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Pharmacodynamic monitoring of calcineurin inhibition therapy : investigation of the calcineurin activity marker

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Citation

Rossum, H. H. van. (2010, April 27). *Pharmacodynamic monitoring of calcineurin inhibition therapy : investigation of the calcineurin activity marker*. Retrieved from <https://hdl.handle.net/1887/15350>

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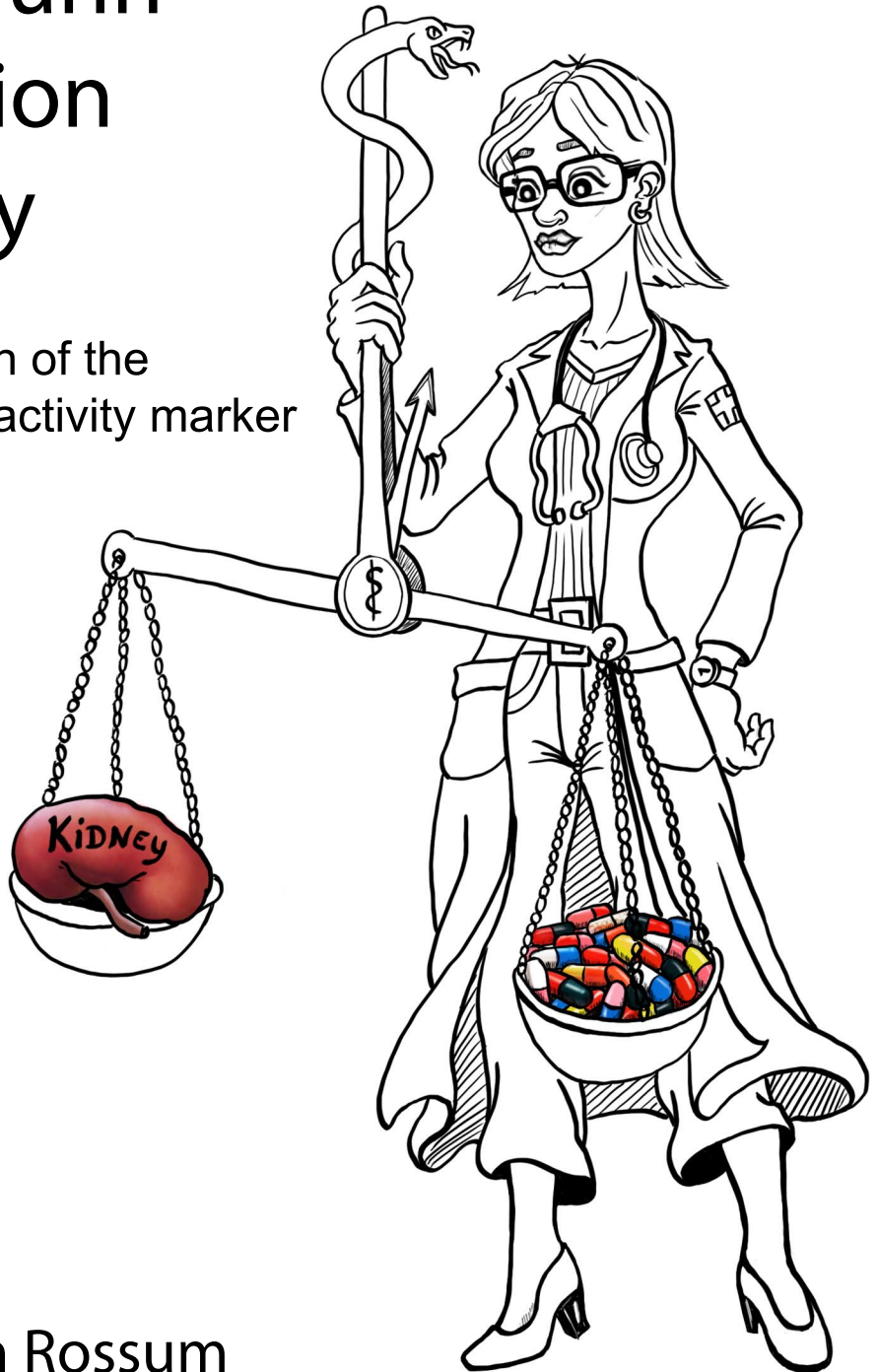
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Pharmacodynamic monitoring of calcineurin inhibition therapy

Investigation of the
calcineurin activity marker



Huub van Rossum

Pharmacodynamic monitoring of calcineurin inhibition therapy

Investigation of the calcineurin activity marker

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof. mr. P.F. van der Heijden,
volgens besluit van het College van Promoties
te verdedigen op dinsdag 27 april 2010
klokke 16:15 uur

door

Huub Harmen van Rossum

geboren te Amsterdam
in 1980

Promotie commissie

Promotor	:	Prof.dr. J.W. de Fijter
Co-promotor	:	Dr. J. van Pelt
Overige Leden	:	Prof.dr. R.J. ten Berge (UvA) Prof.dr. H.J. Guchelaar Prof.dr. F.H.J. Claas Prof.dr. C. van Kooten

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Cover: door Nie Ngo, naar idee van Huub van Rossum
Printed: Proefschriftmaken.nl
Published by: Uitgeverij BOXPress, Oisterwijk
ISBN: 978-90-8891-159-0

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Chapter 1

General introduction

Background

Treatment of end-stage organ failure by organ transplantation has significantly improved over the past 50 years. The necessary surgical skills, peri-operative management and adequate suppression of the immunological response against the allograft have all significantly contributed to better outcomes. In the 1960s and 1970s organ transplantation gained considerable interest and the rate of successful transplantation slowly increased with the use of immunosuppressants such as azathioprine and corticosteroids(1). The introduction of Cyclosporin A (CsA), however, showed adequate suppression of the immunological response and revolutionized allograft transplantation. With the use of CsA the one year graft survival rates of renal transplant recipients increased to over 80 percent and made transplantation of the lung, heart, liver, pancreas, hand, and kidney, feasible in clinical practice as life-saving or life-prolonging therapy(1). Since then, several other small molecule drugs and protein (antibody) preparations, have been developed and used as immunosuppressive agents(2). These new drugs have led to a further decrease of organ rejection episodes, but unfortunately long term allograft survival rates have not improved to the same extent over the past decades(3). These data directly points towards one of the major issues associated with current immunosuppressive protocols in renal allograft recipients; drug related toxicity and/or over-immune suppression that compromise long-term success.

Individualization of the immunosuppressive therapy by tailor-made immunosuppressive protocols is considered a key strategy to further improve long-term graft survival. This means that the immunosuppressive regime and dosing are adjusted to the biological variance between individuals. Monitoring of drug exposure by therapeutic drug monitoring (TDM) is now routinely applied for most immunosuppressive drugs and at the present individual drug exposure is controlled by regular assessment of blood drug concentrations. Since large variation in pharmacokinetics of immunosuppressants has been observed and most drugs have only a small therapeutic window, better control of drug bioavailability has further improved therapy outcome(4). Nevertheless individualization is still warranted and several new strategies such as pharmacogenetics and pharmacodynamics are currently investigated. Pharmacogenetic parameters may predict the pharmacokinetic profile of individual subjects and guide initial dosing to achieve therapeutic target ranges as soon as possible(5). Pharmacodynamic monitoring strategies hold the promise to measure drug effectiveness parallel to

pharmacokinetic parameters and may provide additional information to define new therapeutic target conditions.

Molecular mechanisms of allograft rejection

Allograft recognition

Allograft rejection is the recognition of the transplanted tissue as being non-self, followed by specific targeting of the allograft by the host's immune system. Key molecules involved in the process of recognizing non-self epitopes are the major histocompatibility complex (MHC) proteins. These proteins can be divided into two classes; Class I molecules that are expressed on most cells and present peptides from endogenous proteins, and Class II molecules that have a more restricted distribution and are generally associated with the presentation of peptides from exogenous proteins(6). Cells that express MHC class II proteins are assigned as antigen presenting cells (APCs). Both MHC class molecules are highly polymorphic, especially at sites that determine the peptide binding groove. These differences can directly evoke immune responsiveness of host cells(6). This is observed in hyperacute rejection when preformed antibodies against HLA class I or ABO blood group system are present in the circulation or lymphatic structures of the recipient. It is found that peptides from MHC molecules themselves are prominently presented by MHC molecules. It is therefore thought that a major contribution to allo-recognition via MHC molecules is mediated via presentation of peptides from MHC molecules on either donor (direct) or host (indirect) MHC proteins(6).

For successful allo-recognition, adequate T cell receptor (TCR) binding of allogeneic MHC in the context of co-stimulatory signals is required. A requirement for TCR binding is its association with the CD3 complex that allows surface expression of TCRs and signal transduction following antigen recognition. Other T cell molecules involved in T cell recognition are the CD4 or CD8 proteins. These proteins cross-link either MHC I (CD8) or MHC II (CD4) and play key roles in allowing T cell responsiveness, especially when the affinity of TCR for MHC is low(6). TCR binding itself is insufficient for activation of the T cell and without co-stimulatory signaling, TCR binding leads to anergy(7). For successful T cell activation, a second signal is required that exist of a non-antigen driven interaction between antigen presenting cell and T cell(7). The most potent but not crucial co-stimulatory molecules known are B7s (CD80/86) that are presented by APCs and

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interact with CD28 on T cells(8). When allo-antigen specific T cell clones are successfully activated, biochemical changes are induced that allow transcription of response genes such as cytokines and surface proteins that are involved in controlling adhesion to APC, target and other (immune) cells, endocrine/paracrine signaling and clonal expansion of the activated T cell clones.

T cell activation

Intracellular T cell activation responses are initiated by the activation of the TCR, which leads to activation of tyrosine kinases such as LCK and ZAP70 that phosphorylate several substrates such as SLP76 and LAT(9). These proteins recruit and activate other proteins such as phospholipase C γ . Phospholipases catalyze the hydrolysis of the membrane phospholipid phosphatidylinositol-1,4,5-bisphosphate to inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG)(9). IP₃ binds to IP₃ receptors on the endoplasmic reticulum that result in the release of Ca²⁺ into the cytosolic cell compartment. This initial release of Ca²⁺ itself is not sufficient for T cell activation, but induces the influx of extra-cellular Ca²⁺ via Ca²⁺ release activated Ca²⁺ (CRAC) channels that provides sustained elevated levels of Ca²⁺(10). This Ca²⁺ signaling results in the activation of transcription factors such as NFAT. Only sustained Ca²⁺ levels allow prolonged NFAT translocation and NFAT-dependant gene transcription(9;10).

The Ca²⁺-NFAT signal transduction is mediated via calcineurin. Calcineurin (CN) is the Ca²⁺ and calmodulin dependant serine-threonine protein phosphatase, which becomes activated by an increase of intracellular Ca²⁺(11). The cytosolic NFAT proteins are dephosphorylated by CN at serines within the N terminus of the SP repeats and the serine-rich region(10). Dephosphorylation of these serines is thought to expose nuclear localization sequences, resulting in rapid NFAT translocation to the nucleus(10). Upon co-stimulation, NFAT interacts with members of the AP-1 protein to form a transcription complex that is essential for a productive immunological response(7). The NFAT AP-1 transcription complex transcribes a large number of genes encoding cytokines (e.g. IL-2, IL-4, IFN γ), chemokines (e.g. GM-CSF) and surface proteins (e.g. CD40L, IL2R α) that activate and control the immunological T cell responses(12). The CN inhibitors tacrolimus and cyclosporine block T cell activation by inhibition of CN phosphatase activity and are therapeutically used in allograft recipients to prevent allograft rejection.

Calcineurin and its isoforms

CN is the serine-threonine-specific, Ca^{2+} / calmodulin dependant protein phosphatase and composed of a 59 kDa catalytic subunit also referred to as CN A and a 19 kDa regulatory subunit also referred to as CN B(13). CN A contains a phosphatase domain and this catalytic site contains the metals Zn^{2+} and Fe^{3+} , a CN B-binding helical domain, a calmodulin binding domain and an auto-inhibitory domain(14). This autoinhibitory domain forms a loop that covers the active site in the unligated CN structure. CN B consists of two globular Ca^{2+} -binding domains that have two Ca^{2+} -binding EF hand motifs and are similar to those of calmodulin(14). Ca^{2+} / calmodulin binding to the corresponding binding domain reverses the self-inhibition by inducing conformational changes in the protein structure, resulting in exposure of the active site(15).

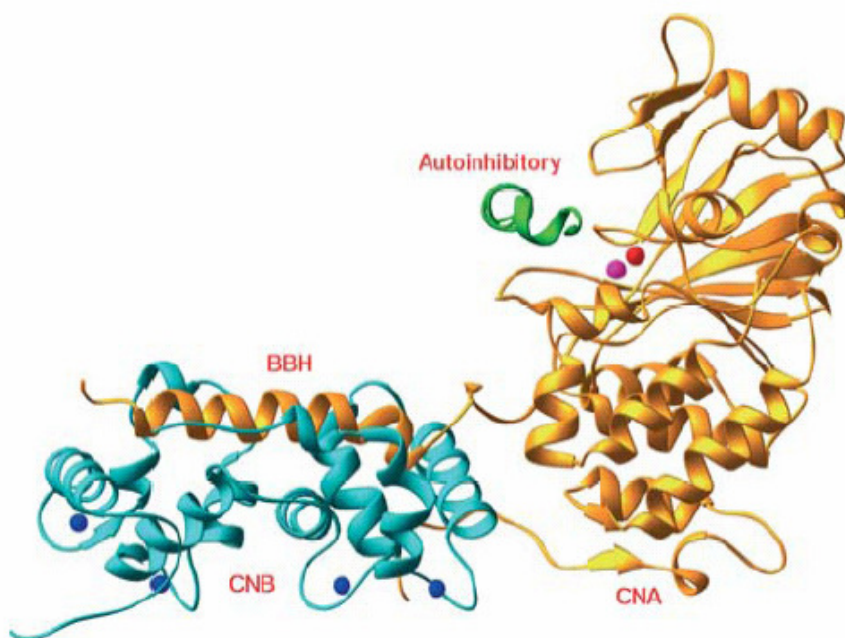


Figure 1.1. Ribbon diagram of calcineurin. Gold is calcineurin A, blue is calcineurin B, green is the autoinhibitory loop and the red, purple and blue balls represent Zn^{2+} , Fe^{3+} and Ca^{2+} respectively. BBH is the calcineurin B binding domain, CNA is Calcineurin A, CNB is Calcineurin B(14).

Three different CN A isoforms (α , β and γ) have been described(13). The expression of CN A γ is restricted to testis and specific parts of the brain, while the CN A α and CN A β isoforms are present in all tissues examined(13). The overall

amino acid sequences of CN A α and CN A β are 81% identical, while the sequence identity within the catalytic region, the CN B binding helix, the Ca²⁺/calmodulin-binding domain and the auto-inhibitory domain is 90%(16). Variations in the amount and ratio of CN A α : CN A β have been identified within and between tissues. In brain, CN A α is more abundant than CN A β . CN A α is more abundant in kidney and its expression is largely restricted to the tubules, while CN A β expression was observed mainly in the glomerular region(13;17).

In T and B cells, CN A β is more abundant(17). Interestingly small differences in kinetic profiles are observed(18). Also differences in physiological roles have been identified, and the absence of CN A α , but not CN A β , was found to be responsible for a defect in normal maturation of the nephrogenic zone and glomeruli, alterations in the cell cycle and impaired kidney function(19).

In mammals two different CN B genes have been identified. One is ubiquitously expressed (CN B1)(13;20), and gene transcripts for the second gene (CN B2) are found in brain, lung, thymus and heart, while the protein has only been detected in testes (21).

Calcineurin; function and pathogenesis of malfunction

Neuronal tissue

CN is probably best known as rate limiting step in T cell activation, but its expression in many tissues and different cell types indicates an important role in many physiological control mechanisms. The highest levels are found in the brain and its localization in neuronal tissue together with its Ca²⁺-binding properties was the basis for its descriptive name “calcineurin”, introduced by Klee et al.(22). CN has several substrates that are located in neuronal tissue including dynamin 1, synapsin 1, Myristoylated alanine-rich C-kinase substrate, neuromodulin and neurogranin.

Dynamin 1 is a neural phosphoprotein and a GTPase that mediates late stages of endocytosis in both neural and non-neural cells(23). High concentrations of intracellular calcium causes association between CN and dynamin 1 as well as the dephosphorylation of dynamin 1 and the delivery of CN to the endocytic protein complex enabling CN to initiate dephosphorylation of other proteins(24).

Synapsins are the most abundant vesicle proteins, synapsin 1 alone accounts for approximately 6% of total vesicle protein(25). Under resting conditions synapsins tether synaptic vesicles to cytoskeletal elements and prevent neurotransmitter

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release, while during synaptic activity synapsins are phosphorylated, dissociate from synaptic vesicles and allow vesicles to mobilize and fuse with the plasma membrane(25). CN inhibition by CsA completely blocked dephosphorylation of certain phosphorylation sites on synapsin 1 that resulted in a prominent increase of the neurotransmitter glutamate(26).

Other substrates of CN are neuromodulin (B50, F1, GAP43), which is localized within axonal membranes of neurons and is believed to play a role in the modulation of membrane signal transduction and axonal regeneration, neurogranin (RC3, p17), which is thought to serve as a "third messenger" substrate of the PKC-mediated pathway during synaptic development and remodeling and myristoylated alanine-rich C-kinase substrate (MARCKS). MARCKS is implicated in the mechanisms of membrane trafficking and regulation of the cell cycle and it has been reported that phosphorylation of MARCKS plays an important role in release of neurotransmitters, such as noradrenaline and serotonin and adrenocorticotropin(27). It is thought that CN reverses protein kinase C induced actions of these proteins(27).

Since CN is important for adequate neuronal functioning, treatment with CN inhibitors may induce severe neurotoxicity resulting in headaches, altered mental status, seizures, cortical blindness, auditory and visual hallucinations, spasticity, paresis and ataxia(28).

CN has also been associated with the pathogenesis of Alzheimer's disease. One of the hallmarks of Alzheimer's disease is aggregation of hyperphosphorylated tau to neurofibrillary tangles in affected neurons(29). Phosphorylation of tau is controlled by tau kinases and phosphatases and it is thought that abnormal tau hyperphosphorylation might be due to defective phosphatase activity(29). CN is an important tau phosphatase and it has been suggested that defective CN activity contributes to the pathogenesis of Alzheimer by allowing hyperphosphorylation of tau(29;30).

Cardiovascular tissue

Two regulators of actin functioning, calponin and cofilin, are substrates of CN. The first is a smooth muscle-specific, thin filament-associated protein, which has been implicated in the regulation of contraction via its interaction with actin and inhibition of the cross-bridge cycling rate. Phosphorylation of calponin causes loss of actin binding and inhibition of the actin-activated myosin MgATPase(31). Cofilin is an essential regulator of actin filament dynamics that is inactivated by phosphorylation and reactivated by dephosphorylation. It is thought that a CN-dependant

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dephosphorylation pathway is responsible for dephosphorylation and thus activation of cofilin(32).

CN is also associated with cardiac hypertrophy a mechanism that was found to be dependant on subtypes of the NFAT transcription family(33). In addition, transgenic mice over-expressing CN showed a profound hypertrophic response that rapidly progressed to dilated heart failure(34).

Renal tissue

In renal tissue, calcineurin is a central regulator of calcium-mediated signaling. It is associated with the regulation of factors that are of particular importance in renal physiology such as TGF β and IGF-I(19). The most illustrative evidence of the relevance of CN activity in the kidney is however the pronounced nephrotoxicity of the selective CN inhibitors TRL and CsA resulting in tubulointerstitial fibrosis and tubular atrophy(19;35).

In renal tissue, CN A isoforms seems to have distinct functions. Mice lacking CN A α , reproduced the characteristics of CN inhibitor nephrotoxicity, while CN A β -null mice did not. *In vitro* experiments showed that NFATc translocation was specifically regulated by the CN A β isoform, whereas CN A α regulated fibronectin and TGF β expression(36).

Regulation of NFAT transcription

Probably the best know substrates of CN are the Nuclear Factor of Activated T Cells (NFAT) transcription factors. The family of NFAT transcription factors consists of 5 members; NFAT1 (NFATp, NFATc2), NFAT2 (NFATc, NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx, NFATc3) and NFAT5. NFAT1-4 are regulated by CN(12). Dephosphorylation of serines at the amino terminal regions of these NFAT proteins exposes nuclear localization sites that lead to rapid nuclear translocation. In immune cells co-operation with other transcription factors such as AP-1 leads to lymphocyte activation and the transcription of cytokines such as IL-2, IL-4, IFN γ , TNF α and many more(11). NFAT self in absence of co-stimulatory signaling can induce a different set of anergy-associated genes that mediate T cell anergy(12). Next to lymphocyte functioning, the calcineurin/NFAT pathway was found to be important in the development and functioning of the cardiovascular system(10), musculoskeletal system(10), perinatal lung(37) and pancreatic β -cells (38), glucose and insuline homeostasis(39), regulation of bone mass in osteoclasts and osteoblasts(40) and the pathogenesis of leukemia(41;42).

Regulators of calcineurin

Several endogenous proteins are known that control CN activity. One is AKAP79 scaffold protein that seems to prevent CN's access to substrates(10). A second inhibitor is cain (cabin-1), that blocks CN activity(43), a third a CN B homolog named CN homologous protein (CHP), that binds to CN A but is not able to induce its activation(44). The last endogenous inhibitor of CN identified is MCIP1 (myocyte-enriched CN interacting protein 1 (also referred to as calcipressin / DSCR1 (Down Syndrome Critical Region 1) / Rcn1)(45), DSCR1L2 and ZAKI-4(46). It is suggested that DSCR1 functions as a feedback inhibitor of CN, since DSCR1 expression is induced by calcium through a CN-dependant mechanism. This loop might be necessary to avoid sustained CN activity in situations of prolonged Ca^{2+} stimulus(46).

An important role of this protein is suggested in the pathogenesis of Down's syndrome(47). Here the trisomy of a part of chromosome 21 that is present in individuals suffering from Down's syndrome contains DSCR1 and DSCR1 is expressed 1.5 times higher than normal. This increase in DSCR1 is thought to destabilize normal regulatory systems of the CN-NFAT pathway. This deregulation of the CN pathway may explain many of the developmental and regulatory features of Down's syndrome(47).

Besides endogenous proteins also some exogenous proteins inhibit CN activity. Some viruses mitigate immune responses by inhibiting CN/NFAT signalling. For instance the A238L protein of the African swine fever virus binds directly to CN, but also other microorganisms as diverse as *Trichoderma*, *Streptomyces* and leukaemia viruses target CN(10).

Calcineurin inhibitors

There are several inhibitors of CN but many are not specific for CN protein phosphatase and lack high affinity. Examples of these are okadaic acid ($\text{IC}_{50} \pm 4 \mu\text{M}$), microcystin LR ($\text{IC}_{50} \pm 200 \text{ nM}$)(13). An example of a selective CN inhibitor is 5-dibenzoyloxymethyl-substituted norcantharidin ($\text{IC}_{50} = 50 \mu\text{M}$) that was computationally designed and shows no inhibition of protein phosphatases 1 and 2A(48).

Inhibition of CN by exogenous molecules is most specific by interaction with immunophilin families. There are two structurally distinct immunophilin-immunosuppressant families that show CN inhibition: Cyclophilin A-Cyclosporin A

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(CsA) and FKBP (FK506 Binding Proteins)-FK506 (Tacrolimus, TRL) (49;50). Both complexes have a large overlap in CN interaction sites and bind to the same composite surface, but also significant differences in specific interaction sites are observed(14).

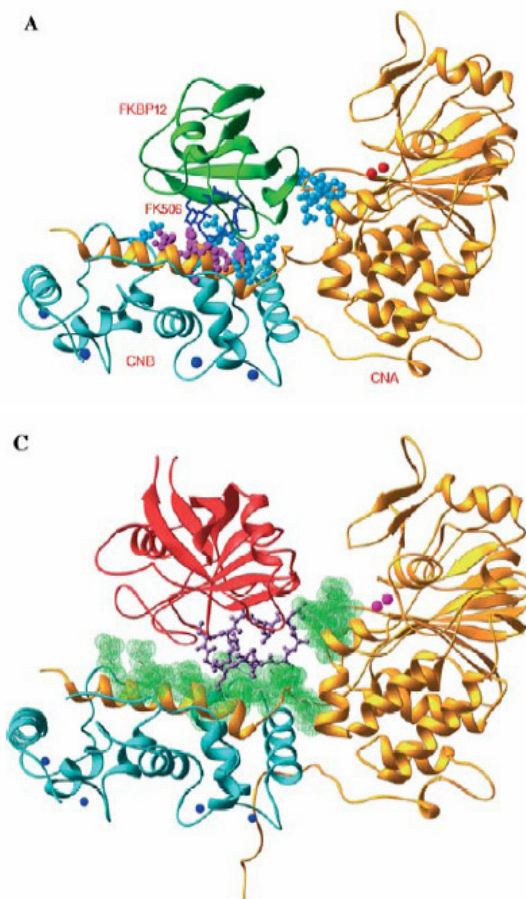


Figure 1.2. Ribbon diagram of immunophilin-immunosuppressant interaction with calcineurin. (A) Interaction of the FKBP-TRL complex with CN. (C) Interaction of the cyclophilin A–CsA complex with CN(14).

Cyclosporine A

Cyclosporine A (CsA) was originally isolated from the fungus *Cylindrocarpon lucidum* and identified as antilymphocytic agent during a screening program at Sandoz (now Novartis). It was found that CsA differed from the then known immunosuppressive agents in that a low degree of myelotoxicity was observed and was identified as the first immunosuppressive agent with more specific anti-T cell activity(51).

CsA consists of a cyclic peptide consisting of 11 amino acids with a molecular weight of 1202.6 Da(51). Seven of its amino acids are methylated and there is one unique aminoacid that contains a N-methyl-(4R)-4-butenyl-4-methylthreonine, see Figure 1.3.

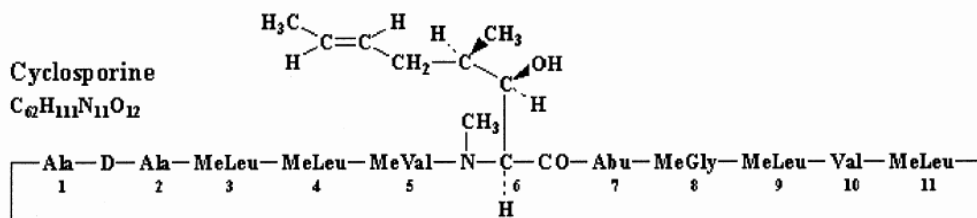


Figure 1.3. Molecular structure of cyclosporin A(55)

Cyclosporine A pharmacokinetics

From the moment the standard oil-based formulation of CsA (Sandimmune) was introduced into clinical practice in the early 1980s, CsA therapy was characterized by poor and unpredictable absorption(52). This was caused by gastrointestinal water-droplets that are formed after oral administration that required emulsification for the release of CsA and made the absorbance of the drug dependant on food intake, bile flow and gastrointestinal motility(52). To overcome these issues, a new micro-emulsion preconcentrate formulation of CsA was developed that ensured independence of bile flow for emulsification and resulted in higher, faster and less variable absorbance of CsA(52). Another factor that significantly affects absorbance of CsA is the drug exporting activity of the enterocytes by the ATP binding cassette protein para-glycoprotein (pgp). Pgp actively pumps CsA back into the gut lumen and reduces its absorbance(53).

After intestinal absorption, most cyclosporine leaves the bloodstream and is concentrated in leukocyte-rich and fat-rich organs(54-56). Within the blood, CsA is highly bound to the erythrocytes (60-70%), lipoproteins and leukocytes (9%) resulting a low free fraction of only 1-1.5%(55;57).

The main metabolizers of CsA are the CYP3A4 and CYP3A5 members of the cytochrome P450 mixed function oxidase system and around 30 metabolites have been identified that all preserve the cyclic structure of CsA(57). Since many drugs are either substrates, inhibitors or inducers of the CYP 3A4/5 enzymes, pharmacokinetic drug-drug interactions at this level can significantly effect the metabolism and clearance of CsA(58). The average half life of CsA is about 19 hours and CsA is excreted over 90% in bile of which less than 1% is unaltered. 6% is excreted by urine of which only 0.1% is unaltered(55).

Tacrolimus

In the course of a search for new immunosuppressive agents in the Fujisawa laboratories in 1982, a strain of *streptomyces tsukubaensis* No. 9993 was found to produce a potent immunosuppressive agent now designated as FK506(59). FK506 is a neutral macrolide that is insoluble in water, slightly soluble in saturated hydrocarbons and highly soluble in lipids and other organic solvents(55). The name tacrolimus, derived from **T**sukuba **macrolide immunosuppressant**, was assigned to the compound(60). Its chemical structure consists of a 23-member ring and is shown in Figure 1.4(61).

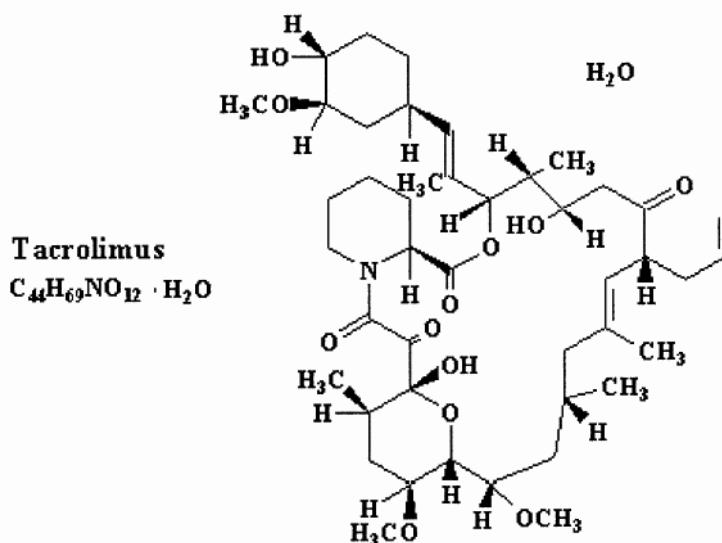


Figure 1.4. Molecular structure of tacrolimus(55)

Tacrolimus pharmacokinetics

Tacrolimus (TRL) is traditionally formulated in the twice daily (Prograf[®]) formulation, although recently a new once daily formulation was introduced (Advagraf[®])(62). The bioavailability of TRL is poor (mean around 25%) and highly variable; it ranges from 4 to 89% in renal, and liver allograft recipients(63). In blood, TRL is highly distributed to the erythrocyte fraction and most of the drug in human plasma is bound to plasma proteins(63). It was found that the drug content per cell is higher in leukocytes and that the drug distribution in blood depends on drug concentration, temperature and hematocrit values(64). TRL is primarily metabolized by the CYP3A subfamily and over 15 metabolites have been identified, some of which showed pharmacological activity(63;65). Less than 1% of

TRL is excreted unchanged and biliary excretion of TRL metabolites is the major pathway of TRL body clearance(63).

Therapeutic monitoring of calcineurin inhibitors

Calcineurin inhibitor (CNI) treatment is associated with significant side effects such as nephrotoxicity, neurotoxicity and hypertension. These side-effects are observed at therapeutic concentrations resulting in a narrow therapeutic index for both CsA and TRL. Since the pharmacokinetics of CsA and TRL are highly variable, controlling of drug exposure by means of therapeutic monitoring is mandatory. The golden standards for controlling CNI treatment is measurement of trough blood concentration (C₀). Unfortunately, C₀ doesn't correlate well to total drug exposure. Peak concentration measurement shows a better correlation with total drug exposure and is therefore also applied(66). Since CNI generally peak 2 hours after drug intake drug concentration measurement at 2 hours after drug intake (C₂) is performed. Unfortunately in the case of slow absorbers, e.g. diabetic patients, the maximum CNI concentrations are found at later time points. There is also a danger that drug exposure in fast metabolizers is overestimated, e.g. CYP3A5 genotype recipients. These cases are estimated to constitute 20% of the renal transplantation population. Advanced pharmacokinetic monitoring such as by estimated AUC could detect such cases and allow proper dosing for these individuals(67). When advanced pharmacokinetic population models are applied, anticipation on small deviations from the standardised sampling and drug intake times is possible.

Whole blood concentrations are currently used as surrogate for effect. Unfortunately, whole blood concentrations of CNI do not necessarily correlate to intracellular concentrations. CNIs are highly distributed to the erythrocyte fraction and bound to lipoproteins. Variation in these concentrations changes the distribution of CNIs in whole blood(68;69). Also the expression of drug efflux transporters such as para-glycoprotein on target cells (T cells) can theoretically effect the cellular distribution for both TRL and CsA(70). In addition, the immunological response of CNI is much more complicated than just drug concentration measurement; sub-cellular distribution of the CNIs could be an issue, CNI require immunophilins for their pharmacological action and immunological drug-drug interaction with other immunosuppressive agents can be expected. To overcome these variables, measurement of drug effect is at least in theory a more accurate alternative and should better reflect the actual immunosuppressive state induced by CNIs(71;72). Insights in individual CNI effectiveness together with their

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pharmacokinetic parameters could therefore, in theory, allow a more tailored dosing regime resulting in better CNi performance.

Efforts are made to monitor immunosuppressive effectiveness of CNIs on the level of target enzyme activity, transcripts and cytokine/ surface receptor expression. All show clear drug concentration vs. effects relations, but could not explain the large inter-individual variation of the markers by relevant clinical endpoints such as acute rejection thus far. Therefore further investigation and understanding of the potential of these techniques for monitoring and control of CNi treatment is very valuable. In this thesis the potential of CN activity as pharmacodynamic marker for monitoring CNi treatment is investigated.

Scope/Outline of this thesis

This thesis constitutes a comprehensive investigation into the analytical variables, fundamental concepts and clinical application of CN activity measurement as pharmacodynamic marker for CN inhibition therapy. First an assay for measurement of CN enzyme activity was developed and validated for its analytical performance (**Chapter 2**). To gain insights in the potential of CN activity as pharmacodynamic marker, CN inhibition, T cell cytokine excretion and their relation was investigated at therapeutic CsA concentrations (**Chapter 3**). Next, blood sample was investigated in order to optimize sample choice (**Chapter 4**). Since it was found that variable sample composition could affect measurement outcome and T cells are the major site of action of the CN inhibitors, a T cell specific calcineurin assay protocol was developed and validated as a novel pharmacodynamic monitoring tool for CsA therapy (**Chapter 6**).

To gain further insights in the opportunities and hurdles for pharmacodynamic strategies of CN inhibition therapy we performed a literature review (**Chapter 5**). To elucidate potential new applications of pharmacodynamic monitoring tools the pharmacological and immunological drug-drug interaction between mTOR inhibitors and TRL were investigated (**Chapter 7**).

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Chapter 2

Spectrophotometric assay for calcineurin activity in leukocytes isolated from human blood

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Abstract

The calcineurin inhibitors, cyclosporine and tacrolimus, still constitute the cornerstone of immunosuppressive regimen after organ transplantation. An efficient and feasible way to measure calcineurin activity and inhibition by these drugs may improve therapeutic monitoring of these drugs in transplant recipients. Calcineurin activity was measured in leukocyte lysates isolated from human blood using spectrophotometric phosphate quantification. The dephosphorylation of a 19-amino acid peptide substrate of calcineurin was determined using the Malachite green phosphate reagent in the presence of okadaic acid and with and without the calcium chelator EGTA. Sample storage and lysis buffer components were among the variables optimized, and the inhibitory effect of calcineurin inhibitors was investigated. Observed loss of calcineurin activity during sample storage was eliminated by adding ascorbic acid to lysis buffer. The final inter- and intraassay variation coefficients were 10 and 4.5%, respectively, and the detection limit was $15 \text{ pmol min}^{-1} \cdot 10^6 \text{ WBC}^{-1}$, where WBC is white blood cells (leukocytes). In vitro IC_{50} values were 212 and 34 $\mu\text{g/L}$ for cyclosporine and tacrolimus, respectively. In vivo calcineurin inhibition was observed when calcineurin activity was measured in transplant recipients on maintenance therapy with cyclosporine and tacrolimus.

Introduction

A quarter-century ago, the launch of the fungal cyclic undecapeptide cyclosporin A (CsA) as a maintenance immunosuppressant revolutionized organ transplantation. This was followed by the introduction of the structurally unrelated but mechanistically similar drug tacrolimus (TRL) and a microemulsion formulation of CsA with a more predictable absorption profile. Both drugs are inhibitors of the calcium-dependent protein phosphatase calcineurin (CN) that ultimately regulates lymphocyte activation-associated cytokine production by dephosphorylation of the nuclear factor of activated T cells(1–3). Despite the marked improvement in acute rejection rates, unfavorable cardiovascular profile and nephrotoxic potential of CsA and TRL question the long-term applicability of these drugs(4). The balance between the prevention of acute rejection episodes and the chronic toxicity associated with high concentrations of these drugs is critical and is described as a narrow therapeutic index. Trough blood calcineurin inhibitor (CNI) concentration is routinely used for therapeutic drug monitoring of CNIs; however, correlation with clinical outcome is unsatisfactory, and cases of acute rejection and chronic nephrotoxicity remain(5). Many attempts are being made to improve this pharmacokinetic monitoring via the use of area under the curve (AUC) models(6) and samples taken 2h after drug administration(5,7). The use of a pharmacodynamic parameter to quantify the intracellular inhibitory effect of the CNIs has been proposed(8), and the first pharmacodynamic analyses of CNIs in transplantation patients have been published(9–13).

Most current techniques that attempt to measure CN phosphatase activity in clinical samples monitor the Ca^{2+} -dependent dephosphorylation of a phosphopeptide substrate derived from the RII subunit of protein kinase A(10–12,14–16). Many groups refer to the method published by Fruman and coworkers that involves an isotopic form of this peptide substrate and chromatographic separation and measurement of the ^{32}P -labeled phosphate product(17). Another technique, published by Enz and coworkers, uses HPLC to separate and quantify the phosphorylated substrate and dephosphorylated product(18).

We aimed to develop a more rapid and feasible CN assay that can be used to measure CN activity routinely in a clinical setting. As a result, we present a new protocol involving a nonradioactive spectrophotometric-based phosphate detection assay that has been optimized to enable monitoring of CN phosphatase activity and its inhibition by the immunosuppressants CsA and TRL in leukocytes isolated from human blood.

Materials and Methods

Materials

The following materials were used: dithiothreitol (DTT), ascorbic acid, Nonidet P40 (NP-40), and soybean trypsin inhibitor from Sigma–Aldrich (Steinheim, Germany); phenylmethylsulfonyl fluoride from Merck (Darmstadt, Germany); leupeptin from Roche (Basel, Switzerland); aprotinin from Bayer (Leverkusen, Germany); phosphate standard concentrate from Fluka (Buchs, Switzerland); CsA microemulsion formulation from Novartis (Basel, Switzerland); and TRL from Fujisawa (Munich, Germany). The assay buffer, ethyleneglycoltetraacetic acid (EGTA) buffer, calmodulin, recombinant CN, okadaic acid, Malachite green reagent, and RII phosphopeptide substrate were obtained from the Biomol Green Cellular Calcineurin Assay Kit PLUS purchased from Biomol (Plymouth Meeting, PA, USA). All other chemicals and reagents were of the highest available grade.

Blood collection

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood from the antecubital veins of healthy volunteers was used for assay optimization and validation experiments. In addition, EDTA anticoagulated blood from the antecubital veins of patients on CsA and TRL maintenance therapy following kidney transplantation was used to study in vivo CN inhibition. Four patients (two males and two females) received CsA (100–125 mg twice a day [BID]) as

part of their immunosuppressive therapy, and four other patients (three males and one female) received TRL (2–4 mg BID) as part of their immunosuppressive therapy. The CsA-treated patients (ages 25–61 years) had undergone transplantation 3 months before, and the TRL-treated patients (ages 27–68 years) had undergone transplantation 6 months to 3 years before. Blood was taken immediately prior to drug intake (C0) and 2 h after drug intake (C2). From one patient on CsA (63-year-old male, 7 days after transplantation, 250 mg CsA BID) and one patient on TRL (47-year-old female, 7 days after transplantation, 6 mg TRL BID), blood was collected at multiple time points after drug intake.

Formal approval from the institutional ethics committee was obtained at the participating sites, and written informed consent was given before enrollment in the study.

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Preparation of total leukocyte lysates

Leukocyte lysates were prepared at 4°C from fresh blood and always within 24 h after sampling. To remove erythrocytes and platelets, 2.5 ml blood was added to 37.5 ml NH₄Cl lysis buffer (8.4 g/L NH₄Cl and 1.0 g/L KHCO₃, pH 7.3) and was incubated on ice for 10 min with inversion every 2 min. Intact leukocytes were spun down (350g, 10 min, 4°C), and supernatants containing erythrocyte and platelet lysates were discarded. Cell pellets were washed twice in 10 ml Hepes-buffered saline (9.0 g/L NaCl and 10 mM Hepes, pH 7.5), spun down (350g, 10 min, 4°C), and then resuspended in 1.5 ml Hepes-buffered saline. A 250-μl aliquot was taken for a leukocyte count performed on a Sysmax XE2100 (Mundelein, IL, USA), and 2 million leukocytes were aliquoted per microcentrifuge tube and were spun down (500g, 10 min, 4°C). Supernatants were removed, and leukocytes were resuspended in 200 μl lysis buffer (50 mM Tris-HCl [pH 7.7], 1.0 mM DTT, 5.0 mM ascorbic acid, 0.02% [v/v] NP-40, 50 mg/L soybean trypsin inhibitor, 50 mg/L phenylmethylsulfonyl fluoride, 5.0 mg/L leupeptin, and 5.0 mg/L aprotinin) and were lysed by three freeze-thaw cycles (liquid N₂/30°C). Cell debris was spun down (10000g, 10 min., 4°C), and supernatant was collected. Lysates were snap-frozen in liquid N₂ and stored at -80°C until they were assayed for CN activity.

CN activity assay

Two phosphate quantifications were performed per sample using a 96-well plate (half area, Costar, Corning): one in the presence of EGTA and the second in the presence of Ca²⁺ and calmodulin.

EGTA wells contained 40 μl of 12.5 mM EGTA, 0.375 mM RII phosphopeptide substrate, 75 mM Tris (pH 7.5), 150 mM NaCl, 9 mM MgCl₂, 0.75 mM DTT, 0.0375% NP-40, 0.625 μM okadaic acid, 0.125 mM CaCl₂, and 5.0 mM ascorbic acid. In the calmodulin/Ca²⁺ wells, EGTA was excluded and replaced by 0.313 μM calmodulin and 0.75 mM CaCl₂. To correct for background absorbance, blanks were performed for both the EGTA and calmodulin/Ca²⁺ wells for every sample. In these wells, substrate was absent. A phosphate series of 0 to 5 nmol was used for calibration, and one aliquot of 50 U (specific activity was determined using Biomol assay kit protocol) pure recombinant human CN was used as an interassay control. Reactions were initiated via the addition of 15 μl thawed leukocyte lysate sample to all wells, resulting in a final assay volume of 55 μl. After 30 min, reactions were stopped by adding 100 μl Malachite green reagent, the plate was mixed, and color was allowed to develop for 50 min at room temperature before absorbance was read at 620 nm using a 96-well plate spectrophotometer (SpectraMax 250,

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Molecular Devices, Downingtown, PA, USA). The Softmax Pro (version 2.4.1) program was used for curve fitting (four-parameter fit).

For CN activity calculation, background absorbance for EGTA and Ca^{2+} /calmodulin wells was subtracted and phosphate was quantified with the calibration curve. The difference in phosphate between EGTA and Ca^{2+} /calmodulin wells (expressed as $\text{pmol min}^{-1} \cdot 10^6 \text{ WBC}^{-1}$, where WBC is white blood cells [leukocytes]) was defined as CN activity.

In vitro CN inhibition

CsA and TRL were diluted to appropriate concentrations using phosphate-buffered saline (154 mM NaCl and 1.4 mM phosphate, pH 7.5). Then 2.5 μl diluted inhibitor stock solution was added to 2.5 ml fresh blood to yield final concentrations of 0 to 5000 $\mu\text{g/L}$ CsA and 0 to 150 $\mu\text{g/L}$ TRL. These samples were incubated for 1 h at 37°C before leukocyte lysate preparation.

Concentrations of CsA and TRL in peripheral blood

CsA concentration was measured in whole blood by fluorescence polarization immunoassay using an Abbott AxSYM system (Abbott Park, IL, USA). TRL concentration was measured in whole blood by microparticle enzyme immunoassay using an Abbott IMx system. The assays were performed according to the manufacturer's instructions.

Statistical analysis

Inhibition curves and IC_{50} values were fitted and analyzed using sigmoidal dose–response curves with GraphPad Prism (GraphPad Software, San Diego, CA, USA) and are described within the 95% confidence interval (CI) range. Other results are reported as mean values, with errors representing 1 standard deviation of the mean. In addition, t tests were used to test significance, and statistical significance was defined as $P < 0.05$.

Results

Optimization of assay conditions

Progress curves were performed to ensure that the initial rate of CN phosphatase activity was measured and to enable optimization of assay duration and cell lysate concentration. During a 30 min reaction using a lysate of $1.5 \cdot 10^5$ WBC, activity rate

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was observed to remain linear and within the range of the phosphate standard curve. These conditions were used in all subsequent experiments (not shown).

Maintaining CN activity during storage

A loss of CN activity was observed repeatedly when leukocyte lysate samples were stored at -80°C prior to assay performance. Ascorbic acid subsequently was added to our lysis buffer in an attempt to maintain CN in its reduced and active state. Samples were lysed and stored in lysis buffer with or without 5.0 mM ascorbic acid and then were assayed for CN activity after 0, 1, 2, 7, and 21 days storage at -80°C (Figure 2.1).

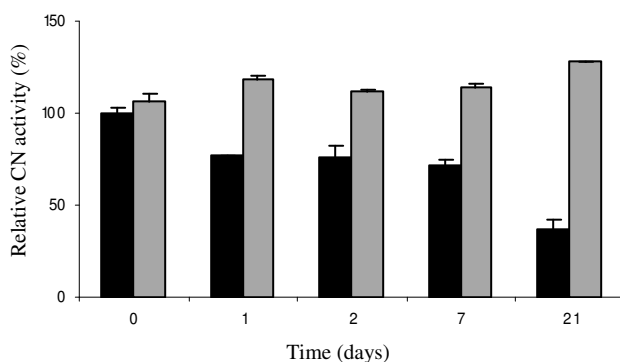


Figure 2.1. CN activity after storage of cell lysate at -80°C . Black bars: without ascorbic acid in the lysis buffer; gray bars: with ascorbic acid (5.0 mM) in the lysis buffer. Columns represent the means of duplicates, and error bars show +1 SD.

The loss of CN activity observed in time was eliminated with the addition of ascorbic acid to the lysis buffer. In this way, the CN activity measured in our samples could be maintained during storage at -80°C for at least 21 days. Subsequent assays always were performed within three weeks of storage.

When lysate samples were added to the reaction mixture during the CN activity assay, the additional ascorbic acid in the lysis buffer was diluted from 5.0 mM to 1.4 mM. To compensate for this, extra ascorbic acid was added directly to the reaction mixture, leading to a 60% increase in the CN activity level. Further observations included nearly total inactivation of CN at ascorbic acid concentrations above 10.0 mM. Ascorbic acid subsequently was added to a final concentration of 5.0 mM in the assay.

A time-dependent loss of CN activity was also observed when whole donor blood was stored at 4°C for a period of 72 h. Aliquots of the stored blood were taken, prepared, and assayed for CN activity after 0 (fresh), 24, 48, and 72 h of storage.

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During the 72-h period, a loss of 47% CN activity was observed despite the presence of ascorbic acid in the lysis buffer. Subsequently, all blood was prepared as soon as possible after sampling and always within 24 h, when activity loss was less than 15%.

Components of the lysis buffer

In addition to ascorbic acid, various other components of the lysis buffer were observed to have a significant effect on CN activity levels. Although many groups add the cation chelators EDTA and EGTA to their lysis buffers to inhibit cation-dependent protease activity, the Ca^{2+} -dependent activity of CN is also affected. The effect of 0.1 mM EDTA or 0.1 mM EGTA in the lysis buffer on CN activity was tested, and it was shown that CN activity was decreased to 21 ± 9 and $52 \pm 12\%$, respectively ($n = 4$), indicating that these cation chelators cause a decrease in activity and must be removed.

The nonionic detergent NP-40 was present in assay buffer and was also observed to increase CN activity when added to our lysis buffer. An optimal increase in CN activity was observed between concentrations of 0.01 and 0.025% (v/v) NP-40 in the lysis buffer. In comparison with the sample lysis without NP-40, increases of 237 ± 30 and $257 \pm 23\%$ in CN activity were achieved by adding 0.01 and 0.025% (v/v) NP-40, respectively ($n = 2$). For subsequent experiments, we chose to add 0.02% NP-40 to our lysis buffer, resulting in an assay NP-40 concentration of 0.033%.

Standard curve

A typical phosphate standard curve is shown in Figure 2.2A. Softmax Pro software was used for curve fitting. To check linearity of our assay, leukocyte lysate was serially diluted and resulted in a linear relation between dilution and CN activity (Figure 2.2B).

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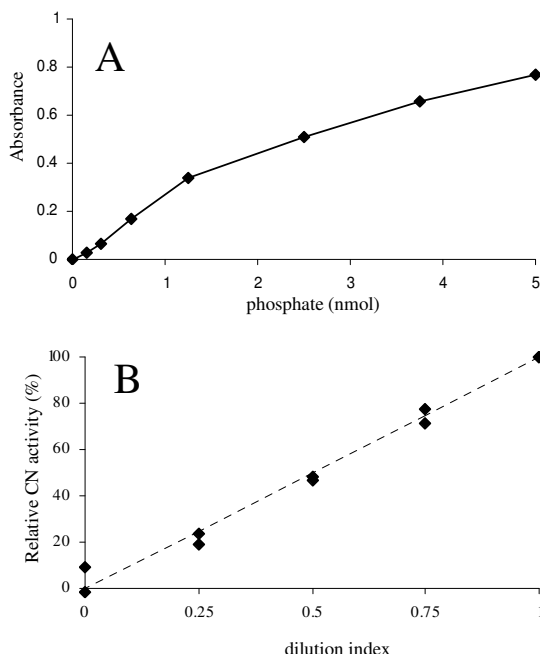


Figure 2.2. (A) Typical phosphate standard curve. (B) Dilution series of sample. The CN activity is calculated with the phosphate standard curve. Diamonds represent sample dilutions, and the dotted line indicates the expected dilution line. This experiment was performed in duplicate.

CN assay validation

To identify the interassay variation, five blood samples of one volunteer taken within a 2-month period were prepared separately and analyzed on different days (including intraindividual variation). This resulted in a coefficient of variation (CV) of 10%. One leukocyte lysate sample was also assayed repeatedly within one experiment at both high and low concentrations to provide intraassay CVs of 4.5 and 2.2%, respectively ($n = 9$).

The detection limit was $15 \text{ pmol min}^{-1} \cdot 10^6 \text{ WBC}^{-1}$, calculated as the mean of the activity measured for sample blanks plus 3 standard deviations ($n = 9$). The lower limit of quantification, calculated as the mean of blanks plus 6 standard deviations, was $31 \text{ pmol min}^{-1} \cdot 10^6 \text{ WBC}^{-1}$.

Analytical recovery was determined by adding recombinant CN to leukocyte solution after erythrocyte lysis, resulting in a recovery of $101 \pm 13\%$ ($n = 3$).

In vitro inhibition of CN activity

The inhibition of CN by CsA and TRL was studied in vitro by adding different concentrations of these immunosuppressive drugs to blood obtained from healthy

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volunteers. The inhibition curves are shown in Figure 2.3. IC_{50} values were calculated to be 212 $\mu\text{g/L}$ CsA (95% CI, 67–668 $\mu\text{g/L}$) and 34 $\mu\text{g/L}$ TRL (95% CI, 13–88 $\mu\text{g/L}$).

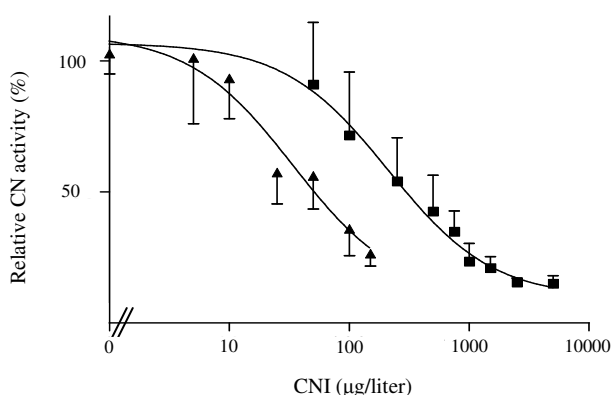


Figure 2.3. In vitro inhibition of CN by CsA (squares) and TRL (triangles). The means $\pm$ 1 SD of four healthy volunteers are displayed. Curves were fitted and analyzed using GraphPad Prism.

In vivo inhibition of CN activity

The in vivo activity and inhibition of CN was also studied using blood samples obtained from kidney transplant patients treated with CN inhibitors CsA and TRL. The CN activities obtained from patients, both prior to (C0) and 2 h after (C2) oral administration of CsA and TRL, are displayed in Figure 2.4.

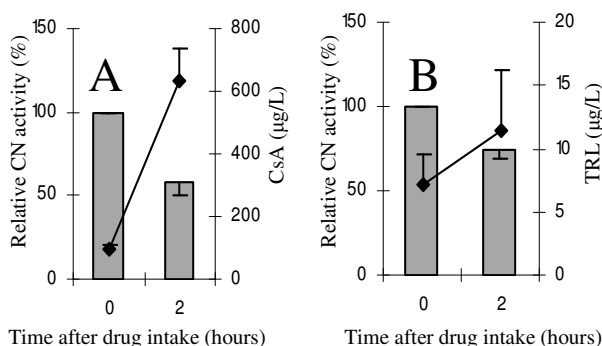


Figure 2.4. In vivo CN inhibition in patients who received CsA and TRL therapy. Bars represent relative CN activity, and diamonds represent full blood drug concentration. (A) C0 and C2 CsA concentrations and relative CN activities of four different patients on CsA therapy. (B) C0 and C2 TRL concentrations and relative CN activities of four different patients on TRL therapy.

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Blood CsA concentration increased from a mean of $94 \pm 13 \mu\text{g/L}$ at C0 to $636 \pm 105 \mu\text{g/L}$ at C2 and resulted in a $42 \pm 8\%$ inhibition of baseline CN activity at C2. Blood TRL concentration increased from a mean of $7.1 \pm 2.4 \mu\text{g/L}$ at C0 to $11.5 \pm 4.8 \mu\text{g/L}$ at C2 and resulted in a $26 \pm 5\%$ inhibition of baseline CN activity at C2.

In addition, *t* tests were performed to identify differences in drug concentrations and CN activities between C0 and C2. For patients treated with CsA as well as patients treated with TRL, significantly higher CN activities were observed at C0 compared than at C2 (*Ps* = 0.004 and 0.01, respectively), whereas significantly lower drug concentrations were observed at C0 than at C2 (*Ps* = 0.0015 and 0.04, respectively). For all patients, higher drug concentrations were observed at C2 than at C0, and inhibition of CN was observed at C2.

To illustrate the potential of this assay for CN monitoring in CNI-treated patients, two CN inhibition curves of patients who had undergone kidney transplantation are shown in Figure 2.5. In these patients, inverse relations between CN activity and drug concentrations are observed.

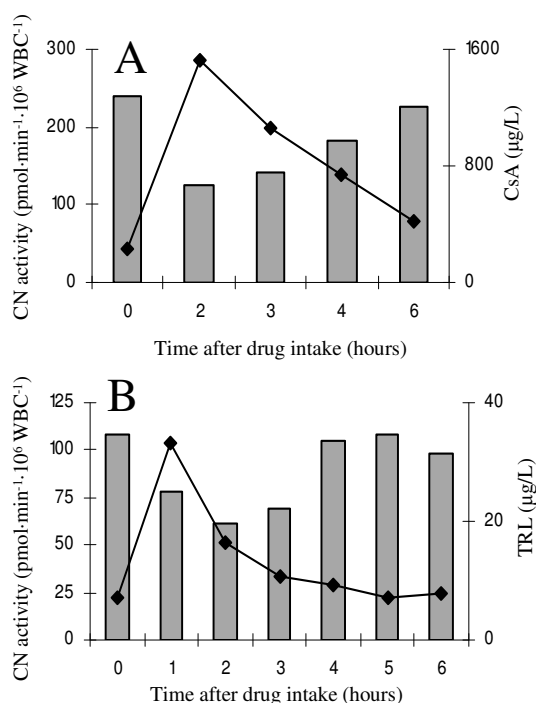


Figure 2.5. In vivo CN monitoring. Bars represent CN activity, and diamonds represent full blood drug concentration. (A) Example of CN inhibition in time by CsA (63-year-old male, 7 days after transplantation, 250 mg BID CsA). (B) Example of CN inhibition in time by TRL (47-year-old female, 7 days after transplantation, 6 mg BID TRL).

Discussion

The main reason for developing the spectrophotometric CN activity assay was to enable assessment of pharmacodynamic CN monitoring in patients treated with the CNIs CsA and TRL. The spectrophotometric detection principle allows us to avoid the use of radioactive materials and organic solvents. A linear increase of phosphate in time was observed for at least 40 min, indicating that substrate is in excess, and we decided to use a 30 min running time.

The concentration of okadaic acid in the final reaction mixture was 0.45 μM , inhibiting protein phosphatase 1 (PP1) and PP2A, which have okadaic acid IC_{50} values lower than 10 nM. Meanwhile, the IC_{50} value of CN exceeds 10 mM(19), resulting in selective inhibition of these other protein phosphatase enzymes. The CN activity was distinguished from okadaic acid-resistant, calcium-independent PP2C activity by subtracting the phosphatase activity measured in the presence of the calcium chelator EGTA from that measured in excess calcium, resulting in calcium-dependent phosphatase activity. PP2C activity is the calcium- independent phosphatase activity measured in the presence of okadaic acid and was found to be less than 5% of the CN activity in healthy volunteers.

Optimization studies involved modifications to the lysis buffer. Because these resulted in significantly higher activity levels and subsequently in lower detection limits, we reduced the requirement for blood and increased lysate storage times. To preserve in vivo conditions, and in particular the state of CN inhibition within the patient, as much as possible, we chose to maintain samples at 4 °C throughout sample preparation. However, the separation of peripheral blood mononuclear cells (PBMCs) using a Ficoll–Hypaque gradient, a technique used by many groups, should be performed at room temperature to maintain the density of the Ficoll solution. Due to this temperature limitation and to the variable amounts of erythrocytes, platelets, and granulocytes observed to remain after Ficoll separation(10), we decided to simply remove erythrocytes and platelets at 4 °C using ammonium chloride lysis buffer and to measure CN activity in total leukocyte lysates that contain PBMCs and granulocyte fraction. This technique requires a lower volume of blood, and separation is less cumbersome and more reproducible than it is with the Ficoll gradient procedure. Support for using material additional to PBMCs comes from work by other groups, such as Halloran and coworkers(20), who have shown that CN inhibition patterns in whole blood and PBMCs are in accord.

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The observed loss of CN activity on sample storage(10,14) was hypothesized to be due to inactivation of CN via oxidation of the binuclear $\text{Fe}^{2+}\text{-Zn}^{2+}$ center found at the CN active site(21,22). This same mechanism was proposed to cause a 10- to 20-fold loss of activity during the purification of CN from human tissue(21,23). Various antioxidants, including ascorbic acid, were found to have an activating effect on CN(23). When ascorbic acid was added to our lysis buffer, the level of CN activity could be maintained during the storage of lysate samples at -80°C for a minimum of 21 days (Figure 2.1). Activity levels were further increased when ascorbic acid was added directly to the reaction mixture and an optimal activity was achieved in the presence of 5.0 mM ascorbic acid, in agreement with data for purified CN published by Sommer and coworkers(23). The effect of the reducing agent DTT on enzyme activity and storage was also investigated, but no significant effect was observed.

The cation chelators EDTA and EGTA originally were included in the lysis buffer of Fruman and coworkers to inhibit cation-dependent proteases(17). That study stated that these subsequently were omitted (in some experiments) because they were found to have no protective effect on cellular phosphatase activity. Considering that the removal of Ca^{2+} is known to inactivate CN and that EGTA is used in this way to define Ca^{2+} -dependent phosphatase activity in the assay, it does not seem sensible to add cation chelators to the reaction mixture.

The nonionic detergent NP-40 was added to the lysis buffer at the concentration at which we observed maximum CN activity. In one study, NP-40 was used to extract CN from the T lymphocyte membrane with which it is reported to be associated(24). The release of membrane-associated CN during lysis would explain the rise in CN activity observed. However, at NP-40 concentrations higher than 0.025% (v/v), the level of CN activity gradually decreases, probably due to partial denaturation.

The phosphate standard curve used for this assay is not linear and becomes insensitive at higher phosphate concentrations. Therefore, we recommend that samples with an absorbance higher than 0.75 be diluted to ensure assay performance. Assay linearity was observed in sample dilution and standard addition experiments. In vitro CN inhibition experiments resulted in CsA and TRL IC_{50} values of 212 and 34 $\mu\text{g/L}$, respectively. These values are similar to those described previously(10,11) and agree with reports that TRL is more potent than CsA(2).

CN activity and inhibition was also measured in samples obtained from four kidney transplantation patients on CsA therapy and four kidney transplantation patients on TRL therapy. In both CsA and TRL patients, CN inhibition is observed given that

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significantly higher drug concentrations at C2 resulted in significantly lower CN activities when compared with C0. Interestingly, no total inhibition of CN activity was observed for any patient, a phenomenon that was observed previously by others(25). The data presented show that in vivo binding of CNIs to their immunophilins and calcineurine is reflected in the CN assay. In vivo measurement of CN activity in patients who undergo immunosuppressive therapy by CNIs has the potential to guide drug dosing and improve clinical outcome, although the benefits of pharmacodynamic monitoring have not yet been proven. The ability of this assay for pharmacodynamic monitoring of CN inhibition therapy is illustrated in Figure 2.5, where CN activity is measured at multiple time points after drug intake. In conclusion, the protocol presented requires 2.5 ml fresh blood and standard laboratory equipment. Multiple samples can be prepared and assayed for CN phosphatase activity within 1 working day with high intra- and interassay reproducibility. The spectrophotometric assay is flexible and can be used to measure CN activity in lysates of leukocytes, PBMCs, and combinations of other blood cell subsets as well as in other cell types. The assay can be used to monitor CN activity in patients that undergo CN inhibition therapy and to assess the role of pharmacodynamic CN monitoring in clinical practice.

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Chapter 3

Calcineurin activity and cytokine secretion at therapeutic cyclosporine whole blood concentrations

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Submitted

Abstract

Background: Calcineurin (CN) activity has the potential to become a pharmacodynamic marker to monitor CN inhibitor (CNI) therapy after organ transplantation. We investigated the relation between CN activity and cytokine secretion at therapeutic cyclosporine (CsA) levels in order to visualize CsA based immunosuppression and the applicability of the CN activity marker.

Methods: Healthy volunteer whole blood incubated with CsA was used as a model for allograft recipient blood. CN activity and cytokine (IFN γ , TNF α) secretion after CD3/CD28 stimulation were determined.

Results: A concentration of 25000 $\mu\text{g/L}$ CsA, used as CsA excess, showed only a minor increase in CN inhibition and no increase in cytokine (IFN γ , TNF α) inhibition when compared to 2500 $\mu\text{g/L}$ CsA. Between therapeutic target concentrations (100, 250, 600, and 1700 $\mu\text{g/L}$ CsA) significant differences in inhibition of cytokine secretion and CN activity were observed. Inhibition of cytokine secretion ranged from 0 to 90% while CN inhibition ranged from 25 to 48%. Additional Cyclophilin A could not increase CN inhibition by CsA in cell lysates, while addition of CsA and both CsA and Cyclophilin A could.

Conclusions: Whole blood concentrations of 2500 $\mu\text{g/L}$ CsA approaches immunosuppressive saturation and is not yet limited by the CyP content. At the therapeutic CsA range, therapeutic target concentrations represented distinct immunosuppressive states. In addition, small increases in CN inhibition resulted in relative larger suppression of cytokine secretion. Altogether, CN activity as well as cytokine secretion after T cell specific stimulation is able to visualize CsA pharmacodynamics at relevant *in vivo* CsA concentrations.

Introduction

The calcineurin inhibitors (CNI), cyclosporine (CsA) and tacrolimus (TRL), are still widely applied in immunosuppressive regimen to prevent allograft rejection after organ transplantation(1). Despite proven efficacy, their apparent lack in selectivity remains a major drawback, resulting in an unfavorable cardiovascular profile and lack of improvement on the long-term(2). To overcome these issues, CNI minimization and avoidance have been investigated with varying success(3-5).

The wide variation in inter- and intra-individual pharmacokinetic parameters requires frequent therapeutic drug monitoring to guide dosing. Further optimization of CNI therapy by new monitoring strategies such as the use of pharmacogenetic and pharamcodynamic biomarkers are currently under investigation(6,7).

CNIs suppress allograft reactivity by blocking the intracellular T cell recognition signaling pathway(1). In T cells, CsA inhibits calcineurin (CN) phosphatase activity that is required for translocation of the transcription factor NFAT into the nucleus. Upon translocation, NFAT can transcribe cytokines, such as IL-2, IFN γ and TNF α that control T cell mediated immune responses(8). CN inhibition by CsA can only occur after dimerization with members of the cyclophilin (CyP) family(9). The CsA/CyP complex blocks substrate excess to the catalytic active binding site of CN and are therefore the true pharmacological inhibitors of CN(10). Since only partial inhibition of CN is observed in transplant recipients and, *in vitro*, excess of CsA only partially inhibited CN activity, it has been suggested that CyPs might limit CN inhibition at higher CsA concentrations(11,12). This view was supported by further *in vitro* experiments showing that addition of Cyclophilin A (CyPA), at high CsA concentrations, could further increase the degree of CN inhibition(12). The relevance of *in vitro* CyP limitation, for allograft recipients remains unknown.

To determine the potential of CN activity as a pharmacodynamic biomarker, it is relevant to gain insights in the relation between the achieved CNI inhibition at therapeutic relevant target levels and the resulting immunosuppressive state, measured by cytokine secretion. We therefore investigated the effect of representative CsA concentrations in whole blood on CN activity and T cell specific cytokine secretion, determined by IFN γ and TNF α measurement after CD3/CD28 stimulation.

Materials and Methods

Healthy volunteers and kidney transplant recipients blood

EDTA-or heparin-anticoagulated blood of healthy volunteers was obtained via Sanquin Bloodbank (Amsterdam, NL). For simulation of recipient blood, 2 mL of EDTA-anticoagulated blood or 1 mL of heparin anticoagulated blood of healthy volunteers was incubated (1h, 37°C) with various CsA stock solutions resulting in final ethanol concentrations of 0.25% (v/v). For investigation of CsA pharmacodynamics, CsA concentrations were used that represented trough and peak concentrations regularly found in renal allograft recipients. These conditions are referred to as T0 and T2 respectively. As representative CsA concentrations: 100 µg/L (late T0), 250 µg/L (early T0), 600 µg/L (late T2) and 1700 µg/L (early T2), were chosen.

Six patients on CsA immunosuppressive therapy after living donor renal transplantation were included in this study. The patients underwent renal allograft implantation within 2 weeks prior to sample date and received a quadruple regimen comprising basiliximab (20 mg, days 0 and 4), prednisolone (10 mg/bid), mycophenolate mofetil (1 g/bid) and CsA starting 4 mg/kg bid. EDTA-anticoagulated blood was taken just prior to dose (T0, 12 hours after the previous dose) and 2 hours after drug intake (T2). Mean CsA whole blood concentrations at T0 was 204 ± 71 µg/L and 983 ± 278 µg/L at T2. Formal approval from the institutional ethics committee was obtained and informed consent for blood sampling was obtained from all participants. CsA concentrations of renal allograft recipient blood were measured by fluorescence polarization immunoassay using an Abbott AxSYM system (Abbott Park, IL, USA). The assays were performed according to the manufacturer's instructions.

Preparation of leukocyte lysate for CN measurement

Leukocyte lysates of CN inhibitor incubated EDTA-anticoagulated blood were prepared as previously described(13). In brief; 2 mL of EDTA-anticoagulated blood was lysed with NH₄Cl lysis buffer (8.4 g/L NH₄Cl, 1.0 g/L KHCO₃, pH 7.3) and washed twice with 10 mL HBS before resuspension in 1.5 mL Hepes buffered saline (HBS, 9.0 g/L NaCl, 10 mmol/L Hepes pH 7.5) for cell count on a Sysmex XE2100.

Cell lyses was performed at a cell concentration of $10 \cdot 10^6$ cells/mL, therefore $2 \cdot 10^6$ cells were resuspended in 200 µL lysis buffer (50 mmol/L Tris HCl pH 7.7, 1.0 mmol/L dithiothreitol, 5.0 mmol/L ascorbic acid, 0.02% (v/v) NP-40, 50 mg/L

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soybean trypsin inhibitor, 50 mg/L phenylmethysulphonyl fluoride, 5.0 mg/L leupeptin and 5.0 mg/L aprotinin) and lysed by 3 freeze thaw cycles (liquid N₂ / 30°C). Cell debris was spun down (10 min, 10000g, 4°C) and supernatants were snap frozen in liquid N₂ before storage at -80°C till cyclophilin A lysate experiments and/or CN activity measurement.

For cyclophilin A (CyPA) / CsA experiment in cell lysate, lysate either or not prepared from CsA pre-incubated whole blood or patient blood was incubated with CsA and/or CyPA. Final ethanol concentrations were 1%. Samples were incubated for 1 hour at 37°C and CN activity was determined directly after incubation.

CN activity measurement

CN enzyme activity was determined by spectrophotometric quantification of phosphate released after incubation with the phosphorylated RII substrate as has been described before(13). In brief, 15 µL of lysate was added to 40 µL of assay buffer (0.313 µmol/L calmodulin, 0.375 mmol/L RII phosphopeptide substrate, 75 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 9 mmol/L MgCl₂, 0.75 mmol/L dithiothreitol, 0.0375% NP-40, 0.625 µmol/L okadaic acid, 0.75 mmol/L CaCl₂ and 5 mmol/L ascorbic acid) in presence and absence of substrate. Phosphatase reaction was run for 30 min at 30°C, the reaction was stopped by adding 100 µL malachite green reagent and the absorbance (620 nm, SpectraMax 250, Molecular Devices, Downingtown, PA, USA)) was read after 50 min incubation at RT. Background absorbance from blank measurement was subtracted and phosphate was quantified out of a 0-1.5 nmol phosphate calibration curve.

IFN γ and TNF α excretion

For cytokine determination, 30 µL of anti-CD3/anti-CD28 stimulation solution was added to the CN inhibitor / Heparin-anticoagulated blood resulting in final concentrations of 0.5 mg/L anti-CD28 and 0.12 mg/L anti-CD3. Samples were incubated for another 4 hours at 37°C under gentile rotation. After centrifugation (1550g, 20 min, 20°C), plasma was collected and stored at -80°C until cytokine concentration determination. IFN γ and TNF α plasma concentrations were determined using ELISA technique and performed according to manufacturers' protocol, based on(14).

Data analysis

Data was normalized and expressed as % of blank measurement in order to correct for inter-individual variation. Data in the text are presented as mean $\pm$ SD.

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Student t-tests were used to test significance and significance was defined as $p < 0.05$.

Materials

The following materials were used; cyclosporine A, dithiothreitol (DTT), ascorbic acid, NP-40, Cyclophilin A (CyPA), and soybean trypsin inhibitor from Sigma Aldrich (Steinheim, Germany); phenylmethylsulfonyl fluoride from Merck (Darmstadt, Germany), Leupeptin from Roche (Basel, Switzerland); aprotinin from Bayer (Leverkusen, Germany); Phosphate standard concentrate from Fluka (Buchs, Switzerland); mouse anti-CD28 and mouse anti-CD3 from BD Biosciences (San Jose, CA, USA); CN assay components from the Biomol Green Cellular Calcineurin Assay Kit Plus from Biomol (Plymouth Meeting, PA, USA). Buffycoats, ELISAs (IFN γ and TNF α) were from Sanquin (Amsterdam, NL).

Results

Extent of CsA mediated immunosuppression

First, the extent of CsA mediated immunosuppression for *in vivo* conditions was investigated. Therefore, the pharmacodynamic effects of high range relevant CsA *in vivo* whole blood concentrations were determined relative to baseline level and a condition of CsA excess. Whole blood from healthy volunteers was incubated with 2500 $\mu\text{g/L}$ and 25000 $\mu\text{g/L}$ CsA. Whole blood incubated with final CsA concentrations of 2500 $\mu\text{g/L}$ represented a relevant high range *in vivo* concentration and 25000 $\mu\text{g/L}$ CsA was used as a condition of CsA excess. CN enzyme activity and plasma cytokine concentrations after CD3/CD28 stimulation were determined, see figure 3.1.

Compared to 2500 $\mu\text{g/L}$ CsA, a minor but significant decrease in CN inhibition was observed for 25000 $\mu\text{g/L}$ CsA ($p = 0.004$). For IFN γ and TNF α no differences in plasma cytokine concentrations were observed between 2500 and 25000 $\mu\text{g/L}$ CsA with $p = 0.18$ for IFN γ and $p = 0.25$ for TNF α . These results indicated that, in the system used, a whole blood CsA concentration of 2500 $\mu\text{g/L}$ approaches the maximum immunosuppressive effect of CsA.

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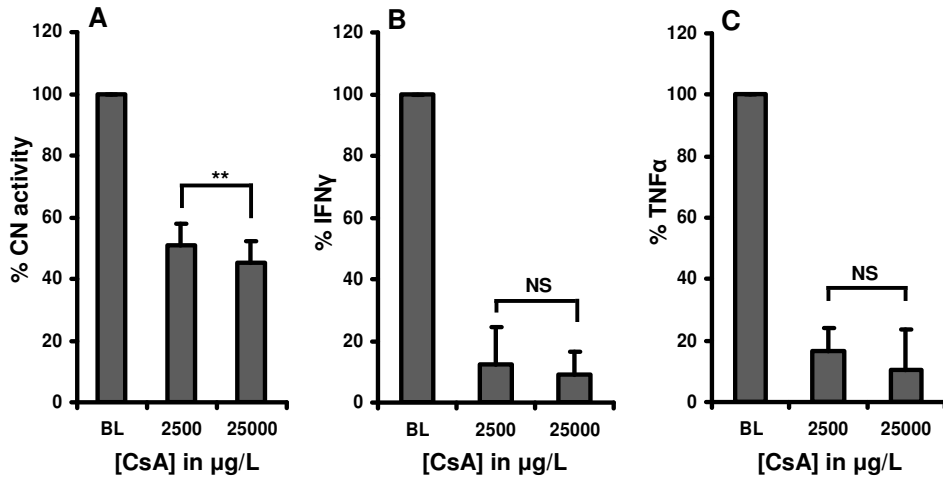


Figure 3.1. The extent of CsA pharmacodynamics. Healthy volunteer whole blood was incubated with final CsA concentrations of 0 (BL), 2500 and 25000 µg/L. A; CN activity (n=14). B; IFN γ (n=6) and C; TNF α (n=6). Data are expressed relative to Baseline (BL) within each volunteer and presented as mean + SD. ** for $p < 0.01$ and NS (not significant) for $p \geq 0.05$.

Pharmacodynamics under representative *in vivo* CsA concentrations

Next, the immunosuppressive dynamics at simulated representative CsA T0 and T2 conditions for allograft recipients at early and late stages after graft implantation were investigated. The results are plotted in figure 3.2.

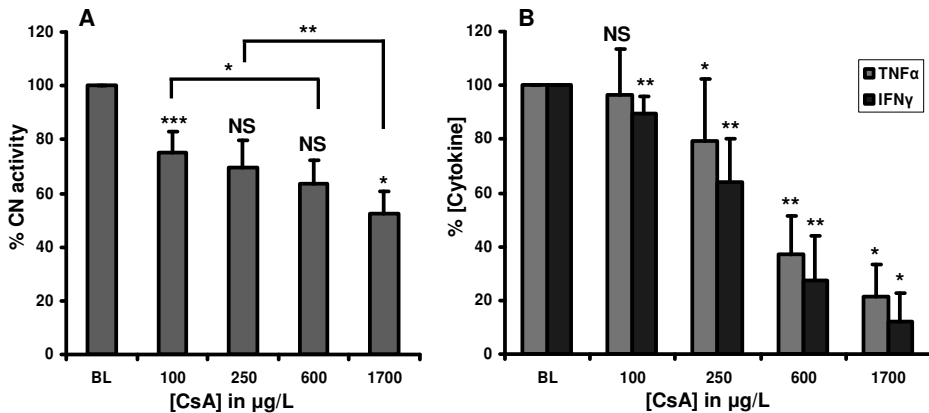


Figure 3.2. Pharmacodynamics of CsA for whole blood target conditions. A; relative CN inhibition (n=8) and B; relative TNF α and IFN γ plasma concentrations (n=7). Data are all expressed relative to baseline (BL) condition per volunteer and are presented as mean + SD. Statistics presented in the figure on the bars represent t-testing with respect to the lower CsA concentration. *** for $p < 0.001$, ** for $p < 0.01$, * for $p < 0.05$ and NS (not significant) for $p \geq 0.05$.

A CN inhibition of $25 \pm 8\%$ was observed at 100 µg/L CsA and a CsA concentration dependant increase in CN inhibition was found with $48 \pm 8\%$ of CN inhibition at

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1700 µg/L. For IFN γ and TNF α the CsA concentration range was responsible for much larger relative dynamic effects between conditions chosen and covered almost the full dynamic range from no inhibition to total inhibition of cytokine excretion. At 100 µg/L CsA a small (IFN γ , $11 \pm 6\%$, $p=0.004$) or no reduction (TNF α , $p=0.27$) was observed, while at 1700 µg/L, cytokine inhibition was $88 \pm 11\%$ for IFN γ and $79 \pm 12\%$ for TNF α . Each increase in CsA concentration led to a further significant decrease in IFN γ and TNF α .

CyP limitation

Since the dynamic range of CN inhibition was more narrow than observed for inhibition of cytokine secretion, we investigated whether cyclophilin content could be responsible for limiting CN inhibition at high CsA concentrations. Limitation of CyP on CsA based CN inhibition at a whole blood concentration of 2500 µg/L CsA was investigated by (cyclophilin A) CyPA and CsA addition experiments in leukocyte lysate. The leukocyte lysate used was obtained from whole blood previously incubated with 2500 µg/L CsA (L2500). The lysates were incubated with and without additional 5000 µg/L CsA and/or 40000 µg/L CyPA.

Addition of CsA to L2500 lysate could further increase CN inhibition ($p=0.027$) and an additional increase in CN inhibition was observed by the addition of CyPA and CsA ($p=0.003$). Addition of CyPA itself did not significantly affected CN activity ($p=0.066$), see figure 3.3.

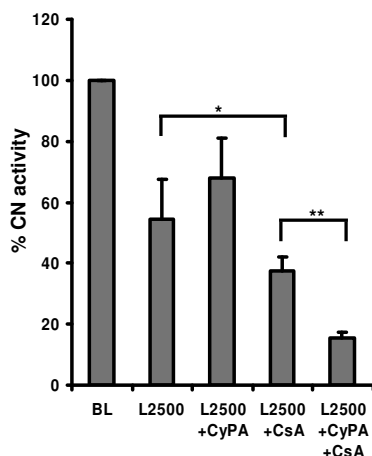


Figure 3.3. Cyclophilin limitation. Sample lysate of full blood incubation with 2500 µg/L CsA. was incubated with 40000 µg/L CyPA and 5000 µg/L CsA. BL is leukocyte lysate of whole blood without CsA and L2500 is leukocyte lysate prepared from whole blood incubated with 2500 µg/L CsA. Data are presented as mean + SD. * is used for $0.05 > p > 0.01$ and ** is used for $p \leq 0.01$.

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Peripheral blood obtained from 6 renal allograft patients treated with CsA was used to investigate the relevance of CN inhibition limitation by cyclophilins for allograft recipients. Patient samples were taken within the first two weeks after transplantation, when whole blood target concentrations or CsA are highest. Sample lysate was prepared and lysate prepared from T2 sample was incubated with or without 20000 µg/L CsA, 40000 µg/L CyPA or both. After addition of either CyPA or CsA to sample lysate, a decrease in CN activity of $8 \pm 6\%$ for CyPA ($p=0.04$) and $30\% \pm 8\%$ for CsA ($p=0.004$) were observed. Addition of both CsA and CyPA led to additional inhibition of CN activity when compared to previous conditions.

Relation between CN inhibition and cytokine inhibition

To investigate the relation between CN inhibition and inhibition of T cell cytokine excretion, we plotted the calcineurin inhibition and cytokine excretion inhibition data from the previous experiments in one graph, see figure 3.4.

Here it is shown that at over a certain CN inhibition threshold, which is located around 100 µg/L CsA, a small increase in CN inhibition resulted in relative large increase in cytokine secretion inhibition. At 2500 µg/L, CsA approaches its maximal immunosuppressive state.

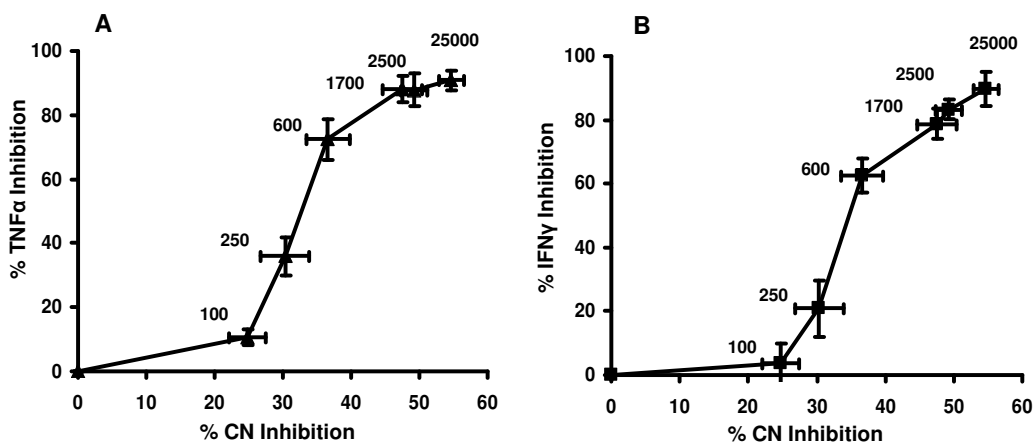


Figure 3.4. Relation between CN and cytokine inhibition. Relative TNFα (A) and IFNγ (B) inhibition is plotted against the CN inhibition observed at different CsA concentrations. The numbers in the graph represent CsA concentration in µg/L. Error bars represent SEM values.

Discussion

To further optimize CNI therapy, pharmacodynamic monitoring strategies are currently investigated as potential tool to guide dosing. Previously we developed a CN assay to monitor CN activity in allograft recipients treated with CNIs(13). In the present study we assessed the relation between CN activity and the decrease in T cell related cytokine secretion at therapeutically relevant CsA whole blood concentrations. The results show that at relevant CsA concentrations for renal allograft recipients, small changes in CN inhibition resulted in a significant change in cytokine secretion and, at high CsA concentrations, approached immunosuppressive saturation.

Complete inhibition of cytokine excretion with only partial inhibition of CN enzyme activity has been reported previously(12). These studies have been very valuable in understanding the pharmacologic effects of CNIs, but since they have been performed in culture systems their clinical relevance and applicability has remained unclear. We chose whole blood incubated with CsA as a model to simulate allograft recipient blood treated with CsA. Due to the absence of relevant CsA binding sites, CsA-related pharmacodynamic parameters under culture conditions may not correspond to these markers in whole blood(15). In addition, by using healthy volunteer blood, the pharmacodynamic responses could be investigated without interference of other concomitantly drugs present and can be expressed relative to their baseline level.

Between the high end of the regular therapeutic range and high excess of CsA, only a small increase in CN inhibition was noted, but no differences in cytokine secretion, indicating that 2500 µg/L CsA represented the optimal immunosuppressive effect in the whole blood system. Using CsA concentrations from 100 to 1700 µg/L, covering the range of relevant T0/T2 conditions for renal allograft recipients, the decrease in secretion of T cell related cytokine reached 90% relative to baseline values. In contract, for CN inhibition the relative differences were much smaller. In addition, 100 µg/L CsA had only a marginal effect on the suppression of cytokine secretion, while already a relative large degree of CN inhibition around 25% was found. This could imply that CN inhibition has to cross a certain threshold, located CsA concentrations around 100 µg/L or a CN inhibition of 25%, before CN inhibition actually results in inhibition of T cell cytokines. Thereafter, however, a small additional increase in CN inhibition resulted in a relative large increase in suppression of cytokine secretion. The cytokine response range was covered by the different representative T0 and T2 conditions

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chosen and responses to these different concentrations were significantly separated. These results indicate that different late and early CsA C0 and C2 target concentrations represent distinct immunosuppressive conditions.

It has been demonstrated that cyclophilins can limit CN inhibition by CsA in cell culture test systems(12). In addition, partial CN inhibition has been observed in all studies that monitored CN activity in allograft recipients(16). These two observations led us to investigate the role of cyclophilin as rate limiting step at high CsA concentrations. Leukocyte lysate prepared from whole blood incubated with 2500 µg/L CsA was incubated with and without additional CsA and CyPA. Addition of CsA resulted in a further increase in CN inhibition, that was not observed when CyPA was added. This confirmed that CyPA did not limit CsA inhibition at clinically relevant concentrations. In agreement with previous observations, however, adding both CyPA and CsA resulted in significantly less CN activity, confirming that, at higher CsA concentrations CyP, may indeed be rate-limiting(12). Therefore our results indicate that up to 2500 µg/L CN inhibition by CsA is not limited by lack of CyPs.

To investigate whether cyclophilin limitation is relevant for allograft recipients, we repeated these experiments in leukocyte lysate isolated from renal transplant recipients treated with CsA-based immunosuppression. Leukocyte lysates were obtained just before drug intake and two hours thereafter, representing trough and peak levels respectively(17). Addition of CsA to T2 lysates resulted in a further decrease in CN activity illustrating that maximum CN inhibition by CsA was not achieved under the corresponding whole blood conditions. The number of patients used here, are however too small to exclude cyclophilin limitation for CsA therapy in allograft recipients.

Even though these data illustrate the immunosuppressive dynamics of CsA in recipients, one important assumption is made, namely that whole blood is a relevant and representative matrix to assess the immunosuppressive state. In peripheral whole blood there is a large fluctuation in CsA concentrations that may not necessarily reflect concentrations at the tissue level.

In summary, despite redundancy in the immune system, and calcineurin inhibition in particular, there was a significant and dose-dependent suppression of cytokine synthesis as assessed by secretory capacity. Thus far, pharmacodynamic biomarkers have mainly contributed to study drug interactions and visualizing the degree of clinical immunosuppression in allograft recipients using culture systems(11,18,19). The results of the present study add to our understanding of biological variances in CN inhibition and revealed that the degree of clinical

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immunosuppression can assessed either by CN activity or T cell cytokine secretion.

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Chapter 4

Variation in Leukocyte Subset Concentrations Affects Calcineurin Activity Measurement: Implications for Pharmacodynamic Monitoring Strategies

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Abstract

Background: In renal transplantation patients, therapeutic drug monitoring of the calcineurin (CN) inhibitor cyclosporin A (CsA) is mandatory because of the drug's narrow therapeutic index. Pharmacodynamic monitoring of CN inhibition therapy could provide a tool to define and maintain the therapeutic efficacy of CsA therapy. We investigated the effect of variation in cell counts of leukocyte subsets on leukocyte CN activity measurement in renal transplant recipients.

Methods: We measured leukocyte CN activity, whole blood CsA concentrations, and leukocyte subset cell counts in 25 renal transplant recipients. Blood was collected before graft implantation and CsA therapy, 1 day before transplantation when CsA therapy was already started, and 5 days after transplantation. Monocyte, granulocyte, CD4+ T cell, CD8+ T cell, B-cell, and natural killer cell CN activities and CsA inhibition sensitivities were determined in vitro by a spectrophotometric CN assay.

Results: Leukocyte CN activity was inhibited after drug intake. Inter- and inpatient variation in leukocyte subset cell counts resulted in variation of sample composition. The mean (SD) CN activity varied among leukocyte cell subsets, ranging from 650 (230) to 166 (26) pmol/min./ 10^6 cells for monocytes and CD4+ T cells, respectively. CsA half maximal inhibitory concentration (IC_{50}) values ranged from 15 to 78 μ g/L for monocytes and B cells, respectively.

Conclusion: Inter- and intraindividual leukocyte subset cell count variation can affect measured CN activity independent of CsA concentration. Cell-specific activity and drug sensitivity should be considered for sample validation to optimize method specificity when pharmacodynamic monitoring strategies are applied in a clinical setting.

Introduction

Immunosuppression by calcineurin (CN) inhibition therapy is essential for prevention of graft rejection early after renal transplantation. The 2 CN inhibitors (CNI) used in transplantation medicine, cyclosporin A (CsA) and tacrolimus, inhibit CN after formation of a complex with their corresponding binding proteins, and result in suppression of lymphocyte activation(1,2). Unfortunately, side effects, such as nephropathy, neuropathy, diabetogenesis, malignancies, and cardiovascular disease occur in CNI-treated patients(3–7). Because of the unpredictable kinetic profiles of CNIs and potential drug-drug interactions, patients receiving CNI therapy require intensive drug monitoring(8). Pharmacokinetic monitoring is performed by measuring blood drug concentrations at different time points after drug intake(9–11). However, more accurate drug monitoring strategies are desired to maximize efficacy of these drugs(12,13).

Pharmacodynamic monitoring of CNI therapy is a new approach for the optimization of CNI monitoring(8), and several methods are available for measurement of CN enzyme activity as a pharmacodynamic marker. All methods use the same substrate, a 19-amino acid peptide (R11-peptide), which corresponds to a part of the regulatory subunit of bovine cAMP dependent protein kinase(14), but methods differ in the detection techniques used, such as HPLC-ultraviolet measurement of dephosphorylated peptide(15,16), radioactive measurement of ³²P-labeled phosphate(1,17,18), and absorbance measurement of malachite green phosphate reagent(19). In addition, various blood fractions, such as whole blood, peripheral blood mononuclear cells (PBMCs), and leukocytes are used as the specimen. Although clear relationships between drug concentration and CN activity have been observed, no clear relationship between clinical outcome and CN activity has been reported(17,20,21). We investigated whether variations in leukocyte subset counts would directly affect CN activity measurement independent of CNI concentration and potentially confound the use of CN activity as a predictive marker. To investigate the effect of sample composition variation on CN activity measured in renal transplantation patients, we determined cell-specific CN activities and CsA inhibition sensitivities for granulocytes, monocytes, CD4+ T cells, CD8+ T cells, B cells, and natural killer (NK) cells.

Materials and Methods

Patients and Samples

Twenty-five patients who underwent living-donor renal transplantation at the Leiden University Medical Center were included in this study. Formal approval from the institutional ethics committee was obtained, and informed consent for blood sampling was obtained from all participants, both patients and healthy volunteers. The patient group consisted of 17 male and 8 female recipients with a mean (SD) age of 49 (13) years at the time of transplantation. All patients received quadruple immunosuppressive therapy with basiliximab, prednisolone, mycophenolate mofetil, and CsA (4 mg/kg twice a day), with the latter therapy started 3 days before transplantation. EDTA-anticoagulated blood samples from the antecubital veins were collected at 3 different time points; 5 days before transplantation (day -5), when no immunosuppressive therapy had begun; 1 day before transplantation (day -1), after initiation of oral CsA therapy but without administration of the other immunosuppressive drugs; and 5 days after transplantation (day 5), when full immunosuppressive therapy was given. On the day before transplantation (day -1), samples were obtained immediately before drug intake (12 hours after previous dose), and 2 and 3 hours after drug intake. On day 5 after transplantation samples were obtained immediately before and 2, 3, 4, and 6 hours after drug intake.

Blood leukocyte and lymphocyte subset measurement

Hematologic profiling was performed on a Sysmex XE2100. Lymphocyte subsets were analyzed by flow cytometry. The lymphocytes gated in the side-scatter/ CD45 dot-plot were analyzed for CD3+/CD4+, CD3+/CD8+, CD3+/CD19+ and CD3+/CD16/56+ populations using fluorescein isothiocyanate (FITC)-labeled CD4 (clone SK3), phycoerythrin (PE)-labeled CD8 (clone SK1), APC-labeled CD3 (clone SK7) or APC-labeled CD19 (clone SJ25Cl), FITC-labeled CD3, and PE-labeled CD16 and PE-labeled CD56 panels on a FACSCalibur (all from BD Biosciences) equipped with CellQuest.

Sample preparation for calcineurin activity measurement

EDTA-anticoagulated blood (2 mL) was treated with erythrocyte lysis buffer (8.4 g/L NH_4Cl , 1.0 g/L KHCO_3 , pH 7.3). The leukocytes were then washed twice with 10 mL Hepes buffered saline (HBS, 9.0 g/L NaCl, 10 mmol/L Hepes pH 7.5) before resuspension in 1.5 mL HBS for cell count on the Sysmex XE2100. Leukocyte cell lysis was performed for $2 \cdot 10^6$ cells that were resuspended in 200 μL lysis buffer [50

Chapter 4: Calcineurin activity in leukocyte subsets

mmol/L Tris-HCl pH 7.7, 1.0 mmol/L dithiothreitol, 5.0 mmol/L ascorbic acid, 0.02% (vol/vol) NP-40, 50 mg/L soybean trypsin inhibitor, 50 mg/L phenylmethylsulfonyl fluoride, 5.0 mg/L leupeptin, and 5.0 mg/L aprotinin] and lysed by 3 freeze-thaw cycles (liquid N₂, 30°C). Cell debris was separated by centrifugation (10 min, 10000g, 4°C), and supernatants were snap-frozen in liquid N₂ and stored at -80°C before calcineurin activity measurement.

Isolation of leukocyte and lymphocyte subsets for absolute CN activity measurement

Granulocyte and PBMC fractions from healthy volunteers were separated by Ficoll density gradient centrifugation. The PBMC fraction was washed with HBS and resuspended in 0.1% BSA in HBS at a final concentration of $15 \cdot 10^6$ to $20 \cdot 10^6$ cells/mL. In the granulocyte fraction erythrocytes and thrombocytes were removed by erythrocyte lysis with NH₄Cl lysis buffer, washed once with HBS, and resuspended in HBS. Purity of granulocyte and PBMC fractions was analyzed and found to be at least 95% for the PBMC fraction and 90% for the granulocyte fraction. Cell fractions were divided into aliquots of $2 \cdot 10^6$ cells and resuspended in 165 μ L lysis buffer for cell disruption.

CD3+/CD4+ (CD4+ T cells), CD3+/CD8+ (CD8+ T cells), CD3+/CD19+ (B cells), CD3+/CD16/56+ (NK cells), and monocyte fractions were sorted from the PBMC fraction using an ARIA sorter (BD Biosciences). The PBMC fraction was incubated for 30 min with FITC-labeled CD4, PE-labeled CD8, and APC-labeled CD3 or APC-labeled CD19, FITC-labeled CD3, and PE-labeled CD16/56 before cell sorting. Isolated fractions were collected in 5-mL BD FACS tubes (BD Biosciences) containing 500 μ L 50% heat inactivated fetal calf serum in Roswell Park Memorial Institute medium. The purity of sorted fractions was analyzed and found to be at least 95% for every fraction. Truecount tubes (BD Biosciences) were used for controlling cell counts. Sorted cells were spun down (10 min, 500g, 4°C), washed once with 250 μ L HBS, and resuspended in 165 μ L lysis buffer for cell disruption.

Leukocyte and lymphocyte subset isolation for CsA inhibition curves

Granulocyte and PBMC fractions were isolated from buffycoats (Sanquin) using Ficoll density gradient separation. PBMC subsets were isolated using CD4, CD8, B cell, NK cell, and monocyte-negative isolation kits (Invitrogen). We incubated $1.5 \cdot 10^6$ to $2 \cdot 10^6$ cells/mL in Roswell Park Memorial Institute medium containing 20% inactivated fetal calf serum and 1% ethanol for 1 hour at 37°C, with final CsA concentrations of 0, 0.1, 0.32, 1, 3.2, 10, 32, 100, 320, 1000, 3200, and 10000 μ g/L. Cells were washed once with HBS before cell lysis.

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Calcineurin activity assay

Calcineurin activity was determined as previously described(19). In brief, 15 μL of lysate was added to 40 μL of assay buffer (0.313 $\mu\text{mol/L}$ calmodulin, 0.375 mmol/L RII phosphopeptide substrate, 75 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 9 mmol/L MgCl_2 , 0.75 mmol/L dithiothreitol, 0.0375% NP-40, 0.625 $\mu\text{mol/L}$ okadaic acid, 0.75 mmol/L CaCl_2 , and 5 mmol/L ascorbic acid) and to 40 μL of assay buffer without substrate to correct for background absorbance. The enzyme reaction was performed at 30°C and terminated after 30 min by adding 100 μL of malachite green reagent, and then the color was allowed to develop for 50 min at ambient temperature. Absorbance was read at 620 nm using a 96-well plate spectrophotometer (SpectraMax 250, Molecular Devices), and phosphate was quantified using a 0–5 nmol phosphate calibration curve using SoftmaxPro (Version 2.4.1) software.

CsA blood concentration

CsA blood concentrations were quantified using a fluorescence polarization immunoassay on the Abbott AxSYM system according to the manufacturer's instructions.

Data analysis

Means and SD are used in the text of this manuscript. Data presented in the Figs. Are shown as either mean, mean (SE), or box plot. Paired 2-tailed Student *t*-tests were performed to test for statistical significance, which was defined as $P < 0.05$. Inhibition curves and IC_{50} (half maximal inhibitory concentration) values were fitted and analyzed using sigmoidal dose–response curves with GraphPad software.

Results

Leukocyte CN activities and blood CsA concentrations

The observed leukocyte CN activities and blood CsA concentrations for 25 renal transplant recipients, measured at the 3 different time points, are shown in Figure 4.1.

CN activities measured in the patients before CsA therapy ranged from 168 to 385 pmol/min./ 10^6 leukocytes. The range of CN activities after oral treatment with CsA was 48 to 464 pmol/min./ 10^6 leukocytes, and blood CsA concentrations ranged from 90 to 2016 $\mu\text{g/L}$.

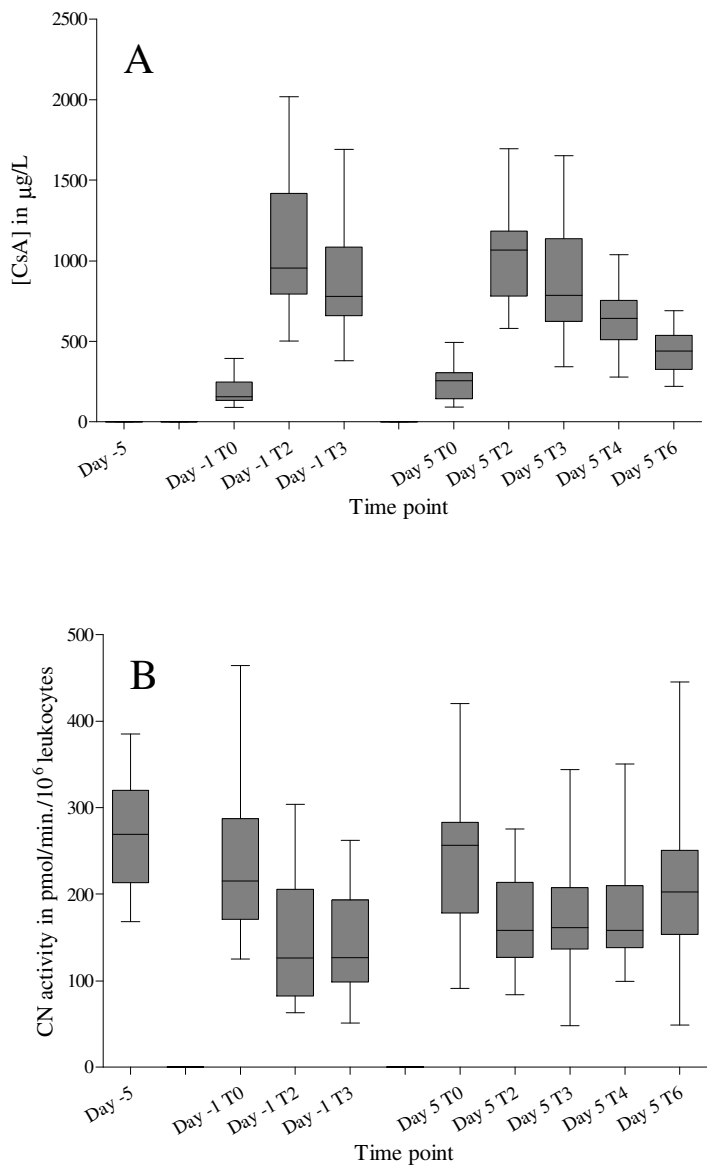


Figure 4.1. Leukocyte CN activity before and shortly after renal transplantation in patients on CsA therapy. Box plots of CsA blood concentration (A) and leukocyte CN activities (B) at 3 different time points: day -5, before CsA treatment and graft implantation; day -1, before graft implantation at initiation of CsA treatment; and day 5 after transplantation. T0, T2, T3, T4, T5 and T6 represent the time (hours) after drug intake.

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An inverse relationship between CsA drug concentration and CN activity was observed on day -1 and day 5. On day -1 and day 5, maximal CN inhibition values of 42% and 31%, respectively, were observed 2 hours after drug intake compared to T0 CN activities, with mean (SD) T0 CsA concentrations of 195 (86) and 245 (100) $\mu\text{g/L}$ and T2 concentrations of 1070 (399) and 1037 (312) $\mu\text{g/L}$, respectively. Compared to pretherapy CN activity, a maximum inhibition of 52% and 38% on day -1 and 5, respectively, was observed 2 hours after drug intake. CN activity 6 hours after drug intake was not significantly different from T0 CN activity on day 5 ($P = 0.12$), whereas at 2 hours ($P < 0.001$), 3 hours ($P < 0.001$), and 4 hours ($P = 0.001$) after drug intake significantly lower CN activities were found compared to T0. When average T0 CN activities were compared to predrug (day -5) CN activities, 15% and 13% lower CN activities were observed on day -1 and day 5 respectively, though only day -1T0 ($P = 0.03$) was found to be significantly lower ($P = 0.08$ for day 5 T0).

Peripheral blood leukocyte and lymphocyte subset concentrations

Leukocyte and lymphocyte subset cell counts were monitored in parallel with CN activity. The mean peripheral blood concentrations for leukocyte subsets at all sampling points are shown in Figure 4.2A and 4.2B.

When the leukocyte subset cell counts before graft implantation (day -5 and day -1 T0) were compared to subset counts after implantation (day 5 T0), an increase of total leukocytes ($P < 0.001$), granulocytes ($P < 0.001$), monocytes ($P < 0.001$), and B cells ($P = 0.009$) was observed. On day -1 no significant changes of leukocyte subsets were observed, whereas on day 5 a significant decrease in PBMC cell counts (starting 4 hours after drug intake, $P = 0.012$), B cells (starting 3 hours after drug intake, $P = 0.040$), CD4+ T cells (starting 3 hours after drug intake, $P = 0.011$), and CD8+ T cells (starting 4 hours after drug intake, $P = 0.026$) were found. All of these changes in peripheral leukocyte subset cell counts affect leukocyte sample composition. Figure 4.2C and 4.2D show the relative fractions of leukocyte subsets that made up a leukocyte and PBMC sample, which is used as sample in other CN activity methods.

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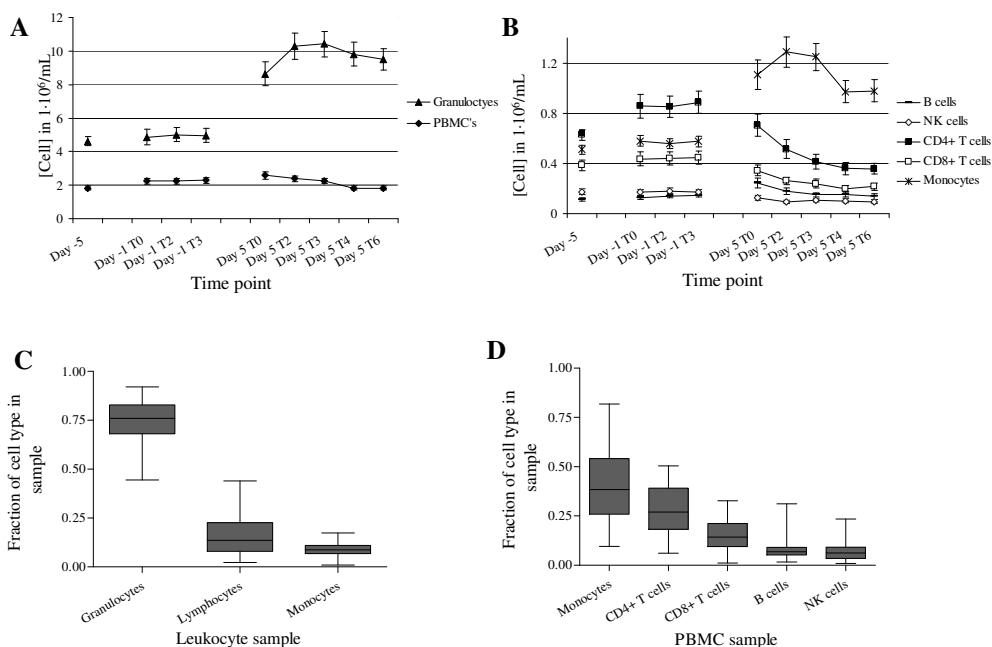


Figure 4.2. Peripheral blood cell counts of leukocyte subsets in renal transplantation patients and the effect on sample composition. Granulocyte and PBMC cell counts in peripheral blood (A) and PBMC subset cell counts in peripheral blood (B). Data points represent mean cell counts of all patients, and error bars represent SE. (C,D), box plots of subset fractions within leukocyte and PBMC sample, respectively. All sampling time points for all patients are included. Sample compositions were calculated using full blood differentiates.

CN activities of different leukocyte and lymphocyte subsets

To investigate whether the changing sample composition within a patient, and the variation of sample composition between patients, affects CN activity measurement, we determined the cell-specific CN activity for the leukocyte subsets. Cell-specific CN activities for 4 healthy volunteers are shown in Figure 4.3.

The PBMC fraction seemed to have higher CN activity than the granulocyte fraction. The monocytes subset had the highest CN activity in at least 3 healthy volunteers. Within the lymphocyte subsets variation in CN was also observed in these volunteers. The NK-cell fraction seemed to have the highest CN activity, and CD8+ T cells had slightly higher CN activity than CD4+ T cells, which had the lowest cell specific CN activity in these individuals.

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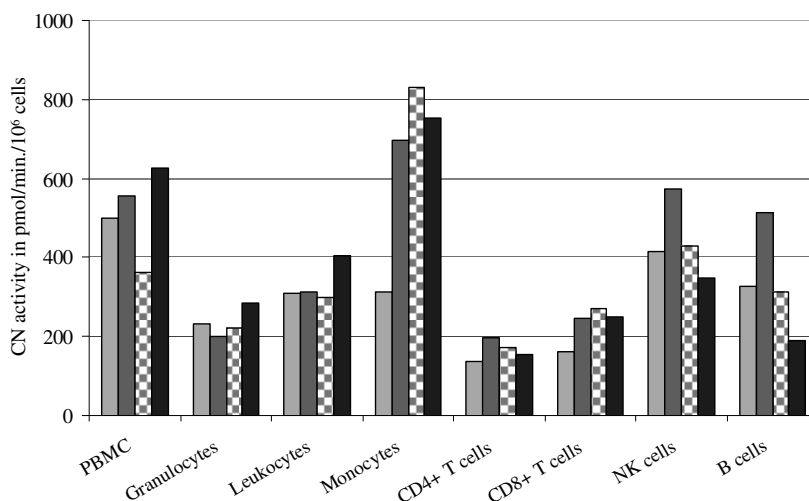


Figure 4.3. Cell-specific CN activity. Cell-specific CN activity determined for 4 healthy volunteers and expressed per cell. Every bar style represents one individual and columns represent the means of duplicates.

CsA inhibition curves for leukocyte subsets

We determined cell-specific CsA inhibition sensitivities to investigate whether the changing sample composition affects the CN inhibition profile of leukocytes. CsA inhibition curves obtained for the different leukocyte subsets are shown in Figure 4.4. Table 4.1 lists CsA IC_{50} concentrations, which were obtained from the inhibition curves in Figure 4.4 for the leukocyte subsets.

Discussion

Investigation of the usefulness of CN activity measurement as a pharmacodynamic marker for CNi therapy requires a clear relationship with therapy outcome. Most studies have focused on the relationship between pharmacokinetics and pharmacodynamics(22–24), or evaluation of CN inhibition profiles by CsA and tacrolimus at different time points after transplantation(25–29). To our knowledge only one study, by Fukudo et al., has provided evidence that CN activity is related to clinical responses in organ transplantation patients(17), and one other study by Sanquer et al. showed that CN activity might be a predictive marker for graft-vs-host-disease in allogeneic stem-cell transplantation(30). Both groups used PBMC samples that were isolated from peripheral blood by Ficoll density gradient.

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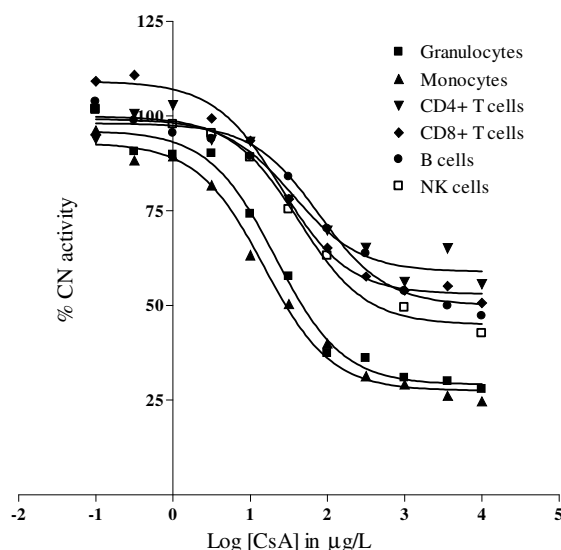


Figure 4.4. Cell specific inhibition of CN by CsA. CsA inhibition curves for different leukocyte subsets fitted with sigmoidal dose–response curves using GraphPad software. CN activity is expressed as percentage of CN activity without CsA. Means of duplicates are displayed.

Leukocyte subset	IC ₅₀ CsA (µg/L)	95% CI (µg/L)
Granulocytes	21	15 - 31
Monocytes	15	11 - 21
CD4+ T cells	36	18 - 71
CD8+ T cells	25	18 - 36
NK cells	43	29 - 62
B cells	78	43 - 142

Table 4.1: CsA IC₅₀ values for different leukocyte subsets. CsA IC₅₀ values and CI were obtained from the inhibition curves in Figure 4.4 analyzed by GraphPad software.

We monitored CN activity in the leukocyte fraction because its preparation requires less blood and is less cumbersome than PBMC isolation. In a previous study we validated our analytical method(19), and as a follow-up we investigated and validated our sample choice by monitoring inter- and inpatient compositional variation of the leukocyte fraction and determination of cell-specific activity and CsA-inhibition sensitivity.

Parallel to leukocyte CN activity, peripheral leukocyte subset cell counts were monitored in 25 renal transplantation patients (Figure 4.2), and large interpatient variation was observed. Significant inpatient changes were also observed before

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and after graft implantation, and in time after drug intake when monitored after implantation. The granulocytosis, monocytosis, and lymphopenia observed after transplantation are caused by surgical trauma and steroid therapy. These changes in peripheral blood leukocyte subset concentrations caused time dependent shifts in sample composition. To investigate the effect of these changes and interindividual variations in leukocyte sample composition on the leukocyte CN activity measured, cell-specific CN activities and CsA inhibition sensitivities were determined. On average, monocytes have the highest CN activity per cell, as has been reported previously(31). Within the lymphocyte fraction, differences in CN activity were observed, with the highest CN activity observed in NK cells and the lowest CN activity in the T cell subsets for our 4 volunteers. CsA inhibition sensitivity profiles for the different leukocyte populations were similar and comparable with cell IC_{50} values reported by Kung et al.(32), a finding that indicates that measurement of leukocyte CN inhibition by CsA is not affected by variation in sample composition. Taken together, these results show that variation in sample composition affects CN activity measured in patient samples, whereas CsA inhibition is not affected. Variation in monocyte cell count could have a particularly influential effect on CN measurement because monocytes have the highest cell-specific CN activity. When linear regression was performed between day -5 (pre CsA) CN activities measured in the renal transplantation patients and the percentage of cell subsets present in the sample, no clear relationship (max $R^2 = 0.15$, for monocyte fraction) was found, and therefore no effect of sample composition on leukocyte CN activity was observed. Variation in monocytes could have a greater effect on PBMC CN activities because monocytes account for a larger proportion of the PBMC sample, and the inter- and inpatient variation in PBMC fraction composition is much larger than that of the leukocyte fraction (see Figure 4.2D). In PBMC samples, monocytes were found to constitute 20% to 80% of the sample, and this large variation, based on the larger cell-specific CN activity, can significantly influence CN measurement. Halloran et al. compared the PBMC fraction with the leukocyte fraction in healthy volunteers and found similar CsA inhibition affinities, but larger and more variable inhibition in the PBMC fraction(26). When leukocytes are used as sample, the majority of the cells [mean (SD) 75% (10%)] are granulocytes, and sample composition changes are relatively small. A disadvantage is that a large proportion of the CN activity used for monitoring CNi pharmacodynamics is from the granulocyte fraction. Ideally, one would like to selectively monitor in the compartment where the drug exerts its effect(21). The effector compartment of CsA therapy is thought to be the lymphocyte or, more specifically, the T cell populations, and monitoring of these fractions could

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theoretically enable avoidance of the influence of changing sample composition on CN activity. Absolute CN activities would then be more characteristic of immunosuppressive status, and observed CN inhibition after drug intake would reflect drug pharmacodynamics within the effector compartment and could therefore be more relevant. In practice the procedure for routinely sorting and preparing these cell fractions for CN activity analyses is very complicated and time consuming. In addition, when T cell depletion by anti-thymocyte globulin is used in, for instance, treatment of acute rejection episodes, collection of these cells from the peripheral blood is very difficult because they are therapeutically removed.

The CN activities observed in 25 renal transplantation patients were inversely related to whole blood CsA concentrations, and for every CN activity time curve except that of 1 patient, lower CN activity was observed at T2 than at T0. Interestingly, on day 5 we observed significant changes in sample composition by time after drug intake that, at least theoretically, could have caused the measured decrease in leukocyte CN activity. However, since no changes in leukocyte composition are observed within day -1, when a decrease in CN was also observed, and as no relationship between leukocyte fraction and CN activity was found by linear regression, we conclude that our CN activity measurements reflect CN inhibition by CsA. The inhibition of CN by CsA in renal transplantation patients is partial and has a temporary duration, as has been observed by other groups(25,33). We show that trough CsA concentrations in these patients are sufficient to cause CN inhibition.

The effect of sample composition changes in renal transplantation patients could also affect other pharmacodynamic monitoring strategies that are used for monitoring CNI therapy, such as cytokine messenger RNA monitoring(34). Also, monitoring of other therapies such as inhibition of inosine monophosphate dehydrogenase activity by mycophenolic acid in these patients could be affected by sample composition variation(35). Investigation of sample composition, cell specific activities, and drug sensitivities could therefore be very useful for optimization of the clinical sensitivity and specificity of these methodologies.

In conclusion, we have shown that large inter- and inpatient variation in peripheral blood leukocyte subset cell counts results in a large variation in sample composition, which depends on sample choice. In vitro measurement of cell-specific CN activities indicate that this variation could affect CN activity measurement in renal transplantation patients. No relationship was observed between sample composition and leukocyte CN activity, and leukocyte CN activity can therefore be used for monitoring CN activity in renal transplantation patients. Whether measurement of CN inhibition is useful to define and maintain the

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therapeutic index of CNi therapy is still unknown and requires investigation by adequate analytical methods. In this respect further insight in analytical confounders is very helpful for optimization of clinical specificity. In future studies, larger populations should be monitored for leukocyte CN activity to define the role of this activity in maintaining the therapeutic index of CNi therapy after organ transplantation.

Acknowledgments

We thank the technicians from the cytometry group of the hematology department in the Leiden University Medical Center, The Netherlands, for their help with the cell-sorting experiments.

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Chapter 5

Pharmacodynamic monitoring of calcineurin inhibition therapy; principles, performance, and perspectives

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Therapeutic Drug Monitoring 2010, 21(1), 3-8

Abstract

Background: The calcineurin inhibitors cyclosporin A and tacrolimus are immunosuppressive drugs used extensively in allograft recipients. These drugs show large inter-individual pharmacokinetic variation and are associated with severe adverse affects, including nephrotoxicity and cardiovascular disease. In current practice, calcineurin inhibitors are combined with other immunosuppressive drugs such as steroids and mycophenolate mofetil. Dosage is titrated based on blood concentration measurement. For further optimization of calcineurin inhibition therapy, new monitoring strategies are required. Pharmacodynamic monitoring strategies constitute novel approaches for optimization of calcineurin inhibitors therapy.

Content: This review focuses on the general aspects of immunosuppressive drug pharmacodynamic monitoring and describes the methodologies used for monitoring calcineurin inhibition therapy. Two different types of pharmacodynamic monitoring strategies can be distinguished: 1) enzymatic strategies, which monitor inhibition of drug-target enzyme activity, and 2) immunological strategies, which measure cellular responsiveness after *in vitro* simulated immunological responses. Enzymatic tests are drug type specific markers, in which calcineurin activity is directly determined. Immunological strategies measure immune responsiveness at several levels, such as mRNA transcripts, (intracellular) concentrations/excretion of cytokines, expression of surface activation markers, and cell proliferation. This review also discusses analytical issues and clinical experience with these techniques.

Summary: The call for new methodologies to evaluate immunosuppressive therapy has led to the development of a large variety of pharmacodynamic monitoring strategies. The first reports of their clinical relevance are available, but further understanding of the analytical and clinical variables involved are required for the development of accurate, reproducible, and clinically relevant markers.

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There is large inter-patient variation in the pharmacokinetic profile of calcineurin inhibitors. These agents also produce various adverse effects, such as nephrotoxicity and cardiovascular disease(2). In order to control drug over- or under-exposure, therapeutic drug monitoring (TDM) is applied. TDM is performed via determination of whole blood drug concentrations at various time points after drug intake (C₀, C₂) and estimation of area under the curve (AUC) values(2). These TDM strategies have led to significant improvement in immunosuppressive treatment by reducing episodes of rejection(2). Unfortunately, over 10% of renal transplant recipients experience at least one acute rejection within the first 6 months post-transplant, and drug toxicity and oversuppression of immune function by these drugs seriously hamper long-term success(2,3). A search for new, successful immunosuppressive drugs is ongoing. Until new agents are available, optimization of current treatments is probably the most effective means to improve immunosuppressive treatment(4). Improved monitoring strategies for these drugs, including the integration of pharmacogenetics or/and pharmacodynamics within the current TDM protocols, could further individualize drug dosing and improve clinical performance of these agents(4). Currently, the pharmacogenetic profile of calcineurin inhibitors is used as an extension of pharmacokinetics to predict individual pharmacokinetic profiles and allow for more rapid and simple achievement of therapeutic target drug levels. Pharmacodynamic monitoring strategies measure drug effectiveness parallel to pharmacokinetics, and could thus provide additional information to define new therapeutic target conditions.

General aspects of pharmacodynamic monitoring strategies for calcineurin inhibitors

Why pharmacodynamic monitoring?

The current TDM approaches are based on concentration measurement, and are surrogate markers of drug effect. They are limited in their ability to measure drug effectiveness at its immunosuppressive site of action. Pharmacodynamic strategies are designed to address this issue. A large amount of evidence supports the concept that T cell suppression is the key mechanism by which calcineurin inhibitors achieve immune suppression. T cells are therefore interesting candidates for pharmacodynamic monitoring strategies(5). Current TDM strategies determine blood drug concentrations in extra-cellular or whole blood fractions. However, measurement of whole blood or extra-cellular blood concentrations does not

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necessarily provide an accurate and reproducible reflection of intra-cellular drug concentrations within T cells. For instance, calcineurin inhibitors in whole blood are highly bound to erythrocytes and lipoproteins. Inter- and intra-patient variations of these parameters have been shown to affect calcineurin inhibitor concentrations in lymphocytes(6,7). Another variable that could significantly affect distribution of immunosuppressive drugs in blood are the drug-efflux transporters. Most immunosuppressive drugs are substrates for one or more drug transporter proteins, such as P-glycoprotein, that are expressed on T cells(8,9). Intracellular drug concentrations can therefore be affected by cellular drug-exporting activity. Pharmacokinetic profiles and drug-drug interactions at this level are not monitored by current TDM strategies. In order to evaluate and adjust drug dosage based on these variables, intracellular T cell drug concentration assays have been developed(10).

Drug concentrations are used as a surrogate marker of effect, but do not necessarily correlate with pharmacological response(11). Effect itself is much more complex and may involve drug-independent mediators, such as the requirement of immunophilins for activity of calcineurin inhibitors and mTOR inhibitors. An advantage of pharmacodynamic monitoring strategies is that pharmacological and immunological drug-drug interactions can be detected(12).

Enzymatic versus immunological monitoring strategies

There are two different approaches applied in pharmacodynamic monitoring of immunosuppressive drugs: 1) enzymatic monitoring by measurement of the drug (main) target enzyme, and 2) immunological monitoring by measurement of *ex vivo* simulated immunological responses. In enzymatic protocols, the intracellular enzyme activity of calcineurin is used to monitor CsA and TRL. This enzyme system is selectively targeted by one class of drugs and will therefore monitor the pharmacodynamics of one immunosuppressive agent (figure 5.2)(13).

In immunological monitoring protocols, stimulated full blood or isolated cell fractions are monitored for T cell activation markers such as cytokine mRNA transcripts, cytokine excretion, and surface activation proteins(12). These responses are more general measures of immune function. Drug pharmacodynamics are determined by quantifying the degree of inhibition of these immunological responses that can be ascribed to the immunosuppressive agents(s)(14). The calcineurin inhibitors are thought to be the major effectors of these parameters, since they are the first in line to interfere with immune functioning(1) and the immunological markers used are primarily controlled by calcineurin(15). Using these strategies, immunosuppressive synergism, additivity,

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or antagonism by combinations of various classes of immunosuppressive agents can be detected(12). As a consequence, it is more difficult to ascribe the immune inhibitory effect to a single component of combination drug therapy.

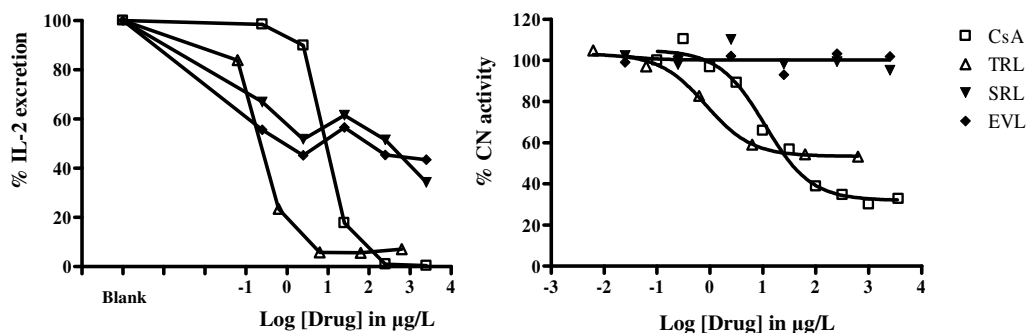


Figure 5.2. Drug specificity of enzymatic vs. immunological strategies. IL-2 excretion inhibition after mitogen stimulation (left) and calcineurin inhibition (right) of CsA, TRL, sirolimus (SRL), and everolimus (EVL) in PBMCs isolated from healthy volunteers. Data represent mean of duplicate and are expressed as percent of blank measurement. Adapted from(13).

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Pharmacology of calcineurin inhibitors in T cells

Calcineurin is the Ca^{2+} -and calmodulin-dependent serine-threonine phosphatase(16). It consists of a regulatory subunit (CNB) and a catalytic subunit (CNA) that becomes available for substrate dephosphorylation after formation of a complex between CNB, calmodulin, and Ca^{2+} (16). Calcineurin inhibitors inhibit calcineurin after binding an intracellular protein (immunophilin). These complexes block the active site for large substrates(17). There are two different classes of immunophilins: 1) cyclophilins, targeted by CsA, and 2) FK506 binding proteins, targeted by TRL(18).

Calcineurin is involved in a variety of homeostatic processes. Its actions include dephosphorylation of multiple phosphoproteins and phosphopeptides, but calcineurin is probably best known for its role in T cell activation. T cell receptor binding results in increased intracellular Ca^{2+} . After binding of calmodulin, this intracellular Ca^{2+} activates calcineurin phosphatase activity. Activated calcineurin dephosphorylates NFAT (nuclear factor of activated T cells) transcription complex, which then translocates to the nucleus(19). Upon co-stimulation, NFAT proteins cooperate with members of the activator protein 1 (AP-1) family of transcription

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factors to transcribe activation-induced genes(20). One of these genes is interleukin 2 (IL-2) whose transcription and excretion is controlled by calcineurin. IL-2 activates various cells of the immune system including helper T-cells, cytotoxic T-cells, NK cells, B cells, and macrophages(21). Other gene products in activated mononuclear cells that are under the control of calcineurin are interferon- γ (IFN- γ), tumor necrosis factor α (TNF- α), and granulocyte-macrophage-colony stimulating factor (GM-CSF)(15,22). IFN- γ is a cytokine predominately produced by activated T and NK cell; it exerts its immuno-modulatory effect by induction of MHC expression(23). The hematopoietic growth factor GM-CSF is produced by a variety of cell types including activated T cells. GM-CSF recruits circulating neutrophils, monocytes, and lymphocytes(24). TNF- α is produced by activated T-cells and macrophages, activates macrophages and lymphocytes, and enhances their cytotoxic potential(25). In addition, transcription of several Th2 cytokines is controlled by calcineurin(15). Among these, IL-4 has a broad range of immune functions, including stimulation of IgE synthesis in B cells.

Calcineurin also controls the expression of T cell surface receptors. Their appearance is associated with T cell activation, and the receptors constitute important immune-response control systems after T cell activation(15). The biological effects of IL-2 are mediated via the IL-2 surface receptors, which is composed of three subunits; the α -chain (CD25), the β -chain (CD122), and the γ -chain (CD132)(21). In normal circulating lymphocytes, the IL-2 receptor α is expressed in less than 5% of cells at very low levels, but is rapidly transcribed and expressed on activated T-cells; this expression is inhibited by CNIs(15,21,26). CD40 ligand (CD40L/CD154) is another surface receptor whose expression is controlled by calcineurin(15,27). Like TNF- α , CD40L is a member of a large family of structurally related ligands that are referred to as the TNF superfamily. These proteins regulate functions such as immune responses and haematopoiesis(28). CD40L is selectively expressed on activated mature T cells and not on resting T cells(29). Its surface expression is controlled by translocation of preformed CD40L and mRNA-mediated CD40L expression after anti-CD3 stimulation. This expression is observed as early as 1-2 hours after stimulation and peaks at 6 to 8 hours(29). The effects of TNF ligands are mediated via TNF receptors. Several of these ligands are expressed on T cells after activation, and act to control duration of T cell responses and T cell survival. One of these ligands is OX40 (CD134), which is crucial for T-cell activation and survival(30). OX40 expression is induced by T cell receptor stimulation. Expression peaks around 2 to 3 days after stimulation and is lost 4 to 5 days after stimulation(31). Another TNF receptor that is selectively expressed on activated T cells is Fas (CD95). Interaction of Fas with

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its ligand (FasL/CD178) triggers rapid apoptosis of activated T cells(32). Other T cell receptors that show expression on activated T cells are involved in cell adhesive interactions. The leukocyte function associated antigen (LFA-1/CD11a) is activated upon TCR stimulation, resulting in increased affinity for its counter-receptors. This interaction is important for T cell adhesion to endothelial cells, and for diapedesis through endothelium. It also acts as a co-stimulatory signal in T cell interactions(33). Two other surface activation receptors are CD69 and CD71. Activation induced molecule (AIM/CD69) is present on activated but not resting T cells, and appears relatively early after T cell activation. CD69 is important in cell activation, proliferation, and differentiation(34). The transferrin receptor (TfR/CD71) binds iron-loaded transferrin and plays a key role in cell proliferation control, since iron is a key nutrient required for cell proliferation. TfR constitutively cycles between the endosomal compartment and plasma membrane. Increases in T cell surface expression are observed after T cell stimulation(35).

Calcineurin activity measurement

Calcineurin enzyme activity is determined in excess of all required reagents. Therefore in vitro enzyme activity does not necessarily reflect the actual intracellular enzyme activity. In this context, this technique may be more correctly referred to as a capacity measurement that reflects binding of calcineurin inhibitor/immunophilin complexes to calcineurin when fully active(5). Calcineurin enzyme activity is regulated intracellularly by Ca^{2+} ; in naïve T cells, no sustained elevation of calcium is present. From an immunological point of view, a lack of sustained intracellular hypercalcemia means there is no activation of T cells via nuclear factor of activated T-cells (NFAT); thus no suppression of the calcineurin pathway is theoretically required. T cells activated by T-cell receptor binding of allo-antigen have sustained elevation in Ca^{2+} concentrations and calcineurin activity. Theoretically, only these cells would require calcineurin inhibition in order to suppress the immune response. Adequate binding to and inhibition of calcineurin in these cells is therefore crucial for successful immunosuppression. In vitro assays approximate these intracellular conditions.

In the calcineurin enzyme activity protocols, the Ser-phosphorylated 19-amino acid peptide Asp-Leu-Asp-Val-Prol-Ile-Pro-Gly-Arg-Phe-Asp-Phe-Asp-Arg-Arg-Val-Ser-Val-Cys-Ala-Glu is used as substrate, which corresponds to residues 81-99 of the bovine cardiac cAMP-dependent protein kinase regulatory subunit (R_{II}). This substrate is not specific for calcineurin, and it can be dephosphorylated by other serine-threonine phosphatases (PP)(36). There are four serine-threonine phosphatase enzymes (PP1, PP2A, calcineurin, and PP2C). For selective

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calcineurin activity determination, okadaic acid is used as a specific inhibitor of PP1 and PP2A. To distinguish calcineurin from PP2C, calcineurin phosphatase activity measurement is defined as CsA-inhibitable or Ca^{2+} dependant phosphatase activity. In the last case, the Ca^{2+} chelator EGTA is used to bind Ca^{2+} , removing free Ca^{2+} from the assay. Interestingly, when these two different approaches are used *in vivo*, differences in relative PP2C activity are observed. When CsA is used to define calcineurin activity, PP2C phosphatase activity is much larger in the calcineurin + PP2C fraction (> 60%(37) of total serine-threonine phosphatase activity) than Ca^{2+} /calmodulin defined calcineurin activity (< 5%(38,39)). This difference can be explained by the mechanism of calcineurin inhibition by CsA. CsA itself does not inhibit calcineurin, but requires cyclophilin. Cyclophilins are not abundant, and at high CsA concentrations, they limit the ability of CsA to inhibit calcineurin(40,41).

For quantification of the R_{II} substrate dephosphorylation, several detection techniques are used. These techniques are based on either determination of the radioactive ^{32}P isotope, HPLC-UV detection of dephosphorylated peptide, or absorbance measurement of phosphate reagent. An overview of the calcineurin assay methodologies is shown in table 5.1.

Sample choice and preparation

Another issue that has been thoroughly investigated and discussed is sample choice for calcineurin activity monitoring. The major therapeutic target compartment of calcineurin inhibitors is thought to be the T lymphocyte fraction, since calcineurin inhibition within these cells is thought to be crucial for their immunosuppressive action. Monitoring calcineurin activity in this fraction would therefore be most relevant. Unfortunately, no protocol for selective measurement in this cell fraction has been developed, though recently an assay for IMPDH activity in CD4+ cells has been described(42). Calcineurin activity and inhibition by calcineurin inhibitors is monitored in whole blood, leukocyte, peripheral blood mononuclear cell (PBMC), and lymphocyte fractions. Two important features of a suitable sampling fraction are that the fraction is representative of the immunosuppressive site of action and that it is compositionally stable in the sense that compositional changes should not affect measurement outcome. Large differences in PBMC sample composition are observed for renal transplant recipients early after transplantation that can directly confound calcineurin measurement based on differences in cell-specific calcineurin activities of leukocyte subsets(43).

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A) Calcineurin activity

Sample	Detection technique	Definition of calcineurin activity	Refs
Whole blood	Phosphate, by released ^{32}P	Phosphatase activity in the presence of OA $\pm$ CsA	11,37
Whole blood	Phosphate, by released ^{32}P	Phosphatase activity in the presence of OA $\pm$ EGTA	46,66
Leukocytes	Phosphate, Malachite Green	Phosphatase activity in the presence of OA $\pm$ EGTA	39
PBMC	Phosphate, by released ^{32}P	Phosphatase activity in the presence of OA $\pm$ CsA	11,67
PBMC	Phosphate, by released ^{32}P	Phosphatase activity in the presence of OA $\pm$ EGTA	38,46
PBMC	Dephosphorylated RII, HPLC	Phosphatase activity in the presence of OA	51,63
PBMC	Phosphate, by released ^{32}P	Phosphatase activity in the presence of OA and Mg	52
Lymphocytes	Dephosphorylated RII, HPLC	Phosphatase activity in the presence of OA	68

B) Cytokine mRNA

Sample	Stimulation agents	Cytokines	References
Leukocytes/PBMC	PMA/ionomycin	IL-2, IFN- γ , GM-CSF	60,69
Leukocytes/PBMC	Anti-CD3/anti-CD28	IL-2, IL-4, TNF α	57,70
Leukocytes	Anti-CD3/anti-CD28	IL-2	59

C) Extracellular cytokine concentration

Sample	Stimulation agents	Cytokines	References
Supernatant stimulation matrix	Anti-CD3/anti-CD28	IL-2	59,70
Blood supernatant	PHA	IL-2, IFN- γ	67,71

D) Intracellular cytokine concentration / cytokine producing cells

Analyte	Stimulation agents	Cytokines	References
CD4+ and CD8+ T cells	PMA/ionomycin	IL-2	63
CD4+ and CD8+ T cells	PMA/ionomycin	IL-2, IFN- γ , TNF α , IL-4	72
T cells	PMA/ionomycin	IL-2, IFN- γ , TNF α , IL-4	14,62,73

E) Surface activation markers

Analyte	Stimulation agents	Markers	References
CD4+ T cells	Anti-CD3/anti-CD28	CD25, CD69	70
T cells	Concanavalin A	CD11a, CD25, CD71, CD95, CD134, CD154	62, 72-74

F) Cell proliferation

Analyte	Stimulation agents	Markers	References
Lymphocytes	Concanavalin A	PCNA	62, 72-74

Table 5.1. Overview of methods used for pharmacodynamic monitoring of calcineurin inhibitor therapy. Methods are listed per strategies (A-F) and are subdivided by characteristics. Abbreviations: CsA, cyclosporin A; EGTA, ethyleneglycoltetraacetic acid; IFN- γ , interferon- γ ; IL-2, interleukin-2; OA, okadaic acid; PBMC, peripheral blood mononuclear cells; PCNA, proliferating cell nuclear antigen; PHA, phytohemagglutinin; PMA, phorbol 12-myristate 13 acetate; TNF α , tumor necrosis factor α .

Sample choice can also affect the inhibition profile of calcineurin inhibitors: inhibition affinities for CsA were comparable for leukocyte subsets, but erythrocytes had significant lower CsA affinity(40).

Another problem that has been highlighted is the problem of calcineurin activity loss due to fractionizing and separation procedures(5) and washing out of drugs during cell preparation or by drugs efflux transporters. To overcome some of these issues, samples have been treated with inhibitors of drug-efflux transporters or have been prepared on ice(10,39).

CN activity *in vivo*

Two comprehensive reviews were recently published that report the clinical performance of current assays in detail(44,45). Here we briefly discuss the general outlines of these studies. When calcineurin activity is monitored in transplantation patients treated with either TRL or CsA, a clear inverse relation between drug concentration and enzyme activity in whole blood, leukocytes, and PBMC fraction is found(39,46,47). A representative example of calcineurin activity and CsA drug concentration profiles in a renal transplantation patient the first 6 months after transplantation is shown in figure 5.3.

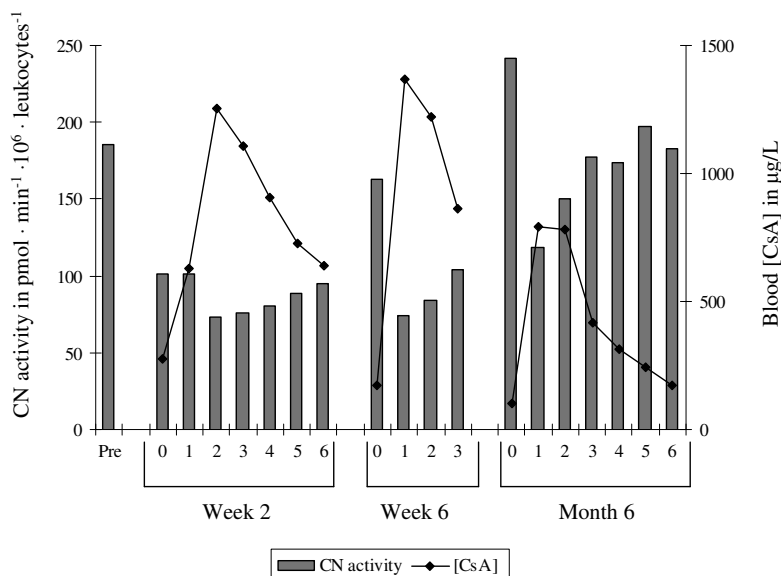


Figure 5.3. Leukocyte calcineurin activity and blood CsA concentration of representative renal transplantation patient. Calcineurin activity and CsA blood concentrations were determined at four time points. Pre-transplant, before starting CsA therapy; week 2 and week 6 after transplantation when reference AUC was 5400 µg/L·h, and 6 after transplantation when reference AUC was 3250 µg/L·h. 1,2,3,4,5, and 6 represent hours after drug intake, 0 being just before drug intake and 12 hours after previous dose. Adapted from(65).

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The inhibition of calcineurin activity by CsA in these fractions is rapidly reversible, and no time lag is observed between CsA drug concentration and calcineurin activity(46,48). Interestingly, in the first hours after drug intake, clear inhibition of calcineurin activity is observed in patients. After six hours, calcineurin activity cannot be distinguished from pre-intake calcineurin activity, while drug concentration is still significantly elevated(46,47). Population PK/PD analysis of living donor liver transplant recipients showed large inter-individual variability in the IC_{50} value of CsA 200 $\mu\text{g/L}$ (CV = 84%) and TRL 26.4 $\mu\text{g/L}$ (CV = 81%)(38). In addition, TRL shows a less dynamic change when compared to CsA in the PBMC fraction, and the maximal inhibitory effect of TRL is lower, findings which correspond to *in vitro* data(38). Another characteristic of calcineurin inhibition by either CsA or TRL is that enzyme activity is only partially inhibited *in vivo*. This partial calcineurin inhibition results in greater inhibition of weak stimulatory signals compared to maximally stimulatory signals, and is thought to be responsible for the presence of immune responsiveness against infections while patients are successfully protected from allograft rejection(49). Immunosuppressant concentrations limit calcineurin inhibition by CsA and TRL at high drug concentrations in *in vitro* systems(41). Whether these effects occur under physiological conditions in transplant recipients remains unclear, since TRL and CsA concentration extrapolations are necessary to translate *in vitro* results to the *in vivo* situation. This translation is necessary due to abundant irrelevant binding sites in blood that are absent in culture medium(50).

In order to prove that pharmacodynamic monitoring strategies act as a functional index of calcineurin inhibitor-based immune suppression, correlation between clinical immunological events and measurement is required. Sanquer et al. showed that patients with acute grade II or higher graft-versus-host-disease (GVHD) had greater calcineurin activity, which could be predicted with a sensitivity of 89% and a specificity of 54%(51). However, others have found a decrease in calcineurin activity in bone marrow transplanted patients with acute GVHD, suggesting that CsA-resistance could be independent of calcineurin inhibition(52). In liver transplantation patients, Fukudo et al. showed that calcineurin activity was increased in both TRL and CsA treated patients who suffered from an acute rejection(38). In renal transplant recipients, baseline calcineurin enzyme inhibition was significantly lower in patients with biopsy-proven acute rejection(53).

Immunological monitoring strategies

Immunological monitoring strategies of calcineurin inhibitor therapy were developed early after the introduction of CsA and well before calcineurin was identified as its pharmacological target protein(54). In these assays, third-party mixed lymphocyte reactions and IL-2 excretion after mitogen stimulation were used as immunologic markers for CsA therapy(54,55). Simulation of an immune response, either by mixed lymphocyte reaction, superantigens, or TCR/co-stimulation directed activation is required for immunological calcineurin inhibitor monitoring strategies. Inhibition of immune responsiveness by immunosuppressant therapy is often used as readout. This inhibition is measured on different levels, including cytokine mRNA, activation surface markers, cytokine producing cells, extracellular cytokine concentration, and cell proliferation (see Figure 1 and Table 1). When patients treated with calcineurin inhibitors are monitored, full blood samples or isolated cell fractions are stimulated and incubated for a period of time to allow cellular responses and the appearance of markers. Distinct kinetic profiles are observed for different markers after stimulation. In addition, the type of stimulus employed, affect the kinetic profile of surface markers(56) and cytokine mRNA(57). When mRNA gene expression programs were analyzed by microarrays, highly similar gene expression patterns were observed for CD3/CD28, phytohemagglutinin (PHA), and ionomycin/phorbol-12-myristate-13-acetate (PMA) stimulation protocols, illustrating the overlapping usefulness of different types of stimulation(58).

For analyzes of cytokine mRNA, mRNA is isolated from leukocyte or PBMC fraction. Quantification of cytokine mRNA is performed by quantitative real-time PCR and expressed with respect to housekeeping mRNA (β -actin, cyclophilin B, or GAPDH)(57,59,60). Several cytokine mRNA transcripts are used as pharmacodynamic markers of calcineurin inhibitor therapy, including IL-2, IFN γ , TNF α , IL-4, and GM-CSF. When blood is incubated with a calcineurin inhibitor, a significant reduction in these transcripts is found. In addition, when cytokine transcripts (IL-2, IFN γ , and GM-CSF) were monitored in renal, cardiac, and liver allograft recipients undergoing immunosuppressive therapy, expression was decreased and showed an inverse relationship with CsA concentrations. Maximum inhibition was observed 2 hours after drug intake corresponding to the peak of CsA drug concentration. As observed for calcineurin enzyme inhibition, gene expression of NFAT-regulated genes 6 hours after drug intake was comparable to through levels(60). In addition, NFAT-regulated gene expression of IL-2, IFN- γ , and GM-CSF was found to be lower in renal allograft patients on CsA that suffered from recurrent infections and malignancies(61).

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Determination of cytokine producing cells and surface markers is performed using flow-cytometry to analyze stained cells. For intracellular cytokine analyzes, secretion inhibitors are used during stimulation protocols. Next, permeabilized cells are stained and analyzed by flow cytometry and expressed as percent of cytokine producing cells(14,62). When monitoring cytokine producing lymphocytes in liver allograft recipients, it was found that the percent of IL-2 producing CD8+ T cells was higher during acute rejection compared to non rejecting recipients, with a positive predictive value of 58% and a negative predictive value of 98%(63). No difference was found between percentage of IL-2 producing CD4+ T cells and total T cells, suggesting an important role for IL-2 producing CD8+ T cells.

When cell surface markers are monitored, the expression of T-cells activation markers is determined using monoclonal antibodies. Positive cell populations are quantified by flow cytometry and expressed as percent of marker positive cells within the T cell population(14,62). These strategies have primarily been used to study immunosuppressive combination protocols for drug interactions(12). When monitored in heart transplanted patients on a CsA-based immunosuppressive regime, reductions of the proliferation marker PCNA as well as the T cell surface markers CD11a, CD25, CD71, CD95, CD134, CD152, and CD154 were observed 2 hours after drug intake(64).

Future perspectives

The current technologies used for pharmacodynamic monitoring of calcineurin inhibition therapy have not proved to be clinically useful markers in the management of patients on these drugs. More specific samples combined with improved understanding and anticipation of analytical variation, particularly concerning biological assays, might be important for the development of more accurate markers. Most pharmacodynamic monitoring techniques used to date have been validated for their analytical performance, but validation methods such as sample composition effects on measurement and drug leakage/dilution during processing are of key importance. For instance, it is required that the *in vivo* conditions are maintained during the cell isolation/preparation protocol in order to measure a representative pharmacodynamic state. It is assumed that pharmacodynamic measurement in blood or blood fractions are representative of the pharmacodynamic effects at the immunological sites responsible for allograft rejection.

An interesting next step would be to monitor calcineurin capacity or immune responses within T cells, which are the specific immunosuppressive target compartment of the calcineurin inhibitors(5). This method should avoid analytical

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variation by sample composition, and absolute values should be more comparable between individuals. These immunosuppressive capacities could provide a threshold under which no functional T cell activation is possible, even in the presence of full T cell receptor binding and maximum intracellular Ca^{2+} influx.

When such new methods have been developed, clinical validation by means of large cohorts is crucial to investigate their efficacy and to determine whether pharmacodynamic monitoring strategies are viable for clinical use.

Conclusions

The call for more accurate and clinically useful methods for monitoring immunosuppressive drugs has led to the development of various pharmacodynamic monitoring strategies for calcineurin inhibitors and other immunosuppressive agents. The study of pharmacodynamic monitoring of immunosuppressive therapy is still in its infancy. Its true efficacy remains to be proven, though the first signs of its clinical utility are emerging. Further understanding and anticipation of the analytical and clinical variables seem to be required for the development of new markers for monitoring calcineurin inhibition therapy in allograft recipients.

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Chapter 6

Validation of a T cell specific calcineurin activity assay for monitoring cyclosporine therapy

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Abstract

To further improve calcineurin (CN) inhibition therapy, pharmacodynamic monitoring is currently investigated as a novel approach. Although clear relations with pharmacokinetics parameters have been observed, its clinical usefulness has not been demonstrated thus far. In order to increase target cell specificity, a T cell specific CN activity assay was developed. T-cells were isolated from whole blood using anti-CD3 coated paramagnetic beads and the isolated cells were analyzed for purity, yield, recovery, cell death and CD4+ and CD8+ subpopulations. T cell isolates were $95.5\% \pm 1.5\%$ pure and no differences were observed for CD4+ and CD8+ population distribution before and after isolation, illustrating that representative T cell preparations were taken from whole blood samples. The CN assay showed linearity and reproducibility and no significant washout of CsA during the isolation procedure was noted. CN inhibition was observed when the protocol was used to monitor CsA-based immunosuppressive therapy in six stable renal allograft recipients.

The described T cell isolation protocol isolates a representative, highly pure T cell sample from whole blood. Adequate analytical performance was observed and the method was found to be suitable to monitor T cell specific CN inhibition in stable renal allograft recipients receiving CsA.

Introduction

Pharmacodynamic monitoring of immunosuppressive therapy offers new potential tools to further improve the therapeutic index of critical dose drugs such as the calcineurin (CN) inhibitors cyclosporine (CsA) or tacrolimus. These drugs share a large inter-individual variability in pharmacokinetic parameters and are associated with considerable adverse effects such as nephrotoxicity, hypertension and diabetes mellitus(1). Advanced pharmacokinetics such as intracellular drug concentrations, pharmacogenetics and pharmacodynamics are currently investigated to increase monitoring specificity and clinical performance of calcineurin inhibitors(2-4).

Several pharmacodynamic monitoring strategies have been described, but only a few have been tested in solid organ allograft recipients. Although definite correlations between drug concentrations and pharmacodynamics have been described(5-7), no clear clinical associations have been reported and further insight in analytical variables may lead to identification of more accurate markers(8). We have recently reported that matrix composition directly confounds calcineurin activity measurement(8). This notion and the generally accepted view that T cells are the major target cells of calcineurin inhibitor therapy led us to the development of a T cell specific calcineurin activity assay(9).

Materials and Methods

Materials

The following materials were used: cyclosporin A, tacrolimus, dithiothreitol (DTT), ascorbic acid, NP-40, and soybean trypsin inhibitor from Sigma-Aldrich, phenylmethylsulfonyl from Merck, leupeptin from Roche, aprotinin from Bayer, phosphate standard from Fluka, biomol green cellular calcineurin assay kit plus from Biomol, fluorescein isothiocyanate (FITC)-labeled CD4 (clone SK3), phycoerythrin (PE)-labeled anti-CD8 (clone SK1), APC-labeled anti-CD3 (clone SK7), APC-labeled anti-CD2 (clone S5.2), PerCP-labeled anti-CD45 (clone 2D1), APC-labeled anti-CD19 (clone SJ25Cl), FITC-labeled anti-CD3, PE-labeled anti-CD16 and anti-CD56 and anti-CD3 coated paramagnetic beads, all from BD Biosciences and RPMI 1640, iFCS, penicillin/streptomycin and paramagnetic anti-CD3-beads from Invitrogen.

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Patients and healthy volunteers

EDTA-anticoagulated blood from the anticubal veins of healthy volunteers and renal allograft recipients receiving a CsA based immunosuppressive regimen was used for validation of the T cell calcineurin activity assay. Formal approval from the institutional ethics committee was obtained, and informed consent for blood sampling was obtained from all participants. The renal allograft recipient group underwent graft implantation 12 to 24 months prior to sample date and received dual immunosuppressive therapy including prednisolone (10 mg daily) and CsA. CsA dosage was controlled by estimated min-AUC(0,2,3 h) aiming at a target AUC(0-12 h) of 3250 $\mu\text{g/L}\cdot\text{h}$ (10).

T cell isolation, sample preparation and calcineurin assay

EDTA-anticoagulated blood (1.5 mL) was incubated with 60 μL of pre-washed (0.1 % BSA, 10 mM Hepes, 9.0 g/L NaCl, pH 7.5) anti-CD3 paramagnetic bead suspension for 25 minutes at 4°C with continuous tilting and rotation. After incubation, samples were placed in a magnet (DynaMac-2, Invitrogen) for 1 min. and supernatant was discarded. T cells were washed 4 times with 1 mL erythrocyte lysis buffer (8.4 g/L NH_4Cl , 1.0 g/L KHCO_3 , pH 7.3). During this washing procedure samples and washing solution were kept on ice. Isolated and washed T cells were directly lysed by addition of 100 μL lysis buffer (50 mM Tris-HCl (pH 7.4), 1.0 mM DTT, 5.0 mM ascorbic acid, 0.02% (v/v) NP-40, 50 mg/L soybean trypsin inhibitor, 50 mg/L phenylmethylsulfonyl fluoride, 5.0 mg/L leupeptin and 5.0 mg/L aprotinin) and three freeze-thaw cycles (liquid N_2 / 30°C). Cell debris and beads were spun down (10.000g, 10 min, 4°C), supernatant was collected and snap frozen in liquid N_2 before storage at -80°C until CN activity measurement.

The calcineurin activity assay used, was previously described, with one adjustment; a 0-1.5 nmol phosphate calibration curve was used for phosphate quantification(11). Calcineurin activity was expressed as nmol phosphate released per minute per μg protein. Protein concentrations of lysates were determined using the Coomassie Plus protein assay (Thermo Scientific, Rockford, IL, USA).

Cellular analysis

T-cell recovery, purity and yield were calculated from hematological analyses before and after T-cell isolation. T-cell concentrations of the starting material (whole blood) were calculated from leukocyte differentiates, performed on a Sysmex XE2100, and lymphocyte T-cell differentiations obtained using flow-cytometry. Lymphocytes gated in the side-scatter / CD45 dot-plot, were analyzed for CD3+, CD3+ /CD4+ and CD3+/CD8+ populations.

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For analyzes of the isolated T-cell population, the paramagnetic beads were detached by resuspension in RPMI culture medium containing 10 % inactivated fetal calf serum, 100 U/mL penicillin and 100 µg/mL streptomycin and overnight incubation in a humidified environment (5%, CO₂). Next day, the soaked off beads were removed by use of magnet. From the supernatant, manual erythrocyte and leukocyte counts were performed. Cell death was determined by trypan blue staining and leukocyte differentiate were determined after May-Grunwald/Giemsa staining of cytopins (Sysmex XE2100).

Determination of the T-cell purity within the lymphocyte population was performed by flow-cytometric analysis of B-, NK- and T-cells. Since the CD3 epitope was absent after detachment of T cells from the CD3-coated paramagnetic beads, we used the CD2 epitope as T cell marker for the isolated lymphocytes. Here, lymphocytes gated in the side-scatter / CD45 dot-plot were analyzed for CD19+, CD16/56+ populations in one panel and CD2+, CD2+/CD4+ and CD2+/CD8+ populations in a second panel.

Data analysis

Data are presented as mean ± SD. One-way ANOVA was used for statistical analysis of the multiple groups in the storage experiments. Statistical difference between two groups was determined by student t-test and statistical difference was defined as p<0.05.

Results

The final T cell purity isolated from healthy volunteer blood (n=15) was 95.5% ± 1.5% with 4 ± 1.2% of erythrocytes present (table 6.1).

erythrocytes (%)	granulocytes (%)	monocytes (%)	Lymphocytes subsets		
			CD19+ (%)	CD15/56+ (%)	CD2+ (%)
4.0 (1.2)	n.d	n.d	0.09 (0.06)	0.3 (0.2)	95.5 (1.5)

Table 6.1. T cell sample composition. Purity of T cell isolate was determined for 15 healthy volunteers. Data are presented as mean % (SD) of contaminants present in isolate. n.d.; non detected.

A yield of $0.86 \pm 0.44 \cdot 10^6$ T cells and a recovery of $42\% \pm 14\%$ was found. Since the recovery was relatively low the CD3 coated beads were up-titrated, but did not increase T cell recovery. Significant cell death after isolation was excluded by trypan blue staining, being only $7\% \pm 2\%$. No changes were observed in CD4+ and

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CD8+ T cell subpopulations before and after the isolation procedure ($p=0.89$ and 0.84 respectively, $n=5$).

Blood was stored at 4°C for 0, 4, 8 and 24 hours ($n=3$) after collection and no decrease in activity was detected ($p=0.78$). Stability of calcineurin activity during lysate storage was investigated for 0,1,2,6 and 15 days and a decrease in calcineurin activity was observed after 2 days (13%, $p=0.046$). Subsequently, sample lysates were stored for maximal 1 day before calcineurin activity measurement.

Linearity was observed when T cell-beads fractions isolated from healthy volunteers were serial diluted with beads without T cells, before T cell lysis (figure 6.1).

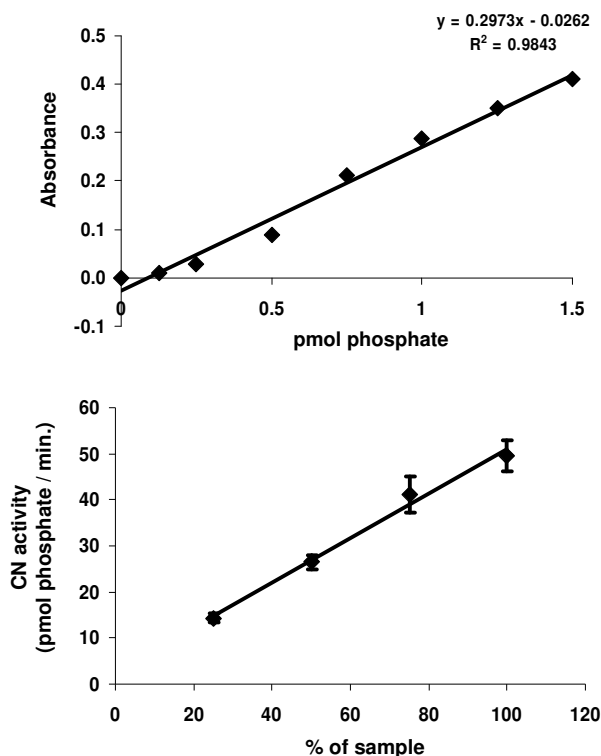


Figure 6.1. Linearity of calcineurin assay. Upper; a typical calibration curve of the calcineurin assay. Lower; linearity of calcineurin assay determined by dilution of T-cells. These results are presented as mean $\pm$ SD ($n=3$) and calculated from calibration curve shown in upper figure.

The detection limit of the phosphate measurement was 0.15 nmol phosphate when calculated as the mean of blank measurement plus 3 standard deviations ($n=8$).

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When blood from one healthy volunteer was taken 4 times over a 4 month period, a reproducibility including intra-individual variation of 7% was found.

To investigate whether CsA is washed out during the isolation procedure, we incubated whole blood with CsA and tested whether doubling of the washing steps, required for T cell isolation, decreased calcineurin inhibition by CsA. We could detect a calcineurin inhibition of about 30% for final whole blood CsA concentrations of 1000 and 2500 $\mu\text{g/L}$ ($p=0.0011$) and no loss of calcineurin activity ($p=0.18$) or washing out of CsA ($p=0.57$) during the isolation procedure (figure 6.2).

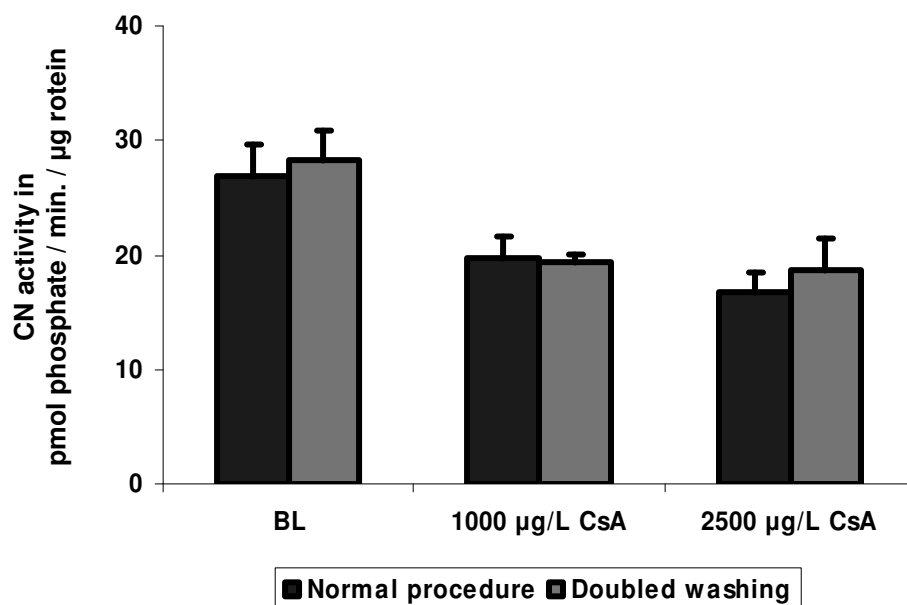


Figure 6.2. Washing out of CsA. Washing out of CsA from the T cells during the isolation procedure was investigated by doubling of the isolation washing steps for 2 whole blood CsA concentrations. The results are presented as mean $\pm$ SD ($n=7$ for BL; $n=4$ for 1000 $\mu\text{g/L}$ CsA; $n=3$ for 2500 $\mu\text{g/L}$). BL (Blank); without CsA

To test whether the T cell calcineurin activity protocol can be used for monitoring CsA therapy, we monitored T cell calcineurin activity in 6 renal allograft recipients that received CsA therapy. The observed T cell calcineurin activities and whole blood CsA concentrations of two representative patients are illustrated in figure 6.3. Significant inhibition of T cell calcineurin activity was observed (14%) when activities just before drug intake (T_0) (23.3 ± 9.6 pmol phosphate/min./ μg protein) were compared to activities 2 hours after drug intake (T_2) values (20.0 ± 7.8 pmol phosphate/min./ μg protein, $p=0.026$) with corresponding CsA concentrations of 105 ± 18 $\mu\text{g/L}$ (T_0) and 736 ± 303 $\mu\text{g/L}$ (T_2 , $p = 0.0015$).

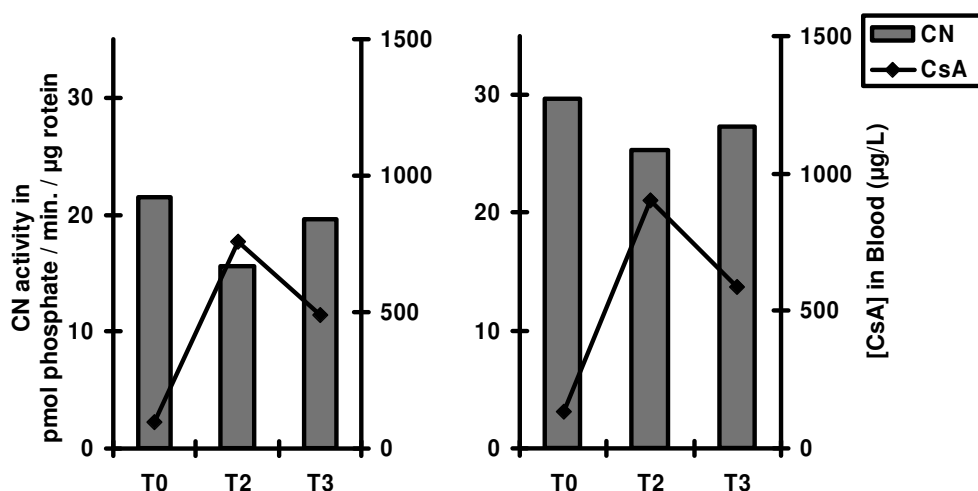


Figure 6.3. T cell calcineurin inhibition and whole blood CsA concentrations in two representative allograft recipients. T cell calcineurin activity was monitored parallel to whole blood CsA concentration. T0, sample taken at time point just before drug intake (12 hours after previous dose) and T2 and T3 are samples taken at 2 and 3 hours after drug intake, respectively.

Discussion

One of the major issues associated with the current pharmacodynamic monitoring strategies for calcineurin inhibition therapy is the large inter-individual variability observed that does not necessarily correlates to clinical endpoints. Part of this variation may be ascribed to either sample choice, sample composition and/or accuracy of the analytical techniques used(8,9). A T cell specific protocol for measurement of calcineurin activity might overcome most of these issues. Final T cell isolates were of high purity, ensuring that calcineurin activity measured after isolation was not significantly confounded by the presence of other cells. For instance, erythrocytes have only low cellular calcineurin activity(12), but they do contain proteins and when calcineurin activity is expressed per protein, large variations in erythrocytes could seriously confound measurement outcome. In addition, no changes in the CD4+ and CD8+ population distribution were observed within the T cell fraction after isolation. This indicated that a representative T cell sample was taken from the blood.

Recovery of T cells was relatively low, but up-titration of the beads did not increase the recovery of T cells. The T cell yield from 1.5 mL of blood from healthy

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volunteers as well as for renal allograft recipients was however sufficient to perform the calcineurin assay with a suitable analytical reproducibility. The most likely cause of the low recovery is cell loss during the bead/T cell washing procedure. This is confirmed by lower calcineurin measurement and lower protein concentration measurement after doubling of the washing steps.

Storage of T cell lysate showed a decrease in calcineurin activity starting already at the 2nd day after initial storage. Previously it was observed that storage of leukocyte lysate using the same lysis buffer could be stored up to 3 weeks(11). The most likely explanation for this discrepancy is the presence of the paramagnetic beads or remainings thereof in the cell lysate. Its possible that remainings of the beads could for instance oxidize and subsequently inactivate calcineurin(13,14). Loss of intracellular CsA during T cell isolation was found to be of only limited relevance, since doubling of the washing steps did not affect calcineurin inhibition by CsA. This indicates that the cellular conditions are maintained throughout the T cell isolation procedure.

In this protocol we couple the T cell isolation protocol to a calcineurin assay for pharmacodynamic monitoring of CsA therapy. The isolation protocol could however also be followed by other enzymatic assays such as mTOR activity, inosine monophosphate dehydrogenase (IMPDH) or cellular drug concentration measurement that could be of interest for allograft recipients. Intracellular T cell analysis could also be of interest in other diagnostic fields; a wide range of other intracellular enzymes and markers can be analyzed in T cells isolated from 1.5 mL of blood.

In conclusion we present a validated protocol for the isolation of highly pure T cell fractions that can be used to monitor calcineurin therapy and other intracellular markers.

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Chapter 7

Everolimus and sirolimus antagonize tacrolimus
based calcineurin inhibition via competition for
FK-binding protein 12

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Abstract

The calcineurin inhibitors cyclosporin A and tacrolimus and the inhibitors of the mTOR, sirolimus and everolimus bind immunophilins that are required for their immunosuppressive action. In contrast to cyclosporin A, tacrolimus and the mTOR inhibitors (MTIs) share common immunophilins, the FK506-binding proteins (FKBPs). We investigated the immunosuppressive interactions of MTIs on tacrolimus based immune suppression, since insights in immunological drug–drug interactions can be very relevant for optimization of immunosuppressive regimens in allograft transplantation medicine.

Isolated peripheral blood mononuclear cells from healthy volunteers were incubated with combinations of MTIs and calcineurin inhibitors and when monitored for calcineurin activity and IL-2 excretion after mitogen stimulation, tacrolimus IC₅₀ concentrations shifted to higher concentrations in the presence of MTIs. This antagonism was absent for cyclosporine A, reproducible for 10 healthy volunteers ($p < 0.001$) and stronger for sirolimus than for everolimus. When cell lysate was treated with and without MTI, tacrolimus and FKBP12, FKBP12 could increase calcineurin inhibition by tacrolimus and reverse the MTI antagonism for both MTIs. These results demonstrate that FKBP12 can be rate limiting for calcineurin inhibition at high tacrolimus concentrations and that the antagonism of sirolimus and everolimus on tacrolimus based immune suppression is mediated via saturation of FKBP12.

Introduction

After organ transplantation, immune suppression to prevent allograft rejection is crucial for successful graft survival. Several immunosuppressive agents, such as the calcineurin (CN) inhibitors tacrolimus (TRL) or cyclosporin A (CsA) and the mammalian target of rapamycin (mTOR) inhibitors sirolimus (SRL) or its derivative everolimus (EVL), are used and combinational therapies are applied to achieve optimal immunosuppressive effect and reduce individual drug toxicity(1). CsA and TRL inhibit CN, a calcium/calmodulin dependent serine/ threonine protein phosphatase that plays an important role in mediating T-cell receptor stimuli into cytokine (IL-2, IFN γ) transcription. SRL and EVL inhibit mTOR, a kinase with a key function in cell cycle progression(1,2). Both classes of drugs inhibit enzyme activity only after binding to specific intracellular binding proteins, so called immunophilins(3,4). CsA binds selectively to the cyclophilin class of immunophilins that are cytosolic peptidyl–prolyl isomerases, while TRL and the mTOR inhibitors (MTIs) bind to the FK506-binding proteins (FKBP) that also exert peptidyl–prolyl isomerase activity(3). SRL, EVL and TRL bind similar FKBP(5,6), while distinct and selective inhibition of respectively mTOR and CN is observed (7). Since similar FKBP are required for successful CN inhibition by TRL and mTOR inhibition by SRL or EVL, pharmacological drug–drug interactions have been investigated by several groups. Dumont et al. has shown that SRL and TRL are reciprocal antagonists(5,8), while others have demonstrated immunosuppressive synergism of these drugs(9,10).

In clinical practice, pharmacokinetic drug–drug interactions on metabolic and drug-transporter proteins are therapeutically avoided or adjusted for by therapeutic drug monitoring. When drug–drug interactions occur on the pharmacologic level and thus inhibit each others effectiveness, concentration measurement becomes less relevant since it is unable to detect this interaction(10). In addition, combinations of immunosuppressive drugs might be associated with immunosuppressive synergism(11). Pharmacodynamic monitoring strategies that can detect these interactions may prove to be useful to further optimize immunosuppressive regimen(12). In this respect we studied the interaction of EVL and SRL on TRL based immunosuppression in peripheral blood mononuclear cells (PBMCs) isolated from healthy individuals. Pharmacological interaction was investigated by measurement of CN enzyme activity. For immunological monitoring, the IL-2 downstream product of CN was used.

Materials and methods

Materials

The following materials and reagents were used: Buffy coat were purchased from Sanquin (Amsterdam, NL). RPMI 1640, heat inactivated fetal calf serum (iFCS), penicillin/streptomycin, phytohemagglutinin (PHA) and IL-2 EASIA kit from Invitrogen (Carlsbad, CA), NP-40, soybean trypsin inhibitor, cyclosporin A, tacrolimus, sirolimus (rapamycin), FKBP12 and phorbol-12-myristate-13-acetate (PMA) from Sigma–Aldrich (Steinheim, Germany). Phenylmethylsulfonyl fluoride from MERCK (Darmstadt, Germany), leupeptin from Roche (Basel, Switzerland), aprotinin from Bayer (Leverkusen, Germany), phosphate standard concentrate and everolimus from Fluka (Buchs, Switzerland) and the malachite green calcineurin activity assay kit from Biomol (Plymouth Meeting, PA). All other chemicals and reagents were of the highest available grade.

Immunosuppressants

The immunosuppressants CsA (MW 1203), TRL (MW 804), EVL (MW 958) and SRL (MW 914) were dissolved in ethanol and serial diluted in ethanol. These immunosuppressant stock solutions were diluted in the experiments resulting in final CsA, EVL and SRL concentrations of 0.025, 0.25, 2.5, 25, 250 and 2500 µg/L and final TRL concentrations of 0.00625, 0.0625, 0.625, 6.25, 62.5 and 625 µg/L. Final ethanol concentrations were 0.25% for single immunosuppressant experiments and 0.5% for the interaction experiments. For the CsA inhibition curve in Figure 7.2, CsA ranged from 0.1 to 3600 mg/L with final ethanol concentrations of 1%. The ethanol concentrations resulted in a 5% increase in CN activity when compared to an ethanol blank (n = 4, P = 0.04).

PBMC isolation and cell culture

Peripheral blood mononuclear cell (PBMC) fraction of healthy volunteer blood was isolated from buffy coats by ficoll density gradient centrifugation. Remaining of erythrocytes and thrombocytes were lysed and washed out by NH₄Cl lysis buffer (8.4 g/L NH₄Cl, 1.0 g/L KHCO₃, pH 7.3) and resuspended in phosphate buffered saline (PBS, 154 mmol/L NaCl, 1.4 mmol/L phosphate pH 7.5) for cell count on a Sysmex XE2100. Cells were centrifuged (10 min., 350g, 4 °C) and resuspended at a concentration of $2 \cdot 10^6$ cells/mL in RPMI 1640 cell culture containing 10% iFCS, 100 U/mL penicillin and 100 µg/mL streptomycin.

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For CN activity measurement, cells were incubated for 1 h at 37°C with immunosuppressant(s). After incubation, samples were centrifuged (10 min, 350g, 4°C) and cell pellets were washed once with 400 µL HEPES buffered saline (9.0 g/L NaCl, 10 mmol/L Hepes pH 7.5), resuspended in 400 µL lysis buffer (50 mmol/L Tris-HCl pH 7.7, 1.0 mmol/L dithiothreitol, 5.0 mmol/L ascorbic acid, 0.02% (v/v) NP-40, 50 mg/L soybean trypsin inhibitor, 50 mg/L phenylmethylsulphonyl fluoride, 5.0 mg/L leupeptin and 5.0 mg/L aprotinin) and lysed by 3 freeze thaw cycles (liquid N₂ /30°C). Cell debris was centrifuged (10 min, 10000g, 4°C) and supernatants were snap frozen in liquid N₂ before storage at -80°C till CN activity measurement(13).

For IL-2 excretion measurement, cells were stimulated by PHA/PMA 1 h after incubation with immunosuppressant(s), and incubated overnight at 37°C in a humidified environment (5%, CO₂). PMA stock solution was 4000 times diluted in cell cultures resulting in a final PMA and DMSO concentration of respectively 5.3 nM and 0.025%, and PHA was 100 times diluted according to manufacturers' protocol. The next day, cells were centrifuged (10 min, 350g, 4°C) and supernatant was stored at -20°C until IL-2 concentration measurement.

Cell lysate experiments

In order to investigate the role of FKBP, we isolated PBMCs from buffy coats obtained from healthy volunteers. Untreated PBMCs were lysed as described before and aliquoted lysate was incubated in duplicate (1 h, 37°C) with and without TRL, MTI and FKBP12 resulting in final lysate concentrations of 25 µg/L TRL, 50 µg/L of SRL/EVL and 2000 µg/L and 10000 µg/L of extra FKBP12. At these concentrations the molar concentrations of the MTIs, TRL and FKBP12 were of the same order of magnitude. Directly after incubation CN activity was determined.

Calcineurin assay

Calcineurin phosphatase activity was measured as described before(13). In brief, CN phosphatase was quantified by measurement of the dephosphorylation of the phosphorylated RII substrate in the presence of okadaic acid. 15 µL of sample lysis was added to 40 µL of assay buffer (0.313 mmol/L calmodulin, 0.375 mmol/L RII phosphopeptide substrate, 75 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 9 mmol/L MgCl₂, 0.75 mmol/L dithiothreitol, 0.0375% NP-40, 0.625 µmol/L okadaic acid, 0.75 mmol/L CaCl₂ and 5 mmol/L ascorbic acid) and substrate blanks were used to correct for background absorbance. The assay was run for 30 min. at 30 °C and stopped by addition of 100 µL of malachite green reagent. Colour was developed

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the next 50 min. before absorbance measurement at 620 nm, and phosphate was quantified by means of a 0–5 nmol calibration curve.

Cytokine concentration measurement

IL-2 concentrations were determined with the EASIA kit (Invitrogen) and performed according to manufacturers' protocol.

Data analysis and statistics

Values are presented as mean of duplicate and blank experiments were performed in quadruple. CN activity and IL-2 concentrations were expressed as % of blank measurement in order to remove inter-individual variation in absolute CN activity and IL-2 excretion. CN inhibition curves and IL-2 cytokine inhibition curves were fitted by sigmoidal dose–response curves using GraphPad software and IC₅₀ concentrations, Emax values and 95% confidence intervals (CI) were obtained from these regression analysis. Student t-tests were used to test significance and statistical significance was defined as $P < 0.05$.

Results

IL-2 inhibition by TRL and CsA in the presence of EVL and SRL

To gain insights whether and under which conditions the immunological interaction between the MTIs and TRL occurred, we first measured the effect of the MTIs on IL-2 inhibition by TRL. PBMCs isolated from healthy volunteers were stimulated using PHA/PMA and IL-2 excretion was measured in the presence of TRL or CsA and various concentrations of EVL or SRL. The results of these experiments are shown in figure 7.1 and the obtained IC₅₀ values of CsA and TRL in the presence of various concentrations of EVL and SRL are listed in table 7.1.

The MTIs inhibited IL-2 excretion in the PHA/PMA stimulated PBMCs themselves at the concentration range used and this inhibition increased with increasing concentrations of MTI, though no clear sigmoidal concentration response relationship was visible as well as no inhibition of total IL-2 excretion. When high TRL concentrations were used in combination with EVL or SRL, low concentration of the MTIs did not significantly effect IL-2 inhibition by TRL.

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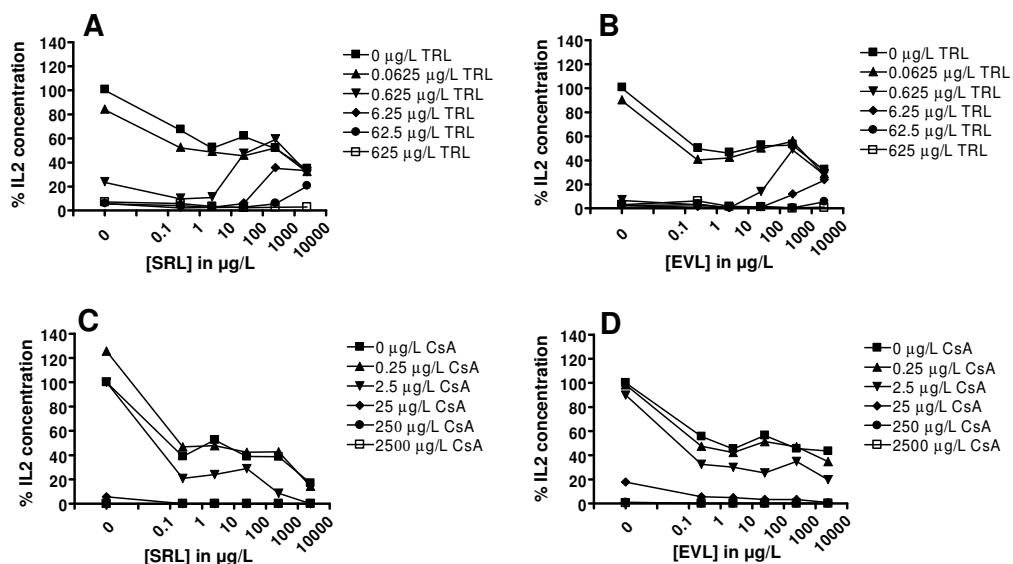


Figure 7.1. IL-2 inhibition by TRL and CsA in the presence of MTIs. IL-2 inhibition by TRL was determined in the presence of various concentrations of SRL (A) and EVL (B). (C) and (D) show the CN inhibition by CsA in the presence of the same concentrations of SRL and EVL respectively. Data show the mean of duplicate measurement for one healthy individual.

	[mTOR inhibitor] in µg/L	Tacrolimus IC ₅₀ in µg/L (95% CI)	Cyclosporin A IC ₅₀ in µg/L (95% CI)
Sirolimus	0	0.20 (0.12 – 0.33)	8.5 (2.6 - 27)
	0.25	0.14 (0.09 – 0.23)	2.7 (1.1 - 6.7)
	2.5	0.22 (0.11 – 0.44)	2.1 (1.1 - 4.1)
	25	1.6 (0.57 - 4.6)	4.7 (2.4 - 9.2)
	250	11 (4.8 - 26)	1.2 (0.35 - 4.1)
	2500	114 (56 – 234)	0.59 (0.05 - 6.8)
Everolimus	0	0.15 (0.06 – 0.36)	9.5 (4.7 - 19)
	0.25	0.13 (0.05 – 0.31)	3.6 (2.1 - 6.3)
	2.5	0.13 (0.05 – 0.38)	4.7 (2.9 - 7.5)
	25	0.28 (0.14 – 0.55)	2.1 (0.84 - 5.0)
	250	2.6 (1.4 - 4.6)	5.6 (3.1 - 10)
	2500	19 (11 - 32)	2.0 (0.84 - 4.9)

Table 7.1. IL-2 IC₅₀ values of TRL and CsA in the presence of various concentrations of SRL and EVL. IC₅₀ values and 95% confidence intervals (CI) were obtained from the sigmoidal concentration–response regressions when the data in Figure 7.1 are presented with the CNI inhibitor on the x-axis and the individual curves are variations in EVL or SRL concentrations.

However at higher concentrations (250 µg/L), significant shifts in IL-2 IC₅₀ values were observed. MTI concentrations of 25 µg/L and higher reversed IL-2 inhibition by TRL though the occurrence and magnitude of this effect is also dependent on

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the TRL concentration and seems to be less for EVL when compared to SRL (figure 7.1A and B). No increasing IC_{50} values and significant differences in IC_{50} values were observed when CsA was used in combination with SRL and EVL (figure 7.1C and D).

CN inhibition by CNIs, MTIs and combinations thereof

To further investigate the origin of the observed IL-2 antagonism, we measured calcineurin activity that is the pharmacological target enzyme of TRL. First, the individual effect of the immunosuppressants on CN activity was investigated and therefore CN inhibition curves were determined for CsA, TRL, EVL and SRL. The data in Figure 7.2 show that SRL and EVL do not inhibit CN activity, while for TRL and CsA a clear dose–response curve was found with an IC_{50} value of 1.0 $\mu\text{g/L}$ (0.51– 2.1 $\mu\text{g/L}$, 95% CI) and an E_{max} of 53% (48–58%, 95% CI) for TRL and an IC_{50} value of 14 $\mu\text{g/L}$ (9.6–21 $\mu\text{g/L}$, 95% CI) and an E_{max} of 31% (26–36%, 95% CI) for CsA.

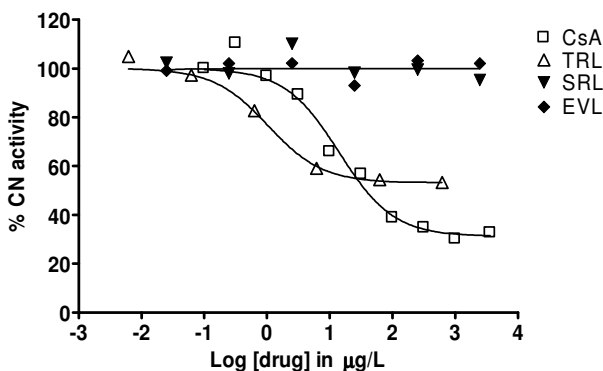


Figure 7.2. CN inhibition by CsA, TRL, SRL and EVL. Data are normalized and expressed as % by dividing through CNI blank measurement (performed in quadruple) and maximum effect was fixed at 100% for the sigmoidal dose–response analysis in GraphPad. Data are presented as mean of duplicate measurement.

Next, we incubated five concentrations of TRL (0.0625–625 $\mu\text{g/L}$) and a TRL blank, each in combination with five concentrations (0.25–2500 $\mu\text{g/L}$) and a blank of either EVL or SRL. As control experiment TRL was replaced by CsA (0.25– 2500 $\mu\text{g/L}$). The obtained CN inhibition curves are shown in figure 7.3 and the calculated IC_{50} concentrations for TRL and CsA in the presence of various concentrations of EVL and SRL are listed in table 7.2.

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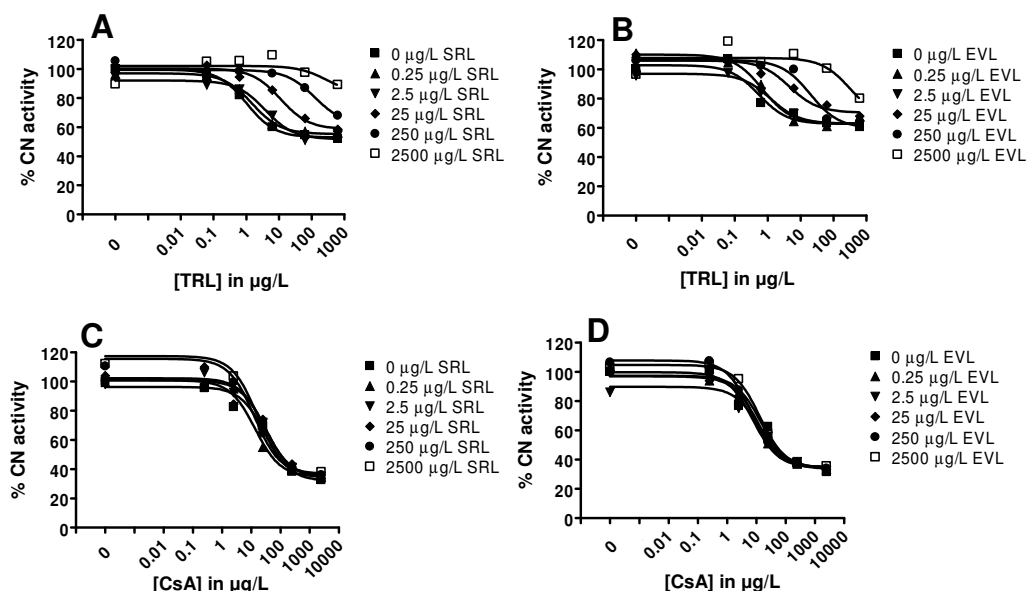


Figure 7.3. CN inhibition by CsA and TRL in the presence of MTIs. CN inhibition by TRL was determined in the presence of SRL (A) and EVL (B). (C) and (D) show the CN inhibition by CsA in the presence of the same concentrations of SRL and EVL respectively. Data show the mean of duplicate of one healthy individual and the lines are the CN inhibition curves of TRL and CsA in the presence of various concentrations of EVL and SRL fitted for sigmoidal dose-response curves by GraphPad software. The IC_{50} values for CsA and TRL obtained from these curves are listed in table 7.2. Data were normalized by dividing through blank measurement in the absence of CNI and MTI. CNI 0 values were set at 10^{-4} µg/L for the regression analysis.

	[mTOR inhibitor] in µg/L	Tacrolimus IC_{50} in µg/L (95% CI)	Cyclosporin A IC_{50} in µg/L (95% CI)
Sirolimus	0	1.0 (0.32 - 3.4)	30 (12 - 74)
	0.25	1.5 (0.69 - 3.4)	13 (7.8 - 23)
	2.5	3.8 (1.6 - 8.8)	31 (15 - 63)
	25	11 (6.7 - 18)	29 (11 - 79)
	250	125 (25 - 620)	13 (5.3 - 31)
	2500	290 (0.08 - $1.1 \cdot 10^6$)	16 (8.5 - 31)
Everolimus	0	0.54 (0.15 - 1.9)	14 (5.1 - 41)
	0.25	0.70 (0.34 - 1.4)	7.6 (3.7 - 16)
	2.5	1.2 (0.39 - 3.9)	13 (4.0 - 43)
	25	4.6 (0.91 - 23)	11 (5.2 - 23)
	250	20 (3.7 - 104)	7.1 (4.0 - 13)
	2500	320 (0.21 - $5.0 \cdot 10^5$)	13 (9.8 - 17)

Table 7.2. CN IC_{50} values of TRL and CsA in the presence of various concentrations of SRL and EVL. IC_{50} values and 95% confidence intervals (CI) were obtained from the sigmoidal concentration-response regressions presented in figure 7.3 by GraphPad software.

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A concentration dependent effect of EVL and SRL on CN inhibition by TRL was observed and significant higher TRL IC₅₀ values were found when 25 µg/L SRL and 250 mg/L EVL or higher, were present in culture medium (figure 7.3A and B). This pattern was not detected for the CsA CN inhibition curves; here no differences in IC₅₀ values were observed by increasing concentration of MTIs (figure 7.3C and D).

Antagonism of MTIs on TRL based immune suppression in 10 healthy volunteers

To determine whether the TRL/MTI interactions are reproducible for other individuals we investigated the interaction on PBMCs isolated from 10 healthy volunteers. For reproduction of the immunological antagonism we reproduced the experiment for 10 healthy volunteers and treated the PBMCs with 0.625 µg/L TRL and 25 µg/L of EVL or SRL, concentrations that previously (figure 7.1) showed to be characteristic for the interaction. The results are shown in figure 7.4.

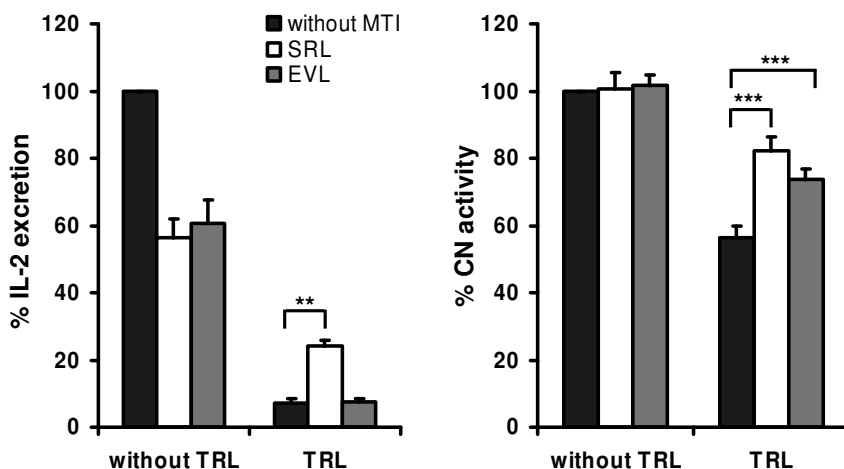


Figure 7.4. MTI antagonism in 10 healthy volunteers. Left: IL-2 excretion after PHA/PMA stimulation for combinations of TRL (0.625 µg/L TRL) and MTIs (25 µg/L). Right: CN activity in the presence/absence of 6.25 µg/L TRL, and 25 µg/L EVL or SRL. Both were tested on PBMCs isolated from 10 healthy volunteers and data were normalized to remove inter-individual variation in absolute IL-2 excretion and CN activity. Data are presented as mean $\pm$ SEM and statistical significance was tested using paired Student t-tests and presented as (***) for $P < 0.001$ and (**) for $P < 0.01$.

All three drugs inhibited IL-2 excretion in our cell culture system, though TRL caused a larger inhibition than SRL ($P = 0.001$) and EVL ($P = 0.002$) that partially inhibited the IL-2 excretion in these concentrations. When combinations of TRL and MTIs were tested, SRL significantly reversed IL-2 excretion inhibition by TRL ($P = 0.004$), while for EVL no difference in IL-2 levels was found ($P = 0.53$). The IL-2

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concentrations observed for the EVL/TRL combination was also significant lower when compared to the combination of SRL/TRL ($P = 0.01$).

Next, we determined CN activity in the presence/absence of TRL (6.25 $\mu\text{g/L}$) and/or EVL/SRL (25 $\mu\text{g/L}$), concentrations that were previously illustrative for the interaction (figure 7.3).

Here no CN inhibition was found for SRL and EVL. TRL caused a mean CN inhibition of $44 \pm 11\%$ (SD) in the PBMC fractions isolated from these individuals. When MTIs were co-incubated with TRL in the culture medium, CN inhibition by TRL was partially lost and final mean inhibition in the presence of SRL and EVL was $18 \pm 14\%$ and $26 \pm 10\%$, respectively. Both were significant lower ($P < 0.001$) when compared to TRL alone. Antagonism of SRL on TRL mediated CN inhibition was significantly larger than that of EVL for these concentrations ($P = 0.01$).

Role of FKBP

To investigate whether the mechanism of antagonism between both mTOR inhibitors and TRL was mediated via competition for saturated FKBP binding sites, we prepared PBMC lysate from untreated cells of 4 healthy volunteers in duplicate and incubated (1 h, 37 °C) these with and without mTOR inhibitor, TRL and FKBP12. CN activity was determined directly after incubation. The results of these experiments are shown in figure 7.5.

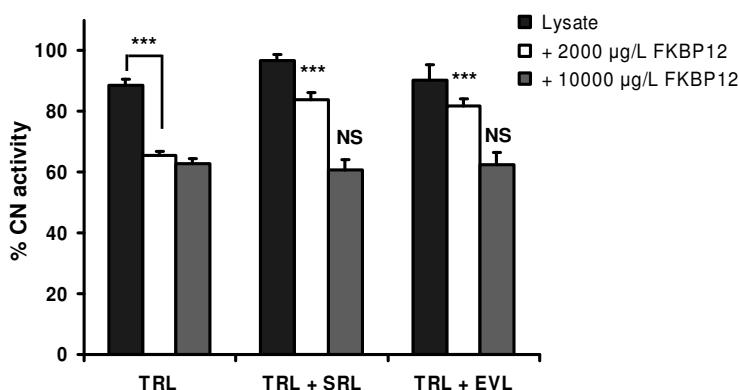


Figure 7.5. CN activity after FKBP12, TRL and MTI addition to cell lysate. CN activity was determined in PBMC lysate in the presence/absence of TRL, FKBP12 and either SRL or EVL and expressed as % of blank measurement. Final concentrations were 25 $\mu\text{g/L}$ TRL, 50 $\mu\text{g/L}$ of SRL/EVL and 0, 2000 and 10000 $\mu\text{g/L}$ of FKBP12 was added to cell lysate. Results were obtained from duplicate measurement of 4 healthy volunteers and presented as mean $\pm$ SEM. Statistical analysis was performed between TRL and TRL + SRL or EVL conditions, except when lines indicate otherwise and presented as (***) for $P < 0.001$ and NS for not significant.

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In this test system no inhibition of CN was found by the MTIs, FKBP12 and combinations thereof (data not shown). Inhibition by TRL in cell lysate was observed though it was much smaller ($\pm 10\%$) compared to the cell systems. Since the addition of 2000 $\mu\text{g/L}$ FKBP12 increased TRL inhibition ($\pm 35\%$, $P < 0.001$) it seemed that the FKBP12 pool from the intracellular compartment was diluted in the cell lysate, limiting CN inhibition by TRL. Addition of SRL to TRL reduced TRL inhibition of CN ($P = 0.005$), while for EVL antagonism under these conditions could not be detected ($P = 0.7$). In the presence of 2000 $\mu\text{g/L}$ FKBP12 antagonism for both SRL and EVL was found (for both $P < 0.001$) and when 10000 $\mu\text{g/L}$ FKBP12 was used, antagonism of both MTIs was no longer observed.

Discussion

In transplant recipients, multiple immunosuppressive agents are normally used in order to optimally prevent acute rejection episodes and to reduce the side effects of the individual drugs(1). The CNI and MTI class of drugs require intracellular binding proteins (immunophilins) to exert their immunosuppressive action. TRL binds to its intracellular binding proteins (FKBPs) and especially the 12 kDa protein FKBP12 is considered critical for the immunosuppressive action of TRL(14). The MTIs SRL and its analog EVL require binding to the same protein though an other immunosuppressive mechanism is observed for these drugs(6,15). Where TRL inhibits CN and block T-cell receptor induced activation/proliferation of T cells, the mTOR inhibitors inhibit IL-2 induced T-cell proliferation(7,15). In this study, using a CN assay, we confirmed that CN enzyme activity is not inhibited by EVL and SRL but selectively by TRL and CsA (figure 7.2), which is in agreement with previous observations.

In studies by Dumont et al., reciprocal antagonism was observed for SRL and TRL which was explained by the use of similar intracellular binding sites of the drugs(5,8). Here we show that both SRL and EVL antagonize TRL mediated CN inhibition and TRL inhibition of IL-2 excretion in PHA/PMA stimulated PBMCs, an effect which is not observed for the CNI CsA. In addition, we show that the antagonism between MTIs and TRL in cell lysate is reversed by the addition of excess of FKBP12 (10000 $\mu\text{g/L}$), indicating that the antagonism is mediated via this protein. The in vitro antagonism observed in our cellular system is significant starting at SRL concentrations of 25 $\mu\text{g/L}$ for CN activity and 250 $\mu\text{g/L}$ for IL-2 excretion and at EVL concentrations of 250 $\mu\text{g/L}$ for both CN inhibition and IL-2

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excretion inhibition by TRL. These relative high concentrations of MTIs in combination with TRL seem to saturate FKBP binding sites that are required for CN inhibition by TRL. Competition between the MTIs and TRL for these binding sites is clearly illustrated by the IL-2 interaction experiments in figure 7.1. Here it is shown that 0.625 µg/L TRL itself inhibits IL-2 excretion, but when increasing concentrations of SRL are introduced, above a certain threshold (25 µg/L SRL), IL-2 inhibition by TRL is lost and the IL-2 response is more comparable to the 25 µg/L SRL response itself.

The MTIs themselves reduce IL-2 excretion in our cell culture system, without showing a clear sigmoidal concentration– response relationship. This effect is probably best explained to be indirect and mediated through inhibition of proliferation/activation of IL-2 producing cells. In our system the lowest MTIs concentrations used already significantly decreased IL-2 excretion. Under these conditions of low MTI concentration, immunosuppressive additivity or even synergism could be very well achieved in patients in combination protocols with CsA, but also TRL; although MTIs are mechanistically antagonists of TRL, antagonism only occurs at saturating conditions for FKBP. Immunosuppressive synergy has been demonstrated for SRL/TRL and SRL/CsA combinations on lymphocyte proliferation and lymphocyte activation markers(10).

25 µg/L of both EVL and SRL partially reversed CN inhibition by 6.25 µg/L TRL though the interference by SRL was significant larger when compared to EVL in 10 healthy volunteers. In addition, only SRL could significantly reverse IL-2 inhibition by 0.625 µg/L TRL. These results indicate relevant differences between both MTIs that could be explained by the weaker binding of EVL to FKBP(12)(6).

Kung and Halloran showed that at high concentrations of CNIs, immunophilins limit CN inhibition for both CsA and TRL(16). It is however not known whether this is relevant for CNI treated patients. When using combination immunosuppressant therapy of both MTI and TRL, saturation of FKBP and thus pharmacological drug–drug interaction is even more likely and this interaction could therefore be clinically relevant. The MTI concentrations for which TRL antagonism was observed (25 µg/L) are of the same magnitude of MTI blood concentrations in allograft recipients treated with these drugs(17). In addition, also the TRL concentrations at which the interference was observed are found in allograft recipients treated with this drug(17). The drug concentrations that are used in our in vitro model can however not directly be translated to full blood drug concentrations, since drug concentrations from cell culture experiments do not necessarily show similar cellular responses compared to drug concentrations in blood due to the presence

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of irrelevant binding sites as has been demonstrated for CsA(18). The true clinical relevance remains therefore unclear.

If CN inhibition is limited by the immunophilin content in patients receiving CNi, this could be of great importance; higher CNi concentrations are ineffective. Especially early after graft implantation, when the blood concentrations are highest, this might become relevant. Consequently, under condition of immunophilin saturation, inter-individual variation in the content of immunophilins could be of great importance for allowing different maximal CN inhibitions.

Finally, blood drug concentrations and also intracellular drug concentrations are not able to detect the limitation of CN inhibition by immunophilins. Only pharmacodynamic strategies are theoretically capable of detecting these. This also holds for detecting the interaction between the MTI and TRL in individuals receiving both drugs.

In conclusion, it is shown that mTOR inhibitors can antagonize TRL and not CsA mediated immunosuppression. FKBP12 that is bound by EVL and SRL, and required for TRL based CN inhibition is a key mediator; saturation of this protein by high concentrations of MTIs and TRL results in competition for FKBP12.

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Chapter 8

Summary and General Discussion

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Over the last decades, restoration of renal function by renal allograft transplantation has evolved into the preferred treatment option for patients with end stage renal disease. The introduction of the calcineurin inhibitors (CNI) cyclosporine (CsA) and tacrolimus (TRL) have significantly contributed to this success. Both CNIs allowed successful suppression of the immunological response and rejection of the allograft by the recipient(1). Unfortunately considerable adverse drug effects are observed in patients treated with CNIs, including nephrotoxicity, hypertension and diabetes mellitus(2-4). This, together with the large inter-individual variation in pharmacokinetics of both drugs necessitates therapeutic drug monitoring (TDM) to control drug exposure(5). Nowadays, TDM of CNI is routinely performed by drug concentration measurement in blood which has led to an improved clinical performance of these drugs. Though acute rejection episodes have been significantly reduced, the incidence of acute allograft rejection episodes is still 10-20% within first year after transplantation(6). In addition, lack of selectivity and over-immunosuppression, at least partly caused by CNIs, hampers long-term allograft and patient survival. CNI withdrawal and avoidance protocols have been investigated with varying results. Early removal of CNIs from the immunosuppressive protocols continues to be problematic mainly due to the occurrence of late acute rejection(7,8). Minimization of CNIs in the immunosuppressive protocols early after transplantation was recently found to be equally effective as compared to the “old standards” with higher CNI target concentrations(9).

Another strategy to improve clinical immunosuppression early after transplantation is improved monitoring to control CNI therapy. TDM for CNI treatment is mandatory since there is a clear relation between drug exposure and incidence of acute rejection and drug toxicity(10,11). Improvement of CNI therapy by better monitoring is an interesting strategy, since in current daily practice TDM is performed under suboptimal monitoring conditions. This includes TDM by through drug concentrations (C₀) and non-standardized sampling protocols. Next to advanced pharmacokinetic monitoring, such as estimated AUC monitoring, the development of pharmacodynamic (PD) markers could theoretically contribute to improve CNI therapy. Pharmacodynamic monitoring strategies, however, are still in an experimental phase and have not proven clinical benefit yet. They carry the theoretical advantage of monitoring the true effectiveness of immunosuppressive therapy including CNIs. In addition, pharmacological and immunological drug-drug interaction can be detected. This led us to investigate PD monitoring as potential

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tool to guide drug dosing. We choose CN activity as PD marker for monitoring and in this thesis, the analytical aspects, fundamental characteristics and insights in clinical usefulness of CN activity measurement as a PD marker for CNi were investigated.

(Bio) analytical aspects of CN enzyme activity measurement

As starting point to research PD monitoring of CN inhibition therapy a CN enzyme activity assay was developed (**Chapter 2**). CN activity was determined in the leukocyte fraction isolated from peripheral blood. CN activity was determined by phosphate quantification after dephosphorylation of the phosphorylated RII substrate. Phosphate concentration was determined using the malachite green phosphate reagent. To adjust for background absorbance, a blank measurement without substrate was included. Ascorbic acid, NP-40 and DTT were titrated in the lysis buffer that resulted in a significant increase in CN activity and conservation of CN activity during sample storage. This was hypothesized to be caused by prevention of CN oxidation that is known to be enzymatically inactivated by oxidation(12,13). Furthermore CN inhibition by both TRL and CsA was found *in vitro* as well as in renal allograft recipients treated with either TRL or CsA.

In **Chapter 3** we investigated the relation between CN inhibition, inhibition of T cell cytokine excretion and their relation at therapeutic CsA concentrations. Blood from healthy volunteers incubated with relevant high range CsA concentrations (2500 µg/L) showed only a minor increase in CN inhibition when CsA was added in excess (25000 µg/L). In addition, no increase in cytokine excretion after CD3/CD28 stimulation was observed. This indicated that in the system used, at 2500 µg/L, CsA approaches immunosuppressive saturation. At relevant target C0/C2 levels early and late after renal transplantation (100, 250, 600 and 1700 µg/L) every condition represented a distinct immunosuppressive state. In addition after an initial CN inhibition threshold, small increases in CN inhibition resulted in relatively large changes in T cell cytokine excretion. No cyclophilin limitation at 2500 µg/L CsA could be detected. These results showed that CN activity could act as a relevant marker to monitor CsA based immunosuppression.

As a follow-up, we investigated whether the leukocyte fraction is a representative matrix for monitoring CsA therapy by CN activity measurement in renal allograft recipients. This is described in **Chapter 4**. Large inter-individual variation in CN activity was observed in renal allograft recipients receiving CsA. In addition, large variation in sample composition was observed when parallel to CN activity,

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leukocyte concentrations of granulocytes, monocytes and lymphocyte subsets including T cells B cells and NK cells in peripheral blood were determined. Next, cell specific CN activities and CsA inhibition curves were determined and revealed cellular differences in CN activity. Similar cellular differences have been observed previously(14). No clear deviations in CsA inhibition profiles for the different leukocyte subsets were observed as has been described before(15). Altogether, these results could indicate that sample composition, at least partially, determines CN activity measurement outcome. When applying CN activity monitoring in clinical practice CN activity could therefore be biased by sample composition. Interestingly, the sample composition effect could also be relevant for other PD markers used such as mRNA transcripts or cytokines excretion markers. Here T cell specific mRNA transcripts isolated from the mononuclear cell fraction are used as readout and expressed against reference transcripts available in all mononuclear cells(16-18). Variation in relative T cell concentration between and within individuals could therefore confound these markers in a similar way. An interesting next step would be to monitor specifically in the target compartment(s) of CNi; the T cells. T cell specific readout assays could eliminate the compositional effects on measurement outcome and in addition, monitor in the immunological relevant cells(19,20).

To anticipate on the suggested higher specificity of T cell specific pharmacodynamic assays, we have developed a T cell specific CN assay for monitoring CsA therapy (**Chapter 6**). In this protocol T cells were isolated from peripheral blood using paramagnetic beads coated with anti-CD3 antibodies. T cells isolates from peripheral blood were highly pure $95\% \pm 1.5\%$ and showed to be a representative sample of whole blood T cells, since no differences in CD4+ and CD8+ subsets were found. When coupled to the CN activity assay protocol, adequate analytical performance was observed and no washing out of CsA during sample preparation. In addition, in 6 renal allograft recipients receiving CsA therapy, inhibition of T cell specific CN activity was observed. In future studies it would be very interesting to investigate the relevance of the T cell specific calcineurin activity marker as tool for monitoring CsA therapy. Measurement of other intracellular T cell markers such as protein concentrations or their activities, drug concentrations and metabolic compounds could be very relevant markers for a large variety of diagnostic fields.

Fundamental properties of PD strategies

To gain insights in the current status of PD monitoring for CNi therapy, a review of

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recent literature on this subject was performed (**Chapter 5**). Two different types of PD monitoring strategies have been investigated. The first type monitors enzyme activity and the second cellular responsiveness after *in vitro* stimulated immunological responses. CN activity has been measured in various cellular fractions such as whole blood, leukocytes and peripheral blood mononuclear cells (PBMC) and is usually performed in conditions of co-reagent excess. It is therefore more appropriate to refer to as CN capacity measurement. For immunological strategies simulation of immunological responses is performed by mitogen or CD3/CD28-directed T cell activation. Immune responsiveness has been investigated at several levels such as mRNA transcripts, (intracellular) concentration/excretion of cytokines, expression of surface activation markers and cell proliferation. So far, clear relations of such markers with drug concentrations have been observed, but no definite clinical benefits have been reported(19,21,22). The large inter-individual variation of pharmacodynamic marker outcome could not be explained by clinical end-points such as acute rejection. Better insight in the large inter-individual variation is relevant to understand and further develop new, more accurate markers. Strategies including pharmacodynamic parameters have proven to be valuable in studying pharmacological drug-drug interactions of combinational immunosuppressive therapy applied in allograft recipients and visualizing *in vivo* immunosuppression(23-26).

We have used pharmacodynamic strategies to investigate the drug-drug interaction between TRL and the mTOR inhibitors sirolimus (SRL) and everolimus (EVL) (**Chapter 7**). Combinational therapies including mTOR inhibitors are increasingly applied in allograft recipients. However, based on the pharmacological mechanism of mTOR inhibition by the mTOR inhibitors and CN inhibition by TRL, pharmacological interactions can be expected. Both drugs bind to FK-binding proteins and complexes of either FK-binding protein with TRL or mTOR inhibitor selectively inhibit CN and mTOR respectively(27-29). We have tested the effect of the mTOR inhibitors SRL and EVL on TRL based CN inhibition in PBMC cell cultures. First, no CN inhibition was observed for both SRL and EVL. Next combinations of mTOR inhibitor with TRL led to increased TRL IC₅₀ values for CN activity as well as IL2 excretion after mitogen stimulation. This was reproduced in 10 healthy volunteers. No antagonism was observed for CsA, which does not require FKBP for CN inhibition. When FKBP12 was added to cell lysates TRL antagonism by SRL and EVL could be reversed. Altogether, these results indicate that competition for FKBP binding sites mediates the antagonism. Since all

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experiments have been performed in culture conditions, the clinical relevance of the antagonism remains unknown. Due to the presence of irrelevant binding sites in whole blood that are absent in culture medium extrapolation of culture concentrations is required, as has been demonstrated for CsA(30). It would be very relevant to investigate whether this antagonism occurs under relevant *in vivo* blood concentrations of mTOR inhibitors and TRL.

Overall conclusions and future perspectives

This thesis shows that pharmacodynamic monitoring strategies, including CN activity, are very helpful in describing immunosuppressive responses and interactions. Especially in well controlled *in vitro* experimental set-ups these assays can be used to investigate drug properties and interactions. CN activity was very useful to illustrate the pharmacological and immunological interaction of TRL with the mTOR inhibitors SRL and EVL. This rate limiting interaction is mediated via competition for the relevant chaperone for both drug classes. A relevant follow-up would be to investigate the clinical relevance of this interaction.

Unfortunately, the pharmacodynamic monitoring strategies are not ready yet for clinical use as marker to guide drug dosing. For the final transition from investigative tools to relevant biomarkers, insight in the analytical and biological variables appears to be of key importance. We have illustrated the importance of sample/matrix choice. Confounding of measurement outcome by sample composition should be avoided or at least adjusted for. Specific measurement in the relevant pharmacological and immunological target compartments of CNi to evade from these methodological confounders.

In this respect we have developed a T cell specific CN assay that showed acceptable analytical performance. T cell specificity could also contribute to improved performance of other pharmacodynamic markers for monitoring CNi therapy such as T cell specific cytokines, mRNA and cell surface T cell activation markers. This could be performed by for instance T cell specific stimulation and includes the activation of T cells by targeting specific T cell epitopes e.g. CD3 and CD28. In such assays, optimization of the time interval should prevent secondary activation of other cells present due to cytokines produced by activated T cells. Finally, T cell specific assays to assess T cell specific CN activity need to be validated as a marker for monitoring CsA therapy in a large cohort of patients treated with CNi in order to proof their clinical relevance.

This thesis has demonstrated that the anticipation on analytical confounders, such

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as sample choice, is crucial for the development of CN activity as a marker that has the intrinsic property to visualize CNI pharmacodynamics.

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Chapter 9

Nederlandse samenvatting

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Nederlandse samenvatting

Bij orgaan transplantatie patiënten is een goede onderdrukking van de immunologische reactie tegen het ontvangen, lichaamsvreemde orgaan van cruciaal belang. Om deze natuurlijke afstotingsreactie te onderdrukken wordt immuunsuppressieve therapie gebruikt. Als deze niet gegeven wordt, of niet toerijkend is, stoot het lichaam het ontvangen orgaan af. Dit betekent dat het lichaam het ontvangen orgaan vernietigd.

Er zijn verschillende medicijnen die gebruikt kunnen worden als immuunsuppressieve therapie. Kort na transplantatie vormt een zogenaamde calcineurine (CN) remmer de basis van de immuunsuppressieve therapie. Er zijn 2 calcineurine remmers in gebruik in de transplantatie geneeskunde; tacrolimus (TRL) en ciclosporine (CsA). Deze calcineurine remmers zorgen voor een goede onderdrukking van de afstotingreactie, maar kunnen ernstige bijwerkingen geven, waaronder niertoxiciteit, hoge bloeddruk en suikerziekte. Een ander probleem van TRL en CsA therapie is dat er grote verschillen tussen personen bestaan in de mate waarop deze medicijnen worden opgenomen in, of uitgescheiden door, het lichaam. De mate waarin een individu TRL of CsA opneemt of uitscheidt is van te voren moeilijk te voorspellen, terwijl een minimale aanwezigheid van CsA of TRL noodzakelijk is voor een goede onderdrukking van het immuunsysteem en een te grote aanwezigheid kan zorgen voor ernstige bijwerkingen. Om de aanwezigheid van CsA of TRL te controleren worden medicijnconcentraties in het bloed gemeten. Deze zijn een maat voor de aanwezigheid van CsA of TRL in het lichaam. Bij te lage CsA of TRL concentraties wordt vervolgens de medicijn dosis verhoogd en bij te hoge concentraties wordt de medicijn dosis verlaagd. Dit zogenoemde farmacotherapeutisch monitoren zorgt dat de blootstelling aan CsA en TRL binnen vastgestelde grenzen blijft.

Ondanks dat calcineurine remming therapie op deze manier gecontroleerd wordt, komen er nog steeds afstotingsreacties voor en vormt de medicijn toxiciteit naast over-immuunsuppressie een groot klinisch probleem. Het beter meten van CsA en TRL therapie in de patiënt zou voor een verdere verbetering van de calcineurine remmings therapie kunnen zorgen. De therapie kan dan beter ingesteld worden, waardoor er minder afstotingsreacties en bijwerkingen optreden. Dit kan gedaan worden door geavanceerde strategieën te gebruiken zoals het schatten van de totale medicijn blootstelling op basis van meerdere concentratie bepalingen na inname (AUC waarden), of het implementeren van farmacogenetica. Bij farmacogenetica kan aan de hand van het genetisch profiel van een persoon een

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inschatting worden gemaakt van de opname en uitscheiding van CsA en TRL. Daarnaast kan er gedacht worden aan monitorings strategieën waarbij men de werking van CsA of TRL meet. Dit wordt ook wel farmacodynamisch monitoren genoemd. De medicijn concentraties zijn wel gecorreleerd aan de werking die ze hebben, maar zijn slechts een indirecte maat. Helaas is het zo dat farmacodynamische monitorings strategieën nog in een experimentele fase zitten en dat ze klinisch nog geen bewezen toegevoegde waarde hebben. Dit heeft ons ertoe bewogen om de toepassing van deze markers voor het monitoren van CsA en TRL therapie bij orgaantransplantatiepatiënten te onderzoeken. Hierbij hebben wij ons vooral gericht op de calcineurine activiteit als farmacodynamische marker.

Om de werking van de calcineurine remmers TRL en CsA te meten werd er een calcineurine activiteits meting ontwikkeld. Deze staat beschreven in **hoofdstuk 2**. Met deze methode (assay) wordt de calcineurine activiteit in de witte bloedcellen gemeten. Er werd voor de witte bloedcel fractie van het bloed gekozen omdat calcineurine remming in deze cellen zorgt voor immuunsuppressie. In de assay wordt het RII substraat specifiek door calcineurine omgezet in vrij fosfaat. De hoeveelheid vrijgekomen fosfaat wordt gemeten met het malachite green kleurreagens. De hoeveelheid fosfaat dat vrij komt in een bepaalde tijd is vervolgens een maat voor de calcineurine activiteit. De assay liet een goede reproduceerbaarheid, lineariteit en calcineurine remming door CsA en TRL zien. Tot slot werd aangetoond dat de calcineurine activiteit geremd wordt in niertransplantatie patiënten die CsA of TRL therapie ontvangen.

In een vervolg studie werd gekeken of de calcineurine activiteit daadwerkelijk een maat is voor de immuunsuppressie bij patiënten die behandeld zijn met CsA. Dit staat beschreven in **hoofdstuk 3**. Er werd gevonden dat verschillende therapeutische CsA concentraties een verschillende onderdrukking van de calcineurine activiteit lieten zien. Dit gold ook voor IFN γ en TNF α secretie na T cel specifieke stimulatie. Deze laatste twee zijn stoffen die bij een immuunreactie vrijkomen uit de witte bloedcellen. Dit betekent dat zowel de calcineurine activiteit als cytokine excretie na T cel specifieke stimulatie in staat zijn om de werking van CsA therapie bij relevante CsA concentraties te laten zien.

Er zijn meerdere klassen van witte bloedcellen, waaronder lymfocyten, granulocyten en monoccyten. Deze hebben ieder een andere rol bij de afweerreactie. De concentratie van deze cellen in het bloed kan behoorlijk variëren. Om de keuze voor de witte bloedcelfractie als monster voor de calcineurine assay

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te evalueren werd gekeken wat het effect is van een samenstellingsverandering van de witte bloedcel fractie op de gemeten calcineurine activiteit. Dit staat beschreven in **hoofdstuk 4**. Dit werd gedaan door bij 25 niertransplantatiepatiënten naast de calcineurine activiteit, tegelijkertijd de leukocyten-samenstelling te bepalen. Bij deze patiënten was er een grote spreiding in de calcineurine activiteit en samenstelling van de leukocyten fractie zichtbaar. De celspecifieke CN activiteit van de individuele witte bloedcel soorten liet ook duidelijke verschillen zien. Zo bleken monocysten een hogere CN activiteit te hebben dan bijvoorbeeld lymfocyten. Dit zou kunnen betekenen dat bij patiënten met relatief veel monocysten er een hogere calcineurine activiteit gevonden kan worden, niet op basis van de activiteit, maar op basis van de aanwezigheid van relatief meer monocysten. Deze problematiek kan omzeild worden door specifiek in die celfractie te meten waar de CsA en TRL hun therapeutische effect genereren; de T lymfocyt.

Naast de door ons ontwikkelde calcineurine assay zijn er verschillende methoden beschikbaar om naar het effect van immuunsuppressieve medicatie en in het bijzonder de calcineurine remmers te kijken. Deze verschillende technieken staan beschreven in **hoofdstuk 5**. Er zijn 2 verschillende methoden beschreven. 1) methoden die de activiteit van het directe doeleiwit meten en 2) technieken die immunologische waarden meten na het nabootsen van een T cel respons. Bij het eerste type wordt de calcineurine activiteit bepaald. Hiervoor zijn verschillende methoden beschreven. Deze verschillen in de manier waarop de calcineurine activiteit wordt gedefinieerd, het monster waarin de activiteit wordt gemeten en de detectie techniek die wordt gebruikt. Meting van het directe enzym heeft als voordeel dat andere soorten immuunsuppressieve medicatie geen direct effect hebben op de meting. Bij het tweede type, worden witte bloedcellen, of specifiek T cellen, gestimuleerd en wordt de reactie van de cel als uitleesmaat genomen. Deze wordt uitgelezen aan de hand van signaal moleculen die de geactiveerde cellen uitscheiden of tot expressie brengen zoals mRNA transcripts, cytokinen, oppervlakte eiwitten en cel deling. Deze methoden kunnen ook effecten van andere klassen van immuunsuppressieve medicatie laten zien. Helaas is er nog geen duidelijke marker die in patiënten een duidelijke relatie laat zien tussen meet waarde en klinische parameters van immuunsuppressie of calcineurine remmer toxiciteit. Meer inzicht in de grote inter-individuele variatie lijkt noodzakelijk voor de ontwikkeling van geschikte markers voor het farmacodynamisch monitoren van CsA en TRL therapie.

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Voor het ontwikkelen van een specifiekere marker voor het monitoren van CsA en TRL therapie is er een T cel specifieke calcineurine assay opgezet. Deze methode staat beschreven in **hoofdstuk 6**. De methode maakt gebruik van microkraaltjes waarop antistoffen tegen de T cel zitten. Deze kraaltjes worden in een magnetisch veld magnetisch en hiermee kunnen de T cellen uit het bloed worden weggevangen. De geïsoleerde T cellen worden vervolgens verder verwerkt volgens de eerder ontwikkelde calcineurine assay. De methode liet een goede zuiverheid van T cellen en goede analytische eigenschappen zien. Daarnaast werd er in zes patiënten die CsA kregen, T cel specifieke calcineurine remming gevonden. Het zou nu zeer relevant zijn om met deze methode de calcineurine activiteit in T cellen van transplantatiepatiënten te monitoren die worden behandeld met CsA.

Tot slot werd gekeken of er met farmacodynamische monitorings technieken medicijn interacties kunnen worden opgepikt (**hoofdstuk 7**). Hiervoor werden de witte bloedcellen geïsoleerd uit bloed van bloeddonoren van de bloedbank. Deze cellen werden geïncubeerd met verschillende TRL, everolimus en sirolimus concentraties en vervolgens werd de farmacologische interactie tussen TRL en de mTOR remmers sirolimus en everolimus bestudeerd. Alle drie de medicijnen binden aan het eiwit FKBP wat nodig is voor hun werking. Bij combinatie therapie van deze medicijnen zou er verzadiging kunnen zijn van FKBP bindingsplaatsen waardoor er competitie en verdringing kan optreden. Calcineurine activiteit en de excretie van cytokinen na witte bloedcel stimulatie werden gebruikt als uitleesmaat. Er werd gevonden dat hoge concentraties van everolimus of sirolimus de calcineurine remming van TRL teniet konden doen. Dit betekende dat combinatietherapie van een mTOR remmer met TRL bij hogere concentraties een ongunstige immuunsuppressieve respons liet zien. Of dit klinisch relevant is was echter niet vast te stellen, omdat deze experimenten zijn uitgevoerd in kweek media en de medicijnconcentraties hierin niet vergelijkbaar zijn met dezelfde concentraties in bloed. Het zou echter erg relevant zijn om dit uit te zoeken.

Samengevat laat dit proefschrift zien dat de calcineurine activiteit geschikt is als marker voor het bestuderen van TRL en CsA. Voor het gebruik van calcineurine activiteit als marker voor het monitoren van transplantatiepatiënten is verder inzicht in de analytische en biologische variatie nodig. Deze kan dan gebruikt worden voor het ontwikkelen van calcineurine assays die klinisch toegevoegde waarde hebben. De ontwikkelde T cel specifieke calcineurine activiteits assay is een interessante

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kandidaat om te toetsen als marker voor het monitoren van CsA therapie in niertransplantatiepatiënten.

Curriculum Vitae

Huub Harmen van Rossum werd op 10 maart 1980 geboren en behaalde in 1998 zijn VWO diploma aan de scholengemeenschap De Rietlanden in Lelystad. Datzelfde jaar begon hij aan het propedeuse jaar van de studies scheikunde en farmacochemie aan de Vrije Universiteit in Amsterdam. In 2004 werd het doctoraal examen van de studie farmacochemie behaald met als afstudeer richting moleculaire toxicologie en in 2005 werd het doctoraal examen van de studie scheikunde behaald met als afstudeer richting, analytische chemie. Hiervoor is er een stage is gelopen op de Kansas University in Lawrence, Kansas bij professor JF Stobaugh. Naast deze opleidingen is hij gedurende deze periode ook werkzaam geweest als student assistent op de afdeling moleculaire toxicologie en heeft hij literatuur onderzoek gedaan naar structuur activiteits relaties van PDE3 remmers voor Altana Pharma onder begeleiding van dr. GJ Sterk. In 2005 werd er begonnen met een promotie onderzoek naar calcineurine remmers op de afdelingen klinische chemie en nierziekten van het Leids Universitair Medisch Centrum te Leiden. Sinds 1 juni 2009 is hij bezig met de opleiding tot klinisch chemicus in het Bronovo ziekenhuis in Den Haag (Dr. A Castel).

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