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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 extend 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
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 Cγ. Phospholypases catalize the hydrolysis of the membrane phospholipid phosphatidylinositol-1,4,5-biphosphate to inositol-1,4,5-triphosphate (IP₃) and diacylglycerol (DAG)(9). IP₃ binds to IP₃ receptors on the endoplasmatic 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, IFNγ), chemokines (e.g. GM-CSF) and surface proteins (e.g. CD40L, IL2Ra) 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, \( \text{Ca}^{2+} / \) 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 \( \text{Zn}^{2+} \) and \( \text{Fe}^{3+} \), 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 \( \text{Ca}^{2+} \)-binding domains that have two \( \text{Ca}^{2+} \)-binding EF hand motifs and are similar to those of calmodulin(14). \( \text{Ca}^{2+} / \) 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).

Three different CN A isoforms (\( \alpha \), \( \beta \) and \( \gamma \)) have been described(13). The expression of CN A\( \gamma \) is restricted to testis and specific parts of the brain, while the CN A\( \alpha \) and CN A\( \beta \) isoforms are present in all tissues examined(13). The overall
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amino acid sequences of CN Aα and CN Aβ 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 Aα : CN Aβ have been identified within and between tissues. In brain, CN Aα is more abundant than CN Aβ. CN Aα is more abundant in kidney and its expression is largely restricted to the tubules, while CN Aβ expression was observed mainly in the glomerular region(13;17).

In T and B cells, CN Aβ 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 Aα, but not CN Aβ, 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, Myristoyoated alanine-rich C-kinase substrate, neuromodulin and neuorogranin.

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 myristoyoated 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
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 TGFβ 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 Aα, reproduced the characteristics of CN inhibitor nephrotoxicity, while CN Aβ-null mice did not. In vitro experiments showed that NFATc translocation was specifically regulated by the CN Aβ isoform, whereas CN Aα regulated fibronectin and TGFβ 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, IFNγ, TNFα 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 insulin homeostasis(39), regulation of bone mass in osteoclasts and osteoblasts(40) and the pathogenesis of leukemia(41;42).
Regulators of calcineurin

Several endogeneous 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 that 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 (IC_{50} ± 4 μM), microcystin LR (IC_{50} ± 200 nM)(13). An example of a selective CN inhibitor is 5-dibenzoyloxymethyl-substituted norcantharidin (IC_{50} = 50 μM) 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
(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).
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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.

![Molecular structure of cyclosporin A](image)

**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 metabolization 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).
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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 Tsukuba 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)](image)

**Tacrolimus pharmacokinetics**
Tacrolimus (TRL) is traditionally formulated in the twice daily (Prograft®) 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
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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 through 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 that 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
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 pharmacodymic 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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