the variation in pharmacokinetic tacrolimus parameters

The characteristics of the early posttransplant (group I) as well as the late posttransplant (group II) recipients that were enrolled in our study are shown in Table 8.1.

Tabel 8.2 Influence of independent clinical and genetic parameters on the pharmacokinetic parameters of an early (group I) and a late (group II) posttransplant recipient group.

Group	Dependent parameter	Independent parameter	Partial r^2	^a P value
I	Tacrolimus dose	CYP3A5 A6986G polymorphism Model r^2 : 0.531	0.531	< 0.001
I	DnC ₀	Time since transplantation Haemoglobin Model r^2 : 0.692	0.613 0.079	< 0.001 < 0.001
I	DnAUC ₀₋₁₂	Time since transplantation Haemoglobin Model r^2 : 0.620	0.528 0.092	< 0.001 < 0.001
I	DnC _{max}	Time since transplantation Haemoglobin Model r^2 : 0.635	0.405 0.230	< 0.001 < 0.001
II	Tacrolimus dose	CYP3A5 A6986G polymorphism Model r^2 : 0.354	0.354	< 0.001
II	DnC ₀	CYP3A5 A6986G polymorphism Body mass index (BMI) Gender Model r^2 : 0.418	0.175 0.157 0.086	0.010 0.001 < 0.001
II	DnAUC ₀₋₁₂	CYP3A5 A6986G polymorphism Body mass index (BMI) Model r^2 : 0.335	0.190 0.145	0.007 0.001
II	DnC _{max}	CYP3A5 A6986G polymorphism Body mass index (BMI) Serum Albumin Model r^2 : 0.412	0.226 0.109 0.077	0.003 0.001 < 0.001

^aP value belonging to the partial r^2 . DnC₀, dose-normalized trough level, dnAUC₀₋₁₂, dose-normalized area under the curve, DnC_{max} dose-normalized maximum concentration. Tested independent parameters were gender, age, body mass index (BMI), haemoglobin, haematocrit, ALAT, serum albumin, serum creatinine, creatinine clearance (Cockcroft-Gault), CYP3A4 A-392G, CYP3A5 A6986G, ABCB1 C1236T, G2677T/A, C3435T.

The influence of different clinical (gender, age, body mass index (BMI), haemoglobin, haematocrit, ALAT, serum albumin, serum creatinine, creatinine clearance) and genetic (CYP3A4 A-392G, CYP3A5 A6986G, ABCB1 C1236T, G2677T/A, C3435T) parameters is examined on the variation in the tacrolimus pharmacokinetic parameters in all posttransplant recipients. Table 8.2 illustrates the influence of independent clinical and genetic parameters on the variability of pharmacokinetic tacrolimus parameters. In the

early posttransplant recipient group the time since transplantation and the haemoglobin concentration correlate significantly with dnC_0 , $dnAUC_{0-12}$ and dnC_{max} whereas the CYP3A5 A6986G polymorphism and the body mass index (BMI) have a significant contribution on the variability of dnC_0 , $dnAUC_{0-12}$ and dnC_{max} in the late posttransplant recipient group. The clinical and genetic parameters: age, haematocrit, ALAT, serum creatinine, creatinine clearance and the polymorphisms CYP3A4 A-392G, ABCB1 C1236T, ABCB1 G2677T/A and ABCB1 C3435T show no significant correlation with any of the pharmacokinetic parameters in both renal transplant recipient groups.

Allele distribution of the different ABCB1 and CYP3A polymorphisms

Table 8.3 and 8.4 show the genotype frequencies of the different CYP3A and CYP3AP1 polymorphisms that were determined for the 26 early and 37 late posttransplant recipients. The genotype frequencies of the two renal transplant recipient groups were not significantly different from that predicted by the Hardy-Weinberg equation.

Table 8.3 Influence of ABCB1, CYP3A and CYP3AP1 allelic variants on the pharmacokinetic tacrolimus parameters of the early posttransplant recipients.

Genotype	N	Allelic status	Dose	dnC_0	$dnAUC_{0-12}$	dnC_{max}
CYP3A4	21	*1A/*1A	0.28 (0.04-0.80) ^a	90 (40-1329)	1782 (824-15721)	383 (89-1535)
A-392G	5	*1A/*1B	0.50 (0.43-0.79) ^a	59 (32-107)	1275 (922-1796)	216 (184-338)
	0	*1B/*1B	-----	-----	-----	-----
CYP3A5	1	*1/*1	0.78	32	922	184
A6986G	9	*1/*3	0.50 (0.35-0.80) ^a	59 (40-107) ^a	1220 (824-1796) ^a	216 (89-380) ^a
	16	*3/*3	0.25 (0.04-0.59) ^a	118 (45-1329) ^a	1975 (839-15721) ^a	435(90-1535) ^a
CYP3AP1	1	G/G	0.78	32	922	184
G-44A	10	G/A	0.49 (0.12-0.80) ^a	60 (40-107) ^a	1248 (824-1796) ^a	236 (89-381) ^a
	15	A/A	0.25 (0.04-0.59) ^a	123 (45-1329) ^a	2030 (839-15721) ^a	454 (90-1535) ^a

Tacrolimus dose (mg/kg/day), dnC_0 , dose-normalized trough concentration (ng/ml per mg/kg), $dnAUC_{0-12}$ (ng × hr/ml per mg/kg) dose-normalized area under the curve, dnC_{max} dose-normalized maximum concentration (ng/ml per mg/kg). Values are indicated as median and (range), ^a $P < 0.05$; (Mann-Whitney).

Effect of ABCB1 and CYP3A polymorphisms on the daily tacrolimus dose and the pharmacokinetic parameters

As is demonstrated in Table 8.5 and Table 8.6, we found that neither the individual ABCB1 polymorphisms nor the ABCB1 haplotypes are associated with the daily tacrolimus dose and the pharmacokinetic parameters; dnC_0 , $dnAUC_{0-12}$ and dnC_{max} .

Table 8.3 shows a trend between the heterozygote carriers of the CYP3A4*1B variant allele in both the daily tacrolimus dose and the pharmacokinetic tacrolimus parameters compared to the homozygote carriers of the CYP3A4*1A allele. However, except for the daily tacrolimus dose (0.28 *versus* 0.50 mg/kg/day; Mann-Whitney, $P = 0.025$) no

significant differences are found between the pharmacokinetic parameters for tacrolimus and the different CYP3A4 A-392G genotypes in the early posttransplant recipient group.

Table 8.4 Influence of ABCB1, CYP3A and CYP3AP1 allelic variants on the pharmacokinetic tacrolimus parameters of the late posttransplant recipients.

Genotype	N No	Allelic status	Dose	DnC ₀	DnAUC ₀₋₁₂	DnC _{max}
CYP3A4	36	*1A/*1A	0.05 (0.02-0.14)	276 (70-669)	4642 (1355-11994)	775 (294-1729)
A-392G	1	*1A/*1B	0.067	121	2766	504
	0	*1B/*1B	-----	-----	-----	-----
CYP3A5	0	*1/*1	-----	-----	-----	-----
A6986G	5	*1/*3	0.10 (0.05-0.14) ^a	87.4 (70-248) ^a	1663 (1355-4057) ^a	461 (294-646) ^a
	32	*3/*3	0.04 (0.02-0.11) ^a	287 (118-669) ^a	4808 (2227-11994) ^a	814 (465-1729) ^a
CYP3AP1	0	G/G	-----	-----	-----	-----
G-44A	8	G/A	0.07 (0.02-0.14) ^a	161 (70-669) ^a	2990 (1355-11994) ^a	544 (294-1729) ^a
	29	A/A	0.04 (0.02-0.11) ^a	283 (118-640) ^a	4761 (2227-11504) ^a	810 (465-1493) ^a

Tacrolimus dose (mg/kg/day), dnC₀, dose-normalized trough concentration (ng/ml per mg/kg), dnAUC₀₋₁₂ (ng×hr/ml per mg/kg) dose-normalized area under the curve, dnC_{max} dose-normalized maximum concentration (ng/ml per mg/kg). Values are indicated as median and (range), ^a *P* < 0.05; (Mann-Whitney).

Additionally, Table 8.3 demonstrates a significant decrease in the pharmacokinetic tacrolimus parameters dnC₀, 118 *versus* 59 ng/ml per mg/kg; dnAUC₀₋₁₂ 1975 *versus* 1220 ng×hr/ml per mg/kg; and dnC_{max} 435 *versus* 216 ng/ml per mg/kg when renal transplant recipients were carrier of none or one CYP3A5*1 allele, respectively. Consequently, the daily tacrolimus dose is significantly higher in heterozygous carriers of the CYP3A5*3 variant allele compared to homozygous carriers of the CYP3A5*3 variant allele (0.50 *versus* 0.25 mg/kg/day; Mann-Whitney, *P* = 0.001). Regarding the late posttransplant recipients included in group II, a similar genetic effect is found for CYP3A5*1 allele in association with the pharmacokinetic tacrolimus parameters and the daily tacrolimus dose, as is shown in Table 8.4. Because there is only one heterozygous carrier of the CYP3A4*1B variant allele among the late posttransplant group, no statistical analyses could be performed between the CYP3A4*1B genotypes and the pharmacokinetic tacrolimus parameters. Although we observed a significant difference in the daily tacrolimus dose as well as in the dnC₀, dnAUC₀₋₁₂ and dnC_{max} for only the CYP3A5*3 variant allele, it is not clear whether the CYP3A4*1B variant allele has, similarly as the CYP3A5*3 variant allele, an important contribution to the pharmacokinetic variability of tacrolimus. Particularly, knowing that in this study all individuals carrying a CYP3A4*1B variant allele also carry at least one CYP3A5*1 allele. To examine the influence of the CYP3A4*1B variant allele solely, we selected those renal transplant recipients in group I that were heterozygous for the CYP3A5*1 allele. One renal transplant patient was heterozygote for the CYP3A4*1B variant allele and homozygote for the CYP3A5*1 allele. Five renal transplant patients were carrier of both one CYP3A4*1B variant allele and one CYP3A5*1 allele. Additionally, another four renal

transplant recipients were homozygous for the CYP3A4*1A allele and heterozygous for one CYP3A5*1 allele. Figure 8.1 illustrates renal transplant recipients who are heterozygous for the CYP3A4*1B and CYP3A5*3 variant allele show no significantly higher daily tacrolimus dose or lower dnAUC_{0-12} compared to patients that were only heterozygous for the CYP3A4*1B variant allele.

Table 8.5 ABCB1 haplotypes related to tacrolimus dose requirement and dose-normalized AUC_{0-12} of the early posttransplant recipients.

ABCB1 haplotypes			N No	Dose (mg/kg/day)	DnAUC ₀₋₁₂ (ng×hr/ml per mg/kg)
C1236T	G2677T/A	C3435T			
CC	GG	CC	3	0.78 (0.23-0.79)	1333 (922-1737)
CC	GG	CT	3	0.23 (0.04-0.49)	2030 (1275-6186)
CC	GA	CC	2	0.34 (0.04-0.65)	8292 (864-15721)
CT	GT	CC	1	0.46	1097
CT	GT	CT	6	0.30 (0.22-0.59)	1570 (1103-3032)
CT	GT	TT	3	0.28 (0.25-0.42)	1920 (1821-2066)
CT	GG	CC	1	0.54	839
CT	GG	TT	1	0.35	1321
TT	TT	TT	6	0.40 (0.07-0.80)	1789 (824-8964)

Values are indicated as median and (range).

Table 8.6 ABCB1 haplotypes related to tacrolimus dose requirement and dose-normalized AUC_{0-12} of the late posttransplant recipients

ABCB1 haplotypes			N No	Dose (mg/kg/day)	DnAUC ₀₋₁₂ (ng×hr/ml per mg/kg)
C1236T	G2677T/A	C3435T			
CC	GG	CC	3	0.03 (0.02-0.04)	8798 (5010-9320)
CC	GG	CT	7	0.06 (0.03-0.08)	4057 (2227-5216)
CC	GG	TT	3	0.04 (0.02-0.06)	4465 (3147-6854)
CC	GA	CC	1	0.07	3215
CT	GT	CC	1	0.07	3081
CT	GT	CT	13	0.05 (0.02-0.13)	4761 (1355-9520)
CT	GT	TT	2	0.09 (0.04-0.14)	4571 (1662-7481)
TT	GT	CT	1	0.05	4511
TT	TT	CT	1	0.02	11504
TT	TT	TT	5	0.04 (0.02-0.07)	4718 (2766-11994)

Values are indicated as median and (range).

Figure 8.1 Boxplot of the distribution of (A) the dose-normalized (Dn)AUC₀₋₁₂ (ng×hr/ml per mg/kg) and (B) the tacrolimus dose (mg/kg/day) clustered according to the combination of CYP3A4 A-392G and CYP3A5 A6986G genotypes. *P* values are given for the pairwise comparisons of each genotype combination.

Discussion

In the early posttransplant recipient group the time since transplantation and the haemoglobin concentration contributes significantly to the variability of the pharmacokinetic tacrolimus parameters dnC₀, dnAUC₀₋₁₂ and dnC_{max} while in the late posttransplant recipient group the CYP3A5 A6986G polymorphism and the body mass index (BMI) have an important influence on the variability of different pharmacokinetic tacrolimus parameters. Previously, Haufroid *et al.*¹² reported a significant contribution of the CYP3A5 A6986G polymorphism and the time since transplantation on the variation of tacrolimus C₀ concentrations and the tacrolimus dose requirement. Additionally, we have also demonstrated in a stable Chinese renal transplant recipient population that the CYP3A5 A6986G polymorphism is the most significant independent variable when considering the daily tacrolimus dose as a dependent variable³⁰. Kuypers *et al.*³² found higher tacrolimus peak concentrations for female renal transplant recipients compared to their male counterparts and a significantly higher tacrolimus AUC₀₋₁₂ in female recipients six months after transplantation. Furthermore, Kuypers *et al.*³² found lower tacrolimus trough concentrations with increasing age. In the present study, a significantly higher allele frequency of the CYP3A4*1B and CYP3A5*1 allele is observed in the early renal transplant recipients compared to the late renal transplant recipients. The higher allele frequency for the CYP3A4*1B variant allele and CYP3A5*1 allele in the early renal transplant recipient group may partly clarify the difficulties in achieving and maintaining an optimal tacrolimus blood concentration. Although earlier studies²⁰⁻²² reported a weak significant association between ABCB1 genotypes or haplotypes with tacrolimus C₀ concentrations of the transplant patients, no differences were found between either the different ABCB1 polymorphisms or the ABCB1 haplotypes and the

pharmacokinetic tacrolimus parameters in the two renal transplant recipient groups. Recently, Fredericks *et al.*³³ reported after examining 206 stable renal transplant recipients that the individual ABCB1 polymorphisms and ABCB1 haplotypes show a relatively minor association with the tacrolimus pharmacokinetics which implies that genotyping for ABCB1 polymorphisms seems to be of minor importance for predicting the daily tacrolimus dose regime. CYP3A enzymes are responsible for the most important metabolic route of tacrolimus, namely its demethylation to 13-O-demethyltacrolimus³⁴⁻³⁷. Due to the lack of CYP3A5 activity caused by the CYP3A5*3 variant allele, transplant patients who were homozygous for the CYP3A5*3 variant allele required an almost twofold lower daily tacrolimus dose and achieved even a higher dnC_0 , dnAUC_{0-12} and dnC_{max} compared to the transplant recipients that carry at least one CYP3A5*1 allele. Although in the present study a trend is observed between the different CYP3A4 A-392G genotypes and the pharmacokinetic tacrolimus parameters, the contribution of this CYP3A4*1B variant allele seemed limited. After selecting two renal transplant recipient groups, one group with the genotype combination CYP3A4 *1A/*1A – CYP3A5 *1/*3 and another group with the genotype combination CYP3A4 *1A/*1B – CYP3A5 *1/*3, no significant differences were observed between these groups and the daily tacrolimus dose as well as the dnC_0 , dnAUC_{0-12} and dnC_{max} . This may indicate that the influence of the CYP3A4 *1B variant allele is restricted and that at least most of the genetic effect is being caused by the CYP3A5 A6986G polymorphism. These data seem to conflict with a previous study in which Hesselink *et al.* reported that the CYP3A4*1B variant allele has a significant influence on the daily tacrolimus dose despite they observed a 80% overlap between the CYP3A4*1B and CYP3A5*1 allele¹⁵. Although tacrolimus is a substrate of CYP3A4, our findings suggest that if there is an influence of this CYP3A4*1B polymorphism, it is probably caused by the high linkage between CYP3A4*1B and CYP3A5*1. Summarized, it appears that carriers of CYP3A5*1 allele included, in either the early or the late postransplant recipient group, show a twofold lower dnC_0 , dnAUC_{0-12} and dnC_{max} for tacrolimus compared to homozygous carriers of a CYP3A5*3 variant allele. Thus carriers of a CYP3A5*1 allele require a twofold higher tacrolimus dose compared to homozygous carriers of a CYP3A5*3 variant allele. Therefore, we conclude that genotyping for the CYP3A5*3 variant allele is of great value to determine the initial and maintenance oral tacrolimus dose. By doing so the risk of under- or over-immunosuppression in individual renal transplant recipients will be minimized.

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Chapter 9

Cytochrome P450 (CYP3A7) *1C allelic variant is associated with the tacrolimus pharmacokinetics in renal transplant recipients

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Abstract

Cytochrome P450 3A4/5 (CYP3A4/5) are mainly responsible for the tacrolimus metabolism although little information is available regarding the contribution of CYP3A7. The CYP3A7*1C allele contains the proximal everted repeat 6 motif of CYP3A4 which implicates that the pregnane x receptor and the constitutively activated receptor bind with a higher affinity to the CYP3A7*1C allele than to the CYP3A7*1 allele, which results in a higher expression of CYP3A7 for individuals carrying a CYP3A7*1C allele. An allele frequency of respectively 2.8% and 0.0% was found after genotyping 70 Caucasian and 103 Chinese renal transplant recipients for the CYP3A7*1C allele using a real-time polymerase chain reaction (PCR) fluorescence resonance energy transfer (FRET) assay. Heterozygous carriers of the CYP3A7*1C allele showed no significant lower trough levels, area under the time tacrolimus concentration curve or maximum concentrations for tacrolimus compared to the carriers of the CYP3A7*1 allele. Based on these findings we conclude that the CYP3A7*1C allele is not associated with lower pharmacokinetic tacrolimus parameters.

Introduction

Tacrolimus, a calcineurin inhibitor used in the transplantation therapy, is characterized by its variable pharmacokinetic characteristics and narrow therapeutic window. Moreover, tacrolimus is predominately metabolised into 13-O-demethyltacrolimus in the liver and the intestines by cytochrome P450 (CYP) 3A¹⁻⁴. The CYP3A subfamily composed of four genes, CYP3A4, CYP3A5, CYP3A7 and CYP3A43 and three pseudogenes CYP3AP1, CYP3AP2 and CYP3AP3, is located on chromosome 7q21-q22.1⁵. The CYP3A concentrations and activities display interindividual variability, which can at least partly explain the variation in the trough (C_0) levels⁶⁻¹⁵ and the other pharmacokinetic parameters of tacrolimus¹⁶⁻¹⁸. Although more than 30 CYP3A4 allelic variants have been reported⁵, most CYP3A4 variants are single nucleotide polymorphisms (SNPs) with low allelic frequencies and many of these SNPs are population specific¹⁹. Due to their low allelic frequency, the contribution of these CYP3A4 SNPs to the interindividual variability of CYP3A4 expression is limited. In contrast to CYP3A4, a few SNPs in CYP3A5 have a major impact on the CYP3A5 expression^{20,21}. However, in Caucasians CYP3A5 expression appears to be mainly regulated by the CYP3A5*3 allelic variant²⁰⁻²². Individuals carrying a CYP3A5*3 (low expressers) variant allele express a significantly lower amount CYP3A5 compared to individuals carrying a CYP3A5*1 allele (high expressers). Transplant patients categorized as low expressers achieve significantly higher pharmacokinetic parameters for tacrolimus (dn C_0 , dnAUC₀₋₁₂ and dn C_{max}) and therefore require a lower daily tacrolimus dose compared to transplant patients included in the high expresser group^{16,17}. Although Kamden *et al.*²³ previously demonstrated with *in vitro* experiments that CYP3A7 compared to CYP3A4 and CYP3A5 plays a minor role in the tacrolimus metabolism, the role of higher CYP3A7 protein levels on the tacrolimus metabolism *in vivo* is not yet been elucidated. CYP3A7 is the most abundant CYP in human liver during fetal development and the first months of postnatal age²⁴. Moreover, CYP3A7 plays an important role in the metabolism of key steroids in the adrenals and gonads of potentially toxic and teratogenic endogenous substrates²⁴⁻²⁷ and of many drugs of abuse that reach the fetus²⁸⁻³¹. Sim *et al.*³² found that the CYP3A7 protein expression is high in a fraction of the human adult livers and is associated partially with the CYP3A7*1C allele. Additionally, Smit *et al.*³³ found that the CYP3A7*1C variant allele is associated with a nearly 50% reduction of the serum dehydro-epi-androsterone sulfate (DHEAS) levels. Considering the relevance of this CYP3A7*1C allelic variant, we developed a real-time PCR fluorescence resonance energy transfer (FRET) assay for this CYP3A7 polymorphism. Moreover, since clinical studies regarding the influence of CYP3A7*1C polymorphism on the pharmacokinetic parameters of tacrolimus are still lacking, two Caucasian and a Chinese renal transplant recipient group are genotyped for the CYP3A7*1C variant allele. Furthermore, the genotypes of the CYP3A7*1C variant allele found in the renal transplant recipient groups are correlated with their corresponding pharmacokinetic tacrolimus parameters.

Materials and methods

Study populations

In total 70 Caucasian and 103 Chinese renal transplant recipients are included in the present study. The Caucasian renal transplant recipients are divided over two different groups of whom in the past for clinical reasons (group I) or for a clinical trial (group II) a 12 hour time tacrolimus concentration curve was performed. A two time point sampling strategy³⁴ was used to calculate the abbreviated AUC_{0-12} in the Chinese renal transplant recipients (group III). Group I included early Caucasian posttransplant patients with a short median time after transplantation and large variability in the tacrolimus AUC_{0-12} compared to the late Caucasian posttransplant recipients of group II. Most pharmacokinetic profiles of the patients included in group I were recorded within six weeks after transplantation. All renal transplant recipients included in group II and III underwent a renal transplantation at least one year (group II) or four months (group III) ago. Moreover, eight patients in group I used a calcium channel blocker that showed interference with tacrolimus. Group II and III included no patients taking medication known to interact with tacrolimus such as calcium channel blockers, anti-epileptics, anti-mycotics and macrolide antibiotics. In addition, patients that suffer from gastrointestinal, liver disease, or other disorders that may alter the absorption of tacrolimus were also disqualified for inclusion. The initial tacrolimus dosage, administrated twice daily, was 0.2 mg/kg per day for patients included in group I and II and 0.3 mg/kg per day for patients included in group III. For patients in group I and II the dose requirement for tacrolimus was assessed three months after transplantation, when the target C_0 concentration was between 10 to 15 ng/ml while the daily tacrolimus dose for patients in group III was adjusted according to the AUC_{0-12} value, which was kept at around 100-150 ng $\times$ hr/ml in the first three months. After three months the target AUC_{0-12} value was decreased to around 80-100 ng $\times$ hr/ml for long term maintenance. Prior to the blood sample collection, there had been no tacrolimus dose change for at least three days in groups I and II and for at least two weeks in group III. After overnight fasting the blood samples were collected immediately pre (C_0) and 0.5 ($C_{0.5}$), 1 (C_1), 2 (C_2), 3 (C_3), 4 (C_4), 5 (C_5), 7.5 ($C_{7.5}$) and 12 (C_{12}) hours after the morning tacrolimus administration for group I and II while blood samples were collected at two time points (C_2 and C_4) to calculate the abbreviated AUC_{0-12} for the patients included in group III. All patients were not allowed to take food until one hour after ingesting the tacrolimus dose and were advised to avoid grapefruit juice after transplantation to prevent alterations in the tacrolimus metabolism. Demographic as well as clinical data were determined at the time of recording the AUC_{0-12} or the abbreviated AUC_{0-12} (Table 9.1). The renal transplant recipients included in group I and II underwent a renal transplantation and a regular follow up in the University Hospital of Maastricht, the Netherlands whereas the renal transplant recipients included in group III had regular follow up in Queen Elizabeth Hospital or Tuen Mun Hospital in Hong Kong. The study was performed in accordance

to the Declaration of Helsinki and its amendments. The protocol was approved by the local Medical Ethics Committees in the Netherlands and Hong Kong and written informed consent for participation in this study was obtained from all patients.

Table 9.1 Demographic characteristics of the three renal transplant recipients groups.

Demographic characteristics	Group I	Group II	Group III
Ethnicity	Caucasians	Caucasians	Chinese
Gender (male/female)	22/11	24/13	58/45
Age (years, mean $\pm$ SD)	42.0 $\pm$ 12.2	51.3 $\pm$ 10.9	43.1 $\pm$ 11.1
Weight (kg, mean $\pm$ SD)	71.7 $\pm$ 16.4	77.4 $\pm$ 13.5	62.5 $\pm$ 11.4
Primary kidney disease			
Glomerulonephritis	5	1	57
Chronic pyelonephritis	2	2	1
IgA nephropathy	3	4	0
Hypertensive nephropathy	4	7	9
Diabetes Mellitus nephropathy	5	0	10
Polycystic kidney disease	2	8	2
Obstructive Uropathy	0	0	3
Unknown	3	4	18
Other	9	11	3
Transplantation number			
First	26	30	97
Second	6	6	6
Third or more	1	1	0
Tacrolimus mono therapy	2	29	0
Tacrolimus dose (mg/kg/day, mean $\pm$ SD)	0.34 $\pm$ 0.233	0.054 $\pm$ 0.029	0.067 $\pm$ 0.032
C ₀ (ng/ml, mean $\pm$ SD)	14.8 $\pm$ 6.60	6.59 $\pm$ 1.39	NA
AUC ₀₋₁₂ (ng $\times$ hr/ml, mean $\pm$ SD)	269.7 $\pm$ 110.9	122.5 $\pm$ 31.1	NA
Abbreviated AUC ₀₋₁₂ (ng $\times$ hr/ml, mean $\pm$ SD)	ND	ND	100.3 $\pm$ 24.2
C _{max} (ng/ml, mean $\pm$ SD)	50.2 $\pm$ 22.8	20.9 $\pm$ 6.5	NA
T _{max} (hr, mean $\pm$ SD)	1.46 $\pm$ 1.21	1.24 $\pm$ 0.43	NA
Use of AZA ^a , MMF ^b , rapamycine, steroids	10/3/4/28	3/4/0/0	78/25/0/103
Current steroid dose (mg, dose, no. patients)			
0 mg/day	5	37	0
4 mg/day	0	0	1
5 mg/day	7	0	33
7.5 mg/day	0	0	64
8 mg/day	2	0	0
10 mg/day	10	0	3
15 mg/day	4	0	1
20 mg/day	3	0	0
> 20 mg/day	2	0	1
Time since Tx (days, median, (range))	20 (3-5359)	1465 (453-4128)	977 (133-4982)
Haemoglobin (mmol/l, mean $\pm$ SD)	6.03 $\pm$ 1.56	8.52 $\pm$ 0.83	12.8 $\pm$ 2.0
Haematocrit fraction (mean $\pm$ SD)	0.27 $\pm$ 0.09	0.41 $\pm$ 0.04	0.38 $\pm$ 0.058
ALAT (U/l, mean $\pm$ SD)	32 $\pm$ 31	24 $\pm$ 13	22 $\pm$ 18
Serum albumin (g/l, mean $\pm$ SD,)	32.5 $\pm$ 5.03	37.0 $\pm$ 3.84	43 $\pm$ 3.2
Serum creatinine (μ mol/l, median $\pm$ SD)	189 $\pm$ 268	125 $\pm$ 29	156 $\pm$ 76

^a azathiopurine; ^b mycophenolate mofetil; ND not determined, NA not available; Abbreviated AUC₀₋₁₂ is the AUC₀₋₁₂ calculated with the following formula: AUC₀₋₁₂ = 16.2 + 2.4 $\times$ C₂ + 5.9 $\times$ C₄.

Tacrolimus concentration determination

The tacrolimus measurements for the three groups were performed in different laboratories. For all groups the tacrolimus blood concentrations were determined in ethylene diamine tetra-acetic acid (EDTA) whole blood, using a microparticle enzyme immunoassay (MEIA) with a monoclonal antibody (IMx II assay; Abbott Laboratories, Abbott Park, IL, USA) for group I and III and a method based on high pressure liquid chromatography (LC) tandem mass spectrometry (MS/MS) for group II. All laboratories participate in the International Tacrolimus Proficiency Testing Scheme. The tacrolimus C_0 concentration, the peak blood concentration (C_{max}) during the assessed time interval and the time at which the highest blood concentration was observed (T_{max}) were determined directly from the time *versus* tacrolimus blood concentration data. Additionally, the area under the time tacrolimus concentration curve (AUC_{0-12}) was calculated from the time *versus* tacrolimus concentration plot using the linear trapezoidal rule in MWPharm 3.50 (Mediware, Groningen, the Netherlands). The two tacrolimus blood concentrations determined for the patients included in group III were used to calculate the abbreviated AUC_{0-12} according to the equation based strategy as described by our group³⁴: $AUC_{0-12} = 16.2 + 2.4 \times C_2 + 5.9 \times C_4$. DnC_0 , $dnAUC_{0-12}$ and dnC_{max} were calculated by dividing the C_0 , AUC_{0-12} and C_{max} respectively by the corresponding morning dose on a milligram per kilogram basis.

Real-time PCR FRET assay for CYP3A7*1C allelic variant

Genomic DNA was isolated from 70 Caucasian and 103 Chinese renal transplant recipients by using 200 μ L EDTA anti coagulated blood for isolation with a Qiagen blood mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturers' instructions. Genotyping for the CYP3A7*1C allelic variant was performed using a real-time PCR FRET assay on the LightCycler (Roche Diagnostics, Almere, the Netherlands). Since the different members of the CYP3A subfamily show a high sequence homology (> 90%), the position of the primers is carefully selected. Moreover, the CYP3A7*1C variant allele consists of seven different polymorphisms within a 60 base pair (bp) range in the CYP3A7 promoter region, which narrows the position options of the probe that covers one of these polymorphisms. Specific primers for CYP3A7 and a sensor probe covering the T-188G polymorphism in the CYP3A7 promoter region are used in the real-time PCR FRET assay developed for the CYP3A7*1C variant allele. The primers F41: 5'-CCA TAg AgA CAA gAg gAg AgT TAA T-'3 (sense; 110611-635) and R41: 5'-gAg gCT TCT CCA CCT Cg-'3 (anti-sense; 110790-806) were used to amplify a 196 bp part from the CYP3A7 gene which covers the T110718G polymorphism (corresponding to position -188 of the CYP3A7 promoter; Genbank acces no: AF280107)³⁵. Detection was carried out using the anchor probe CYP3A7A41: 5'-ggg AgT CCA Agg gTT CTg gg-'3 (anti-sense; 110677-696) which was labelled at the 5' end with LCRed640 and phosphorylated at the 3'-end to block extension and the sensor probe CYP3A7P41: 5'-ATC ATA CAC AAC TCA ATC AAT gTT ACT-'3 (anti-

sense; 110698-724) which is complementary to the CYP3A7*1 allele. This 3'-fluorescein labelled sensor probe, in which the polymorphic nucleotide is underlined, binds with a distance of one base 5' to the detection probe. The PCR mixture contained 3.2 μ l sterile water; 3.0 mmol/l $MgCl_2$; 1.0 μ l LC Faststart DNA Master Hybridization Probes (Roche Diagnostics GmbH, Mannheim, Germany); 0.50 μ mol/l of each primer and 0.20 μ mol/l of both anchor and sensor probe (TIB MOLBIOL, Berlin, Germany). After adding 1.0 μ l genomic DNA to the PCR mixture, the total volume is 10 μ l. The PCR protocol included the following steps: a) denaturation for ten minutes at 95°C b) 45 cycles at 95°C for 10 seconds; 55°C for ten seconds and 72°C for five seconds. After amplification was completed, a melting curve was recorded by heating to 95°C (20°C/second), holding at 95°C for 30 seconds then cooling to 40°C (20°C/second), holding at 40°C for one minute and then heating slowly to 85°C at 0.2°C/second. During the slow heating procedure of the melting curves, fluorescence was measured continuously to monitor the dissociation of the fluorophore-labelled detection probe. The fluorescence signals were then converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature *versus* temperature ($-dF_2/dT$ *versus* T). The melting point of the sensor probe was 61°C when hybridised to the wild type CYP3A7*1 allele and 56.5°C when hybridised to the CYP3A7*1C variant allele. A heterozygous carrier of the CYP3A7*1C variant allele shows both melting points (Figure 9.1). The real-time PCR FRET assay for the CYP3A7*1C variant allele was validated by sequencing a heterozygote sample and a wild type sample of the CYP3A7*1C variant allele according to a direct sequence procedure performed with the capillary sequencer ABI 3100 (Applied Biosystems, Fostercity, USA) using the Bridge version 1.1 sequence kit (Applied Biosystems, Fostercity, USA).

Figure 9.1 Genotyping of the CYP3A7 T-188G polymorphism with allele specific fluorescent probes by derivative melting curve plots. The derivative melting curves are read in channel 2 for the detection of the CYP3A7 fragments. The derivative melting curve is plotted for a sample homozygous for the -188G allele (1; T_m 61.0°C); and a heterozygous sample (2; T_m 56.5°C and 61.0°C). Melting analysis of a no template control was also performed (3).

Statistical analysis

Statistical analysis of the data was performed with use of the statistical software SPSS 11.0 for windows (Chicago, IL, USA). Patients' genotypes were used as categorical independent variables for analysis of continuous clinical and pharmacological variables. To examine the population homogeneity of the patients, the genotype frequencies of the CYP3A7*1C polymorphism were tested against Hardy-Weinberg equilibrium by the Pearson's goodness-of-fit test. For analysis of the daily tacrolimus dose (mg/kg/day), dose-normalized (dn) C_0 (ng/ml per mg/kg), dose-normalized (dn)AUC₀₋₁₂ (ng × hr/ml per mg/kg), dose-normalized (dn) C_{max} (ng/ml per mg/kg) and T_{max} (hr), groups were compared using non parametric statistical tests. To compare the two groups we used the Mann-Whitney test. *P* values less than 0.05 were considered statistically significant. All values are expressed as median and range unless stated otherwise.

Results

In total 70 Caucasian renal transplant recipients divided over 33 early (group I) and 37 late (group II) posttransplant recipients and 103 Chinese renal transplant recipients (group III) were genotyped for the CYP3A7*1C variant allele. In group I and II, respectively one and three heterozygous carriers were found for the CYP3A7*1C variant allele while no CYP3A7*1C variant allele was found in the Chinese renal transplant recipients of group III. The previous findings result in an allele frequency for the CYP3A7*1C variant allele of 1.5%, 4.1% and 0.0% in group I, II and III, respectively. The allele frequencies found in the Caucasian renal transplant recipients are in line with previous studies^{20,35}. Since no CYP3A7*1C variant allele was found in the 103 Chinese renal transplant recipients examined, it is likely that the frequency of this CYP3A7 polymorphism in the Chinese population is very low. Renal transplant recipient groups I and II showed no significantly different distribution of the CYP3A7*1C variant allele from that predicted by the Hardy-Weinberg equilibrium (*P* > 0.05). Moreover, no significant difference in the allele frequency for the CYP3A7*1C variant allele was found between the two Caucasian renal transplant recipient groups examined. All heterozygous carriers for the CYP3A7*1C variant allele were homozygous carrier of the CYP3A4*1A and CYP3A5*3 allele. Since one renal transplant recipient in group I was carrier of the CYP3A7*1C variant allele, a statistical analysis was only performed with the 37 Caucasian renal transplant recipients that were included in group II. Renal transplant recipients carrying a CYP3A7*1C variant allele showed no significant lower dn C_0 levels (404 *versus* 264 ng/ml per mg/kg; Mann-Whitney *P* = 0.290), dnAUC₀₋₁₂ (6376 *versus* 4539 ng × hr/ml per mg/kg; Mann-Whitney *P* = 0.504), dn C_{max} (1179 *versus* 743 ng/ml per mg/kg; Mann-Whitney *P* = 0.469) or T_{max} (1.00 *versus* 1.00 hr; Mann-Whitney *P* = 0.312), compared to the renal transplant recipients carrying a wild type CYP3A7*1 allele.

Additionally, Figure 9.2 illustrates that heterozygote carriers of the CYP3A7*1C variant allele showed no difference in the 12 hour pharmacokinetic tacrolimus profile.

Figure 9.2 The influence of the CYP3A7 T-188G polymorphism on the 12 hour area under the time tacrolimus concentration curve (AUC_{0-12}) in 37 late Caucasian posttransplant patients. The boxplot of the dose-normalized AUC_{0-12} is clustered according to the CYP3A7 genotype. *P* values for the pairwise comparisons of each genotype are given. The open circles indicate an outlier value at more than 1.5 box lengths above the box.

Highlights

- Tacrolimus is predominantly metabolised into 13-O-demethyltacrolimus by cytochrome P450 3A (CYP3A) iso-enzymes. Recently, a study described that the catalytic efficiency for CYP3A5 with tacrolimus is 60% higher than that for CYP3A4. The contribution of the CYP3A7*1C allele, responsible for a higher CYP3A7 expression, to the tacrolimus metabolism in transplant patients is not elucidated.
- The CYP3A7*1C variant allele consists of seven different polymorphisms located in a 60 basepair promoter region of CYP3A7. The proximal everted repeat 6 motif present in the CYP3A7*1C variant allele binds with a higher affinity the pregnane X receptor and the constitutively activated receptor than the CYP3A7*1 allele, which results in a higher CYP3A7 expression for individuals carrying a CYP3A7*1C allele.
- A novel real-time PCR FRET assay on the LightCycler is described for the CYP3A7*1C allelic variant. This real-time PCR FRET assay is validated by sequencing a heterozygous sample and a homozygous wild type DNA sample.
- In total 70 Caucasian and 103 Chinese renal transplant recipients are genotyped for the CYP3A7*1C variant allele. The Caucasian renal transplant recipients were divided in 33 early and 37 late post transplant patients. The allele frequency for the CYP3A7*1C variant allele in the Caucasian and the Chinese renal transplant recipients is 2.8% and 0.0%, respectively.

- The CYP3A7*1C variant allele is not associated with any dose-normalized pharmacokinetic parameter (C_0 , AUC_{0-12} , C_{max}) recorded in the late posttransplant recipient group. Our findings indicate that a higher CYP3A7 expression has no influence on the tacrolimus metabolism.

Discussion

The initial and maintenance dosage regime of tacrolimus in transplant patients is difficult to assess due to its highly variable pharmacokinetic characteristics and narrow therapeutic range. Although several clinical studies^{16,17} demonstrated that the variation in the pharmacokinetic characteristics of tacrolimus may be explained by the CYP3A5*3 variant allele, there is still a large variation within the patient groups carrying a different CYP3A5 genotype for this CYP3A5*3 variant allele. Sattler *et al.*¹ suggested that other members of the CYP3A subfamily might be involved than CYP3A4. Bader *et al.*³⁶ later confirmed that CYP3A5 plays an important role in the 13-O-demethylation of tacrolimus. More recently, Kamden *et al.*²³ demonstrated with *in vitro* experiments that the demethylation of tacrolimus into 13-O-demethyltacrolimus in hepatocytes is mainly performed by CYP3A4 and CYP3A5. Although Kamden *et al.*²³ suggested that CYP3A7 played no role in the tacrolimus metabolism *in vivo*, clinical studies confirming these results are still lacking. Sim *et al.*³² found that the CYP3A7 protein expression contributes 9 to 36% of the total CYP3A levels in 10% of 59 adult livers. Additionally, they demonstrated that the CYP3A7*1C variant allele is partly responsible for these higher CYP3A7 expression levels. The CYP3A7*1C allele contains the proximal everted repeat 6 (ER6) motif of CYP3A4 which implicates that the pregnane X receptor (PXR) and the constitutively activated receptor (CAR) bind with a higher affinity to the CYP3A7*1C allele than to the CYP3A7*1 allele. Consequently only the promoter constructs of CYP3A7 containing this CYP3A4-ER6 motif, CYP3A7*1C, will be transactivated by PXR and CAR. Previously, Burk *et al.*³⁵ reported that the presence of the ER6 motif of CYP3A4 mediates the high expression of CYP3A7 in individuals carrying a CYP3A7*1C allele. Within the late posttransplant recipient group, no significant effect on both the pharmacokinetic tacrolimus parameters and the daily tacrolimus dose was observed between carriers of the wild type CYP3A7*1 allele or the CYP3A7*1C variant allele. Previously, Kamden *et al.*²³ demonstrated with *in vitro* experiments that CYP3A7 played a minor role in the tacrolimus metabolism. Our findings indicate that a higher CYP3A7 protein expression *in vivo* caused by the CYP3A7*1C variant allele has no significant effect on the tacrolimus metabolism. In conclusion, in the present study a reliable and reproducible real-time PCR FRET assay is described for the CYP3A7*1C variant allele. Additionally, we found that the CYP3A7*1C variant allele, responsible for higher CYP3A7 protein levels, has a (very) low frequency in a Caucasian and a Chinese renal transplant population and is not associated with the pharmacokinetic parameters of tacrolimus.

Outlook

In the last decade pharmacogenomics has gained considerable interest among several medical disciplines. Especially, the immunosuppressive drugs cyclosporin and tacrolimus are of special importance due to their highly variable pharmacokinetic characteristics and narrow therapeutic window. Cyclosporin and tacrolimus are substrates of the cytochrome P450 3A (CYP3A) family. Although CYP3A4 and CYP3A5 both show a great affinity for cyclosporin and tacrolimus, several clinical studies demonstrated that a part of the variation in the pharmacokinetic tacrolimus parameters can be explained by a single nucleotide polymorphism (CYP3A5 A6986G) present in the CYP3A5 gene. However, even within a group transplant patients carrying the same genotype for this CYP3A5 polymorphism, there is still a large variation in the pharmacokinetic tacrolimus parameters. To achieve a better individualized immunosuppressive therapy with a lower occurrence of subtherapeutic or toxic side effects of these immunosuppressive drugs, minimizing the variation in the pharmacokinetic tacrolimus parameters is certainly a prerequisite. Therefore, future studies are necessary to examine the role of other enzymes, transporters or transcriptional regulators involved in the tacrolimus metabolism and whether polymorphisms in these genes have an important impact on the pharmacokinetic parameters and thus on the daily dose requirement.

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Chapter 10

Influence of allelic variants of uridine
diphosphateglucuronosyltransferase (UGT) 2B7
on the tacrolimus exposure

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Abstract

Tacrolimus is predominantly demethylated into 13-O-demethyltacrolimus although identification of tacrolimus glucuronides demonstrates that conjugation also plays a role in the tacrolimus metabolism. Uridine 5'-diphosphateglucuronosyltransferase (UGT) 2B7 is identified as the most relevant member of the UGT family responsible for tacrolimus glucuronidation. Single nucleotide polymorphisms (SNPs) in the UGT2B7 gene may have a contribution on the interindividual variation of tacrolimus blood concentrations. A two time point sampling strategy is used to calculate the AUC_{0-12} for 103 Chinese renal transplant recipients. In the present study real-time polymerase chain reaction (PCR) fluorescence resonance energy transfer (FRET) assays were developed and used to determine the genotype of three UGT2B7 polymorphisms in Chinese renal transplant recipients. The effect of these UGT2B7 polymorphisms on both the dose-normalized area under the time tacrolimus concentration curve ($dnAUC_{0-12}$) and the daily tacrolimus dose is examined. The variant alleles of UGT2B7 polymorphisms G-79A, T-66C and C816T with an allele frequency of respectively, 0.0%, 5.8% and 29.6% showed no significant differences on both the $dnAUC_{0-12}$ and the daily tacrolimus dose compared to carriers of the wild type UGT2B7 alleles when the patients were categorized based on their CYP3A5 A6986G, ABCB1 G2677T/A and ABCB1 C3435T genotype. Therefore, genotyping transplant patients for these UGT2B7 polymorphisms before using tacrolimus is irrelevant.

Introduction

Tacrolimus is usually administered orally and has a rather unpredictable and variable absorption rate. Therefore close monitoring of the tacrolimus blood levels is required to prevent both subtherapeutic and toxic blood concentrations. Although cytochrome (CYP) 3A enzyme polymorphisms and possibly also adenosine triphosphate-binding cassette B1 (ABCB1) polymorphisms clarify a considerable part of the variation in tacrolimus trough (C_0) levels¹⁻¹³, there is still a large interindividual variation in the tacrolimus C_0 levels. The analysis of tacrolimus metabolites in humans has provided evidence not only for CYP3A catalysed metabolism but also for the formation of glucuronides¹⁴. Strassburg *et al.*¹⁵ examined the glucuronidation activities of different uridine 5'-diphosphate glucuronosyltransferase (UGT) (sub)families for both tacrolimus and cyclosporin and found that UGT2B7 showed the highest glucuronidation activity in the human gastrointestinal tract. The UGT family, a group of proteins responsible for the glucuronidation of several endogene and exogene compounds in humans, is mainly present in the liver. However, UGTs are also found throughout the gastrointestinal tract, where they are an integral part of prehepatic first-pass metabolism. In addition, UGTs also work in the kidneys, brain, placenta and in several other locations in the human body¹⁶⁻¹⁸. The major function of glucuronidation is to increase the polarity of the target compound, a process which facilitates their detoxification and excretion. However, glucuronidation can also result in compounds which are biologically active or demonstrate increased toxicity. After glucuronidation, intestinal bacterial β -glucuronidases break down glucuronidation products and release the unconjugated drugs via enterohepatic recirculation. This "recycling" system slowly clears conjugated compounds and releases glucuronides for re-use¹⁹⁻²³. Although Strassburg *et al.*¹⁵ reported that hepatic and extrahepatic glucuronidation may influence the therapeutic efficacy of immunosuppressants, the genetic influence of known UGT2B7 polymorphisms on the pharmacokinetic parameters of immunosuppressants has never been examined. In the present study, we have developed real-time polymerase chain reaction (PCR) fluorescence resonance energy transfer (FRET) assays for three UGT2B7 polymorphisms and examined whether the interindividual variation in the tacrolimus blood levels can be explained more exactly by genotyping the transplant recipients for these UGT 2B7 polymorphisms.

Materials and methods

Study populations

A total of 103 Chinese renal transplant recipients who received tacrolimus as part of the immunosuppressive therapy and had regular follow up in the Queen Elizabeth Hospital or Tuen Mun Hospital in Hong Kong were included in this retrospective study. There

was no change in the daily tacrolimus dose for at least two weeks. Patients who were taking medication known to have interaction with tacrolimus, such as calcium channel blockers, anti-epileptics, anti-mycotics and macrolide antibiotics were excluded from this study. Additionally, patients who suffered from gastrointestinal tract disease, liver disease or other disorders that may alter the absorption of tacrolimus were also excluded. Apart from tacrolimus and steroids, the patients were normally put on azathioprine, however some patients preferred to use mycophenolic acid on advice of their private physician. The dosage of azathioprine was 1.5 mg/kg/day, while the dosage of mycophenolic acid was 0.5 gram twice daily. The initial tacrolimus dosage, administered twice daily, was 0.3 mg/kg per day for all patients. The daily tacrolimus dose was then adjusted according to the AUC_{0-12} value, which was kept at around 100 - 150 ng×hr/ml during the first 3 months. After three months the target AUC_{0-12} value was decreased at around 80 - 100 ng×hr/ml for long term maintenance. These AUC_{0-12} values were based on our previous study²⁴ and the experiences obtained in our transplantation centers. The steroid regimen for the first month was 30 mg/day of oral prednisolone, progressively tapered by 2.5 mg every two weeks until a daily maintenance dose of 5 mg. Demographic as well as clinical data were collected at the time of the tacrolimus blood sample collection.

Determination whole blood tacrolimus concentrations

Blood samples were collected 2 (C2) and 4 (C4) hours after the morning tacrolimus administration. Tacrolimus blood concentrations were determined in ethylene diamine tetra-acetic acid (EDTA) whole blood using a semi-automated microparticle enzyme immunoassay (MEIA) on an IMx II clinical analyser (Abbott Laboratories, Abbott Park, IL, USA). The two tacrolimus blood concentrations determined were used to calculate the AUC_{0-12} according to the equation based strategy as described earlier: $AUC_{0-12} = 16.2 + 2.4 \times C2 + 5.9 \times C4^{24}$. Dose-normalized AUC_{0-12} (dn AUC_{0-12}) were calculated by dividing the AUC_{0-12} by the corresponding 24-hour dose on a milligrams per kilogram basis.

DNA isolation

Genomic DNA was extracted from 103 Chinese renal transplant recipients by using 200 µl EDTA anti coagulated blood for isolation with a QIAamp blood mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturers' instructions.

UGT2B7 G-79A, T-66C and C816T primers and probes

The real-time PCR fluorescence resonance energy transfer (FRET) assays were designed and optimised in our laboratory. Genotyping for the UGT2B7 G-79A and T-66C polymorphisms was performed using the primers F30: 5'-TTg CAT gTC CAT ACA AgA TCC T-3' (sense; 1150–171) and R30: 5'-ATT TCA CAg ACA TCC Tgg TgC-

'3 (anti-sense; 1432–412) to amplify a 273 bp part from the UGT2B7 gene which covers both G-79A and T-66C polymorphisms in the promoter region of the UGT2B7 gene (Genbank acces no: AF282881). Detection of the UGT2B7 G-79A polymorphism was carried out using the anchor probe 79A30: 5'-Tgg CAA AgA gAC AAA TgA Agg TAA ATg ATC TgT TC-3' (anti-sense; 1265 - 231) which was labelled at the 5'-end with LCRed640 and phosphorylated at the 3'- end to block extension from the sensor probe 79P30: 5' CAT TAA ATC AAC AgT CTg AgC ATg Tgg 3' (anti-sense; 1293-267) which is complementary to the UGT2B7 -79G polymorphism. This 3'-fluorescence-labelled sensor probe, in which the polymorphic nucleotide is underlined binds with a distance of one base 5' to the detection probe. Detection of the UGT2B7 T-66C polymorphism was carried out using the anchor probe 66A30: 5'-Agg gTT ACA TTT TAA CTT CTT ggC TAA TTT ATC TT- 3' (sense; 1283-316) which was labelled at the 5'-end with LCRed640 and phosphorylated at the 3'- end to block extension from the sensor probe 66P30: TTg ATT TAA TgA CAT TgT ATg TAC TTT gAC TTA T (sense; 1283-316) which is complementary to the UGT2B7 -66C polymorphism. This 3'-fluorescence-labelled sensor probe, in which the polymorphic nucleotide is underlined binds with a distance of one base 5' to the detection probe. The PCR mixture for both real-time PCR FRET assays contained: 3.2 µl sterile water; 3.0 mM MgCl₂; 1.0 µl LC Faststart DNA Master Hybridization Probes (Roche Diagnostics GmbH, Mannheim, Germany); 0.5 µM of each primer and 0.2 µM of both sensor and anchor probe (TIB MOLBIOL, Berlin, Germany). After adding 1.0 µl containing 50-100 ng genomic DNA to the PCR mixture, the total volume was 10 µl. The PCR protocol included the following steps: denaturation for ten minutes at 95°C; 45 cycles at 95°C for ten seconds, 55°C for ten seconds and 72°C for 11 seconds. After amplification was completed, a melting curve was recorded by heating to 95°C (20°C/second) holding at 95°C for 30 seconds; subsequently cooling to 45°C (20°C/second) holding at 45°C for 1 minute and then heating slowly to 75°C at 0.2°C/second. Genotyping for the UGT2B7 C816T polymorphism was performed using the primers F31: 5'-ACC TTT TTT TTT TCT ATT CCT gT-3' (sense; 1-23) and R31: 5'-CAA AAT AAA ACC AAC AAA AgT ATg-3' (anti-sense; 204-181) to amplify a 204 bp part from the UGT2B7 gene which covers the T815C and C816T polymorphisms in exon 1 (Genbank acces no: AF282881). Detection of the UGT2B7 C816T polymorphism was carried out using the anchor probe A31: 5'-CCA AAT gTT gAT TTT gTT ggA ggA CTC C-3' (sense; 119-146) which was labelled at the 5'-end with LCRed640 and phosphorylated at the 3' end to block extension from the sensor probe, P31: 5'-gAA TTT TCA gTT TCC ATA TCC ACT CTT-3' which is complementary to the UGT2B7 816T polymorphism. This 3'-fluorescence-labelled sensor probe, in which the polymorphic nucleotide is underlined binds with a distance of one base 5' to the detection probe. The PCR mixture for the real-time PCR FRET assay contained: 3.2 µl sterile water; 3.0 mM MgCl₂; 1.0 µl LC Faststart DNA Master Hybridization Probes (Roche Diagnostics GmbH, Mannheim, Germany); 0.5 µM of each primer and 0.2 µM of both sensor and anchor probe (TIB MOLBIOL, Berlin, Germany). After adding 1.0 µl containing 50-100 ng genomic DNA to the PCR mixture the total volume was 10 µl. The PCR protocol

included the following steps: denaturation for ten minutes at 95°C; 45 cycles at 95°C for ten seconds, 55°C for ten seconds and 72°C for eight seconds. After amplification was completed, a melting curve was recorded by heating to 95°C (20°C/second) holding at 95°C for 30 seconds; subsequently cooling to 62°C (0.5°C/second) holding at 62°C for one minute and finally cooling to 45°C for 0 seconds (20°C/second) and then heating slowly to 80°C at 0.1°C/second. All real-time PCR and melting curve analyses were performed on a LightCycler (Roche Diagnostics, Almere, the Netherlands). Additionally, sequencing is performed on a capillary sequencer ABI Prism 3100 using the Bridge version 1.1 sequence kit (Both products from Applied Biosystems, Fostercity, USA) and is used for identifying the presence of the expected UGT2B7 polymorphisms. The control samples that are sequenced are also used as controls in the different real-time PCR FRET assays.

Statistical analysis

The statistical analysis of the data was performed with use of the statistical software SPSS 11.0 for windows (Chicago, IL, USA). Patients genotypes were used as categorical independent variables for analysis of continuous clinical and pharmacological variables. To examine the population homogeneity of the patients, the genotype frequencies of the UGT2B7 polymorphisms were tested against Hardy-Weinberg equilibrium by the Pearson's goodness-of-fit test. For analysis of the daily dose of tacrolimus (mg/kg/day) and the dnAUC_{0-12} ($\text{ng} \times \text{hr/ml}$ per mg/kg), groups were compared using non parametric statistical tests. To compare two groups we used the Mann Whitney test, and to compare several groups, the Kruskal Wallis test. *P* values less than 0.05 were considered statistically significant. All values are expressed as median and range unless stated otherwise.

Results

Real-time PCR FRET assays for the UGT2B7 G-79A, T-66C, C816T polymorphisms

The melting point of the sensor probe for the UGT2B7 G-79A polymorphism was 59.5°C when hybridised to the UGT2B7 -79G allele and 57.5°C when hybridised to the UGT2B7 -79A allele, whereas for the UGT2B7 T-66C polymorphism, the melting point of the sensor probe was 52°C when hybridised to the UGT2B7 -66T allele and 59°C when hybridised to the UGT-66C allele. A heterozygote sample for the UGT2B7 G-79A and T-66C polymorphisms showed both melting peaks (Figure 10.1A and 10.1B). In addition, for the UGT2B7 C816T polymorphism, the melting point of the sensor probe was 62.5°C for the UGT2B7 816T allele and 57.5°C when hybridised to the UGT2B7 816C allele. A heterozygote sample for the UGT2B7 C816T polymorphism showed both

melting peaks (Figure 10.1C). The presence of the expected polymorphisms was confirmed by sequencing the allelic variants of UGT2B7 according to a direct sequence procedure on a capillary sequencer ABI Prism 3100 using the Bridge version 1.1 sequence kit (Both products from Applied Biosystems, Fostercity, USA). A heterozygote DNA sample was found for the UGT2B7 G-79A polymorphism among anonymized healthy Caucasian volunteers using a real-time PCR FRET assay for this UGT2B7 G-79A polymorphism. Additionally, this control sample was sequenced to confirm that this sample is heterozygous for the UGT2B7 G-79A polymorphism.

Figure 10.1 Genotyping of UGT2B7 G-79A, T-66C and C816T with allele specific fluorescent probes by derivative melting curve plots. The derivative melting curves are read in channel 2 for the detection of the two UGT2B7 fragments. A. The derivative melting curve is plotted for a sample homozygous for the UGT2B7 -79G allele (1; T_m 59.5°C) and a heterozygous sample (2; T_m 57.5°C and 59.5°C). B. The derivative melting curve is plotted for a sample homozygous for the UGT2B7 -66T allele (1; T_m 52°C) and a heterozygous sample (2; T_m 52°C and 59°C). C. The derivative melting curve is plotted for a sample homozygous for the UGT2B7 816C allele (1; T_m 57.5°C); a sample homozygous for the UGT2B7 816T allele (2; T_m 62.5°C) and a heterozygous sample (3; T_m 57.5°C and 62.5°C) Melting analysis of a no template control was also performed for all assays (3 or 4).

Influence of the different UGT2B7 allelic variants on the pharmacokinetic tacrolimus profiles

Table 10.1 illustrates the demographic characteristics of 103 Chinese renal transplant recipients included in the present study. After genotyping 103 renal transplant patients, no variant allele was found for the UGT2B7 G-79A polymorphism whereas the frequency of the UGT2B7 -66TT genotype was found to be 88% ($n = 91$) and for the UGT2B7 -66TC the frequency was 12% ($n = 12$). In addition, 49% ($n = 51$) of the patients were carrier of the UGT2B7 816CC genotype while 42% ($n = 43$) was heterozygous carrier of the UGT2B7 816CT genotype and 9% ($n = 9$) homozygous for the UGT2B7 816TT variant allele. The allele frequencies of the Chinese renal transplant patients examined were found to be 0.0% (-79A), 5.8% (-66C) and 29.6% (816T), which is not in line with those published in previous studies^{25,26}. According to the Pearson's

goodness-of-fit test the genotype frequencies of these UGT2B7 polymorphisms differed not significantly from the Hardy-Weinberg equilibrium ($P > 0.05$).

Table 10.1 Demographic characteristics of the renal transplant recipients.

Demographic characteristics	
Gender (male/female)	58/45
Age (years, mean $\pm$ SD, (range))	43.1 $\pm$ 11.1 (21-77)
Weight (kg, mean $\pm$ SD, (range))	62.5 $\pm$ 11.4 (37.4-87.8)
Tacrolimus dose (mg/kg/day, mean $\pm$ SD, (range))	0.067 $\pm$ 0.032 (0.01-0.18)
Tacrolimus conc. 2 hour post dose (ng/ml, mean $\pm$ SD, (range))	13.5 $\pm$ 4.30 (5.7-29.4)
Tacrolimus conc. 4 hour post dose (ng/ml, mean $\pm$ SD, (range))	8.7 $\pm$ 2.60 (2.3-16.4)
AUC ₀₋₁₂ (ng $\times$ hr/ml, mean, (range))	100.3 (47.8-183.5)
DnAUC ₀₋₁₂ (ng $\times$ hr/ml per mg/kg, mean, (range))	1940 (300-11125)
Use of azathiopurine/mycophenolate mofetil	78/25
Current steroid dose (mg, mean $\pm$ SD, (range))	6.98 $\pm$ 2.37 (4-25)
Time since transplantation (days, mean, (range))	977 (133-4982)
Serum creatinine (μ mol/l, mean $\pm$ SD, (range))	156 $\pm$ 76 (62-462)

A significant higher dnAUC₀₋₁₂ (1685 *versus* 1276 ng $\times$ hr/ml per mg/kg; Mann-Whitney, $P = 0.026$) was observed for carriers of the UGT2B7 -66TT genotype compared to carriers of the UGT2B7 -66TC genotype while no significant difference (Kruskal Wallis, $P = 0.817$) was observed between carriers of a different UGT2B7 C816T genotypes. This is illustrated in Figure 10.2A and 10.3A. However, previously we demonstrated in the same renal transplant patient group an important impact of the CYP3A5 A6986G polymorphism whereas a minor association was reported between both ABCB1 polymorphisms G2677T/A and C3435T and the tacrolimus blood concentrations¹³. To exclude influence of one of these polymorphisms, the renal transplant recipients were based on their CYP3A5 and ABCB1 G2677T/A, C3435T genotype categorized in order to examine solely the effect of the two UGT2B7 T-66C and C816T polymorphisms on the dnAUC₀₋₁₂ and the daily tacrolimus dose. As is illustrated in Figure 10.2B - 10.2D, there is no significant influence of UGT2B7 T-66C polymorphism on the dnAUC₀₋₁₂ of tacrolimus after categorizing the patients for respectively CYP3A5 A6986G, ABCB1 G2677T/A and ABCB1 C3435T genotype. Additionally, after categorizing the patients, Figures 10.3B - 10.3D illustrate that there is, as expected, also no significant effect of the UGT2B7 C816T polymorphism on the dnAUC₀₋₁₂ of tacrolimus. Regarding the daily tacrolimus dose requirement similar results were obtained for both UGT2B7 polymorphisms.

Figure 10.2 Influence of the UGT2B7 T-66C and C816T genotypes on the area under the time – tacrolimus concentration curve (AUC_{0-12}) that were recorded in 103 stable Chinese renal transplant recipients. Figure 10.2 illustrates the boxplots of the distribution of the dose normalized AUC_{0-12} ($ng \times hr/ml$ per mg/kg) clustered according to the (A) UGT2B7 T-66C genotype, (B) the combination of respectively CYP3A5 A6986G and UGT2B7 T-66C genotypes, (C) ABCB1 G2677T/A and UGT2B7 T-66C genotypes and (D) ABCB1 C3435T and UGT2B7 T-66C genotypes. Open circles indicate an outlier value at more than 1.5 box lengths above the box, while asterisks indicate an extreme value at more than 3 box lengths above the box.

Figure 10.3 This figure illustrates the boxplots of the distributions of the AUC_{0-12} ($\text{ng} \times \text{hr/ml}$ per mg/kg) clustered according to the (A) UGT2B7 C816T genotype, (B) the combination of respectively CYP3A5 A6986G and UGT2B7 C816T genotypes, (C) ABCB1 G2677T/A and UGT2B7 C816T genotypes and (D) ABCB1 C3435T and UGT2B7 C816T genotypes. Open circles indicate an outlier value at more than 1.5 box lengths above the box, while asterisks indicate an extreme value at more than 3 box lengths above the box.

Highlights

- Tacrolimus is predominately demethylated into 13-O-demethyltacrolimus by CYP3A iso-enzymes. However, identification of tacrolimus glucuronides demonstrate that besides demethylation also conjugation influences the tacrolimus metabolism.
- Uridine 5'-diphosphate glucuronosyltransferase (UGT) 2B7 mainly present in the liver and intestines is the major UGT isoform responsible for the glucuronidation of tacrolimus.
- Several single nucleotide polymorphisms (SNPs) identified in the UGT2B7 gene may besides the cytochrome 3A (CYP3A) and adenosine diphosphate-binding

cassette B1 (ABCB1) polymorphisms also explain the interindividual differences in the tacrolimus pharmacokinetics.

- A two time point sampling strategy is used to calculate the area under the time tacrolimus concentration curve (AUC_{0-12}) and the dose-normalised (dn) AUC_{0-12} are correlated with the corresponding UGT2B7 genotypes in 103 Chinese renal transplant recipients.
- After eliminating the influence of the CYP3A5 and ABCB1 polymorphisms, no significant differences were found in the $dnAUC_{0-12}$ and daily tacrolimus dose between carriers of different UGT2B7 genotypes.
- Our findings indicate that SNPs in the UGT2B7 gene have no significant impact on the tacrolimus blood concentration and tacrolimus dosage. Therefore, we conclude that polymorphisms in the UGT2B7 gene have no important contribution on the interindividual variability of the tacrolimus pharmacokinetics.

Discussion

Previously, we reported a strong allele-dependent effect between the CYP3A5 A6986G polymorphism and both the $dnAUC_{0-12}$ and the daily tacrolimus dose, while a less pronounced effect was found for the ABCB1 G2677T/A and C3435T polymorphisms in this Chinese renal transplant recipient population¹³. However, there is still variation in the tacrolimus blood concentrations among the renal transplant patients carrying the same CYP3A5 and ABCB1 genotypes. Although identification of tacrolimus¹⁴ glucuronides demonstrates that conjugation also plays a role in the metabolism of this immunosuppressant, less information is available regarding the UDP-glucuronosyltransferases (UGT) involved in the tacrolimus metabolism. Strassburg *et al.*¹⁵ found that UGT2B7 is the member of the UGT family that is responsible for the tacrolimus glucuronidation. Large inter-individual differences in the mRNA expression levels of UGT2B7²⁷, which could be caused to a degree by polymorphisms, may possibly explain a part of the inter-individual pharmacokinetic variation of tacrolimus. A common polymorphism in the UGT2B7 gene (UGT2B7 C816T) has been found in Caucasians and Asians²⁶. Although one study²⁸ found no remarkable function difference between the different carriers of the UGT2B7 C816T polymorphism, another study²⁹ recently reported that UGT2B7 816T variant allele showed significantly higher morphine-6-O-glucuronide/morphine ratios than that with the wild type UGT2B7 allele. More recently, Duguay *et al.*³⁰ discovered the functional polymorphism -79A in the UGT2B7 promoter with significant impact on the promoter activity while the variation at position -66 resulted in a non-significant alteration of promoter activity compared to the wild type promoter. However, no significant difference in the ratios of morphine-6-glucuronide/morphine and morphine-3-glucuronide/morphine were found between 6 heterozygous carriers of the UGT2B7 -79A variant allele and 175 wild type carriers among cancer patients receiving long term oral morphine. Although Strassburg *et al.*¹⁵

demonstrated that tacrolimus glucuronidation is performed exclusively by UGT2B7, the present study demonstrated that the UGT2B7 polymorphisms T-66C and C816T have no influence on the dnAUC_{0-12} of tacrolimus and the daily tacrolimus dose when categorizing patients in different groups for the CYP3A5 A6986G, ABCB1 G2677T/A and C3435T genotype. Since tacrolimus is predominately demethylated into 13-O-demethyltacrolimus it seems that tacrolimus glucuronidation plays a neglectable role in the tacrolimus metabolism process.

Outlook

The calcineurin inhibitor tacrolimus is generally used as an immunosuppressive drug in solid organ transplantations. However, it is difficult to maintain an appropriate blood concentration of tacrolimus due to its narrow therapeutic index and significant intra- and inter-individual variety. Tacrolimus is predominantly metabolized into 13-O-demethyltacrolimus by CYP3A4 and CYP3A5 in the liver and intestines, and subsequently pumped out by P-glycoprotein, an ATP dependent efflux pump, encoded by the adenosine triphosphate-binding cassette B1 (ABCB1) gene. Therefore, these proteins are considered to have a major influence on the first-pass effect in the liver and the intestines and on the variation in tacrolimus pharmacokinetics. Several studies already reported the impact of some CYP3A and ABCB1 polymorphisms on the tacrolimus trough (C_0) levels, pharmacokinetic tacrolimus parameters and the daily tacrolimus dose in transplant patients. However, identification of tacrolimus glucuronides demonstrates that conjugation also plays a role in the tacrolimus metabolism. Uridine 5'-diphosphate glucuronosyltransferase (UGT) 2B7 is the most important UGT isoform responsible for the glucuronidation of tacrolimus. Single nucleotide polymorphisms (SNPs) in the UGT2B7 gene may have an effect on the interindividual variation in the tacrolimus pharmacokinetics. In the present study we calculated an area under the time tacrolimus concentration curve (AUC_{0-12}) based on a two time point sample strategy for 103 Chinese renal transplant recipients and demonstrated that the UGT2B7 polymorphisms T-66C and C816T have no significant role on the variation of the dose-normalized AUC_{0-12} and the daily tacrolimus dose. Therefore, genotyping renal transplant patients for these UGT2B7 polymorphisms will not contribute to less variation in the tacrolimus blood concentrations and a better individualized immunosuppressive therapy.

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Chapter 11

Pregnane X receptor (PXR) polymorphisms involved in the tacrolimus pharmacokinetics

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Abstract

Cytochrome P450 (CYP) 3A4/5 are responsible for metabolizing tacrolimus while the P-glycoprotein pump (ABCB1) modulates its bioavailability. Differences in gene expression can not be fully explained by CYP3A4/5 and/or ABCB1 polymorphisms. The pregnane X receptor (PXR) is a transcriptional regulator of the CYP450 and the ABCB1 expression. Real-time PCR FRET assays determine the genotypes of four PXR polymorphisms in 103 Chinese renal transplant recipients to examine its influence on dose-normalized AUC_{0-12} ($dnAUC_{0-12}$). Individuals carrying a variant allele for PXR polymorphisms A7635G, C8055T, A11156C and T11193C, associated with increased expression of CYP3A4 or ABCB1, had lower $dnAUC_{0-12}$ for tacrolimus compared to individuals carrying the wild type variant for these PXR polymorphisms (respectively, Mann-Whitney; $P = 0.038$, $P = 0.088$, $P = 0.038$ and $P = 0.038$). These PXR polymorphisms are associated with a trend towards a lower $dnAUC_{0-12}$ of tacrolimus which confirms a role of PXR in the CYP3A and ABCB1 expression and thereby on the tacrolimus metabolism.

Introduction

Tacrolimus has a narrow therapeutic index which requires close monitoring of the drug concentration to achieve an optimal efficiency and minimizing the risks of acute rejection and drug overdose. The polymorphisms present in the cytochrome P450 3A5 (CYP3A5) iso-enzyme and adenosine triphosphate-binding cassette gene B1 (ABCB1) can partly explain their variability in expression and activity and thus clarify to a certain extent the interindividual differences in the tacrolimus pharmacokinetics. However, cytochrome P450 3A4 (CYP3A4) expression shows a large interindividual variation that can not be explained solely by polymorphisms identified in this gene¹. The pregnane X receptor (PXR)² also known as SXR³, hPAR⁴ or NR1I2 has been identified as a transcriptional regulator of CYP3A4^{2,4} and ABCB1^{5,6}. PXR binds as a heterodimer with the 9-*cis* retinoic acid receptor (RXR; NR2B) to previously characterized xenobiotic response elements in CYP3A gene promoters, and importantly, PXR is activated by the spectrum of chemicals that are known to induce CYP3A gene expression^{7,8}. Single nucleotide polymorphisms (SNPs) in the PXR gene can influence the PXR activity and thereby the CYP3A4 and ABCB1 expression. Zhang *et al.*⁹ identified 38 different polymorphisms in the PXR gene and determined their allelic frequency in a Caucasian (n = 75) and African-American (n = 11) population. Two other studies also screened the PXR gene for polymorphisms and determined their allele frequency in a Caucasian population^{10,11}. The polymorphisms A7635G and C8055T located in correspondingly intron 5 and intron 6 of the PXR gene have a high allelic frequency in two different Caucasian populations. Bosch *et al.*¹⁰ reported that 32% and 15% of the Caucasians were carrier of, respectively, PXR 7635G and 8055T variant alleles while Zhang *et al.*⁹ found that 35% and 16% of the Caucasians was carrier of correspondingly PXR 7635G and 8055T variant alleles. In addition, Zhang *et al.*⁹ observed that 16% of the Caucasians was carrier of the 11156C or 11193C variant allele which is located in the 3'-untranslated region of the PXR gene. The PXR polymorphisms A7635G and C8055T have been shown to have a higher magnitude of induction of the intestinal CYP3A4 by rifampicin while the homozygous carriers of the PXR 11156C or 11193C variant alleles had lower P-glycoprotein levels in gut biopsies compared to those with homozygous 11156A and 11193T alleles⁹. In the present study, a 12-hour area under the time concentration curve (AUC₀₋₁₂) calculated with a two time point sample strategy is correlated with the corresponding PXR genotypes for 103 Chinese renal transplant patients to elucidate whether these PXR polymorphisms can contribute to a decrease within the interindividual variability of the tacrolimus pharmacokinetics.

Patients and methods

Study population

A total of 103 Chinese renal transplant recipients who received tacrolimus as part of the immunosuppressive therapy and had regular follow up in the Queen Elizabeth Hospital or Tuen Mun Hospital in Hong Kong were included in this retrospective study. There was no change in the daily tacrolimus dose for at least two weeks. Patients who were taking medication known to have interaction with tacrolimus, such as calcium channel blockers, anti-epileptics, anti-mycotics and macrolide antibiotics were excluded from this study. Additionally, patients who suffered from gastrointestinal tract disease, liver disease or other disorders that may alter the absorption of tacrolimus were also excluded. Apart from tacrolimus and steroids, the patients were normally put on azathioprine, however some patients preferred to use mycophenolic acid on advice of their private physician. The dosage of azathioprine was 1.5 mg/kg/day, while the dosage of mycophenolic acid was 0.5 gram twice daily. The initial tacrolimus dosage, administrated twice daily, was 0.3 mg/kg per day for all patients. The daily tacrolimus dose was then adjusted according to the AUC_{0-12} value, which was kept at around 100-150 ng $\times$ hr/ml during the first three months. After three months the target AUC_{0-12} value was decreased at around 80-100 ng $\times$ hr/ml for long term maintenance. These AUC_{0-12} values were based on our previous pilot study¹² and the experiences obtained in our transplantation centers. The steroid regimen for the first month was 30 mg/day of oral prednisolone, progressively tapered by 2.5 mg every two weeks until a daily maintenance dose of 5 mg. Demographic as well as clinical data were collected at the time of the tacrolimus blood sample collection.

Determination whole blood tacrolimus concentrations

Blood samples were collected 2 (C2) and 4 (C4) hours after the morning tacrolimus administration. Tacrolimus blood concentrations were determined in ethylene diamine tetra-acetic acid (EDTA) whole blood using a semi-automated microparticle enzyme immunoassay (MEIA) on an IMx II clinical analyser (Abbott Laboratories, Abbott Park, IL, USA). The two tacrolimus blood concentrations determined were used to calculate the AUC_{0-12} according to the equation based strategy as described earlier¹²: $AUC_{0-12} = 16.2 + 2.4 \times C2 + 5.9 \times C4$. Dose-normalized AUC ($dnAUC_{0-12}$) were calculated by dividing the AUC_{0-12} by the corresponding 24 hour dose on a milligrams per kilogram basis.

DNA isolation

Genomic DNA was extracted from 103 Chinese renal transplant recipients by using 200 μ l EDTA anticoagulated blood for isolation with a QIAamp blood mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturers' instructions.

Primers and probes for the PXR A11156C and T11193C polymorphisms

The real-time PCR fluorescence resonance energy transfer (FRET) assays were designed and optimized in our laboratory. Genotyping for the PXR A11156C and T11193C polymorphisms was performed using the primers F22: 5'-CAA TCA gTT AAA CAC ACC ggA g-3' (sense; 81370-391) and R22: 5'-TTT TAT gTT CTT ACg CCg gAg T-3' (anti-sense; 81766-745) to amplify a 397 bp part from the PXR gene which covers both A11156C and T11193C polymorphisms in the 3'-untranslated region (3' UTR) of the PXR gene (Genbank acces no: AF364606). Detection of the PXR A11156C polymorphism was carried out using the anchor probe 56A22: 5'-TTT Tgg gAA ATg TAG CCC Tgg gT-3' (sense; 81553-575) which was labelled at the 5'-end with LCRed640 and phosphorylated at the 3'-end to block extension from the sensor probe 56P22AL: 5'-CAT TCC ACA CCT AXA gAA CTA-3' (sense; 81532-551) which is complementary to the PXR 11156A polymorphism. This 3'-fluorescence labelled sensor probe, in which the polymorphic locked nucleic acid (LNA) nucleotide is underlined binds with a distance of one base 5' to the detection probe. A locked nucleic acid (indicated as XA in the sensor probe 56P22AL) was necessary to discriminate both melting peaks¹³. Detection of the PXR T11193C polymorphism was carried out using the anchor probe 93A22: 5'-ACC CAg ggC TAC ATT TCC CAA AA-3' (anti-sense; 81575-553) which was labelled at the 5'-end with LCRed640 and phosphorylated at the 3' end to block extension from the sensor probe 93P22: 5'-CTT TTg CCT TgA TTT ggC ATT A-3' (anti-sense; 81598-577) which is complementary to the PXR 11193C polymorphism. This 3'-fluorescence labelled sensor probe, in which the polymorphic nucleotide is underlined binds with a distance of one base 5' to the detection probe. The PCR mixture for both real-time PCR FRET assays contained: 3.2 µl sterile water; 3.0 mM MgCl₂; 1.0 µl LC Faststart DNA Master Hybridization Probes (Roche Diagnostics GmbH, Mannheim, Germany); 0.5 µM of each primer and 0.2 µM of both sensor and anchor probe (TIB MOLBIOL, Berlin, Germany). After adding 1.0 µl containing 50-100 ng genomic DNA to the PCR mixture, the total volume was 10 µl. The PCR protocol included the following steps: denaturation for ten minutes at 95°C; 45 cycles at 95°C for ten seconds, 55°C for ten seconds and 72°C for 16 seconds. After amplification was completed, a melting curve was recorded by cooling to 45°C (20°C/second) holding at 45°C for one minute and then heating slowly to 70°C at 0.2°C/second.

Primers and probes for the PXR A7635G and C8055T polymorphism

Additionally, a real-time PCR FRET assay was designed and optimised for the PXR A7635G polymorphism. Genotyping for the PXR A7635G polymorphism was performed using the primers F23: 5'-gAg CTg TCT gCT ggg TTg Tg-3' (sense; 77928-974) and R23: 5'-ggT CCT CgA Tgg gCA AgT C-3' (anti-sense; 78136-118) to amplify a 209 bp part from the PXR gene which covers the A7635G polymorphisms in intron 5 (Genbank

access no: AF364606). Detection of the PXR A7635G polymorphism was carried out using the anchor probe A23: 5'-ATT ATg ggA Tgg CTg CTg gTg CCg gC-'3 (sense; 78039-064) which was labelled at the 5'- end with LCRed640 and phosphorylated at the 3'- end to block extension from the sensor probe, P23: 5'-CCT CTC ACC CCC AAC TTC T-'3 which is complementary to the PXR 7635A polymorphism. This 3'-fluorescence labelled sensor probe, in which the polymorphic nucleotide is underlined binds with a distance of one base 5' to the detection probe. Finally, a real-time PCR FRET assay was designed, optimised and validated for the PXR C8055T polymorphism. Genotyping for the PXR C8055T polymorphism was performed using the primers F24: 5'-gCT ggA TTA AAg CAT gTA CTT CA-'3 (sense; 78312-330) and R24: 5'-ggC CTg CAT CAg CAC ATA CT-'3 (anti-sense; 78558-539) to amplify a 246 bp part from the PXR gene which covers the C8055T polymorphisms in intron 6 (Genbank access no: AF364606). Detection of the PXR C8055T polymorphism was carried out using the anchor probe A24: 5'-TCC ACA ggT ggC TTC CAg CAA CTT CTA CTg-'3 (sense; 78454-483) which was labelled at the 5'- end with LCRed705 and phosphorylated at the 3'- end to block extension from the sensor probe. P24: 5'-CTg CCC CTC CAT CCT gTT ACC-'3 which is complementary to the PXR 8055C polymorphism. This 3'-fluorescence labelled sensor probe, in which the polymorphic nucleotide is underlined binds with a distance of one base 5' to the detection probe. The PCR mixture for the real-time PCR FRET assays for the PXR A7635G and C8055T polymorphisms contained: 5.2 µl sterile water; 2.0 mM MgCl₂; 1.0 µl LC Faststart DNA Master Hybridization Probes (Roche Diagnostics GmbH, Mannheim, Germany); 0.6 µM of each primer and 0.2 µM of both sensor and anchor probe (TIB MOLBIOL, Berlin, Germany). After adding 1.0 µl containing 50-100 ng genomic DNA to the PCR mixture the total volume was 10 µl. The PCR protocol for the two PXR polymorphisms A7635G and C8055T included the following steps: denaturation for ten minutes at 95°C; 40 cycles at 95°C for 10 seconds, 55°C for ten seconds and 72°C for ten seconds. After amplification was completed, a melting curve was recorded by heating to 95°C (20°C/second) holding at 95°C for 30 seconds; subsequently cooling to 40°C (20°C/second) holding at 40°C for one minute and then heating slowly to 75°C at 0.1°C/second. All real-time PCR and melting curve analyses were performed on a LightCycler (Roche Diagnostics, Almere, the Netherlands). Additionally, sequencing is performed on a capillary sequencer ABI Prism 3100 using the Bridge version 1.1 sequence kit (Both products from Applied Biosystems, Fostercity, USA) and is used for identifying the presence of the expected PXR polymorphisms. The sequenced samples are used as controls in the different real-time PCR FRET assays.

Statistical analysis

The statistical analysis of the data was performed with use of the statistical software SPSS 11.0 for windows (Chicago, IL, USA). Patients' genotypes were used as categorical independent variables for analysis of continuous clinical and

pharmacological variables. To examine the population homogeneity of the patients, the genotype frequencies of the PXR polymorphisms were tested against Hardy-Weinberg equilibrium by the Pearson's goodness-of-fit test. For analysis of the daily dose of tacrolimus (mg/kg/day) and the dnAUC_{0-12} ($\text{ng} \times \text{hr/ml}$ per mg/kg), groups were compared using non-parametric statistical tests. To compare two groups we used the Mann-Whitney test, and to compare several groups, the Kruskal Wallis test. *P* values less than 0.05 were considered statistically significant. All values are expressed as median and range unless stated otherwise.

Results

Real-time PCR FRET assays

After analysing 103 DNA samples obtained from Chinese renal transplant recipients the melting point of the sensor probe for the PXR A11156C polymorphism was $58.7 \pm 0.08^\circ\text{C}$ ($n = 113$) when hybridised to the PXR 11156A allele and $51.9 \pm 0.11^\circ\text{C}$ ($n = 93$) when hybridised to the PXR 11156C allele, whereas for the PXR T11193C polymorphism, the melting point of the sensor probe was $56.4 \pm 0.16^\circ\text{C}$ ($n = 113$) when hybridised to the PXR 11193T allele and $61.3 \pm 0.14^\circ\text{C}$ ($n = 93$) when hybridised to the PXR 11193C allele. In addition, for the PXR A7635G polymorphism, the melting point of the sensor probe was $61.2 \pm 0.19^\circ\text{C}$ ($n = 95$) when hybridised to the PXR 7635A allele and $55.0 \pm 0.11^\circ\text{C}$ ($n = 111$) for the PXR 7635G allele, whereas for the PXR C8055T polymorphism, the melting point of the sensor probe was $64.7 \pm 0.32^\circ\text{C}$ ($n = 116$) when hybridised to the PXR 8055C allele and $58.1 \pm 0.33^\circ\text{C}$ ($n = 90$) when hybridised to the PXR 8055T allele. Heterozygote samples for all studied polymorphisms showed both melting peaks. The presence of the expected polymorphisms was confirmed by sequencing the different allelic variants of the PXR gene according to a direct sequence procedure on a capillary sequencer ABI Prism 3100 using the Bridge version 1.1 sequence kit (Both products from Applied Biosystems, Fostercity, USA).

Distribution of allele frequencies

Table 11.1 illustrates the demographic characteristics of 103 Chinese renal transplant recipients included in the present study while Table 11.2 shows the distribution of the allele frequencies and genotypes of the PXR polymorphisms among Chinese transplant recipients examined in the present study. Moreover, Table 11.2 summarised the allele frequencies from Caucasians and African-American individuals.

Table 11.1 Demographic characteristics of the renal transplant recipients

Demographic characteristics	
Gender (male/female)	58/45
Age (years, mean $\pm$ SD, (range))	43.1 $\pm$ 11.1 (21-77)
Weight (kg, mean $\pm$ SD, (range))	62.5 $\pm$ 11.4 (37.4-87.8)
Primary kidney disease	
Glomerulonephritis	57
Chronic pyelonephritis	1
Diabetic nephropathy	10
Hypertensive nephropathy	9
Polycystic kidney disease	2
Obstructive Uropathy	3
Unknown	18
Other	3
Transplantation number	
First	97
Second	6
Tacrolimus dose (mg/kg body weight/day, mean $\pm$ SD, (range))	0.067 $\pm$ 0.032 (0.01-0.18)
Tacrolimus conc. 2 hour post dose (ng/ml, mean $\pm$ SD, (range))	13.5 $\pm$ 4.30 (5.7-29.4)
Tacrolimus conc. 4 hour post dose (ng/ml, mean $\pm$ SD, (range))	8.7 $\pm$ 2.60 (2.3-16.4)
AUC ₀₋₁₂ (ng $\times$ hr/ml, mean, (range))	100.3 (47.8-183.5)
DnAUC ₀₋₁₂ (ng $\times$ hr/ml per mg/kg body weight, mean, (range))	1940 (300-11125)
Use of azathiopurine/mycophenolate mofetil	78/25
Current steroid dose (mg, mean $\pm$ SD, (range))	6.98 $\pm$ 2.37 (4-25)
Time since transplantation (days, mean, (range))	977 (133-4982)
Haemoglobin (mmol/l, mean $\pm$ SD, (range) ref. ♂ 8.2-11.0, ♀ 7.3-9.7)	12.8 $\pm$ 2.0 (8.5-19)
Haematocrit fraction (mean $\pm$ SD, (range) ref. ♂ 0.41-0.52, ♀ 0.36-0.48)	0.38 $\pm$ 0.058 (0.25-0.57)
ALAT (U/l, mean $\pm$ SD, (range) ref. ♂ < 45, ♀ < 35)	22 $\pm$ 18 (8-147)
Serum albumin (g/l, mean $\pm$ SD, (range) ref. 34-45)	43 $\pm$ 3.2 (34-50)
Serum creatinine (μ mol/l, mean $\pm$ SD, (range) ref. ♂ 71-110, ♀ 53-97)	156 $\pm$ 76 (62-462)

Ref. are the reference values applied in the clinical chemistry and haematology laboratories of the University Hospital in Maastricht. ♂ male, ♀ female.

In total, 79.6% (n = 82) of the Chinese renal transplant recipients have a similar haplotype for these four PXR polymorphisms. The allele frequencies of the Chinese renal transplant patients examined were found to be 45.1% (11156C and 11193C), 53.9% (7635G) and 43.7% (8055T) which is not in line with those published in previous studies^{9,10}. However, these studies determined the allele frequencies in Caucasians or African Americans which may explain the differences. According to the Pearson's goodness-of-fit test the genotype frequencies of these PXR polymorphisms differed not significantly from the Hardy-Weinberg equilibrium ($P > 0.05$).

Table 11.2 Distribution allele frequencies/genotypes PXR polymorphisms.

PXR Polymorphisms	Genotype n (%)	Allele frequency n (%)	Allele frequencies observed in other studies ^{9,10}
A7635G	AA: 20 (19.4%)	AA: 95 (46.1%)	AA: 65% ^a , 23% ^a , 32% ^b
	AG: 55 (53.4%)		
	GG: 28 (27.2%)		
C8055T	CC: 33 (32.0%)	CC: 116 (56.3%)	GG: 35% ^a , 77% ^a , 68% ^b CC: 85% ^a , 82% ^a , 84%
	CT: 50 (48.5%)		
	TT: 20 (19.4%)		
A11156C	AA: 31 (30.1%)	AA: 113 (54.9%)	TT: 15% ^a , 18% ^a , 16% AA: 84% ^a , 67% ^a
	AC: 51 (49.5%)		
	CC: 21 (20.4%)		
T11193C	TT: 31 (30.1%)	TT: 113 (54.9%)	CC: 16% ^a , 33% ^a TT: 84% ^a , 67% ^a
	TC: 51 (49.5%)		
	CC: 21 (20.4%)		

^a Zhang *et al.*⁹ determined for 150 alleles originated from Caucasians and 22 alleles originated from African Americans the allele frequencies of these PXR polymorphisms. ^b Bosch *et al.*¹⁰ determined for 200 alleles obtained from healthy Caucasian volunteers the allele frequencies of these PXR polymorphisms.

Influence of the different PXR allelic variants on the pharmacokinetic tacrolimus profiles

Since identical genotype distributions were found among the Chinese renal transplant recipients for the PXR polymorphisms A11156C and T11193C, the difference observed between the dnAUC₀₋₁₂ was also the same. A significant higher dnAUC₀₋₁₂ (1864 *versus* 1334 ng × hr/ml per mg/kg; Mann-Whitney, *P* = 0.038 and 1918 *versus* 1342 ng×hr/ml per mg/kg; Mann-Whitney, *P* = 0.038) was observed for carriers of respectively the PXR 7635AA and 11156AA/11193TT genotypes compared to the PXR 7635GG and 11156CC/11193CC genotypes while a strong trend towards a lower dnAUC₀₋₁₂ (1882 *versus* 1390 ng × hr/ml per mg/kg; Mann-Whitney, *P* = 0.088) was observed between carriers of the PXR 8055CC genotype compared to carriers of the PXR 8055TT genotype. Additionally, similar results were obtained for the daily tacrolimus dose, a significantly higher daily tacrolimus dose (0.052 *versus* 0.070 mg/kg/day; Mann-Whitney, *P* = 0.042 and 0.051 *versus* 0.070 mg/kg/day; Mann-Whitney, *P* = 0.061) was observed for carriers of respectively the PXR 7635AA and 11156AA/11193TT genotypes compared to the PXR 7635GG and 11156CC/11193CC genotypes while a strong trend towards a higher daily tacrolimus dose (0.052 *versus* 0.070 mg/kg/day; Mann-Whitney, *P* = 0.108) was observed between carriers of the PXR 8055CC genotype compared to carriers of the PXR 8055TT genotype. Figure 11.1A-D illustrate the effect of the different PXR polymorphisms on the dnAUC₀₋₁₂ recorded in 103 Chinese renal transplant recipients. Furthermore, the PXR haplotypes consisting of the four polymorphisms are associated with the dnAUC₀₋₁₂, as is shown in Figure 11.1E.

Figure 11.1 Influence of the PXR A7635G, C8055T, A11156C and T11193C genotypes on the area under the time tacrolimus concentration curve (AUC_{0-12}) that were recorded in 103 stable Chinese renal transplant recipients. Figure 11.1 illustrates the boxplots of the distribution of the dose normalized AUC_{0-12} (ng x hr/ml per mg/kg) clustered according to the (A) PXR A7635G genotype, (B) PXR C8055T genotype, (C) PXR A11156C genotype, (D) PXR T11193C genotype and (E) the PXR haplotypes consisting of the PXR polymorphisms A7635G, C8055T, A11156C and T11193C. The open circles indicate an outlier value at more than 1.5 box lengths above the box, while the asterices indicate an extreme value at more than 3 box lengths above the box.

However, previously we¹⁴ demonstrated in the same renal transplant patient group an important impact of the CYP3A5 A6986G polymorphism on the tacrolimus concentrations whereas a minor association was reported between both ABCB1 polymorphisms G2677T/A and C3435T and the tacrolimus concentration. To exclude influence of one of these polymorphisms, the renal transplant recipients were categorized on their CYP3A5 and ABCB1 G2677T/A, C3435T genotype in order to examine solely the effect of the four PXR polymorphisms on the dnAUC_{0-12} and the daily tacrolimus dose. After categorizing the patients for respectively the CYP3A5 A6986G, ABCB1 G2677T/A and ABCB1 C3435T genotype there is, as is illustrated in Figure 11.2A-C, a tendency towards a lower dnAUC_{0-12} within the groups of the combination ABCB1 polymorphisms and the PXR A7635G polymorphism. Although these data are not shown, similar results were observed for the PXR polymorphisms C8055T, A11156C and T11193C. Additionally, comparable results were achieved for the daily tacrolimus dose requirement and the four PXR polymorphisms.

Figure 11.2 Boxplots of the distribution of the dose normalized AUC_{0-12} (ng x hr/ml per mg/kg) clustered according to (A) the combination of respectively CYP3A5 A6986G and PXR A7635G, (B) ABCB1 G2677T/A and PXR A7635G genotypes and (C) ABCB1 C3435T and PXR A7635G genotypes. The open circles indicate an outlier value at more than 1.5 box lengths above the box, while the asterisks indicate an extreme value at more than 3 box lengths above the box.

Discussion

Tacrolimus is predominantly metabolized by CYP3A iso-enzymes into 13-O-demethyltacrolimus in the liver and the intestine¹⁵⁻¹⁹. Although CYP3A4 and CYP3A5 are the most important members of the CYP3A family, the CYP3A5 A6986G polymorphism is the only relevant polymorphism in the CYP3A4 and CYP3A5 genes which has a relative high allele frequency in different populations and a significant impact on the tacrolimus pharmacokinetics. Although CYP3A4 expression shows a large interindividual variation, this can not only be explained by the polymorphisms in the CYP3A4 gene¹. Elucidation of the mechanisms underlying CYP3A gene expression may be important for the role of the interindividual variation in the tacrolimus pharmacokinetics. Moreover, there is also conflicting evidence regarding the role of the individual ABCB1 polymorphisms and ABCB1 haplotypes in the tacrolimus pharmacokinetics. Several studies reported a significant impact of the ABCB1 polymorphisms or haplotypes on the tacrolimus trough (C_0) concentrations²⁰⁻²⁶ while other studies found no association between the different genotypes of the ABCB1 polymorphisms or ABCB1 haplotypes and the tacrolimus C_0 concentrations²⁷⁻³² or pharmacokinetics^{14,33,34}. Since PXR is emerged as a transcriptional regulator of the expression of different members of the CYP450 family, polymorphisms in the PXR gene may influence the PXR activity and thereby the expression of these CYP450s. PXR binds as a heterodimer with RXR to the functional direct repeat-3 (DR-3) response element and to the functional everted repeat-6 (ER-6) response element in the proximal promoter region of the CYP3A4 gene while the CYP3A5 gene only contains the functional ER-6 response element in the promoter. In addition, the PXR/RXR heterodimer also binds to the functional direct repeat-4 (DR-4) response element in the ABCB1 promoter^{5,6}. Zhang *et al.*⁹ found that the 7635A to G transition in intron 5 of the PXR gene was associated with higher magnitude of induction of intestinal CYP3A by rifampicin. Individuals homozygous for the 7635G allele had a twofold higher induction of CYP3A compared to those homozygous for 7635A. An 8055C to T substitution was also associated with intestinal CYP3A phenotype that was more inducible. Individuals carrying at least one T-allele had twofold higher levels of intestinal CYP3A following rifampicin treatment compared to individuals homozygous for the 8055C allele. The 3'-untranslated region (3' UTR) polymorphisms 11156A to C and 11193T to C showed that those individuals heterozygous with at least one 11156C or one 11193C allele had 1.45-fold lower P-glycoprotein levels in gut biopsies compared to those with homozygous 11156A and 11193T alleles. Since no CYP3A4*1B variant allele is found in this Chinese renal transplant patient group¹⁴, there is no influence of the CYP3A4*1B variant allele on the CYP3A4 expression and thereby on the dnAUC_{0-12} of tacrolimus. However, to exclude the effect of the CYP3A5*3 variant allele and the individual ABCB1 polymorphisms G2677T/A and C3435T or ABCB1 haplotypes, the renal transplant population is categorized according to the CYP3A5 A6986G, ABCB1 G2677T/A and C3435T genotype. In the present study a significant difference was observed regarding

the PXR polymorphisms A7635G, A11156C and T11193C when the dnAUC_{0-12} was plotted *versus* the corresponding PXR polymorphisms of the 103 Chinese renal transplant patient recipients while a trend was observed for the PXR C8055T polymorphism. These results underline the *in vitro* findings demonstrated by Zhang *et al.*⁹. However, after excluding the effect of the CYP3A5 A6986G polymorphism and the ABCB1 polymorphisms G2677T/A and C3435T, only a trend is observed within the patients that were carrier of a similar genotype for the CYP3A5 A6986G or ABCB1 G2677T/A, ABCB1 C3435T polymorphisms. Probably due to the presence of at least eight different groups with other genotype combinations it is likely that the small number of patients included in one group has a relevant impact on these findings. Additional studies with larger patient numbers included in each group could possibly confirm our preliminary results.

Conclusion

We described real-time PCR FRET assays for four PXR polymorphisms and determined the allele frequency of these four PXR polymorphisms in a Chinese renal transplant recipient group. Moreover, a strong trend towards a lower dnAUC_{0-12} is found for individuals carrying a variant PXR allele suggesting that these PXR polymorphisms may play a role in the tacrolimus metabolism.

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Chapter 12

Influence of pregnane X receptor (PXR)
polymorphisms on the pharmacokinetics of
tacrolimus in renal transplant recipients

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Abstract

Tacrolimus has a narrow therapeutic index and shows substantial interindividual variability in its pharmacokinetic characteristics. The pregnane X receptor (PXR) identified as a transcriptional regulator of cytochrome P450 3A4 may play a role in the tacrolimus metabolism. In this study, the role of PXR polymorphisms is examined in an early and a late posttransplant patient group with and without corticosteroids.

Twenty-six early and thirty-seven late posttransplant patients were genotyped for PXR polymorphisms. The dose-normalised trough concentrations, area under the time concentration curves (dnAUC_{0-12}) and maximum concentrations were determined and correlated with the corresponding PXR genotypes.

Early posttransplant patients carrying PXR variant alleles have significantly lower dnAUC_{0-12} (1858 *versus* 1243 $\text{ng} \times \text{hr/ml}$ per mg/kg ; Mann-Whitney, $P = 0.040$) compared to patients that carry PXR wild type alleles while in the late posttransplant patient group no difference is observed regarding the dnAUC_{0-12} .

Early posttransplant patients carrying PXR variant alleles using corticosteroids have lower pharmacokinetic parameters compared to patients carrying PXR wild type alleles.

Introduction

Tacrolimus, a member of the calcineurin inhibitor family, has highly variable pharmacokinetic characteristics and a narrow therapeutic index which complicates its daily dose requirement. Achieving optimal exposure is of critical importance especially during the initial period after transplantation, when the risk of rejection or toxicological dosing is the greatest. Therefore, identification of predictive parameters for an optimal tacrolimus dosage would be of great value in clinical practice to determine the best possible therapeutic tacrolimus concentration. The CYP3A iso-enzymes mainly represented by CYP3A4 and CYP3A5 have been identified as the most important enzymes responsible for the tacrolimus metabolism¹. Although a number of polymorphic CYP3A4 alleles have been identified (<http://www.imm.ki.se/CYPAlleles/cyp3a4.htm>), these are rare and do not contribute significantly to the CYP3A4 expression variability². In contrast to CYP3A4, the expression of CYP3A5 is determined predominantly by polymorphisms³⁻⁹. Tacrolimus is also a substrate for P-glycoprotein (P-gp), the product of the adenosine triphosphate-binding cassette gene B1 (ABCB1) which is also known as multidrug resistance gene-1 (MDR1)¹⁰. P-gp is an ATP-dependent efflux pump that contributes to the protection of the body from environmental toxins by limiting their absorption from the gut lumen or increasing their biliary and urinary excretion and is thus able to modulate the bioavailability of tacrolimus. The nuclear hormone receptors are transcription factors regulating the induction of many genes. Kliewer¹¹ and Blumberg¹² demonstrated that one member of this family, the pregnane X receptor/steroid and xenobiotic receptor (PXR/SXR) mediates steroid and drug induction of detoxification genes including the drug metabolizing enzyme CYP3A and the drug transporter ABCB1. Since CYP3A4 catalyses the oxidative metabolism of one-half of the clinically administered drugs and ABCB1 effluxes many drugs from human enterocytes and hepatocytes, administration of PXR ligands leads to accelerated clearance of co-administered CYP3A4 and ABCB1 substrates and are thus a major basis for drug interactions. Moreover, there is significant variation among individuals in the extent to which rifampicin can induce hepatic and intestinal CYP3A4 and ABCB1 expression in humans¹³. Single nucleotide polymorphisms (SNPs) in PXR may be a major contributor in the induction variability of CYP3A4 and ABCB1. Zhang *et al.*¹³ identified 38 polymorphisms in the PXR gene and found that the 7635A to G transition, and the 8055C to T substitution in respectively intron 5 and 6 of the PXR gene was associated with a higher magnitude of induction of intestinal CYP3A by rifampicin. In addition, the 3'-untranslated (3' UTR) region polymorphisms 11156A to C and 11193T to C showed that those individuals heterozygous with at least one 11156C or one 11193C allele had 1.45-fold lower P-glycoprotein levels in gut biopsies compared to those with homozygous 11156A and 11193T alleles. Moreover, Bosch *et al.*¹⁴ recently identified 8 new allelic variants in the PXR gene and determined the allele frequencies of 24 PXR polymorphisms in 100 Dutch volunteers. Additionally, several studies already described an interaction between corticosteroids and tacrolimus however the exact mechanism

behind this interaction has never been elucidated¹⁵⁻¹⁷. In the present study, 26 early using corticosteroids and 37 late using no corticosteroids posttransplant patients are genotyped for PXR polymorphisms A7635G, C8055T, A11156C and T11193C. The genotypes of these renal transplant patients are associated with their corresponding pharmacokinetic parameters (dose-normalised trough (C_0) concentration, dose-normalised area under the time concentration curve (dnAUC₀₋₁₂) and dose-normalised maximum concentration (dnC_{max}) to examine whether these PXR polymorphisms eventually in combination with the use of corticosteroid have an influence on the interindividual variability in the tacrolimus pharmacokinetics.

Patients and methods

Study population

In total 63 Caucasian renal transplant recipients divided over two different groups of whom in the past for clinical reasons (group I) or for a clinical trial (group II) a 12 hour time tacrolimus concentration curve was performed, were included in this study. Group I included early posttransplant patients that used corticosteroids with a short median time after transplantation and a large variability in the tacrolimus AUC₀₋₁₂ compared to the late posttransplant recipients of group II that used no corticosteroids. Table 12.1 illustrates that most pharmacokinetic profiles of the patients included in group I were recorded within 6 weeks after transplantation while group II included patients that underwent a renal transplantation at least 1 year ago. Patients were not allowed to take food until 1 hour after ingesting the tacrolimus dose and were not allowed to take grapefruit juice after transplantation to prevent alterations in the tacrolimus metabolism. In addition, patients that suffer from gastrointestinal, liver disease or other disorders that may alter the absorption of tacrolimus were also disqualified for inclusion in both groups. Prior to the blood sample collection, there had been no tacrolimus dose change for at least three days in both groups. After overnight fasting the blood samples were collected immediately pre (C_0) and 0.5 ($C_{0.5}$), 1 (C_1), 2 (C_2), 3 (C_3), 4 (C_4), 5 (C_5), 7.5 ($C_{7.5}$) and 12 (C_{12}) hours after the morning tacrolimus administration. Demographic as well as clinical data were determined at the time of recording the 12 hour time tacrolimus concentration curve. The renal transplant recipients of both groups underwent a renal transplantation in the University Hospital of Maastricht, the Netherlands.

Table 12.1 Demographic characteristics of the both renal transplant recipients groups.

Demographic characteristics	Group I	Group II
Gender (male/female)	18/8	24/13
Age (years, mean $\pm$ SD)	43.0 $\pm$ 13.2	51.3 $\pm$ 10.9
Length (cm, mean $\pm$ SD)	172 $\pm$ 8.2	174 $\pm$ 8.4
Weight (kg, mean $\pm$ SD)	69.1 $\pm$ 15.9	77.4 $\pm$ 13.5
Body Mass Index (kg/m ² , mean $\pm$ SD)	23.3 $\pm$ 4.40	25.6 $\pm$ 3.42
Primary kidney disease		
Glomerulonephritis	4	1
Chronic pyelonephritis	2	2
IgA nephropathy	3	4
Hypertensive nephropathy	4	7
Diabetes Mellitus nephropathy	4	0
Polycystic kidney disease	1	8
Unknown	1	4
Other	7	11
Transplantation number		
First	20	30
Second	5	6
Third or more	1	1
Tacrolimus mono therapy	1	29
Tacrolimus dose (mg/kg/day, mean $\pm$ SD)	0.39 $\pm$ 0.231	0.054 $\pm$ 0.029
C ₀ (ng/ml, mean $\pm$ SD)	16.8 $\pm$ 5.83	6.59 $\pm$ 1.39
AUC ₀₋₁₂ (ng $\times$ hr/ml, mean $\pm$ SD)	305.0 $\pm$ 96.8	122.5 $\pm$ 31.1
C _{max} (ng/ml, mean $\pm$ SD)	56.3 $\pm$ 21.3	20.9 $\pm$ 6.5
T _{max} (hr, mean $\pm$ SD)	1.46 $\pm$ 1.33	1.24 $\pm$ 0.43
Use of azothiopurine, MMF ^a , rapamycine, steroids	11/3/4/26	3/4/0/0
Current steroid dose (mg, dose, no. patients)		
0 mg/day	3	37
5 mg/day	2	0
8 mg/day	2	0
10 mg/day	10	0
15 mg/day	4	0
20 mg/day	3	0
> 20 mg/day	2	0
Time since transplantation (days, median, (range))	18 (3-74)	1465 (453-4128)
Haemoglobin (mmol/l, mean $\pm$ SD)	5.43 $\pm$ 1.08	8.52 $\pm$ 0.83
Haematocrit fraction (mean $\pm$ SD)	0.25 $\pm$ 0.07	0.41 $\pm$ 0.04
ALAT (U/l, mean $\pm$ SD)	34 $\pm$ 34	24 $\pm$ 13
ASAT (U/l, mean $\pm$ SD)	22 $\pm$ 18	17 $\pm$ 10
Serum albumin (g/l, mean $\pm$ SD,)	30.7 $\pm$ 3.86	37.0 $\pm$ 3.84
Serum creatinine (μ mol/l, median $\pm$ SD)	331 $\pm$ 293	125 $\pm$ 29
Creatinine (Cockcroft-Gault; ml/min, median $\pm$ SD)	37.5 $\pm$ 28.4	56.0 $\pm$ 26.6

^a mycophenolate mofetil

Ethics

The study was performed in accordance to the Declaration of Helsinki and its amendments. The protocol was approved by the local Medical Ethics Committee and written informed consent for participation in this study was obtained from all patients.

Tacrolimus concentration determinations

The tacrolimus blood concentrations were determined in ethylene diamine tetra-acetic acid (EDTA) whole blood, using a microparticle enzyme immunoassay with a monoclonal antibody (IMx II assay; Abbott Laboratories, Abbott Park, IL, USA) for group I and a method based on HPLC tandem mass spectrometry (MS/MS) for group II. Both methods were performed in laboratories that participate in the International Tacrolimus Proficiency Testing Scheme. The tacrolimus C_0 concentration, the peak blood concentration ($C_{\max}$) during the assessed time interval and the time at which the highest blood concentration was observed ($T_{\max}$) were determined directly from the time *versus* tacrolimus blood concentration data. Additionally, the area under the time concentration curve (AUC_{0-12}) was calculated from the time *versus* tacrolimus concentration plot using the linear trapezoidal rule in MWPharm 3.50 (Mediware, Groningen, the Netherlands). DnC_0 , $dnAUC_{0-12}$ and $dnC_{\max}$ were calculated by dividing the C_0 , AUC_{0-12} and $C_{\max}$ respectively by the corresponding morning dose on a milligram per kilogram basis.

DNA isolation

Genomic DNA isolation was performed by using 200 μ l EDTA anticoagulated blood for isolation with a QIAamp blood mini kit (Qiagen, Leusden, the Netherlands) according to the manufacturers' instructions.

Genotyping of PXR polymorphisms

Real-time PCR fluorescence resonance energy transfer (FRET) assays on the LightCycler (Roche Diagnostics, Almere, the Netherlands) were used for genotyping, PXR polymorphisms A7635G, C8055T, A11156C and T11193C using the same primers and probes compared to a previous publication (Op den Buijsch *et al.*, submitted). For each polymorphism, heterozygote samples were sequenced according to a direct sequence procedure on a capillary sequencer ABI Prism 3100 using the Bridge version 1.1 sequence kit (both products from Applied Biosystems, Fostercity, USA) and used in every real-time PCR FRET assay run as control sample.

Statistical analysis

Statistical analysis of the data was performed with use of the statistical software SPSS 11.0 for windows (Chicago, IL, USA). To examine the population homogeneity of the patients, the genotype frequencies of the PXR polymorphisms were tested against Hardy-Weinberg equilibrium by the Pearson's goodness-of-fit test¹⁸. For analysis of the daily tacrolimus dose (mg/kg/day), dnC_0 (ng/ml per mg/kg), $dnAUC_{0-12}$ (ng $\times$ hr/ml per mg/kg), $dnC_{\max}$ (ng/ml per mg/kg) and $T_{\max}$ (hr), groups were compared using non parametric statistical tests. To compare two groups we used the Mann-Whitney test, and to compare several groups the Kruskal Wallis test. *P* values less than 0.05 were

considered statistically significant. All values are expressed as median and range unless stated otherwise.

Results

The distribution of allele frequencies of four PXR polymorphisms

The clinical characteristics of the early posttransplant group using corticosteroids (group I) and a late posttransplant group using no corticosteroids (group II) that were enrolled in our study are shown in Table 12.1. Table 12.2 and 12.3 show the genotype frequencies of the different PXR polymorphisms that were determined. After genotyping the renal transplant patients for four PXR polymorphisms, the frequency of the 7635AA genotype is 36.5% ($n = 23$), for the 7635AC genotype 50.8% ($n = 32$) and for the 7635GG 12.7% ($n = 8$). In addition, the frequency of the 8055CC genotype is 68.3% ($n = 43$), for the 8055CT genotype 30.2% ($n = 19$) and for the 8055TT genotype 1.5% ($n = 1$) whereas the genotypes for the PXR polymorphisms A11156C and T11193C are in complete concordance with the genotypes of the PXR C8055T polymorphism. There is a complete haplotype between the following three PXR polymorphisms C8055T, A11156C and T11193C. The allele frequencies of the Caucasian renal transplant patients examined were found to be 31.7% (7635G) and 16.7% (8055T, 11156C and 11193T) which is in line with those published in previous studies^{13,14}. The genotype frequencies of both renal transplant recipient groups were not significantly different from that predicted by the Hardy-Weinberg equation.

Table 12.2 Influence of PXR allelic variants on the pharmacokinetic tacrolimus parameters of the early posttransplant recipients in group I.

Genotype	N	Allelic status	Dose (mg/kg/day)	DnC ₀ (ng/ml per mg/kg)	DnAUC ₀₋₁₂ (ng×hr/ml per mg/kg)	DnC _{max} (ng/ml per mg/kg)
PXR	6	A/A	0.464 (0.25-0.79)	83.7 (40-123)	1526 (824-2066)	236 (187-454)
A7635G	16	A/G	0.259 (0.04-0.80)	90.9 (42-1329)	1801 (839-15721)	400 (89-1535)
	4	G/G	0.420 (0.34-0.78)	60.8 (32-76)	1298 (922-1423)	332 (184-380)
PXR	16	C/C	0.271 (0.04-0.79)	110.5 (40-1329) ^b	1858 (824-15721) ^a	402 (176-1535) ^b
C8055T	10	C/T	0.435 (0.07-0.80)	63.7 (32-421) ^b	1244 (839-5954) ^a	293 (89-686) ^b
	0	T/T	----	----	----	----
PXR	16	A/A	0.271 (0.04-0.79)	110.5 (40-1329) ^b	1858 (824-15721) ^a	402 (176-1535) ^b
A11156C	10	A/C	0.435 (0.07-0.80)	63.7 (32-421) ^b	1244 (839-5954) ^a	293 (89-686) ^b
	0	C/C	----	----	----	----
PXR	16	T/T	0.271 (0.04-0.79)	110.5 (40-1329) ^b	1858 (824-15721) ^a	402 (176-1535) ^b
T11193C	10	T/C	0.435 (0.07-0.80)	63.7 (32-421) ^b	1244 (839-5954) ^a	293 (89-686) ^b
	0	C/C	----	----	----	----

Values are indicated as median and (range), ^a $P < 0.05$; ^b $P < 0.10$ (Mann-Whitney).

Table 12.3 Influence of PXR allelic variants on the pharmacokinetic tacrolimus parameters of the late posttransplant recipients in group II.

Genotype	N	Allelic	Dose	DnC0	DnAUC0-12	DnCmax
	No	status	(mg/kg/day)	(ng/ml per mg/kg)	(ng×hr/ml per mg/kg)	(ng/ml per mg/kg)
PXR	17	A/A	0.051 (0.02-0.13)	254 (70-669)	4973 (1355-11994)	820 (294-1729)
A7635G	16	A/G	0.049 (0.02-0.14)	286 (87-640)	4642 (1662-11504)	642 (308-1493)
	4	G/G	0.042 (0.03-0.07)	257 (121-598)	4488 (2766-9320)	814 (504-1086)
PXR	27	C/C	0.044 (0.02-0.13)	275 (70-669)	4761 (1355-11994)	795 (294-1729)
C8055T	9	C/T	0.052 (0.02-0.14)	278 (87-416)	4510 (1662-6854)	646 (308-981)
	1	T/T	0.038	223	4465	810
PXR	27	A/A	0.044 (0.02-0.13)	275 (70-669)	4761 (1355-11994)	795 (294-1729)
A11156C	9	A/C	0.052 (0.02-0.14)	278 (87-416)	4510 (1662-6854)	646 (308-981)
	1	C/C	0.038	223	4465	810
PXR	27	T/T	0.044 (0.02-0.13)	275 (70-669)	4761 (1355-11994)	795 (294-1729)
T11193C	9	T/C	0.052 (0.02-0.14)	278 (87-416)	4510 (1662-6854)	646 (308-981)
	1	C/C	0.038	223	4465	810

Values are indicated as median and (range).

Influence of the different PXR allelic variants on the pharmacokinetic tacrolimus profiles

The early posttransplant recipients carrying a PXR 8055T, 11156C and 11193T variant allele have a significantly lower and clinical relevant dnAUC₀₋₁₂ (1858 *versus* 1243 ng×hr/ml per mg/kg; Mann-Whitney, *P* = 0.040) or a strong tendency towards a lower dnC₀ (111 *versus* 64 ng/ml per mg/kg; Mann-Whitney, *P* = 0.073) or lower dnC_{max} (402 *versus* 293 ng/ml per mg/kg; Mann-Whitney, *P* = 0.073) compared to the carriers of the PXR 8055C, 11156A and 11193T wild type allele while in contrast no significant difference is observed regarding the pharmacokinetic parameters between carriers of the different PXR genotypes included in the corticosteroid group (group II). Figure 12.1A-D illustrate the effect of the different PXR polymorphisms on the dnAUC₀₋₁₂ recorded in 26 early and 37 late posttransplant recipients.

Furthermore, the PXR haplotypes consisting of four polymorphisms are associated with the dnAUC₀₋₁₂ for the early as well as the late posttransplant patients, as is shown in Figure 12.1E and 12.1F.

However, previously Op den Buijsch *et al.* (Op den Buijsch *et al.*, submitted) demonstrated in the same renal transplant patient group an important impact of the CYP3A5 A6986G polymorphism on the tacrolimus concentrations whereas no association was reported between both ABCB1 polymorphisms G2677T/A and C3435T and the tacrolimus concentration. To exclude influence of one of these polymorphisms, the renal transplant recipients were based on their CYP3A4 A-392G and CYP3A5 A6986G genotype categorized in order to examine solely the effect of the four PXR polymorphisms on the dnAUC₀₋₁₂. As is illustrated in Figure 12.2, there is a trend visible

of the PXR C8055T, A11156C and T11193C after categorizing the patients for respectively the CYP3A4 A-392G and CYP3A5 A6986G polymorphisms. Additionally, Table 12.4 and 12.5 illustrate the influence of the combination of respectively CYP3A4 A-392G and CYP3A5 A6986G with the two PXR polymorphisms A7635G and C8055T.

Table 12.4 Influence of the combination of PXR and CYP3A4, CYP3A5 allelic variants on the pharmacokinetic tacrolimus parameters of the early posttransplant recipients in group I.

Genotype combinations	N	Allelic status	Dose (mg/kg/day)	DnC ₀ (ng/ml per mg/kg)	DnAUC ₀₋₁₂ (ng×hr/ml per mg/kg)	DnC _{max} (ng/ml per mg/kg)
PXR/CYP3A4	3	AA-AA	0.28 (0.25-0.72)	114 (40-123)	1920 (824-2066)	386 (187-454)
A7635G/A-392G	16	AG-AA	0.26 (0.04-0.80)	91 (42-1329)	1801 (839-15721)	400 (89-1535)
	2	GG-AA	0.35 (0.34-0.35)	69 (63-76)	1372 (1321-1423)	353 (327-380)
	3	AA-AG	0.50 (0.43-0.79)	60 (55-107)	1333 (1220-1796)	216 (212-256)
	2	GG-AG	0.63 (0.49-0.78)	46 (32-59)	1098 (922-1275)	261 (184-338)
PXR/CYP3A5	1	AA-GG	0.78 [*]	32 [*]	922 [*]	184 [*]
A7635G/A6986G	4	AA-AG	0.61 (0.43-0.79) [*]	58 (40-107) [*]	1277 (824-1796) [*]	214 (187-256) [*]
	3	AG-AG	0.64 (0.45-0.80) [*]	50 (42-65) [*]	1042 (864-1167) [*]	172 (89-260) [*]
	2	GG-AG	0.42 (0.35-0.49) [*]	61 (59-63) [*]	1298 (1275-1321) [*]	359 (338-380) [*]
	3	AA-GG	0.28 (0.25-0.54) [*]	114 (45-123) [*]	1920 (839-2066) [*]	386 (90-454) [*]
	12	AG-GG	0.23 (0.04-0.59) [*]	174 (51-1329) [*]	2455 (1103-15721) [*]	473 (176-1535) [*]
	1	GG-GG	0.34 [*]	76 [*]	1423 [*]	327 [*]
PXR /CYP3A4	13	CC-AA	0.25 (0.04-0.72) ^{**}	123 (40-1329)	2030 (824-15721)	454 (176-1535) ^{**}
C8055T/A-392G	9	CT-AA	0.42 (0.07-0.80) ^{**}	65 (42-421)	1321 (839-5954)	327 (89-686) ^{**}
	3	CC-AG	0.50 (0.43-0.79) ^{**}	60 (55-107)	1333 (1220-1796)	216 (212-256) ^{**}
	1	CT-AG	0.78 ^{**}	32	922	184 ^{**}
PXR/CYP3A5	1	CT-AA	0.78 [*]	32 [*]	922 [*]	184 [*]
C8055T/A6986G	5	CC-AG	0.50 (0.43-0.79) [*]	59 (40-107) [*]	1275 (824-1796) [*]	216 (187-338) [*]
	4	CT-AG	0.55 (0.35-0.80) [*]	53 (42-65) [*]	1132 (864-1321) [*]	213 (89-380) [*]
	11	CC-GG	0.25 (0.04-0.59) [*]	167 (51-1329) [*]	2066 (1103-15721) [*]	471 (176-1535) [*]
	5	CT-GG	0.34 (0.07-0.54) [*]	90 (45-421) [*]	1715 (839-5954) [*]	381 (90-686) [*]

Values are indicated as median and (range), ^{*} $P < 0.05$; ^{**} $P < 0.10$ (Kruskal Wallis).

Table 12.5 Influence of the combination of PXR and CYP3A4, CYP3A5 allelic variants on the pharmacokinetic tacrolimus parameters of the late posttransplant recipients in group II.

Genotype combinations	N No	Allelic status	Dose (mg/kg/day)	DnC ₀ (ng/ml per mg/kg)	DnAUC ₀₋₁₂ (ng×hr/ml per mg/kg)	DnC _{max} (ng/ml per mg/kg)
CYP3A4/PXR	17	AA-AA	0.05 (0.02-0.13)	254 (70-669)	4973 (1355-11994)	820 (294-1729)
A7635G/A-392G	16	AG-AA	0.05 (0.02-0.14)	286 (87-640)	4642 (1662-11504)	642 (308-1493)
	3	GG-AA	0.04 (0.03-0.05)	289 (223-598)	4511 (4465-9320)	818 (810-1086)
	1	GG-AG	0.07	121	2766	504
PXR/CYP3A5	1	AA-AG	0.13	70**	1355**	294**
A7635G/A6986G	3	AG-AG	0.10 (0.05-0.14)	87 (87-248)**	1663 (1662-4057)**	461 (308-646)**
	1	GG-AG	0.07	121**	2766**	504**
	16	AA-GG	0.05 (0.02-0.11)	264 (0.02-0.11)**	4991 (2227-11994)**	842 (495-1729)**
	13	AG-GG	0.04 (0.02-0.07)	295 (128-640)**	4761 (2673-11504)**	643 (465-1493)**
	3	GG-GG	0.04 (0.03-0.05)	290 (223-598)**	4511 (4465-9320)**	818 (810-1086)**
PXR /CYP3A4	26	CC-AA	0.04 (0.02-0.13)	279 (70-669)	4867 (1355-11994)	808 (294-1729)
C8055T/A-392G	9	CT-AA	0.05 (0.02-0.14)	278 (87-416)	4511 (1662-6854)	646 (308-981)
	1	TT-AA	0.04	223	4465	810
	1	CC-AG	0.07	121	2766	504
PXR/CYP3A5	3	CC-AG	0.10 (0.07-0.13)**	87 (70-121)*	1663 (1355-2766)*	461 (294-504)*
C8055T/A6986G	2	CT-AG	0.10 (0.05-0.14)**	168 (87-248)*	2859 (1662-4057)*	477 (308-646)*
	24	CC-GG	0.04 (0.02-0.11)**	289 (118-669)*	4992 (2227-11194)*	842 (495-1729)*
	7	CT-GG	0.05 (0.02-0.07)**	290 (128-416)*	4567 (2673-6854)*	676 (465-981)*
	1	TT-GG	0.04**	223*	4465*	810*

Values are indicated as median and (range), * $P < 0.05$; ** $P < 0.10$ (Kruskal Wallis).

Figure 12.1 Influence of the PXR A7635G, C8055T, A11156C and T11193C genotypes on the area under the time tacrolimus concentration curve (AUC_{0-12}) that were recorded in 26 early and 37 late posttransplant patients. Figure 12.1 illustrates the boxplots of the distribution of the dose-normalized AUC_{0-12} (ng $\times$ hr/ml per mg/kg) clustered according to the PXR A7635G genotype, PXR C8055T, A11156C and T11193C genotype for the early posttransplant patient group (A and B) and the late posttransplant patient group (C and D). Figure 12.1 E and F show the boxplots of the distribution of the dose-normalized AUC_{0-12} (ng $\times$ hr/ml per mg/kg) clustered according to the PXR haplotypes consisting of the PXR polymorphisms A7635G, C8055T, A11156C and T11193C in respectively the early and the late posttransplant patient group. Open circles indicate an outlier value at more than 1.5 box lengths above the box while asterisks indicate an extreme value at more than 3 box lengths above the box.

Figure 12.2 Figure 12.2 illustrates the boxplots of the distribution of the dose-normalized AUC₀₋₁₂ (ng x hr/ml per mg/kg) clustered according to respectively (A) the combination of CYP3A4 A-392G and PXR A7635G, (B) the combination of CYP3A5 A6986G and PXR A7635G, (C) the combination of CYP3A4 A-392G and PXR C8055T and (D) the combination of CYP3A5 A6986G and PXR C8055T in the early posttransplant patient group. * indicates that the PXR polymorphisms A11156C and T11193C show exactly the same figures. Open circles indicate an outlier value at more than 1.5 box lengths above the box while asterisks indicate an extreme value at more than 3 box lengths above the box.

Discussion

After genotyping 63 renal transplant patients divided into 26 early posttransplant recipients and 37 late posttransplant recipients it appeared that there is a complete PXR haplotype consisting of the polymorphisms C8055T, A11156C and T11193C. Although the genotypes of the four PXR polymorphisms differed not significantly between the early posttransplant recipient group and the late posttransplant recipient group, there is a difference in response between the different allelic variants of the PXR polymorphisms. The carriers of variant PXR alleles in the early renal transplant recipients showed a significant trend towards lower pharmacokinetic parameters (dnC₀,

dnAUC₀₋₁₂ and dnC_{max}) compared to carriers of wild type PXR alleles. However, patients included in the late posttransplant group that carry a variant PXR allele showed no trend towards lower pharmacokinetic parameters for tacrolimus compared to the patients that carried wild type alleles. Almost all renal transplant recipients included in group I used steroids as an additive immunosuppressivum while no of the 37 renal transplant recipients included in group II used corticosteroids. Previously, van Duijnhoven *et al.*¹⁵ first described an increase of the tacrolimus C₀ concentration after corticosteroid withdrawal while two other studies^{16,17} also described a similar interaction between corticosteroids and tacrolimus. Induction of PXR by corticosteroids may lead to a higher expression of PXR and thereby to a higher expression of CYP3A4 and ABCB1 which may result in a higher daily tacrolimus dosage to maintain the pharmacokinetic parameters of tacrolimus within the therapeutic range. Differences in the tacrolimus C₀ concentrations after administrating corticosteroids or withdrawing corticosteroids in transplant patients may be explained by polymorphisms present in the PXR gene. Zhang *et al.*¹³ demonstrated for the variant allele of the two PXR polymorphisms A7635G and C8055T a trend towards a higher of intestinal CYP3A after induction with rifampicin.

In addition, a tendency was reported to lower P-glycoprotein concentrations regarding carriers of the 3'-untranslated region variant alleles of the PXR polymorphisms 11156A to C and 11193T to C.

Our findings indicate that there is an inclination towards a lower dnC₀, dnAUC₀₋₁₂ and dnC_{max} for patients who are carrier of at least one variant allele of the PXR polymorphisms examined. Remarkably, this trend was only observed in the early posttransplant patient group while the genotypes of the different PXR polymorphisms showed no trends towards lower pharmacokinetic parameters in the late posttransplant patient group. Due to the lack of corticosteroid use in the late posttransplant recipient group, the influence of PXR polymorphisms on the pharmacokinetic parameters of tacrolimus appears to be minor which may explain the discrepancy between the early and the late posttransplant recipient group. Summarized, tacrolimus is predominantly metabolised by the CYP3A iso-enzymes while the ABCB1 modulates its bioavailability. Although there is a high interindividual variation in the expression of CYP3A4 and ABCB1, genetic polymorphisms can not exclusively explain the difference in CYP3A4 and ABCB1 expression. PXR has been identified as a transcriptional regulator of CYP3A4 and ABCB1. In the present study, four variant alleles of PXR polymorphisms A7635G, C8055T, A11156C and T11193C show a trend towards lower pharmacokinetic parameters however only in the early posttransplant patient group and not in the late posttransplant patient group. Since PXR polymorphisms demonstrate an effect in the early posttransplant group we hypothesized that the PXR polymorphisms have only an effect on the pharmacokinetic parameters of tacrolimus after induction by e.g. corticosteroids. Our findings indicate that PXR polymorphisms may play a role in tacrolimus metabolism although their role is dependent of the presence and/or of inductive drug or chemical components in the transplant patients.

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Chapter 13

General discussion

General discussion

On 31 december 2006, in total 1394 patients were waiting for a kidney (1054), heart (44), lung (141) or liver (155) donor organ although in 2006 only 360 kidney, 37 heart, 52 lung and 83 liver transplantations were performed in the Netherlands¹. Once the patients have received a well-functioning donor organ, the quality of life will improve significantly. However, all patients that receive a donor organ have to cope with a lifelong treatment by immunosuppressants like the calcineurin inhibitors: cyclosporin and/or tacrolimus, the mTOR inhibitors: sirolimus and/or everolimus, mycophenolic acid and azathiopurin, which all have highly variable pharmacokinetic characteristics and a small therapeutic index. The clinical consequences and impact for a patient with a sub-therapeutic or toxic tacrolimus blood level can be very serious as was discussed in CHAPTER 2. Therefore, therapeutic monitoring of the immunosuppressant blood concentrations currently performed by trough (C_0) concentrations is inevitable. In the last five year many studies appeared regarding tacrolimus trough (C_0) levels of renal, liver and heart transplant recipients in association with the genotypes of the CYP3A4 A-392G, CYP3A5 A6986G and ABCB1 C1236T, G2677T/A, C3435T polymorphisms². Although all studies described a significant impact of the CYP3A5 A6986G polymorphism on the tacrolimus blood levels, conflicting results are reported about the contribution of the individual ABCB1 polymorphisms or ABCB1 haplotypes. Due to the large variation in the transplant patient populations that have been examined in the different studies, it is possible that a minor influence of the ABCB1 polymorphisms has not been noticed. The studies differ in the type of transplantation performed, number of patients included, time after transplantation, co-medication received by the patients and whether a trough level or a complete 12-hour AUC is determined. So it is likely that a polymorphism, that has a minor influence on the tacrolimus blood concentrations, due to this large variety in transplant patient population demonstrates contrasting results among these studies.

Tacrolimus is for the most part metabolised into 13-O-demethyltacrolimus by members of the CYP3A family and specifically CYP3A4 and CYP3A5. Additionally CHAPTER 7, 8 and 9 clearly point out that the members CYP3A4 and CYP3A5 of the cytochrome P450 3A family are involved the most in the tacrolimus metabolism process whereby CYP3A5 is able to metabolise tacrolimus with a 60% higher efficiency than CYP3A4. Recently, Kamden *et al.*³ demonstrated with *in vitro* experiments that the CYP3A4 and CYP3A5 members of the CYP3A iso-enzyme family are predominantly responsible for the metabolism of tacrolimus. However, Strassburg *et al.*⁴ demonstrated that tacrolimus is mainly glucuronidated by UGT2B7. Within the UGT2B7 gene several single nucleotide polymorphisms (SNPs) have been identified. One of the first UGT2B7 polymorphisms identified is the polymorphism H268Y (C802T). Since UGT2B7 is responsible for the metabolism of the two most important metabolites of morphine, several studies⁵⁻⁸ examined whether there is an association between patients carrying a wild type or variant allele for the UGT2B7 C802T polymorphism. Conflicting evidence

has been reported regarding the contribution of this UGT2B7 C802T polymorphism on the blood concentrations of morphine. Differences between the results of these studies may be caused by the drug administration of morphine (oral or intravenous), the numbers of patients included and if co-medication is used by the patients. Duguay *et al.*⁶ recently identified two novel polymorphisms located in the promoter area of the UGT2B7 gene. Although two novel UGT2B7 polymorphisms in the UGT2B7 promoter area are identified, only for the UGT2B7 -79G variant allele a difference in UGT2B7 expression is found. However, no differences in the glucuronidation ratios of morphine have been found, after genotyping 50 cancer patients receiving morphine on a long-term basis. In CHAPTER 10 three UGT2B7 polymorphisms are correlated to the tacrolimus exposure in a Chinese renal transplant population. Although quantitative evidence regarding the amount of tacrolimus glucuronidation is still lacking, it remains interesting to see that these UGT2B7 polymorphisms may have a significant contribution on the variability of the tacrolimus exposure. Despite the fact that CYP3A4 and CYP3A5 are mainly responsible for the metabolism of tacrolimus only one polymorphism present in the CYP3A5 gene namely the CYP3A5 6986G or CYP3A5*3 variant allele has a high allelic frequency in different ethnic populations and has a significant contribution on the tacrolimus C_0 concentrations or other pharmacokinetic tacrolimus parameters. Even though there are several other functional CYP3A5 polymorphisms identified⁹, the allele frequency is very low in different ethnic populations and thus the contribution of these polymorphisms on the tacrolimus exposure is minor. Although CYP3A4 plays, like CYP3A5, an important role in the tacrolimus metabolism only the CYP3A4*1B variant allele has a relatively high allele frequency in Caucasians. However, due to a high linkage equilibrium between the CYP3A4*1B and the CYP3A5*1 it is difficult to examine whether the gene-dose contribution obtained is solely due to the CYP3A4*1B variant allele, or that the effect is caused by the high linkage with the CYP3A5*1 allele. Since polymorphisms in the CYP3A iso-enzymes can only partly explain the variation in the tacrolimus blood concentrations, it is likely that other genes are also (in)directly involved in the tacrolimus metabolism. The pregnane X receptor (PXR) is a member of the nuclear receptor (NR) family of ligand-activated transcription factors that include the steroid, retinoid and thyroid hormone receptors, as well as many orphan receptors from which physiological ligands have yet to be identified^{10,11}. PXR is highly expressed in the liver and intestine, where it binds as a heterodimer with the 9-*cis* retinoic acid receptor (RXR; NR2B) to previously characterized xenobiotic response elements in CYP3A gene promoters and importantly, PXR is activated by the spectrum of chemicals that are known to induce CYP3A gene expression¹². Previously, Zhang *et al.*¹³ identified 38 polymorphisms in the PXR gene of which only eight polymorphisms present in either the 3'-untranslated (UTR) or intronic region seem to demonstrate an effect on the erythromycin breath test, CYP3A4 mRNA/villin or ABCB1 mRNA/villin after rifampicin induction. However, this study was performed with a low number of patients. Op den Buijsch *et al.* (*Submitted*; CHAPTER 11) demonstrated after genotyping 103 Chinese renal transplant recipients for four PXR polymorphisms, that these PXR polymorphisms

have a significant influence on the tacrolimus exposure. Additionally, Op den Buijsch *et al.* (Submitted; CHAPTER 12) examined the influence of these four PXR polymorphisms on the pharmacokinetic tacrolimus parameters C_0 , AUC_{0-12} , C_{max} , and T_{max} in both an early using corticosteroids and a late using no corticosteroids renal transplant recipient group. Although the PXR genotypes of these early and late posttransplant recipient group did not significantly differ from each other, a significant contribution for the PXR polymorphism is only found in the early transplant recipient group which used corticosteroids. Previously, three different studies reported an increase of the tacrolimus C_0 concentration when the corticosteroid therapy was stopped¹⁴⁻¹⁶. Possibly, PXR polymorphisms are responsible for the mechanism behind the difference in tacrolimus C_0 concentration observed.

Conclusions based on the research presented in this thesis are:

The commercial DNA isolation kits (QIAamp blood mini kit, Roche High Pure PCR Template Preparation Kit and Puregene) have an extraction efficiency of approximately 30%.

Real-time PCR FRET assays developed and validated in our laboratory can be used perfectly for genotyping in a clinical setting. Most limited sampling strategies for tacrolimus that already appeared in literature give a better indication of the tacrolimus exposure than the morning tacrolimus C_0 concentrations used nowadays.

The single nucleotide polymorphism (SNP) A6986G in the CYP3A5 gene has a major impact on the tacrolimus exposure calculated with a limited sampling strategy, and on the dnC_0 , $dnAUC_{0-12}$, and dnC_{max} . This CYP3A5 A6986G polymorphism can explain the variation observed in the AUC_{0-12} calculated with a two time point limited sampling strategy for approximately 35% in a Chinese renal transplant population.

Conflicting results were observed for the ABCB1 polymorphisms C1236T, G2677T/A and C3435T in the Chinese and the Caucasian renal transplant recipient group which is in line with literature.

Although it is known that tacrolimus is also glucuronidated mainly by UGT2B7, the UGT2B7 polymorphisms G-79A, T-66C and C802T have no influence on the tacrolimus exposure in a Chinese renal transplant patient group that is calculated with a two time point limited sampling strategy.

An interesting finding was observed for the PXR polymorphisms A7635G, C8055T, A11156C and T11193C. In the Chinese renal transplant recipients a significant

difference was observed for the PXR polymorphisms A7635G and A11156C, T11193C while a trend was observed for the PXR polymorphism C8055T. Although after categorizing the renal transplant patients in different groups based on their CYP3A5 and ABCB1 genotype no significant difference was observed, a trend regarding a lower tacrolimus exposure for renal transplant patients carrying a variant allele for these PXR polymorphisms remained.

The fact that these PXR polymorphisms only have an effect on the tacrolimus exposure in early posttransplant patients that use high corticosteroid dosages indicates that induction of PXR by corticosteroids is a prerequisite for a contribution of PXR polymorphisms on the tacrolimus exposure. This finding also confirms the results of several studies that found lower tacrolimus concentration in early transplant patients using a high daily corticosteroid dose while a higher tacrolimus concentration is observed for the late posttransplant patient using a lower daily corticosteroid dose.

Directions for future research

Because all patients that receive a donor organ have to undergo lifelong treatment by immunosuppressants, an accurate follow up with close monitoring of the immunosuppressant blood concentrations of these patients is required. By receiving a donor organ the patients' health and their quality of life increases enormously, although these patients have to ingest at least one immunosuppressant twice a day, with often several other medications. Due to their highly variable pharmacokinetic characteristics, all immunosuppressants, like the calcineurin inhibitors tacrolimus and cyclosporine, mTOR inhibitors sirolimus and everolimus, mycophenolic acid and azathiopurin all require close monitoring of the blood concentrations on a regular basis. Recently, Hoogtanders *et al.*¹⁷ demonstrated a dried blood spot (DBS) method which is able to determine the tacrolimus concentration with capillary blood which can be obtained with a fingerprick by the patients themselves. Additionally, Wijnen *et al.* (*Submitted*) described a non-commercial DBS method for isolating DNA from capillary blood. Both DBS methods have a lot of advantages regarding the patient, logistics, storage, time and total costs. Recently, several studies reported that a limited sampling strategy consisting of two or three time sample points within 4 hours after the morning tacrolimus ingestion, have firstly a better correlation with the complete 12-hour area under the time tacrolimus concentration (AUC_{0-12}) curve and secondly according to a comprehensive study of Op den Buijsch *et al.* (*Submitted*) the absolute prediction error (APE) of most limited sampling strategies are better than the APE of the trough concentrations C_0 and C_{12} . Therefore, we concluded that all limited sampling strategies that already appeared in literature could generate a reliable prediction of the complete 12-hour AUC_{0-12} although in new settings careful evaluation of their reliability with an own transplant population is advisable. It is known that the immunosuppressant blood concentrations

are most variable in the early period after transplantation. A better indication of the initial starting dose for each individual patient may contribute to a decrease in the variation of the tacrolimus blood concentrations and thus to a lower incidence of subtherapeutic or toxic blood concentrations, which ultimately lead to rejection or adverse drug reactions. Tacrolimus is mainly metabolized due to cytochrome P450 (CYP450) 3A iso-enzymes into 13-O-demethyltacrolimus, which has almost no immunosuppressive activity. The most relevant single nucleotide polymorphism (SNP) in the CYP3A iso-enzymes is the CYP3A5 A6986G polymorphism. Kamden *et al.*³ recently demonstrated that the CYP3A subfamily is the only CYP450 iso-enzyme that has a significant contribution *in vitro* on the tacrolimus metabolism. Additionally, they demonstrated that CYP3A5 is able to metabolise tacrolimus with a 60% higher efficiency rate compared to CYP3A4. Regarding the contribution of the adenosine triphosphate binding cassette B1 (ABCB1) polymorphisms C1236T, G2677T/A and C3435T or ABCB1 haplotypes there is conflicting evidence on their role in the tacrolimus metabolism. Although a few studies found a significant association between the individual ABCB1 polymorphisms or ABCB1 haplotypes, no unambiguous evidence is found for their contribution. Several reasons can justify a discrepancy in the results of the studies that already appeared such as a pharmacokinetic (C_0 , C_2 , C_4 ; or AUC_{0-12}) or genetic (SNP *versus* haplotype) analyses that were performed. Moreover, the sample size of the study cohorts and the time after transplantation points at which a possible ABCB1 genotype effect was assessed, differed between studies. Previously, Strassburg *et al.*⁴ demonstrated that cyclosporine and tacrolimus are also glucuronidated especially by UGT2B7. More recently, Bhasker *et al.*¹⁸ identified the UGT2B7 C816T polymorphism and Dugauy *et al.*⁶ reported about two novel UGT2B7 polymorphisms which are located in the UGT2B7 promoter area. However, Op den Buijsch *et al.* (Pharmacogenomics, submitted) first described that three possible relevant polymorphisms in the UGT2B7 gene have no significant influence on the tacrolimus exposure in a Chinese renal transplant population. Because, polymorphisms present in the different CYP3A iso-enzymes, the UGT2B7 gene and the ABCB1 gene can only partly elucidate the variation in the tacrolimus blood concentration, other genes may possibly also have a significant contribution to the tacrolimus blood concentration. However, even when transplant patients achieve therapeutic drug concentrations of the immunosuppressive drugs, rejection and drug toxicity are not eliminated¹⁹. Recently, Op den Buijsch *et al.* (Submitted) demonstrated that polymorphisms located in the PXR gene may also explain the variability in the tacrolimus exposure. Especially, since the PXR polymorphisms seem to have influence in patients only early after transplantation when the daily corticosteroid dose is highest. These studies indicate that genes are not only directly, but also indirectly involved in the tacrolimus metabolism may contribute to the variability of the tacrolimus blood concentration. More recently, a study examining new onset diabetes after transplantation in tacrolimus treated renal transplant patients, showed that the CYP3A5*3, ABCB1 3435T and 2677T/A polymorphisms did not appear to be risk factors. This was, however, a small cohort of 70 subjects, only 10 of which developed

new onset diabetes after transplantation and therefore may not have been sufficiently powerful statistically²⁰. Polymorphisms in target proteins have been previously identified: FK binding protein for tacrolimus and mTOR inhibitors²¹, cyclophilin for cyclosporine²² and inosine monophosphate dehydrogenase for mycophenolic acid²³. However, there are as yet no published studies on any potential pharmacogenetic effect. After identification of a number of polymorphisms in several genes that are involved in the tacrolimus metabolism a microarray with all these polymorphisms included, may identify those transplant patients that need an aberrant daily tacrolimus dose or even another type of immunosuppressant. In the end this multi-pharmacogenetic approach will result in a better immunosuppressive pharmacotherapy for each individual transplant patients and lesser rejections and adverse drug reactions are likely to occur.

But even when patients are genotyped for polymorphisms in all these genes involved in both the metabolism (pharmacokinetics) and the drug receptor interaction (pharmacodynamics) of tacrolimus, still a large area of unsolved mysteries will remain. Numerous exogenic and endogenic compounds have a minor to large inhibitory or inductive effect on the CYP3A iso-enzymes, the UGT2B7 enzyme and the drug transporter P-glycoprotein which all are involved in the tacrolimus metabolism. To obtain an idea of the total amount of CYP3A iso-enzymes that are active in the liver an erythromycine breath test is developed^{1,24-26}. By using this erythromycine breath test one is able to determine the actual amount of CYP3A present in the patients' liver. This CYP3A amount determined by the erythromycin breath test may provide a better indication of the CYP3A activity than solely genotyping for CYP3A polymorphisms so that the tacrolimus pharmacokinetics for each individual patient may be predicted more accurately.

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Summary

Summary

In 2006, a total of 1054 patients were waiting for a donor kidney in the Netherlands, while in the same year only 360 kidney transplantations were performed. Not only the life expectancy but also the quality of life increases enormously after the patient has received a well-functioning donor kidney. However, ten years after transplantation only approximately half of all donor kidneys is still functioning well. The loss of transplanted kidneys is caused by a not well-functioning transplanted kidney (chronic transplant disfunctioning) and more often by the decease of the patient despite a well-functioning transplant kidney. The patients' death is, in that case, often caused by heart and circulation diseases.

However, all patients that receive a donor organ have to cope with a lifelong treatment of immunosuppressants, which all have highly variable pharmacokinetic characteristics and a small therapeutic index. The maintenance immunosuppressive therapy consisted, from 1980 until 1995, of cyclosporine (Neoral®) with corticosteroids and azathiopurin (Immunan®). In 1995 a few new immunosuppressants became available: tacrolimus (Prograf®) a drug which can be used as a cornerstone for the pharmacotherapy, mycophenolate mofetil (Cellcept®) and sirolimus (Rapamune®), which can be used as additive immunosuppressive drugs. When tacrolimus is administered instead of cyclosporine, acute rejections appeared to occur less often. This is the case for corticosteroid-sensitive as well as for corticosteroid-insensitive rejections. Due to the fact that acute rejection and especially corticosteroid-insensitive acute rejection is associated with a higher risk of chronic transplant-disfunction, it was expected that the use of tacrolimus will lead to a better transplantation survival than cyclosporine usage. In the meantime there are strong indications that these expectancies are justified. Furthermore the use of tacrolimus instead of cyclosporine leads to an improvement of several lipoproteins (HDL, LDL) and lipids (triglycerides) in the blood and to a lower blood pressure. These factors are also associated with chronic transplant disfunction and thereby with mortality as a result of heart and circulation diseases after transplantation. However, after using tacrolimus it is also assessed that the incidence of diabetes mellitus after transplantation occurs more often than with the use of cyclosporine. This would, in the end, cancel all the previously mentioned advantages regarding the increased survival of the transplant kidney and the increased survival of the patient.

After in CHAPTER 1 the outline and the aim of this thesis is described, CHAPTER 2 gives a complete review of the tacrolimus metabolism, (absorption, distribution, metabolism and elimination). The high variable pharmacokinetic characteristics of tacrolimus in combination with a small therapeutic index emphasized the need for close monitoring of the tacrolimus blood concentrations. Additionally, CHAPTER 2 describes different methodologies used nowadays to measure the tacrolimus exposure, pharmacokinetic parameters, trough (C_0) levels, abbreviated area under the time tacrolimus concentration curve AUC_{0-12}) or complete 12-hour AUC_{0-12} and the apparatus

used (IMx II or LC-MS/MS). Furthermore, the genetic and non-genetic factors that may play a role on the tacrolimus metabolism are summarized and finally the clinical consequences and impact on a patient with a sub-therapeutic or toxic tacrolimus blood level is discussed.

Before real-time polymerase chain reaction (PCR) can be performed using fluorogenic probes, DNA isolation from the ethylene diamine tetra-acetic acid (EDTA) whole blood samples is required. Although several commercial DNA isolation kits are available to extract DNA from whole blood samples, less information is available regarding the extraction efficiency and the quality of the isolated DNA. CHAPTER 3 presents a study that compares three commercially available DNA isolation kits, QIAamp blood mini kit (Qiagen, Leusden, the Netherlands), Roche High Pure DNA Preparation kit (Roche Diagnostics, Mannheim, Germany), Puregene (Biozyme, Landgraaf, the Netherlands) regarding to their ability to extract DNA from whole blood samples as well as the DNA quality.

In the early nineties, the real-time PCR technology introduced a new dimension in the PCR technology, amplification and detection is possible in one single PCR cup, plate or capillary. The defined cycle threshold (C_t) values can be used to determine the quantity of DNA that is present in the PCR cup, whereas a difference between wild type and variant allele DNA in probe attachment can be used for genotyping using the real-time PCR technology. Due to its speed, less contamination risk, less hands on time and a PCR and detection method in one, real-time PCR has gained an enormous interest among molecular biologists. An application that is used widely in real-time PCR is the fluorescence resonance energy transfer (FRET) methodology. A feature of this real-time PCR FRET technology is that two individual fluorescent probes are complimentary to the PCR product near the polymorphic position. Another characteristic of the FRET technology are the melting curves. After the final cyclus, the generated PCR product is cooled down to a certain temperature after which it is denaturated once more. The double stranded PCR product is heated with a speed of 0.1 to 0.2°C/second until the desired temperature. During this heating procedure the fluorescence signal is measured continuously. If the nucleotide located on the polymorphic position in the sensor probe is complementary to the nucleotide located at the polymorphic position in the multiplied DNA, there is a relatively strong bond between these nucleotides and the fluorescence signal will decrease in strength only at a high temperature. This in contrast to a not complementary nucleotide in the PCR product that will result in a less stable bond and consequently will result in a fluorescence signal decreasing at a lower temperature. By calculating the negative derivative of this decrease in fluorescence signal, a melting peak for the wild type and the variant allele samples is obtained.

Due to the composition of some of the DNA sequence, e.g. a long A/T rich area, it is necessary to design a sensor probe, the probe that covers the polymorphic position, that is long enough in order to obtain a melting temperature that is sufficiently high. As a result of the increased length of such a sensor probe, the influence of the none complementary nucleotide becomes significantly less compared to a shorter sensor

probe. CHAPTER 4 demonstrates that the incorporation of a locked nucleic acid (LNA) significantly improved the genotyping of the PXR A11156C polymorphism. Especially, heterozygotes could be discriminated clearly by showing two melting peaks, whereas the probes without a LNA were not able to separate melting peaks in the melting curve analysis. Therefore, we conclude that a LNA within a sensor probe may improve the discriminating power of FRET assays significantly.

In order to set up a fast and consistent assay for the organic anion transporting polypeptide 1B1 (OATP1B1) polymorphisms, in CHAPTER 5 rapid speed PCR FRET assays on the LightCycler are developed for these OATP1B1 polymorphisms. A LNA on the polymorphic location within the sensor probe was necessary to discriminate both alleles of the OATP1B1 T521C polymorphism. To confirm the reliability of both real-time PCR FRET assays, these new methods were validated by genotyping 120 samples using a PCR restriction fragment length polymorphism (RFLP) assay and an allele-specific PCR. The results of the real-time PCR FRET assays were completely in line with the conventional PCR methods, indicating that the real-time PCR FRET assays are appropriate for clinical settings.

In most transplant centres monitoring of the tacrolimus blood concentration is performed by C_0 concentrations after the morning tacrolimus administration. However, conflicting evidence is present regarding the usefulness of these morning tacrolimus C_0 concentrations. Knowing this, several studies recently developed a limited sampling strategy (LSS) for tacrolimus using stepwise multiple regression analysis or Bayesian fitting to predict the AUC_{0-12} as accurate as possible, since the AUC_{0-12} is considered to be the gold standard of measuring the total tacrolimus exposure.

Most of these studies that developed a LSS for tacrolimus used no independent transplant patient population to validate their limited sampling strategies. In CHAPTER 6 several LSS that are recently published, are evaluated using an independent well-characterised stable renal transplant patient population. The performance of 24 LSS in 37 renal transplant patients with known AUC_{0-12} has been evaluated and the results were also compared with the predictability of the trough concentrations, C_0 and C_{12} . The criterion was an absolute prediction error (APE%) that differs less than 15% from the complete AUC_{0-12} . Thirteen of the 18 (72%) LSS based on regression analysis were capable of predicting at least 90% of the 37 individual AUC_{0-12} within an APE of 15%. Predictions based on C_0 and C_{12} and Bayesian fitting were lower than 90%: 62% and 67%, respectively. In contrast to a recent publication we observed no clear differences in the predictive capacities when a LSS was developed, using 12-hour pharmacokinetic profiles recorded from heart or lung transplant recipients in our renal transplant recipients. Therefore, LSS using C_0 , C_2 and/or C_4 are in our opinion a better alternative for the C_0 concentration, used nowadays for monitoring tacrolimus. The different LSS available in current literature for tacrolimus monitoring could generate adequate predictions although cautious evaluation of the reliability of these LSS is mandatory.

Tacrolimus is predominantly metabolised by cytochrome P450 (CYP450) 3A isoenzymes into 13-O-desmethyltacrolimus, which has a neglectable immunosuppressive

activity. CHAPTER 7 and 8 demonstrate in a Chinese and Caucasian renal transplant patient population, that the CYP3A5 A6986G polymorphisms is the most relevant polymorphism present in the CYP450 3A iso-enzyme family, with a significant impact on both the tacrolimus exposure and the daily tacrolimus dose. In CHAPTER 7 the association of CYP3A and ABCB1 polymorphisms with the AUC_{0-12} calculated using a two time point sample strategy is evaluated. The CYP3A and ABCB1 genotypes are determined by real-time PCR FRET assays in 103 Chinese renal transplant recipients and consequently related to their $dnAUC_{0-12}$. A significant allele-dependent effect (Kruskal-Wallis; $P < 0.001$) is observed between the CYP3A5*3 polymorphism and the dose-normalized (dn) AUC_{0-12} . Multiple regression analysis showed that the CYP3A5*3 polymorphism is the most significant independent variable and explained 35% of the dose requirement variability in relation to tacrolimus use. Regarding the ABCB1 G2677T/A and C3435T polymorphisms, a trend is observed between the different genotypes and the $dnAUC_{0-12}$. CHAPTER 8 described two different renal transplant recipient groups which are used to examine the influence of CYP3A and ABCB1 polymorphisms on the daily tacrolimus dose and several pharmacokinetic parameters. In total 63 Caucasian renal transplant recipients divided into 26 early and 37 late post transplant recipients were genotyped for CYP3A and ABCB1 polymorphisms. The pharmacokinetic parameters of tacrolimus were determined for all renal transplant recipients and correlated with their corresponding genotypes. A significant difference in allele frequencies of the CYP3A4*1B ($P = 0.028$) and CYP3A5*1 ($P = 0.022$) alleles was observed between the early and late posttransplant recipient group. Significant higher dnC_0 , $dnAUC_{0-12}$, and dnC_{max} were observed for carriers of the CYP3A5*3 variant allele in both renal transplant patient groups. Except for the daily tacrolimus dose ($P = 0.025$) no significant differences were observed for carriers of the CYP3A4*1B variant allele. Neither the individual ABCB1 polymorphisms nor the ABCB1 haplotypes were associated with any pharmacokinetic parameter. Additionally, in CHAPTER 9 the allele frequency of the CYP3A7*1C variant allele in a Caucasian and a Chinese renal transplant patient population is examined. Since CYP3A7*1C variant alleles were only found among the Caucasian renal transplant recipients only these patients were used to study the association between the CYP3A7*1C variant allele and the different pharmacokinetic parameters for tacrolimus. An allele frequency of respectively 2.8% and 0.0% was found after genotyping 70 Caucasian and 103 Chinese renal transplant recipients for the CYP3A7*1C allele using a real-time PCR FRET assay. Heterozygous carriers of the CYP3A7*1C allele showed no significant lower C_0 levels, AUC_{0-12} or C_{max} for tacrolimus compared to the carriers of the CYP3A7*1 allele. The variant alleles of UGT2B7 polymorphisms G-79A, T-66C and C816T with an allele frequency of respectively, 0.0%, 5.8% and 29.6% showed no significant differences on both the $dnAUC_{0-12}$ and the daily tacrolimus dose compared to carriers of the wild type UGT2B7 alleles when the patients were categorized based on their CYP3A5 A6986G, ABCB1 G2677T/A and ABCB1 C3435T genotype. Despite the fact that tacrolimus is extensively glucuronidated by UGT2B7, according to a previous study, the three relevant

polymorphisms present in the UGT2B7 gene seem to have no influence on the tacrolimus exposure in the Chinese renal transplant population, which is in line regarding the results obtained from the another *in vitro* study.

However, also polymorphisms in genes that are indirectly involved in the tacrolimus metabolism seem to play an important role. The pregnane X receptor (PXR) plays an important role in the regulation of the expression of CYP450 iso-enzymes, 5'-uridine diphosphate glucuronosyltransferase (UGT) enzymes and the drug transporter ABCB1 which are directly involved in the tacrolimus metabolism. In CHAPTER 11 the influence of four individual PXR polymorphism and PXR haplotypes is examined on the tacrolimus exposure in a Chinese renal transplant population. Individuals carrying a variant allele for the PXR polymorphisms A7635G, C8055T, A11156C and T11193C, associated with increased expression of CYP3A4 or ABCB1, had a lower dnAUC_{0-12} for tacrolimus compared to individuals carrying the wild type variant for these PXR polymorphisms (respectively, Mann-Whitney; $P = 0.038$, $P = 0.088$, $P = 0.038$ and $P = 0.038$). The results in CHAPTER 11 suggests that the four PXR polymorphisms are associated with a strong trend towards a lower dnAUC_{0-12} of tacrolimus which confirms the role of PXR in the CYP3A and ABCB1 expression and thereby on the tacrolimus metabolism. Additionally, CHAPTER 12 describes the influence of both the daily corticosteroid dosage and these four PXR polymorphisms on the different pharmacokinetic tacrolimus parameters in an early and a late Caucasian posttransplant recipient group. However, non-genetic factors like, gender, age, haematocrit and time after transplantation seem also to play a role in the variability of the tacrolimus exposure between renal transplant recipients. Up until now therapeutic drug monitoring of tacrolimus is still performed by measuring C_0 concentrations, although recently several studies point out that a LSS consisting of two or three sample time points within 4 hour after the morning tacrolimus ingestion, gives a significantly better reflection of the complete AUC_{0-12} . In order to decrease the variability in the tacrolimus exposure of transplant recipients it is also recommendable to look for possible drug interactions with tacrolimus and possible interactions with other CYP450 inducers or inhibitors like St. John's wort or grapefruit juice.

Summarized the research presented in this thesis reflects on the comparison of several commercially available DNA isolation kits with respect to their isolation capacities, several real-time PCR FRET assays for different polymorphisms that are designed, optimised and validated in our laboratory and additionally in renal transplant recipients with a different ethnic origin (Caucasian and Chinese) the influence of several genetic (CYP3A, ABCB1, UGT2B7 and PXR polymorphisms) and non-genetic factors (gender, age, haematocrit and time after transplantation) are examined on the tacrolimus exposure using a different methodology to determine the tacrolimus exposure (abbreviated AUC_{0-12} and complete AUC_{0-12}).

Samenvatting

Samenvatting

In 2006 wachtten in Nederland 1054 patiënten op een donornier, terwijl in hetzelfde jaar slechts 360 niertransplantaties werden uitgevoerd. Niet alleen de levensverwachting maar ook de kwaliteit van leven verbetert enorm nadat de patiënt een donornier heeft ontvangen. Echter 10 jaar na transplantatie functioneert nog maar ongeveer de helft van alle donornieren. Het verlies van getransplanteerde nieren wordt veroorzaakt door niet goed functionerende getransplanteerde nieren en nog vaker door het overlijden van de patiënt ondanks een goed functioneerde nier. Het overlijden van deze patiënten wordt vaak veroorzaakt door hart- en vaatziekten. Alle patiënten die een donororgaan ontvangen moeten hun hele leven behandeld worden met immunosuppressiva, die allemaal sterk variërende farmacokinetische karakteristieken en een smalle therapeutische index hebben. De immunosuppressieve onderhoudstherapie bestaat van 1980 tot 1995 uit cyclosporine (Neoral[®]) met corticosteroïden en azathiopurine (Immunan[®]). In 1995 kwamen enkele nieuwe immunosuppressiva beschikbaar: tacrolimus (Prograf[®]) een geneesmiddel dat gebruikt kan worden als een hoeksteen voor de farmacotherapie, mycofenolaat mofetil (Cellcept[®]) en sirolimus (Rapamune[®]), welke gebruikt kunnen worden als aanvullende immunosuppressieve geneesmiddelen. Wanneer in plaats van cyclosporine tacrolimus wordt gegeven komen er minder vaak acute afstotingen voor. Dit is het geval voor zowel corticosteroïd-gevoelige als corticosteroïd-ongevoelige acute afstotingen. Doordat acute afstoting en zeker corticosteroïd-ongevoelige acute afstoting geassocieerd wordt met een hoger risico op chronische transplantaat-disfunctie, was het te verwachten dat het gebruik van tacrolimus zou leiden tot een betere transplantatie overleving dan met cyclosporine gebruik. Ondertussen zijn er sterke aanwijzingen dat deze verwachtingen zijn gerechtvaardigd. Verder leidt het gebruik van tacrolimus, in plaats van cyclosporine tot een verbetering van diverse lipoproteïnen (HDL, LDL) en lipiden (triglyceriden) in het bloed en tot een lagere bloeddruk. Deze factoren zijn ook geassocieerd met chronische transplantaat disfunctie en daardoor met mortaliteit als resultaat van hart- en vaatziekten na transplantatie. Bij tacrolimus gebruik is ook vastgesteld dat de incidentie van diabetes mellitus na transplantatie vaker voorkomt dan bij cyclosporine gebruik. Dit zou uiteindelijk de eerder beschreven voordelen ten aanzien van een vergrote overleving van de getransplanteerde nier en de verhoogde overleving van de patiënt teniet doen. Nadat in HOOFDSTUK 1 het overzicht en het doel van dit proefschrift is beschreven, geeft HOOFDSTUK 2 een compleet overzicht van het tacrolimus metabolisme (absorptie, distributie, metabolisatie en eliminatie). De sterk variërende farmacokinetische karakteristieken van tacrolimus in combinatie met een smalle therapeutische index benadrukt de noodzaak tot het nauwkeurig volgen van de tacrolimus bloed concentraties. Vervolgens beschrijft HOOFDSTUK 2 verschillende methoden die tegenwoordig gebruikt worden om de tacrolimus blootstelling te meten, farmacokinetische parameters, dalspiegels (C_0), verkorte oppervlakte onder de tijd tacrolimus concentratie curve (AUC_{0-12}) of complete 12-uurs AUC_{0-12} en de apparatuur welke gebruikt worden (IMx II of LC-MS/MS) om de tacrolimus spiegels te meten.

Verder zijn de genetische en niet-genetische factoren die een rol zouden kunnen spelen samengevat en tenslotte is er een discussie over de klinische consequenties en de impact van sub-therapeutische of toxische tacrolimus bloedspiegels voor de patiënt.

Voordat de real-time polymerase kettingreactie (PCR) kan worden uitgevoerd, die gebruikmaakt van fluorescentie probes, is DNA isolatie uit ethyleen diamine tetra azijnzuur (EDTA) volbloed monsters vereist. Alhoewel verschillende commerciële DNA isolatie kits beschikbaar zijn om DNA te extraheren uit volbloed monsters, is er weinig informatie beschikbaar ten aanzien van de extractie efficiënties en de kwaliteit van het geïsoleerde DNA. HOOFDSTUK 3 vergelijkt drie commercieel beschikbare DNA isolatie kits, QIAamp blood mini kit (Qiagen, Leusden, Nederland), Roche High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Duitsland) en Puregene (Biozyme, Landgraaf, Nederland) ten aanzien van hun capaciteit om DNA te isoleren uit volbloed monsters en de verkregen DNA kwaliteit. In de begin jaren 90, introduceerde de real-time PCR technologie een nieuwe dimensie binnen de PCR technologie, omdat amplificatie en detectie mogelijk werd in één enkel PCR cupje, plaat of capillair. De gedefinieerde drempel cyclus (C_t) waarde kan gebruikt worden om de DNA kwantiteit, welke aanwezig is in het PCR cupje te bepalen, terwijl een verschil tussen het wild type en variant allel DNA in de probe aanhechting kan worden gebruikt voor genotypering met real-time PCR technologie. Door zijn analysesnelheid, verminderd risico op contaminatie, geringe arbeidstijd en een PCR en detectiemethode in één, heeft real-time PCR een enorme interesse gewekt onder de moleculair biologen. Een toepassing die algemeen wordt gebruikt in de real-time PCR is de "fluorescence resonance energy transfer" (FRET) methodologie. Een kenmerk van deze real-time PCR FRET technologie is dat twee individuele fluorescentie probes complementair zijn aan het PCR product dichtbij de polymorfe positie. Een ander karakteristiek van de FRET technologie zijn de smeltcurves. Na de laatste cyclus, wordt het PCR product nadat het is afgekoeld tot een bepaalde temperatuur nogmaals gedegeneerd. Het dubbelstrengs PCR product wordt verwarmd met een snelheid van 0.1 tot 0.2°C/seconde tot de gewenste temperatuur. Gedurende deze opwarmingsprocedure wordt het fluorescentie-sigitaal continue gemeten. Als het nucleotide dat gepositioneerd is op de polymorfe positie in de sensor probe complementair is aan het nucleotide gepositioneerd in de polymorfe positie in het vermenigvuldigde DNA, is er een relatief sterke binding tussen deze nucleotiden. Het fluorescentiesigitaal zal alleen bij een hoge temperatuur afnemen in sterkte. Dit in tegenstelling tot een niet complementair nucleotide in het PCR product dat een minder stabiele binding tot gevolg heeft en uiteindelijk zal resulteren in een afname in het fluorescentiesigitaal bij een lagere temperatuur. Door het berekenen van de negatieve afgeleide van de afname in dit fluorescentiesigitaal, wordt een smeltpiek voor het wild type en de variant allel monsters verkregen. Door de samenstelling van de DNA sequentie, bijv. een lang A/T rijk gebied, is het noodzakelijk om een sensor probe te ontwerpen, de probe die de polymorfe positie omvat en die lang genoeg is om een voldoende hoge smelttemperatuur te krijgen. Als resultaat van de vergrote lengte van zo'n sensor probe wordt de invloed van het niet complementaire nucleotide significant minder in vergelijking met een kortere sensor probe. HOOFDSTUK 4 toont aan dat het

inbouwen van een “locked nucleic acid” (LNA) het genotyperen van de PXR A11156C polymorfisme significant verbeterde. Vooral heterozygoten zijn duidelijker te onderscheiden door het verschijnen van twee smeltpieken, terwijl de probes zonder een LNA bij een smeltcurve analyse niet in staat zijn om deze smeltpieken te scheiden. Daarom concluderen we dat een LNA binnen een sensor probe het discriminerend vermogen van een FRET assay kan verbeteren. In HOOFDSTUK 5 zijn snelle en consistente assays voor organic anion transporting polypeptide 1B1 (OATP1B1) polymorfismen door middel van snelle PCR FRET assays op de LightCycler ontwikkeld. Een LNA op de polymorfe locatie binnen de sensor probe was noodzakelijk om de twee allelen van het OATP1B1 T521C polymorfismen te onderscheiden. Om de betrouwbaarheid van beide real-time PCR FRET assays te bevestigen, werden deze nieuwe methoden gevalideerd door 120 monsters te genotyperen met een PCR “restriction fragment length polymorphism” (RFLP) assay en een allel-specifieke PCR. De resultaten van de real-time PCR FRET assays komen volledig overeen met de conventionele PCR methoden wat aangeeft dat de real-time PCR FRET assays geschikt zijn voor een klinische omgeving.

In de meeste transplantatie centra wordt het monitoren van de tacrolimus bloed concentraties uitgevoerd met C_0 concentraties na de ochtend tacrolimus inname, ondanks dat er bewijs beschikbaar is dat de bruikbaarheid van deze ochtend tacrolimus C_0 spiegels niet optimaal is. Dit wetende, hebben verschillende studies een “limited sampling strategy (LSS)” ontwikkeld met stapsgewijze multiple regression analysis of Bayesian fitting om de AUC_{0-12} zo nauwkeurig mogelijk te voorspellen. Dit omdat de AUC_{0-12} gezien wordt als de gouden standaard voor het meten van de totale tacrolimus concentratie. Veel van deze studies die een LSS ontwikkelde voor tacrolimus gebruikten geen onafhankelijke transplantatie patiënten populatie om de LSS te valideren. In HOOFDSTUK 6 worden verschillende LSS die recentelijk zijn gepubliceerd geëvalueerd met een onafhankelijke goed gekarakteriseerde stabiele niertransplantatie patiënten populatie. De prestaties van 24 LSS in 37 niertransplantatie patiënten met een bekende AUC_{0-12} is geëvalueerd en de resultaten worden tevens vergeleken met de voorspelbaarheid van de dalspiegels (C_0 en C_{12}). Het criterium was een absolute voorspelbaarheidsfout (APE%) die minder dan 15% verschilt van de complete AUC_{0-12} . Dertien van de 18 (72%) LSS zijn gebaseerd op regressie analyse en in staat om tenminste 90% van de 37 individuele AUC_{0-12} binnen een APE% van 15% te voorspellen. Voorspellingen gebaseerd op C_0 en C_{12} en Bayesian fitting zijn lager dan 90%: respectievelijk, 62% en 67%. In tegenstelling tot een recente publicatie hebben we in onze resultaten geen duidelijke verschillen waargenomen in de voorspellende capaciteiten, wanneer een LSS was ontwikkeld door een studie op basis van 12-uurs farmacokinetische profielen, die opgenomen zijn van hart of long transplantatie patiënten bij validatie in onze niertransplantatie patiënten groep. Daarom zijn LSS met C_0 , C_2 en/of C_4 naar onze mening een beter alternatief voor de C_0 concentratie. De verschillende LSS beschikbaar in de huidige literatuur voor het monitoren van tacrolimus zouden adequate voorspellingen kunnen genereren alhoewel een voorzichtige evaluatie voor de betrouwbaarheid van deze LSS verplicht is. Tacrolimus wordt voornamelijk gemetaboliseerd door cytochroom P450 (CYP450) 3A iso-enzymen in 13-O-desmethyltacrolimus, die een

verwaarloosbare immunosuppressieve activiteit heeft. In de HOOFDSTUK 7 en 8 tonen we in een Chinese en Caucasische niertransplantatie patiënten populatie aan, dat het CYP3A5 A6986G polymorfisme het meest relevante polymorfisme is in de CYP450 3A iso-enzym familie, met een significante impact op zowel de tacrolimus blootstelling als de dagelijkse tacrolimus dosering. In HOOFDSTUK 7 wordt de associatie tussen CYP3A en ABCB1 polymorfismen en de AUC_{0-12} , berekend met een tweetijds punt strategie, geëvalueerd. De CYP3A en ABCB1 genotypes zijn bepaald met real-time PCR FRET assays in 103 Chinese niertransplantatie patiënten en vervolgens gerelateerd aan de dosis genormaliseerde (dn) AUC_{0-12} . Een significant allel-afhankelijk effect (Kruskal-Wallis; $P < 0.001$) is waargenomen tussen het CYP3A5*3 polymorfisme en de $dnAUC_{0-12}$. Multiple regression analyse toonde aan dat het CYP3A5*3 polymorfisme de meest significante onafhankelijke variabele is en 35% van de variabiliteit in de dosering verklaarde in relatie tot het tacrolimus gebruik. Ten aanzien de ABCB1 G2677T/A en C3435T polymorfismen is er een trend waargenomen tussen de verschillende genotypen en de $dnAUC_{0-12}$. HOOFDSTUK 8 beschrijft twee verschillende niertransplantatie patiënten groepen welke worden gebruikt om te onderzoeken wat de invloed is van CYP3A en ABCB1 polymorfisme op de dagelijkse tacrolimus dosering en verschillende farmacokinetische parameters. In totaal zijn 63 Kaukasische niertransplantatie patiënten verdeeld in 26 vroege en 37 late posttransplantatie patiënten gegenotypeerd voor CYP3A en ABCB1 polymorfismen. De farmacokinetische parameters van tacrolimus worden bepaald voor alle niertransplantatie patiënten en gecorreleerd aan hun corresponderende genotypes. Een significant verschil in allel frequenties wordt waargenomen voor CYP3A4*1B ($P = 0.028$) en CYP3A5*1 ($P = 0.022$) allelen tussen de vroege en late posttransplantatie patiënten groep. Significante hogere dnC_0 , $dnAUC_{0-12}$ en dnC_{max} werden waargenomen voor dragers van het CYP3A5*3 variant allel in beide niertransplantatie patiënten groepen. Behalve voor de dagelijkse tacrolimus dosering ($P = 0.025$) werd geen significant verschil waargenomen voor dragers van de CYP3A4*1B variant allel. Zowel de individuele ABCB1 polymorfismen als de ABCB1 haplotypen zijn geassocieerd met iedere farmacokinetische parameter. Vervolgens is in HOOFDSTUK 9 de allel frequentie van het CYP3A7*1C variant allel onderzocht en tevens de invloed van CYP3A7*1C op de verschillende farmacokinetische parameters van tacrolimus. Een allel frequentie van respectievelijk 2.8% en 0.0% werd gevonden na het genotyperen van 70 Kaukasische en 103 Chinese niertransplantatie patiënten voor het CYP3A7*1C variant allel met een real-time PCR FRET assay. Heterozygote dragers van het CYP3A7*1C allel vertoonden geen significant lagere dnC_0 spiegels, $dnAUC_{0-12}$ of dnC_{max} voor tacrolimus in vergelijking met de dragers van het CYP3A7*1 allel. In HOOFDSTUK 10 vertoonde de variant allelen van de UGT2B7 polymorfismen G-79A, T-66C en C816T met een allel frequentie van respectievelijk, 0.0%, 5.8% en 29.6% geen significante verschillen met zowel de $dnAUC_{0-12}$ als de dagelijkse tacrolimus dosering vergeleken met dragers van wild type UGT2B7 allelen wanneer de patiënten worden gecategoriseerd op basis van hun CYP3A5 A6986G, ABCB1 G2677T/A en ABCB1 C3435T genotype. Ondanks het feit dat tacrolimus wordt gegluconideerd door UGT2B7 vertonen de drie relevante polymorfismen in het UGT2B7 gen geen invloed op

de tacrolimus blootstelling in de Chinese niertransplantatie patiënten groep. Hoewel polymorfismen in genen die indirect betrokken zijn bij het tacrolimus metabolisme ook een belangrijke rol schijnen te spelen. De pregnane X receptor (PXR) speelt een belangrijke rol in de expressie regulatie van CYP450 iso-enzymen, 5'-uridine diphosphate glucuronosyltransferase (UGT) enzymen en de geneesmiddeltransporter ABCB1, welke direct zijn betrokken in het tacrolimus metabolisme. In HOOFDSTUK 11 wordt de invloed van vier individuele PXR polymorfismen en PXR haplotypen onderzocht op de tacrolimus blootstelling in een Chinese niertransplantatie patiënt populatie. Individuen die drager zijn van een variant allel voor de PXR polymorfismen A7635G, C8055T, A11156C en T11193C, worden geassocieerd met een hogere expressie van CYP3A4 of ABCB1 in vergelijking met individuen die drager zijn van de wild type variant (Mann-Whitney; respectievelijk, $P = 0.038$, $P = 0.088$ $P = 0.038$ en $P = 0.038$). De resultaten in HOOFDSTUK 11 suggereren dat de vier PXR polymorfismen geassocieerd zijn met een sterke trend naar een lagere $dnAUC_{0-12}$ van tacrolimus, wat de rol bevestigt van PXR bij de CYP3A en ABCB1 expressie en daardoor ook op het tacrolimus metabolisme. Vervolgens wordt in HOOFDSTUK 12 de invloed van zowel de dagelijkse corticosteroid dosering en deze vier PXR polymorfismen op de verschillende farmacokinetische tacrolimus parameters in een vroege en een late Kaukasische posttransplantatie patiënten groep beschreven. Alhoewel niet genetische factoren zoals geslacht, leeftijd, hematocriet en tijd na transplantatie ook een belangrijke rol schijnen te spelen in de variabiliteit van de tacrolimus blootstelling tussen niertransplantatie patiënten. Tot nu toe wordt het therapeutisch monitoren van tacrolimus nog steeds uitgevoerd door het meten C_0 concentraties, hoewel recentelijk verschillende studies aangeven dat de LSS die bestaan uit twee of drie tijdstippen waarop monster worden afgenomen binnen vier uur na de ochtend tacrolimus inname, een significant betere afspiegeling geeft van de complete AUC_{0-12} . Om de variabiliteit in de tacrolimus blootstelling van de transplantatie patiënten te verlagen is het ook aan te bevelen om te kijken naar mogelijke geneesmiddelinteracties met tacrolimus en mogelijke interacties met andere CYP450 inducers en remmers, zoals St. Janskruid of grapefruitsap.

Samengevat, het onderzoek dat wordt gepresenteerd in dit proefschrift omvat de vergelijking van verschillende commercieel verkrijgbare DNA isolatie kits ten aanzien van hun isolatie capaciteiten, diverse real-time PCR FRET assays voor verschillende polymorfismen werden ontworpen in ons laboratorium, geoptimaliseerd en gevalideerd en verder is in niertransplantatie patiënten van een verschillende etnische oorsprong (Kaukasisch en Chinees) de invloed van verschillende genetische (CYP3A, ABCB1, UGT2B7 en PXR polymorfismen) en niet genetische factoren (geslacht, leeftijd, hematocriet en tijd na transplantatie) onderzocht op de tacrolimus spiegels met verschillende methodologieën om de diverse farmacokinetische parameters voor tacrolimus te bepalen (verkorte AUC_{0-12} en complete AUC_{0-12}).

Abbreviations

Abbreviations

A^{260}/A^{280}	ratio absorption at 260 nm and 280 nm
ABCB1	adenosine triphosphate binding cassette B1 gene
ABCC1	adenosine triphosphate binding cassette C1 gene
ALAT	alanine aminotransferase
ANOVA	analysis of variance between groups
ARMS	amplification refractory mutation system
ASAT	aspartate aminotransferase
AUC_{0-12}	12 hour area under the time concentration curve
bp	base pair
C_0	trough tacrolimus level (concentration)
C_1	tacrolimus blood concentration 1 hour after tacrolimus administration
C_2	tacrolimus blood concentration 2 hour after tacrolimus administration
C_3	tacrolimus blood concentration 3 hour after tacrolimus administration
C_4	tacrolimus blood concentration 4 hour after tacrolimus administration
C_5	tacrolimus blood concentration 5 hour after tacrolimus administration
$C_{7.5}$	tacrolimus blood concentration 7.5 hour after tacrolimus administration
C_9	tacrolimus blood concentration 9 hour after tacrolimus administration
C_{12}	tacrolimus blood concentration 12 hour after tacrolimus administration
C_{max}	the maximum tacrolimus concentration reached
cAMP	cyclic adenosine monophosphatase
CAR	constitutive androstane receptor
c-MOAT	canalicular-Multispecific Organic Anion Transporter
CREB	cAMP-response element binding protein
Ct	Cycle threshold
CYP3A4	cytochrome P450 3A4 iso-enzyme
CYP3A5	cytochrome P450 3A5 iso-enzyme
CYP3A7	cytochrome P450 3A7 iso-enzyme
DNA	deoxyribonucleic acid
$dnAUC_{0-12}$	dose-normalized tacrolimus area under the time concentration curve
dnC_0	dose-normalized tacrolimus trough level (concentration)
EDTA	ethylene diamine tetra-acetic acid
FAM	fluorescein
FKBP-12	FK506-binding protein-12
FKBP-52	FK506-binding protein-52
FRET	fluorescence resonance energy transfer
GR	glucocorticosteroid receptor
HPLC	high performance liquid chromatography
IL	interleukin
INF	interferon
LNA	locked nucleic acids

MDR1	multidrug resistance 1
MMF	mycophenolate mofetil
mRNA	messenger ribonucleic acid
MRP2	multidrug resistance-associated protein 2
MS	mass spectrometry
NF-ATc	nuclear factor of activated T-cells
NR1I2	nuclear receptor 1I2
NR1I3	nuclear receptor 1I3
NR1H4	nuclear receptor 1H4
OATP1B1	organic anion transporting polypeptide 1B1
OATP-C	organic anion transporting polypeptide C
OD	optical density
PCR	polymerase chain reaction
PNA	peptide nucleic acid
PXR	pregnane X receptor
r	multiple regression correlation coefficient
RFLP	restriction fragment length polymorphism
r_p	Pearson correlation coefficient
r_s	Spearman correlation coefficient
RXR	retinoid X receptor
SLCO1B1	solute carrier organic anion transporter family, member 1B1
SNP	single nucleotide polymorphism
SXR	steroid and xenobiotic receptor
$t_{1/2}$	half life time tacrolimus
TAMRA	tetramethylrhodamine
T_{max}	time required to reach the maximum tacrolimus concentration
TNF	tumor necrosis factor
UV spectrometry	ultraviolet spectrometry
UDP	uridine diphosphate
UGT2B7	uridine diphosphate glucuronosyltransferases 2B7
WBC	white blood cells

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13. **Op den Buijsch RAM**, Cheung CY, Wijnen PAHM, van Dieijen-Visser MP and Bekers O. Influence of Allelic Variants of Uridine Diphosphate-Glucuronosyltransferase (UGT) 2B7 on the Tacrolimus Pharmacokinetics. Accepted as a poster presentation on Euromedlab, Monday, June 4th 2007, RAI Congress Centre Amsterdam.
14. **Op den Buijsch RAM**, Cheung CY, de Vries JE, Wijnen PAHM, van Dieijen-Visser MP and Bekers O. Pregnane X Receptor (PXR) Polymorphisms involved in the Tacrolimus Pharmacokinetics. Accepted as a poster presentation on Euromedlab, Monday, June 4th 2007, RAI Congress Centre Amsterdam.
15. **Op den Buijsch RAM**, van de Plas A, Bekers O, Christiaans MHL, van Hooff JP, van Dieijen-Visser MP and Stolk LML. Evaluation of Limited Sampling Strategies for Tacrolimus. Accepted as an oral presentation on the Wetenschappelijke mededelingendag klinische farmacologie, vrijdag 30 maart 2007, Jaarbeursgebouw Utrecht.

16. **Op den Buijsch RAM**, Wijnen PAHM, van der Heijden J, Hoogtanders K, Stolk LML, van Dieijen-Visser MP, Neef C and Bekers O. Genotyping with a dried blood spot method: a useful technique for application in Pharmacogenomics. Accepted as a poster presentation on the Wetenschappelijke mededelingendag klinische farmacologie, vrijdag 30 maart 2007, Jaarbeursgebouw Utrecht.
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Dankwoord

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Curriculum vitae

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Robert Antonius Maria Op den Buijsch werd op 15 oktober 1973 geboren in Nederweert. In 1992 behaalde hij het hoger algemeen voortgezet onderwijs (HAVO) diploma aan de Philips van Horne Scholengemeenschap te Weert, waar hij in 1994 ook zijn atheneum diploma kreeg uitgereikt. Nadat hij was uitgeloot voor de studie geneeskunde werd in september 1994 begonnen met de opleiding farmacie aan de Universiteit Utrecht. Na het behalen van zijn doctoraal farmacie in 1999 volgde hij de postdoctorale apothekersopleiding welke in november 2000 werd afgerond.

Vervolgens begon hij in februari 2001 als tweede apotheker bij apotheek Pluymaekers in Utrecht waar hij een ongeveer een jaar heeft gewerkt. Tijdens de studie farmacie was de interesse voor analyse, toxicologie en klinische chemie gewekt. Gedurende de periode dat hij werkzaam was als openbaar apotheker heeft hij gezocht naar opleidingsplek voor klinische chemicus. Toen duidelijk werd dat er betere perspectieven voor zo'n opleidingsplek zijn als je gepromoveerd bent, was de assistent in opleidingsplaats (AIO) op het klinisch chemisch laboratorium in het academisch ziekenhuis Maastricht precies wat hij zocht. In januari 2001 werd dan ook onder supervisie van prof. dr. M.P. van Dieijen-Visser en dr. O. Bekers gestart met deze onderzoeksplaats.

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