**University of Alberta**

# Study of calcineurin interaction with inhibitor-1 and natural products. Towards novel calcineurin inhibitors

by

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I dedicate this thesis to my parents,
Danuta and Marek Raszek,
and my supervisor Charles Holmes.

# Abstract

Calcineurin (CaN) is an eukaryotic Ser/Thr protein phosphatase that plays an important role in lymphocyte activation. Inhibition of CaN leads to immune system suppression, a necessary procedure in organ transplantation. However, use of CaN inhibitors results in toxic side effects. The search for more specific immunosuppressants is therefore an important and ongoing endeavor. The focus of this thesis was to investigate methods that could lead to the discovery of novel CaN inhibitors.

CaN is closely related to protein phosphatase-1 (PP-1) but both phosphatases retain distinct substrates, regulatory proteins, and inhibitors. Two such inhibitors, okadaic acid (OA) and microcystin-LR (MCLR), potently inhibit PP-1 but are markedly less effective against CaN. Mutagenesis of CaN was undertaken to generate a form of CaN more sensitive to OA and MCLR. The optimal construct was a Y159I:F160Y:L312C:Y315L quadruple point mutant that showed 600-fold and 37-fold increased sensitivity to MCLR and OA, respectively. These studies provide the basis for chemical engineering to generate analogs of OA and MCLR that are CaN specific.

Multiple proteins use the PXIXIT amino acid sequence to interact with CaN, most notably the nuclear factor of activated T-cells (NFAT). NFAT dephosphorylation by CaN is necessary for immune system activation. The second chapter of results demonstrates that inhibitor-1 (I-1), a CaN substrate,

contains the PXIXIT motif used for CaN binding. Disruption of the PXIXIT motif of I-1 reduced its dephosphorylation by CaN. I-1 therefore is a suitable substrate for identification of novel immunosuppressants that could block the interaction between CaN and the PXIXIT motif of NFAT proteins.

A new method of bioassay-guided isolation of novel CaN inhibitors from extracts of marine organisms was established in the final chapter of results. Monitoring the inhibition of CaN activity using the I-1 substrate during extract purification led to identification of three compounds: halisulfate-7, hipposulfate C, and a novel one, irregularsulfate. These compounds were identified as equipotent μM inhibitors of CaN and PP-1. The method of bioassay-guided isolation of CaN inhibitors is applicable to searching for potential drugs that could block access to the active site of CaN, or to the PXIXIT motif binding groove.

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# Abbreviations

Abu – 2-aminobutyric acid

Adda – 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid

ADP – adenosine diphosphate

AI – autoinhibitory domain

AKT – protein kinase B

AP-1 – activator protein-1, a Jun/Fos transcription factor complex

ATP – adenosine triphosphate

BBH – calcineurin B-binding α helix

BME – 2-mercaptoethanol

BSA – bovine serum albumin

CaM – calmodulin

cAMP – cyclic AMP

CaN – calcineurin protein phosphatase, also termed PP-2B or PP-3

CaN-A – catalytic subunit of calcineurin

CaN-B – required 19 kDa regulatory subunit of calcineurin

CBD – calmodulin binding domain

CD4 – cluster of differentiation-4, surface glycoprotein of helper T-cells

CD8 – cluster of differentiation-8, surface glycoprotein of killer T-cells

CD28 – cluster of differentiation-28, required for costimulation of T-cells

CPI-17 – C-kinase-activated PP-1 inhibitor of 17 kDa

CPI-17-P – phosphorylated form of CPI-17

CRAC – $Ca^{2+}$-release-activated $Ca^{2+}$ channel

CsA – cyclosporine A

DAG – diacylglycerol

DARPP-32 – 32 kDa dopamine and cyclic AMP-regulated phosphoprotein

D-Masp – D-erythro-β-methyl aspartic acid

DSP – diarrhetic shellfish poisoning

DTT – dithiothreitol

FK506 – code number of calcineurin inhibitor also known as tacrolimus

FKBP12 – FK506 binding protein 12

GSK-3 – glycogen synthase kinase-3

HIV – human immunodefficiency virus

HPLC – high performance liquid chromatography

I-1 – protein phosphatase inhibitor-1

I-1-P – phosphorylated form of I-1

IFN-γ – interferon-γ

IL – interleukin

IL-2R – interleukin-2 receptor

INCA – inhibitors of NFAT-calcineurin association

$IP_3$ – inositol 1,4,5-trisphosphate

$IP_3R$ – $IP_3$ receptor, endoplasmic reticulum $Ca^{2+}$-channel

IPTG – Isopropyl β-D-1-thiogalactopyranoside

JAK3 – Janus kinase-3

kDa – kilodaltons

KO – gene knock-out

LTD – long term depression of postsynaptic potential

LTP – long term potentiation

LXVP – calcineurin binding motif found in NFAT proteins

MCLA-2H – dihydromicrocystin-LA, unable to covalently bind phosphatases

MCLL – microcystin-LL

MCLR – microcystin-LR

Mdha – *N*-methyldehydroalanine

MeBmt – *N*-methlyl-2(S)-amino-3(R)-hydroxy-4(R)-methyl-6(E)-octenoic acid

MeVal – *N*-methylvaline

MHCII – major histocompatibility complex class II

Mle – *N*-methylleucine

MyPhoNE – myosin phosphatase N-terminal element

MYPT1 – myosin phosphatase target subunit 1

NCE – $Na^{+}/Ca^{2+}$ ion exchanger channel

NFAT – nuclear factor of activated T-cells

NF-κB – nuclear factor-κB

NHD – NFAT homology domain

NLS – nuclear localization signal

NMR – nuclear magnetic resonance

NUAK1 – AMP-activated protein kinase-related kinase 5 (abbreviation derived from a gene name)

NUAK1-P – phosphorylated form of NUAK1

OA – okadaic acid

PCR – polymerase chain reaction

Phos a – glycogen phosphorylase a, common PP-1c *in vitro* assay substrate

PI3K – phosphatidylinositol 3'-kinase

PICK1 – protein interacting with C kinase 1

PKA – protein kinase A

PKC – protein kinase C

PLC – phospholipase C

PMSF – phenylmethylsulphonyl fluoride

*p*NPP – *para*-Nitrophenyl phosphate

PP-1c – catalytic subunit of protein phosphatase-1

PP-2Ac – catalytic subunit of protein phosphatase-2A

PP-2B – protein phosphatase-2B, commonly referred to as calcineurin

PP-2C – protein phosphatase-2C

PPIP30 – 30 residue fragment of I-1 containing amino acids 12-41

PPIP30-P – phosphorylated form of PPIP30

PPIP46 – 46 residue fragment of I-1 containing amino acids 9-54

PPIP46-P – phosphorylated form of PPIP46

PPIP54 – 54 residue fragment of I-1 containing amino acids 1-54

PPIP54-P – phosphorylated form of PPIP54

PPM – $Mg^{2+}$-dependent phosphoprotein phosphatases

PPP – phosphoprotein phosphatases

PTP – tyrosine phosphoprotein phosphatases

PXIXIT – calcineurin binding motif found on regulatory proteins and substrates

$R_{II}$ – peptide substrate of calcineurin, derived from PKA regulatory subunit

RHR – Rel homology region of NFATs

RVXF – PP-1c binding motif found on PP-1c regulatory proteins

RyR – ryanodine receptor $Ca^{2+}$ channel

Sar – sarcosine (*N*-methylglycine)

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SERCA – sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ ATPase

Ser/Thr – serine and threonine

TCA – trichloroacetic acid

TCR – T-cell receptor

TFA – trifluoroacetic acid

TonEBP – tonicity-responsive enhancer binding protein

TPR – tetratricopeptide repeat

VIVIT – 14-amino acid peptide that inhibits NFAT binding to calcineurin

WT – wild-type

# Chapter 1: Introduction. Regulation of protein phosphatases by endogenous proteins and exogenous inhibitors.

## 1.1 Protein phosphatases as vital components of eukaryotic cells

Reversible protein phosphorylation is one of the fundamental mechanisms that eukaryotic cells utilize to control the function of various proteins in a myriad of signal transduction pathways that respond to extracellular stimuli. The majority of these phosphorylation events need to be reversed. This cyclic phenomenon of phosphorylation and dephosphorylation occurs primarily on three residues of cellular proteins: serine, threonine and tyrosine. The covalent attachment of phosphate to proteins is an absolutely essential modification for the survival of the cell, uses adenosine triphosphate as a phosphate donor, and is performed by protein kinases. In turn, the phosphate is removed by protein phosphatases (see Figure 1.1, p. 2).[54, 223, 257]

Reversible protein phosphorylation controls virtually every function of the eukaryotic cell, and many members of both kinases and phosphatases are absolutely essential for cell survival.[57, 66, 147, 223, 257] Approximately 70% of all eukaryotic proteins are controlled by reversible phosphorylation.[234] Due to their involvement in many vital cellular roles, particularly cell signaling and growth control, protein kinases constitute one of the major drug target groups pursued by

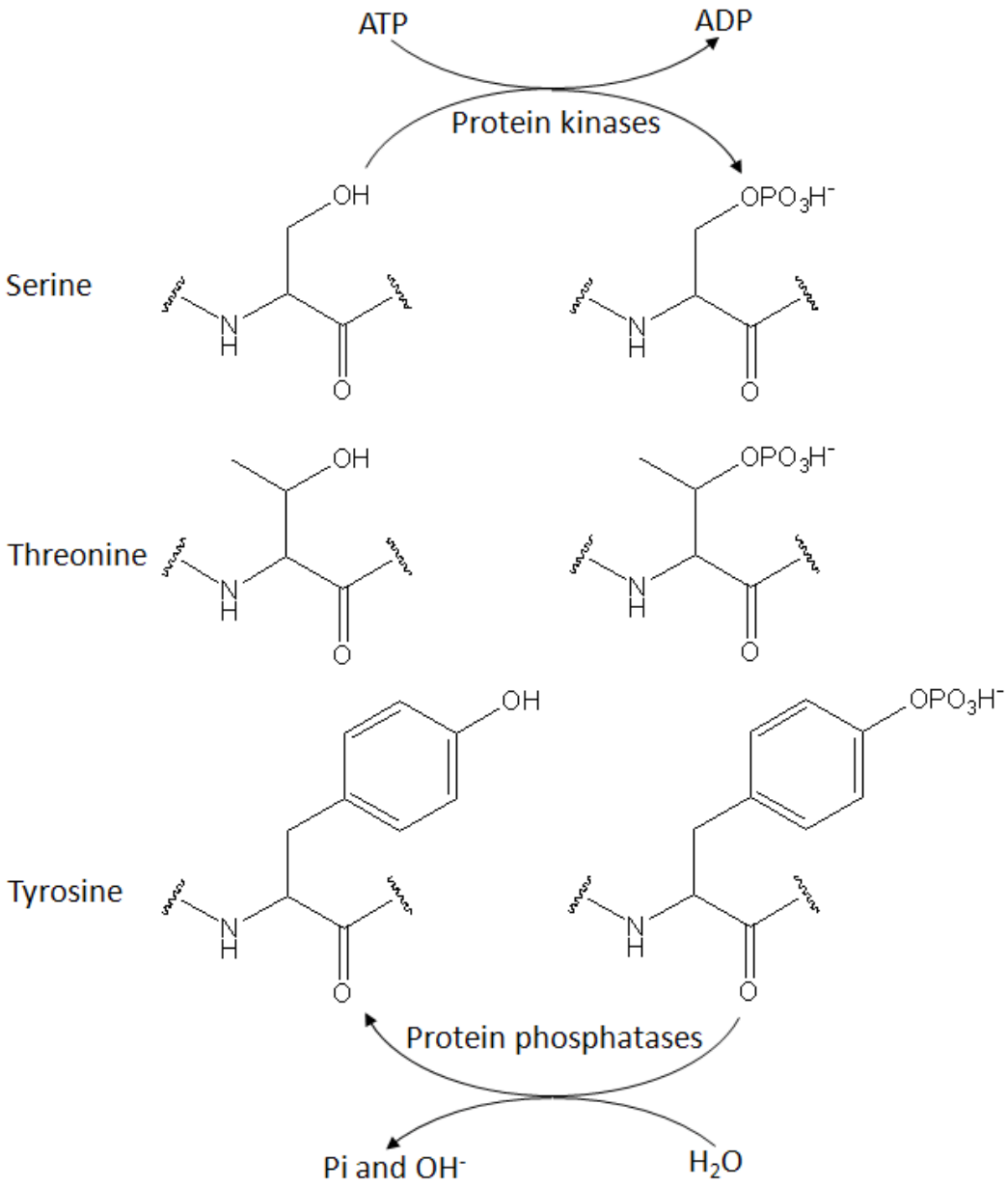


**Figure 1.1: Schematic representation of protein modulation by phosphorylation and dephosphorylation.** Protein phosphorylation is performed by protein kinases which transfer phosphate from adenosine triphosphate (ATP) to the protein. The reverse reaction is catalyzed by protein phosphatases which require hydroxide ions from water molecules to remove the phosphate groups (Pi) from the substrates. The wavy line indicates that these amino acids are constituents of polypeptide chains which are the substrates of the protein kinases and phosphatases. ADP = adenosine diphosphate.

the pharmaceutical industry.[223] Protein phosphatases, if defined as catalytic subunits encoded by a specific gene, are far fewer in numbers than kinases. These few catalytic subunits, however, can interact with a great number of regulatory proteins.[133, 229, 248]

The regulatory protein subunits target phosphatases to specific intracellular compartments or to specific substrates. These regulatory subunits can also directly affect the catalytic activity of protein phosphatases, by temporally activating or inhibiting the multiprotein-complex phosphatases towards a specific subset of substrates. Thus the regulatory subunits allow relatively few catalytic subunits of protein phosphatases to participate in numerous signal transduction pathways.[12, 54, 219, 223] Such intricate regulation of protein phosphatases has made it difficult to uncover the different functions of these enzymes, or developing drugs that could specifically target these functions. However, the vital importance of protein phosphatases has been demonstrated by the fact that they are a target of many natural products, they function as tumor suppressors and are important in cell survival and cell death, as well as cell-cycle progression.[12, 54, 219, 223, 227] Involvement of protein phosphatases in essential cell functions has implicated their role in numerous health related problems, such as neurological and metabolic disorders, immunosuppression, or cancer disease. Protein phosphatases have since become important targets of drug manipulation.[12, 54, 67, 147, 215, 219, 223]

## 1.2 Historical classification of protein phosphatases: an expanding family of enzymes

98% of all protein phosphorylation occurs on serine and threonine (Ser/Thr) amino acids.[57, 66, 147, 223, 257] In eukaryotic cells the Ser/Thr protein phosphatases represent a unique class of enzymes that catalyze the dephosphorylation of phosphoserine or phosphothreonine residues in target substrate proteins. Classically, these enzymes have been divided into four types; PP-1, PP-2A, PP-2B (calcineurin or CaN) and PP-2C, based on their sensitivity to inhibitors, requirement for cations and *in vitro* substrate specificity.[65, 157, 158] Current classification divides the Ser/Thr protein phosphatases into two families: phosphoprotein phosphatases (PPP) and $Mg^{2+}$-dependent phosphoprotein phosphatases (PPM).[54, 147] CaN, PP-1, and PP-2A, are part of the PPP gene family that also comprises PP-4, PP-5, PP-6 and PP-7 (see Table 1.1, p. 5).[56, 147, 153, 223] PP-2C was later confirmed not to be related to this subgroup but rather belongs to the PPM family.[147]

There are ~520 protein kinases in the human genome, comprising ~2% of the genome, as opposed to ~22 protein phosphatases in the PPP family (including 14 isolated protein phosphatases, see Table 1.1 (p. 5), with the remaining number predicted to be encoded by human DNA) and 18 in the PPM family, together comprising <0.1% of the genome.[219, 223, 229] With the overwhelming numerical superiority of protein kinases, it is not surprising that regulation of the relatively few protein phosphatases had to become complex. The PPP family of

**Table 1.1: Classification of the PPP family of Ser/Thr protein phosphatase catalytic subunits.**

| Protein Phosphatase | Catalytic Subunit Isoforms | Major Signal Transduction Pathway Functions | Inhibitors ($IC_{50}$)[a] | Refs |
|---|---|---|---|---|
| PP-1 | α, β, $\gamma_1$, $\gamma_2$ | glycogen metabolism, synaptic plasticity, cell cycle progression, regulation of receptors, ion pumps and channels, muscle contraction | OA = ~30 nM<br>MCLR = 0.1 nM<br>I-1 = ~0.4-1 nM | 54, 71, 144, 146-148, 208, 239, 261 |
| PP-2A | α, β | cell cycle control, growth and proliferation, DNA replication, viral transformation, | OA = ~0.1 nM<br>MCLR = ~0.1 nM | 147, 227, 257, 261 |
| CaN (PP-2B/ PP-3) | α, β, γ | lymphocyte activation, skeletal muscle differentiation, cardiac development, brain development, memory formation cell cycle progression | OA = 5 µM<br>MCLR = 100 nM<br>CsA = 5 nM<br>FK506 = 0.5 nM | 12, 26, 60, 115, 142, 208, 250 |
| PP-4 (PPX/ Ppp4) | one isoform | small nuclear/nucleolar ribonucleoprotein maturation, hepatic gluconeogenesis | OA = 0.1 nM<br>MCLR = 0.15 nM | 72, 130, 313 |
| PP-5 (Ppp5) | one isoform | cell cycle progression, glucocorticoid receptor signaling, regulation of Raf-1 activation, mammalian circadian clock rhythm regulation | OA = 1.4 nM<br>MCLR = 15 nM | 56, 57, 137, 170, 255 |
| PP-6 (Ppp6) | one isoform | DNA repair, mitotic spindle formation, inflammation suppression | not determined | 147, 168, 316, 321 |
| PP-7 (Ppp7) | two isoforms | not determined | OA = >1 µM<br>MCLR = >1 µM | 147 |

[a] $IC_{50}$ refers to the concentration of inhibitor which inhibits 50% of enzyme activity. CsA = cyclosporine A; FK506 = code number of CaN inhibitor, also known as tacrolimus; I-1 = protein phosphatase inhibitor-1; OA = okadaic acid; MCLR = microcystin-LR. Inhibitors of the protein phosphatases are discussed further in the text.

phosphatases can interact with a large number of regulatory proteins, resulting in a large number of different holoenzyme combinations that can equal in number those of protein kinases.[54, 261] Therefore Ser/Thr protein phosphatases have evolved to possess a wide range of functional diversity, as important as the role of kinases in the regulation of cellular protein phosphorylation.

In contrast to Ser/Thr protein phosphatases, even though less than 2% of protein phosphorylation occurs on tyrosine residues, the family of tyrosine phosphoprotein phosphatases (PTP) is large, outnumbering that of PPP and PPM combined.[54, 147, 223, 260]

### 1.2.1 The PPP family of protein phosphatases

CaN (PP-2B or PP-3) is a heterodimer protein composed of the 58–64 kDa catalytic subunit, CaN-A, and a 19 kDa regulatory subunit, CaN-B. CaN-A exists as three isoforms: α, β, and γ, encoded by three separate genes. These isoforms share 83–89% amino acid identity in humans, with the majority of variation found in the C- and N-terminal regions.[142, 147, 181] The β isoform is broadly distributed, whereas the γ and α isoforms are testis and brain specific, respectively.[147, 181]

For full activation of CaN, CaN-B and the regulatory protein, calmodulin (CaM), must bind to the CaN-A subunit. CaM itself needs to bind $Ca^{2+}$ prior to interacting with CaN, thus making CaN the principal phosphatase known to participate in $Ca^{2+}$-dependent signal transduction pathways.[12, 147]

In mammalian cells CaN regulates ion channels, the activity of other signaling enzymes (such as PP-1c), brain and heart development, memory formation, cell cycle progression, activation of lymphocytes, and skeletal muscle differentiation (see Table 1.1, p.5).[12, 60, 142, 250]

CaN-B appears to have additional functions besides activation of CaN-A. CaN-B has been shown to take part in processing and maturation of caspase-3, promoting apoptosis in a CaN phosphatase independent manner.[251] CaN-B can also act as a scaffolding protein, shown to interact with PICK1 (protein interacting with C kinase 1), forming a regulatory complex of CaN activity in the central nervous system.[156] CaN-B deletion in forebrains of adult mice revealed impaired working memory, and behavioural abnormalities reminiscent of schizophrenia.[221, 315] Interestingly, PICK1, a scaffolding protein located at neuronal synapses, is associated with increased risk of schizophrenia.[89]

The catalytic subunit of PP-1 (PP-1c) exists as four isoforms: α, β, $\gamma_1$, and $\gamma_2$. PP-1c$\gamma_1$ and PP-1c$\gamma_2$ are encoded by a single gene but are produced by alternative splicing. All isoforms have >90% identity in humans. PP-1c$\gamma_2$ is expressed mainly in testis.[147] PP-1c, a 37 kDa enzyme, dephosphorylates many substrates, shows no specific consensus sequence selectivity, and interacts with over 200 regulatory proteins.[34, 133] Numerous roles have been assigned to PP-1c, including regulation of glycogen metabolism, synaptic plasticity in the brain, cell cycle progression or smooth muscle contraction/relaxation (see Table 1.1, p. 5).[54]

An example of an inhibitory protein regulator of PP-1c is protein phosphatase inhibitor-1 (I-1). I-1 needs to be phosphorylated (I-1-P) to inhibit PP-1c.[159, 274, 275] Inhibition of PP-1 by I-1-P plays an important role in the β-adrenergic pathway in cardiomyocyte cells, and heart hypertrophy can result when this pathway is unregulated.[52, 54, 98, 99, 127, 216] I-1-P is also a substrate of CaN, therefore CaN can regulate activity of PP-1c by modulating the levels of I-1-P.[97, 159, 181, 274] Such upstream regulation of PP-1 by CaN can be described as a phosphatase cascade. How I-1-P interacts with CaN is not known, and this is the focus of third chapter of this thesis.

The catalytic subunit of PP-2A (PP-2Ac) exists as two isoforms: α and β, which share >97% identity in humans.[147] PP-2Ac shows no obvious consensus sequence for substrate binding. In mammals, the catalytic subunit forms a heterocomplex with an A-subunit, and one of at least 14 B-type subunits. The A-subunit acts as a scaffolding protein that can interchangeably interact with different B-subunits.[147, 227] Specific roles for these holoenzymes have not been defined, but as observed with PP-1c, PP-2Ac complexes act on many substrates, with different holoenzyme combinations specific for certain substrates.[147, 257]

Notably, PP-2Ac is involved in control of the cell cycle, growth and proliferation, DNA replication, morphogenetic events, and regulation of metabolism.[257] Recent evidence suggests that suppression of PP-2A activity is an important step in cell transformation.[227]

CaN-A, PP-1c and PP-2Ac are closely related catalytic subunits with high levels of amino acid identity (49% between PP-1c and PP-2Ac, and 39% between PP-1c and CaN-A; see Figure 1.2, p. 10).[69, 261]

PP-4 is a 35 kDa enzyme with one isoform and shares 65% amino acid identity with PP-2Ac.[130, 147] PP-4 has been found predominantly in the nucleus with lower expression in the cytoplasm. PP-4 was found to bind centrosomes during mitosis, suggesting it plays a role in microtubule nucleation.[130] It is also involved in hepatic gluconeogenesis regulation.[313]

PP-5 is a 56 kDa enzyme with one isoform in all human tissues examined.[56, 147] The C-terminal region of the protein is conserved in the PPP family, showing 38–40% identity with PP-1c, 38% with PP-2Ac, and 36–38% with CaN-A. The N-terminal region of PP-5 contains four tetratricopeptide (TPR) repeats. TPR repeats are degenerate 34 residue-long sequence motifs present in tandem arrays, together comprising a TPR domain. TPR domains can interact with one-another; thus they act as scaffolds that allow interaction with "TPR-docking" sites in other proteins.[56, 57, 84, 147] The basal activity of PP-5 is very low and can be improved by removal of the N-terminal TPR domain. Thus the TPR domain also acts as an autoinhibitory domain against PP-5 enzyme activity.[57, 84, 147] PP-5 has been implicated in cell cycle progression, lipid metabolism, Raf-1 kinase signaling regulation, regulation of the mammalian circadian clock rhythm, as well as regulation of $K^+$-channels function.[57, 137, 170, 255]

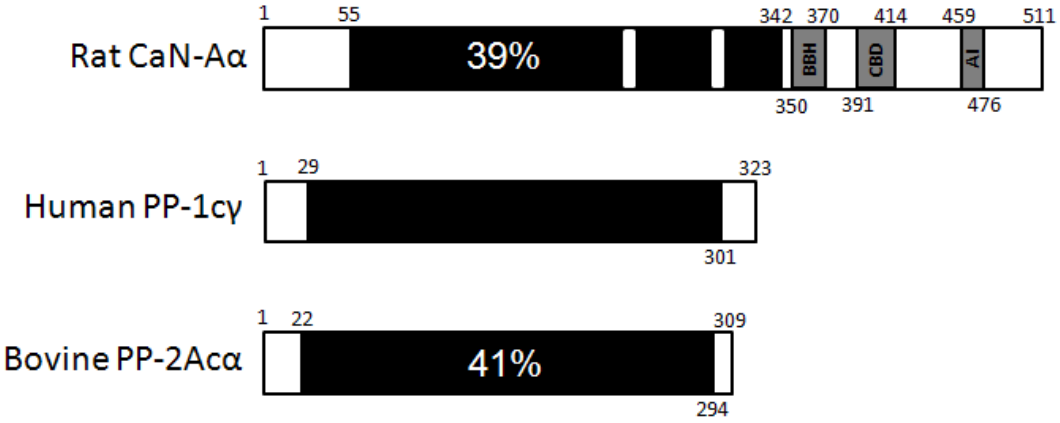


**Figure 1.2: Structural comparison between three of the PPP family of protein phosphatases.** The three primary representatives of the PPP family of phosphatases, CaN, PP-1c and PP-2Ac, are shown in schematic depiction. Catalytic domains, as defined by Barton et al. (1994) are indicated in black. Percent of amino acid identity shared between the PPP protein phosphatases, relative to PP-1c, is indicated. Additional regulatory domains within CaN-A are shaded in grey, and designate the following secondary structures: BBH = CaN-B binding α helix; CBD = CaM binding domain; AI = autoinhibitory domain. The numbers indicate the position of amino acids that outline different domains of the phosphatases. The choice of protein phosphatase isoforms illustrated reflects the protein phosphatases used in this thesis work. The sequence of rat CaN-Aα is identical to human CaN-Aα, except that the human isoform contains an additional ten amino acids at residue 414. The sequence of bovine PP-2Acα is identical to human PP-2Acα.[20]

PP-6 is a 36 kDa enzyme with one isoform that shares 40–41% homology with PP-1c, 56–57% with PP-2Ac, 34% with CaN-A, and 65% with PP-4.[21, 111, 147] PP-6 is required for repair of DNA double-strand breaks, regulation of mitotic spindle formation, and suppression of TAK1 kinase-dependent inflammatory response.[168, 316, 321]

PP-7 exists as two isoforms encoded by two separate genes and contains both N- and C-terminal extensions believed to be involved in targeting and regulation of enzymatic activity. PP-7 shares 28–35% identity or 35–44% homology with the catalytic cores of the PPP family, and is unique amongst protein phosphatases in that it is expressed only in the retina, developing fetal brain and primary sensory neurons.[147]

The C-terminal region of PP-7 is structurally different from the PPP family of protein phosphatases, containing five $Ca^{2+}$-binding EF-hand-like motifs. The enzymatic core region contains a 43 amino acid insert not present in other PPP enzymes, the function of which is not known.[153]

### 1.2.2 The PPM family of protein phosphatases

PPM is a family of phosphatases with members found both in prokaryotes and eukaryotes. In humans, this family of protein phosphatases is primarily involved in stress response regulation. Although PPM protein phosphatases share no sequence similarity with PPP family members, they are structurally similar.

PP-2C is the most well-known member of the PPM family. The crystal structure of PP-2C with a phosphate group present at the active site, suggested that PPM protein phosphatase catalysis may occur in a similar manner to that of the PPP family.[19, 85]

### 1.2.3 The PTP family of protein phosphatases

PTP protein phosphatases consist of 107 protein members, forming the largest subgroup of all protein phosphatases, comprising both cytosolic and membrane-bound enzymes.[54, 147, 223, 260] Growth factors and hormones that lead to cell growth and transformation, initiate their physiological actions via downstream activation of transmembrane proteins that possess tyrosine protein kinase functions. PTP phosphatases can reverse this regulation and lead to inactivation of a receptor kinase activity.[260] Discrete cellular roles of most tyrosine protein phosphatases are determined by a specific modular domain they contain.[223] Comparisons of primary structures of PTP phosphatases with the PPP family show that these enzyme families are unrelated, and most likely have evolved independently.[260]

## 1.3 Calcineurin is a unique phosphatase within the PPP family

In the majority of known cases, CaN acts exclusively as the sole protein phosphatase towards its target substrates. Only in rare instances can other PPP phosphatases fulfill the same role as CaN.[12] This attests to the likely importance of the enzyme in cell regulation. In addition, CaN plays a role in many medical

complications, some of which are summarized in Table 1.2, p. 14.

Significantly, with respect to this thesis, endogenous and exogenous inhibitors found to affect the enzymatic activity of other members of the PPP family are remarkably less potent or ineffective toward CaN (see Table 1.1, p. 5). Conversely, potent CaN inhibitors have little to no effect on the remainder of the PPP phosphatases.[144, 213]

### 1.3.1 Calcineurin structure

CaN was the first member of the Ser/Thr protein phosphatases to have its three dimensional structure determined (see Figure 1.3, p. 15). These studies, published by Griffith et al. (1995) and Kissinger et al. (1995), helped to elucidate the catalytic mechanism of the PPP phosphatase enzymes, and provided important information on how CaN is specifically inhibited by the natural product FK506.[126, 180]

CaN-A is ellipsoidal, comprising a β-sandwich motif, surrounded by six α helices on one side, and a mix of three α-helices and 3 β-strands on the other. The β-sandwich is made of two β-sheets, forming open and closed ends. At the latter, the two β-sheets extend over one another at an angle of 30°. The inner core of the β-sandwich contains mainly hydrophobic residues. C-terminal loops connecting β-strands (L2, L3, L4 and L6) and the C-termini of β2 and β3 strands form the active site, also marked by the presence of $Fe^{3+}$ and $Zn^{2+}$ metal ions (see Figures

**Table 1.2: Calcineurin's involvement in health related complications.**

| Condition | Calcineurin role | Refs |
|---|---|---|
| Parkinson's disease | In dopaminergic neurons, DARPP-32 (32 kDa dopamine and cyclic AMP-regulated phosphoprotein) is phosphorylated in response to receptor activation. Phosphorylated DARPP-32 is an inhibitor of PP-1c. Knock-out (KO) of DARPP-32 in mice leads to reduced response to dopamine and locomotor defects equivalent to those seen in Parkinson's disease. Inhibition of PP-1 by DARPP-32 could counter the effects of Parkinson's disease. Since phosphorylated DARPP-32 is a substrate of CaN, targeting CaN function would lead to increased PP-1 inhibition. | 12, 132, 219 |
| Memory loss | Learning and memory requires long-term potentiation (LTP), an enhancement of synaptic efficacy, as well as long-term depression (LTD), a reduction of synaptic efficacy. Stimuli that generate LTP require phosphorylation of protein phosphatase inhibitor-1 (I-1) which inhibits PP-1c. Stimuli that generate LTD require CaN which dephosphorylates I-1. Thus CaN can be targeted to regulate PP-1c activity. Overexpression of CaN in mice leads to deficits in long-term memory and constraints on LTP generation. Conversely, inhibition of CaN improved learning and memory capacity. | 12, 219, 226, 262 |
| Alzheimer's disease | In the frontal cortex of individuals with Alzheimer's disease, both PP-2A and CaN had reduced activity towards τ protein. Hyperphosphorylation of τ protein is a signal indicative of Alzheimer's disease generation. CaN-Aα KO mice showed increased phosphorylation of τ protein. Targeting of CaN activity ameliorates the neurodegenerative processes observed during the disease progression. | 1, 219, 262 |
| Brain ischemia | Exogenous CaN inhibitor, FK506, crosses the blood-brain barrier whereas cyclosporine A (CsA), another CaN inhibitor, does so more poorly. Only patients that undertook the CsA regimen developed brain ischemia. FK506 also reduced neural damage in rats suffering from cerebral artery occlusion, thus preventing neurotoxicity and brain damage. | 219, 262, 267 |
| HIV | Human immunodeficiency virus (HIV) can lead to T-cell activation in a CaN-dependent manner which results in activation of virus propagating genes. Inhibition of CaN with CsA/FK506 leads to downregulation of these genes. | 109, 219 |
| Carcinoma cell invasion | Inhibition of CaN activation of nuclear factor of activated T-cells (NFAT) transcription factors resulted in diminished *ex vivo* cell invasion by carcinoma cells, whereas overexpression of CaN had the opposite effect. Tissues from breast cancer patients with invasive ductal carcinoma showed increased NFAT expression in the carcinoma cells compared with normal breast tissue. Regulator of CaN 1 (RCAN1) was found to negatively impact cancer cell migration via inhibition of CaN. | 105, 165 |
| Heart hyper-trophy | CaN is important for heart development and is also responsible for cardiac hypertrophy, a process requiring NFAT proteins. | 135, 210, 262 |

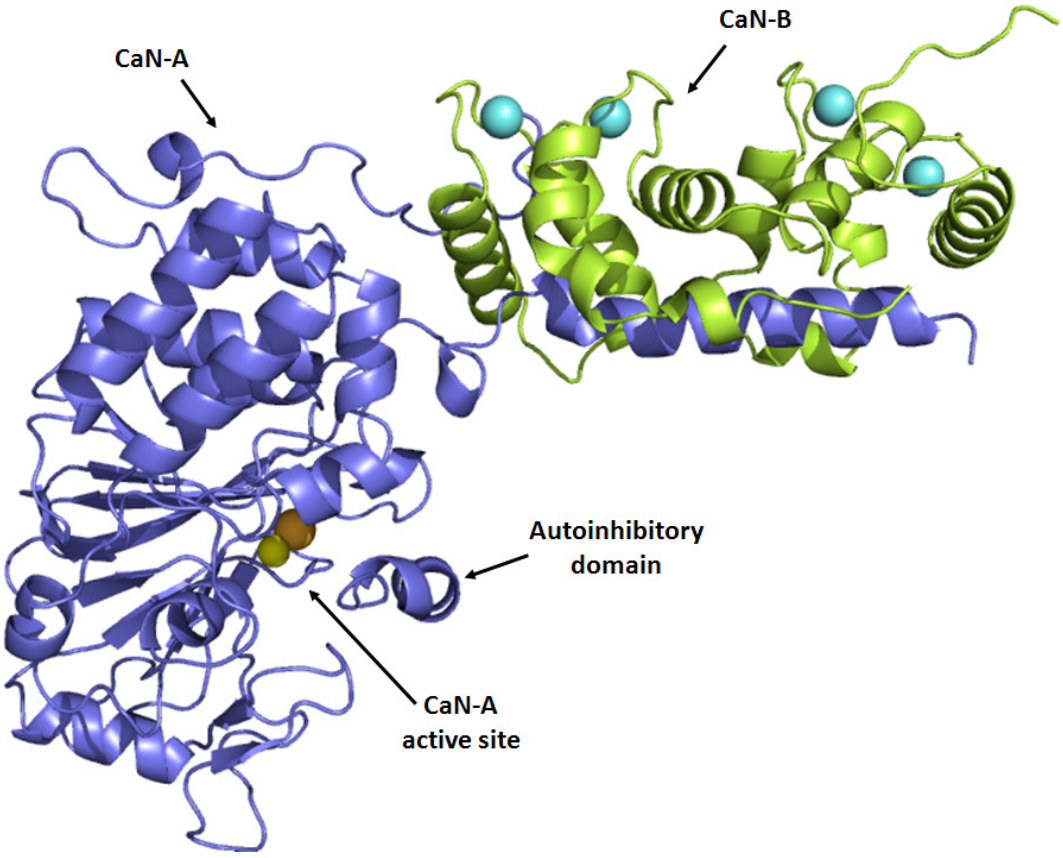


**Figure 1.3: The crystal structure of recombinant human calcineurin.** The catalytic subunit of CaN-Aα, as determined by Kissinger et al. (1995), is shown in slate color, with the active site highlighted by the presence of $Fe^{3+}$ and $Zn^{2+}$ atoms, shown as orange and yellow spheres, respectively. The CaN-B regulatory protein, containing four $Ca^{2+}$ ions (aquamarine spheres) is shown in limon color. Occluding the active site is the autoinhibitory domain of CaN-A, with the remainder of the CaN-A catalytic subunit being unordered.[180]

1.3, p. 15 and 1.4, p. 17).[126, 180] Overall tertiary architecture of the active site is completed by a flexible loop between the β12–β13 strands (termed L7 in CaN, see Figure 1.4, p. 17). The L7 loop is important for substrate catalysis, autoinhibition, as well as interaction with exogenous inhibitors of CaN.[293]

After the β14 strand, a linker of 24 residues connects to CaN-B-binding α helix (BBH, residues 350–370) that binds the CaN-B regulatory protein that is required for full activation of CaN. This CaN-B binding region comprises of an α-helix of 22 residues, 40 Å in length (see Figure 1.3, p. 15).[126, 180]

CaN-B contains two globular $Ca^{2+}$-binding domains, where each domain consists of two $Ca^{2+}$-binding helix-loop-helix motifs termed EF hands. The two $Ca^{2+}$-binding domains form a hydrophobic groove that surrounds the upper surface of BBH which itself is completely nonpolar. The N-terminal glycine residue of CaN-B is covalently linked to a 14-carbon saturated fatty acid, myristate.[126, 180] Myristoylation of CaN-B is not required for enzyme activity.[257]

In the Kissinger et al. (1995) structure, residues 1–13, 374–468 and 487–521 were not visible, and this included the calmodulin (CaM)-binding domain that is found between residues 391 and 414. However, an 18 amino acid long segment of the CaN-A C-terminal region (residues 469-SFEEAKGLDRINERMPR-485), made of two α-helices and consisting of part of the autoinhibitory (AI) domain, was found bound to the active site (see Figure 1.3, p. 15). In total, ten residues

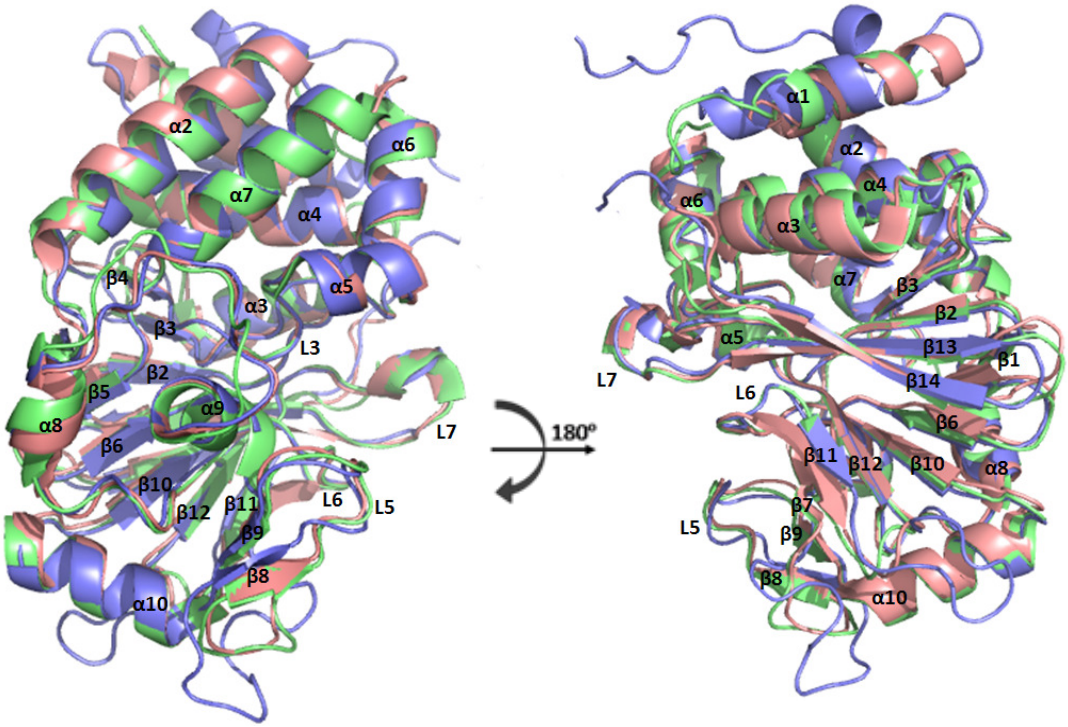


**Figure 1.4: Overlay of the structures of the PPP family of phosphatases reveal close resemblance between the enzymes.** α carbon backbone alignment of human CaN-Aα (slate), human PP-1cγ (lime), and human PP-2Acα (salmon color). Secondary structures of the phosphatases, such as α-helices (α), β-sheets (β), or loops (L), are indicated with a number that determines the order in which they are found in the structure. The L7 loop of CaN-A corresponds to an equivalent loop denoted in PP-1c and PP-2Ac as the β12–β13 loop.[180, 217, 305]

from the AI (470, 473,474,477, 480–485) interact with twenty residues of the catalytic domain. Asp-477 and 481-ERMP-484 residues from the AI participate in seven hydrogen bonds with the CaN-A active site.[180]

1.3.2 Model of calcineurin activation: lessons from *in vitro* and live cell studies

Determination of CaN structures allowed Griffith et al. (1995) to propose a model of CaN activation.[126] CaN when not bound to CaM is inhibited by its AI domain directly at the active site. Autoinhibition prevents access to the active site by substrates, or binding by active site-directed inhibitors. Activation of CaM by $Ca^{2+}$ facilitates binding of CaM to CaN-A. This interaction removes autoinhibition by the AI domain, allowing either substrates to enter the active site, or inhibition by active site-directed inhibitors.[126]

In brain tissue extracts, displacement of the AI domain by $Ca^{2+}$/CaM eventually leads to time-dependent inactivation of the CaN. This is because activation of CaN by $Ca^{2+}$/CaM exposes the CaN-A metal ions to damaging inactivating effects of superoxide anion that can lead to $Fe^{2+}$ ion oxidation. This effect can be prevented by either the presence of ascorbate (Vitamin C), which has antioxidant properties, or superoxide dismutase.[181] This process is described diagrammatically in Figure 1.5, p. 19. In contrast, deletion of CaN regulatory domains leads to a hyperactive form of enzyme that can be 10–100-fold more active than the full length protein.[12, 142, 300, 302, 309]

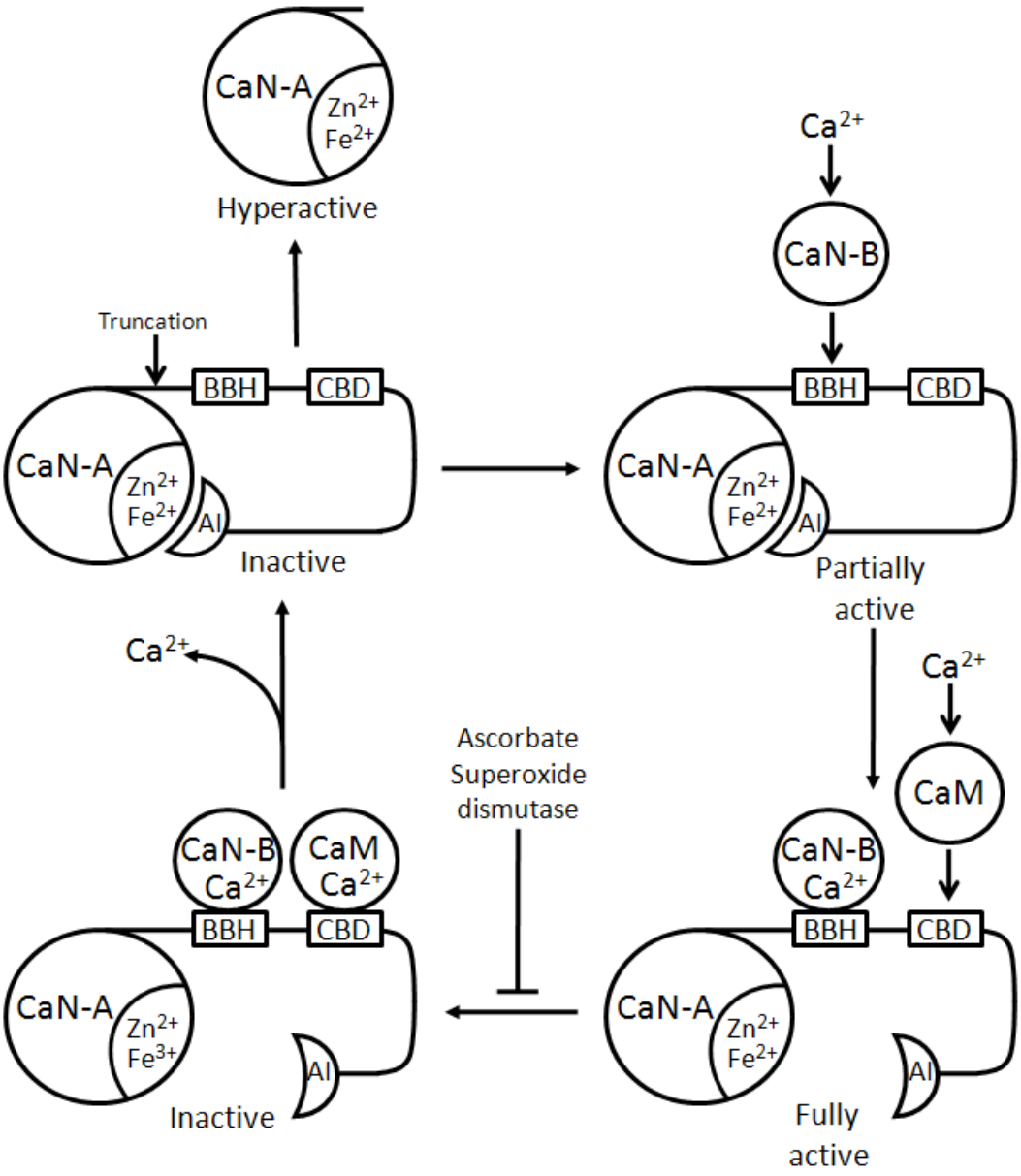


**Figure 1.5: The regulatory domains and active site metal ions of calcineurin govern its activity.** The figure depicts a scheme, whereby in its inactive state, the catalytic subunit of CaN (CaN-A) has its regulatory domains exposed, while its autoinhibitory (AI) domain occludes the active site, denoted by the presence of the metal ions, $Zn^{2+}$ and $Fe^{2+}$. Upon exposure to $Ca^{2+}$, CaN-B binds to the binding domain (BBH) on CaN-A. This confers partial activity on the enzyme. Fully functional phosphatase is formed when $Ca^{2+}$-bound CaM binds to its binding domain (CBD), resulting in removal of the AI domain from the active site. Oxidation can play a role in inhibiting CaN by altering the electron state of the iron metal ion. This can be prevented by superoxide dismutase or by addition of ascorbate. *In vitro* studies have shown that removal of the BBH, CBD and AI regulatory domains leads to the hyperactive form of enzyme.[12, 126, 142, 181, 196, 296, 300, 302, 309]

1.3.3 Structural similarities between the PPP phosphatases

The crystal structure of the PP-1 and PP-2A catalytic subunits closely resembles that of CaN-A, and the backbones of all three protein phosphatases superimpose very closely (see Figure 1.4, p. 17).[59, 94, 124, 292, 305] The metal ions occupying the active site of PP-1c were assigned as $Mn^{2+}$, and either $Fe^{2+}$ or $Fe^{3+}$. With the high level of structural similarities observed between catalytic subunits of the PPP family of phosphatases, it is the discrete differences imposed by the primary structure, and presence of additional regulatory domains that confer the specificity of regulation, substrate binding, or inhibition by exogenous compounds for each of the protein phosphatases.

The active site of PP-1c is at a trifurcation point of an extended Y-shaped cleft formed by a hydrophobic groove (also found in PP-2Ac and CaN), an acidic groove comprising several acidic side chains, and a C-terminal groove which is formed in part by the C-terminal region of PP-1c (see Figure 1.6, p. 21).[94, 124, 292]

The structure of the catalytic subunit of PP-5 has also been determined and is architecturally similar to other PPP family members. The C-terminal end of PP-5 terminates in a twenty residue-long α-helix, termed αJ, that is unique to PP-5.[308] The N-terminal end of PP-5 comprises a TPR domain that makes an extensive interface with the phosphatase catalytic domain, completely blocking access to the active site. The predominant interaction is hydrogen bonding

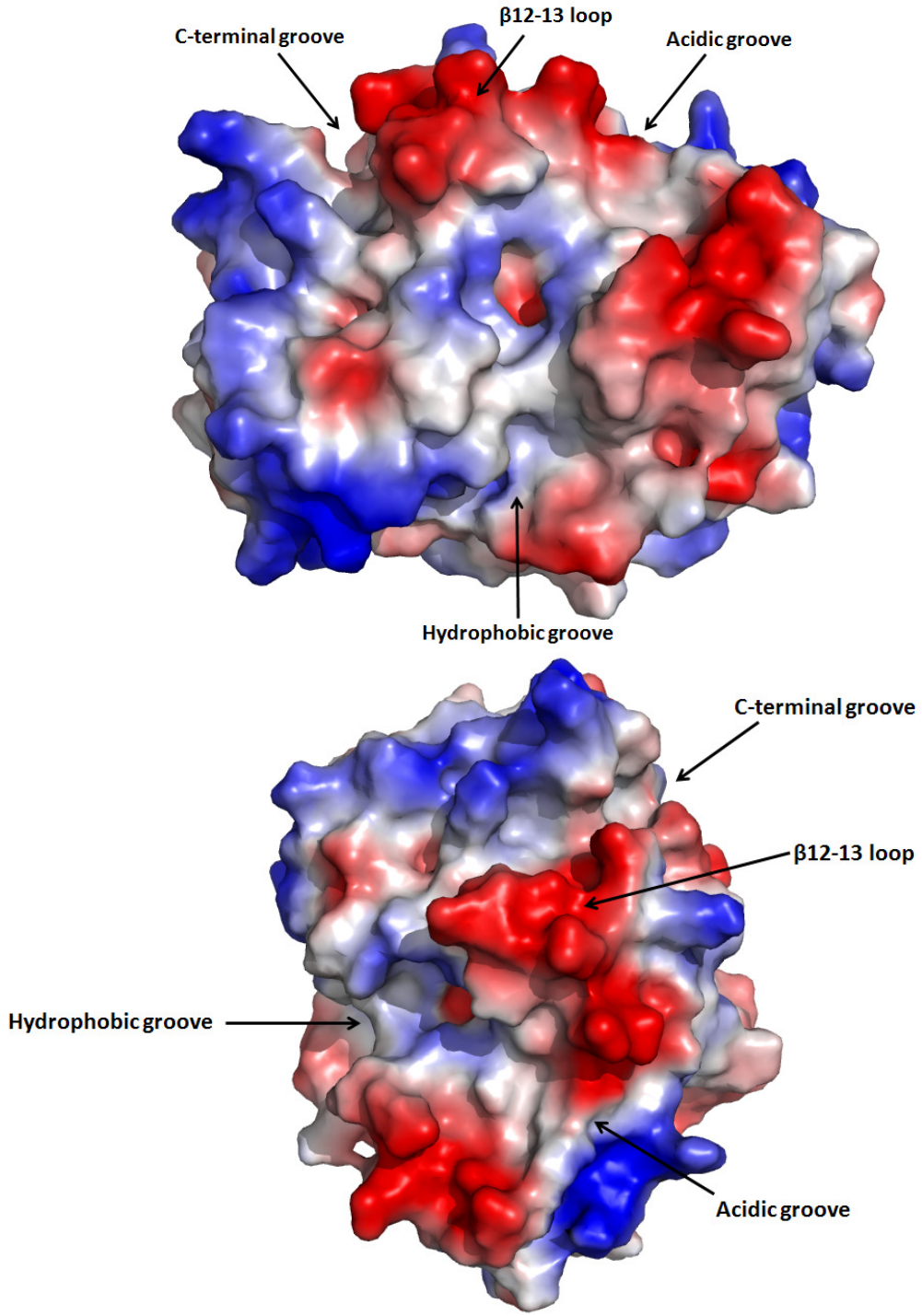


**Figure 1.6: The trifurcation of C-terminal, acidic and hydrophobic grooves forms the active site in PP-1c.** The catalytic domain of human PP-1cγ is shown as a putative electrostatic potential of its surface (red represents the negatively charged regions; blue represents the positively charged regions; white represents the uncharged regions). Top panel was rotated -50º along the abscissa, -10º along the ordinate and 90º along the z-axis to obtain the image in the bottom panel. The β12–13 loop that also is involved in the formation of the active site is labeled.[217]

between Glu-76 of the TPR domain and Arg-275 in the active site of PP-5, as well as Tyr-451, present in the β12-13 loop. The C-terminal αJ helix aids in stabilizing the TPR structure in the autoinhibitory conformation.[308]

Insertion of Glu-76 of the TPR domain into the PP-5 catalytic cleft resembles the mechanism of autoinhibition in CaN-A, resulting in blocking access to the catalytic center. In CaN-A, it is also a glutamic acid residue (Glu-481) that occludes the active site.[180, 308] The similarity between autoinhibition of CaN-A and PP-5 is illustrated in Figure 1.7, p. 23.

### 1.3.4 The catalytic mechanism of PPP phosphatases

Bioinformatic sequence analysis of the PPP phosphatase family by Barton et al. (1994) has shown that all Ser/Thr protein phosphatases contain invariable residues that are either involved in substrate catalysis, or play an important role in structural shaping of the active site.[20]

The current hypothesis for PPP family catalysis was first described by Griffith et al. (1995), using CaN structure as supporting evidence. Metal ions, bridged by a water molecule, and supported by His-92, Asp-90, Asp-118, His-281, His-199 and Asn-150, act as ligands to bind two oxygen groups of the substrate, as well as generate a hydroxide nucleophile that will cleave the phosphate from the substrate. The phosphate group of the substrate itself is stabilized by interaction with Arg-122, Arg-254 and His-281. His-151 serves as a

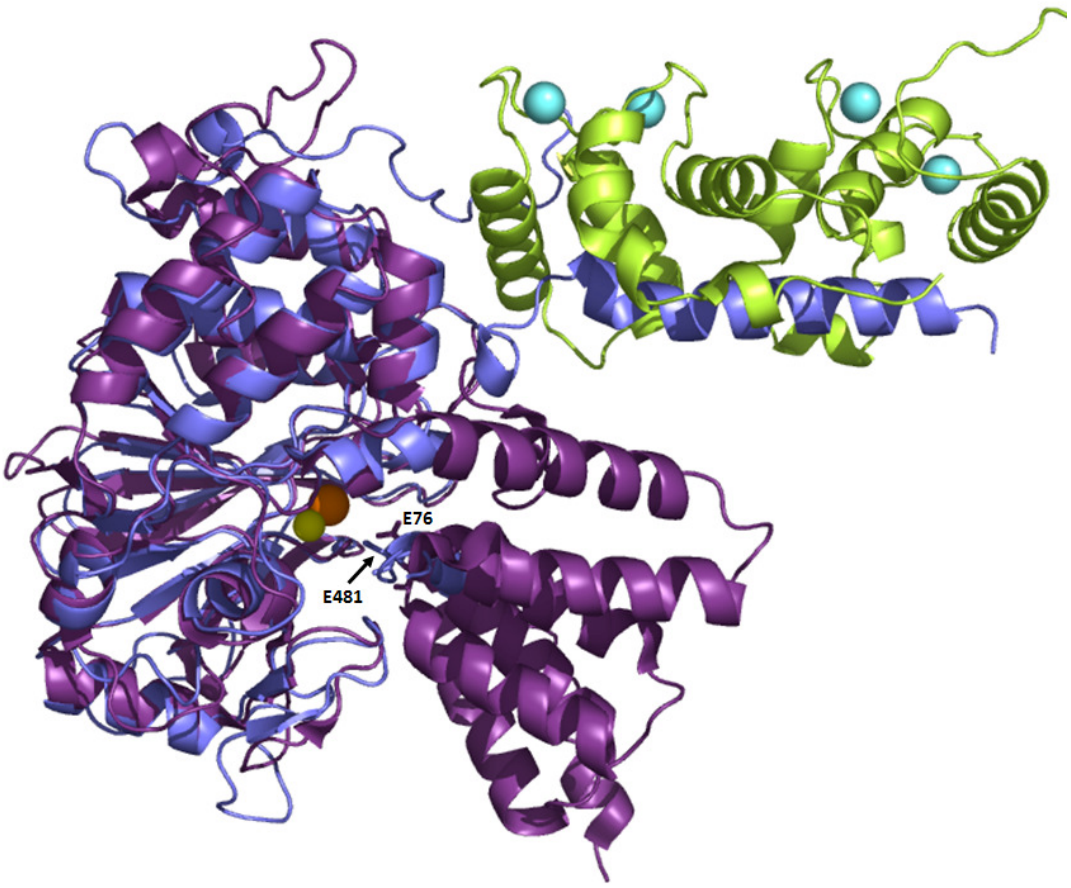


**Figure 1.7: Similar modes of autoinhibition between calcineurin and PP-5.** This figure depicts the overlaid structures of human CaNα (slate color) and human PP-5 (violet color), demonstrating a similar mode of blocking the active site of each enzyme. The active site for both enzymes is marked by the presence of $Fe^{3+}$ and $Zn^{2+}$ atoms, shown as orange and yellow spheres, respectively, as observed in the CaN structure. The N-terminal tetratricopeptide repeat domain of PP-5 can be seen obstructing its active site, with Glu-76 residue making closest molecular contact. In CaN-A, the autoinhibitory domain inserts Glu-481 into the active site.[180, 308]

proton donor to the leaving group. His-151 itself is stabilized by hydrogen bonding to Asp-121 (see Figure 1.8, p. 25). All residues involved in the substrate catalysis by CaN are conserved between members of the PPP phosphatase family.[94, 126, 317]

## 1.4 Interaction of the PPP family of protein phosphatases with exogenous inhibitors

Much of the current knowledge of the structure and function of proteinphosphatases has stemmed from studies involving their inhibition by exogenous inhibitors. The most prominent and informative inhibitors described so far have been okadaic acid, microcystin, cyclosporine A (CsA) and FK506.[70, 147, 261] CsA and FK506 are CaN specific inhibitors. PP-1c and PP-2Ac are potently inhibited by the natural toxins, okadaic acid (OA) and microcystin.[147, 219] CaN is markedly less sensitive to these inhibitors (see Table 1.1, p. 5), the molecular basis of which is not fully understood.[26, 70, 147, 148, 208] Mutagenesis studies of CaN that aid to elucidate the reasons behind CaN's decreased potency to OA and microcystin are the focal point of the second chapter of this thesis. Substitution of only a few specific residues within the active site and the L7 loop of CaN with corresponding residues found either in PP-1c/PP-2Ac, is responsible for the increased resistance of CaN to OA and microcystin natural toxins.

### 1.4.1 Cyclosporine A and FK506: their function and current use

With the discovery of CsA in 1976, the importance of the compound as a

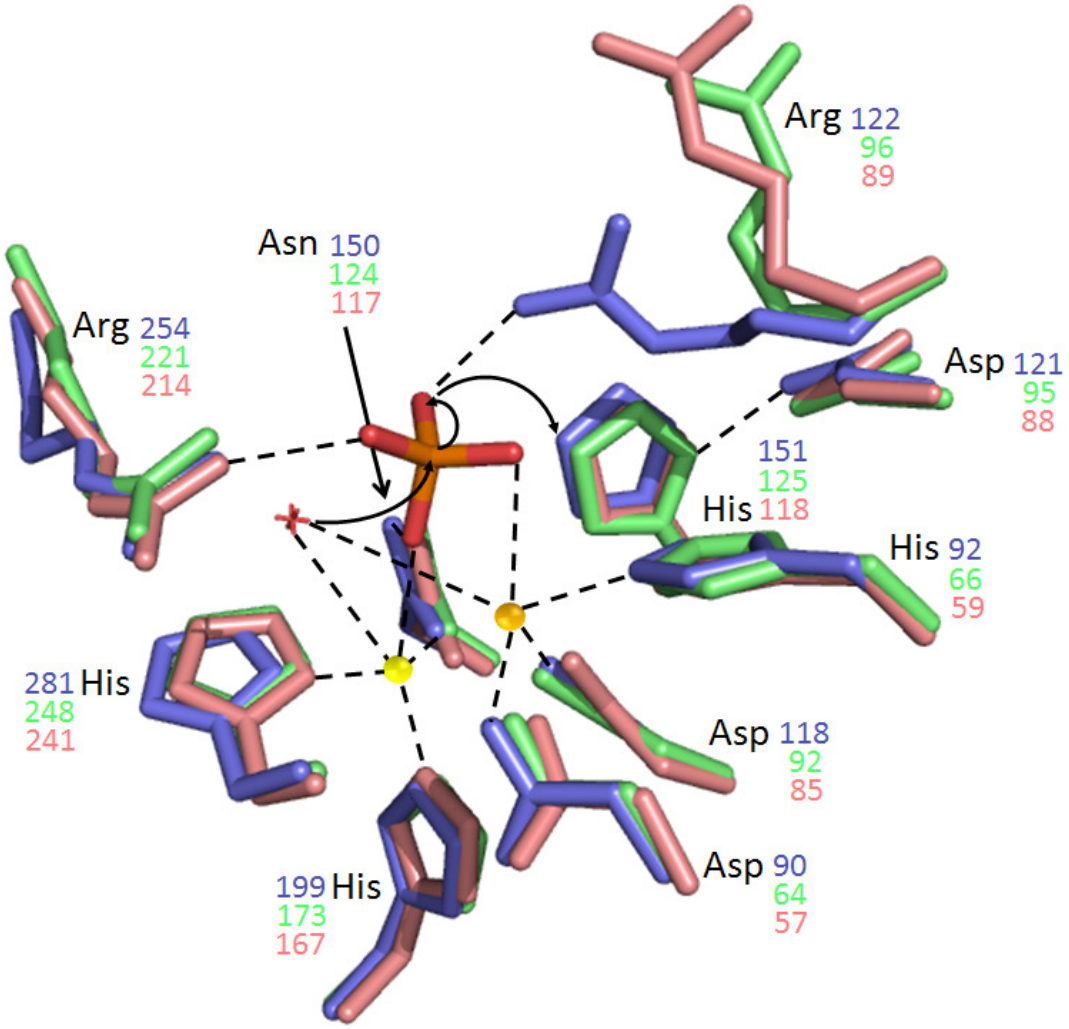


**Figure 1.8: Proposed catalytic mechanism for the PPP family of phosphatases.** This figure depicts the overlaid conserved residues of human CaN-Aα (slate), human PP-1cγ (lime) and human PP-2Acα (salmon color) that are known to be important in substrate catalysis. The corresponding residue numbers for each enzyme are annotated in their respective colors. The $PO_4^{3-}$ is shown in dark orange, with its oxygen atoms highlighted in red. $Fe^{3+}$ and $Zn^{2+}$ metal ions are designated as orange and yellow spheres, respectively. Dashed lines indicate potential electrostatic interactions. The water molecule believed to act as a catalytic nucleophile is shown as a red asterisk. Closed-end arrows follow the proposed pathway of a nucleophilic attack and the electron transfer. Note the CaN-A Arg-122 residue being shifted to accommodate the interaction with the $PO_4^{3-}$. This shift is not seen in the PP-1c and PP-2Ac structures, which did not have bound $PO_4^{3-}$ ion at their active sites.[20, 94, 126, 217, 291, 305]

powerful immunosuppressant tool was immediately recognized.[35-37, 47-49, 286] The most common need for such inhibition is organ transplantation, where foreign tissue is introduced into a host.

CsA is a highly hydrophobic eleven residue cyclic peptide from *Cylindrocarpon lucidum* fungus (see Figure 1.9A, p. 27).[41 178] Prior to the immunosuppressants that targeted CaN, patient survival undergoing transplantation was poor, with 50% survival in the first year post operation.[47, 272] CsA improved patient survival rate dramatically, with at least 80% survival in the first year after transplantation.[41, 48, 49, 129, 272]

A decade later another immunosuppressant of bacterial origin, FK506, was isolated. FK506 is a highly hydrophobic macrolide isolated from *Streptomyces tsukubaensis* (see Figure 1.9B, p. 27). FK506 is approximately ten-fold more potent than CsA, and prolongs the survival of organ grafts in various animal models and man. Unlike CsA, FK506 could reverse cardiac and renal allograft rejection in animals.[145, 209] In comparison to CsA, FK506 is poorly absorbed, thus both compounds appear to have certain advantages, and many organ transplant cases often require a combination of both immunosuppressants.[145] These two drugs have become the clinical foundation for most organ transplantation treatment today.[129, 178, 179, 270]

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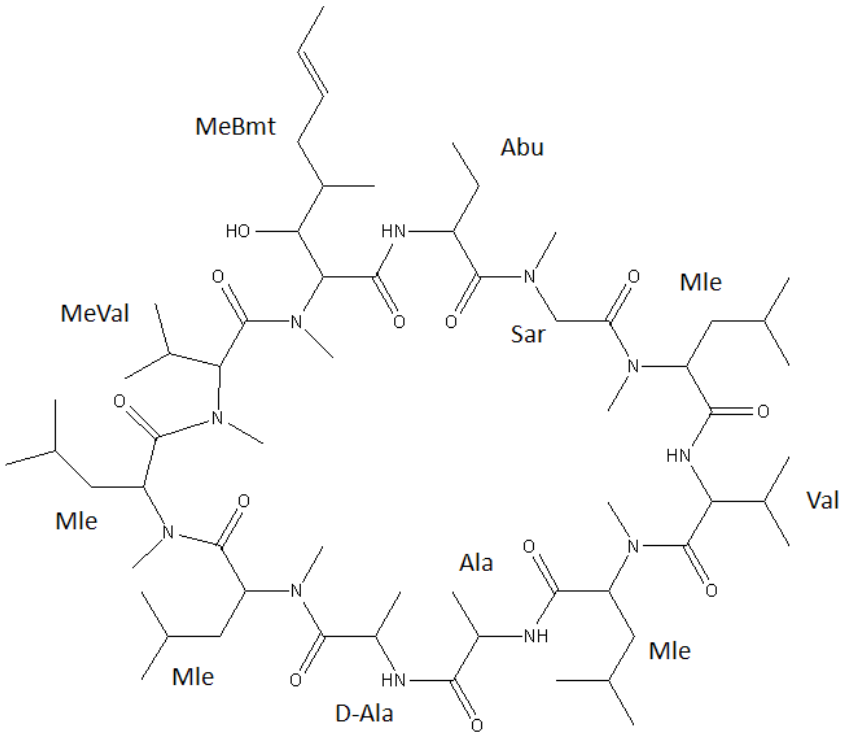


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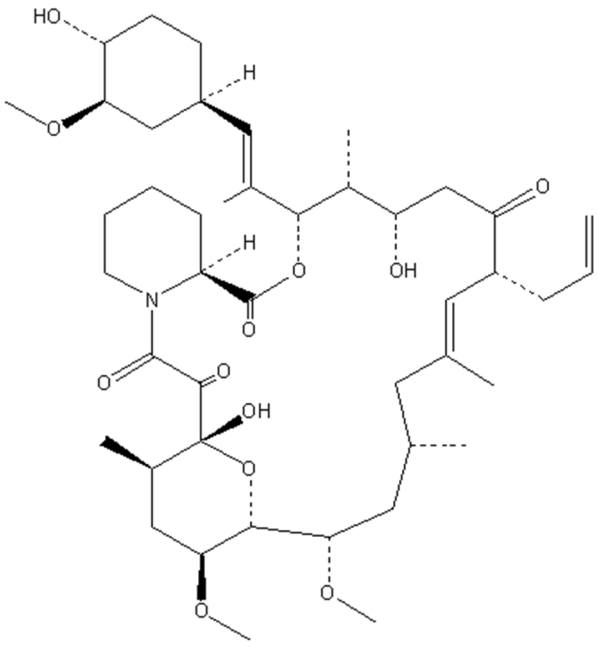



**Figure 1.9: Chemical structures of calcineurin inhibitors.** (A) This panel depicts cyclosporine A shown with its amino acids labeled. Uncommon amino acids are labeled as follows: Abu = 2-Aminobutyric acid; MeBmt = *N*-Methyl-2(S)-amino-3(R)-hydroxy-4(R)-methyl-6(E)-octenoic acid; MeVal = *N*-Methylvaline; Mle = *N*-Methylleucine; Sar = *N*-Methylglycine (sarcosine). (B) This panel depicts FK506.

1.4.2 Calcineurin inhibition by immunophilin-drug complexes

CsA and FK506 exert their immunosuppressive effects via inhibition of CaN phosphatase activity.[199] CsA and FK506 inhibit CaN indirectly via interactions with the endogenous immunophilins, cyclophilin A and FK506 binding protein-12 (FKBP12), respectively. Inhibition of CaN by drug/immunophilin complex therefore occurs by occlusion of the active site, preventing access of substrate to the active site (see Figure 1.10, p. 29).[126, 149, 166, 180] Immunophilins themselves have an endogenous rotamase enzymatic activity that changes the conformation of proline residues from *cys* to *trans* isomers. Thus immunophilins are suspected to have a role in protein folding.[267]

When T-cells are engaged by antigen presenting cells, several signal transduction pathways are activated that lead to T-cell activation and proliferation, amounting to an immune response. Principal among these pathways is one that links changes in genetic expression in response to cellular change in $Ca^{2+}$ levels. This $Ca^{2+}$-dependent signal transduction pathway begins with activation of phospholipase C-$\gamma_1$ (PLC-$\gamma_1$) which can breakdown plasma membrane lipid phosphatidyl inositol biphosphate into the second messengers, inositol 1,4,5-triphosphate ($IP_3$) and diacylglycerol (DAG).[12, 109, 119, 233] While DAG plays an important part in initiating a series of cellular events that also alter gene expression of the cell, $IP_3$ leads to intracellular store $Ca^{2+}$ release, followed by capacitative $Ca^{2+}$ entry and CaN activation via $Ca^{2+}$-binding CaM (see Figure 1.11, p. 30).[12, 109, 119, 233, 290] Activated CaN can then dephosphorylate the substrate

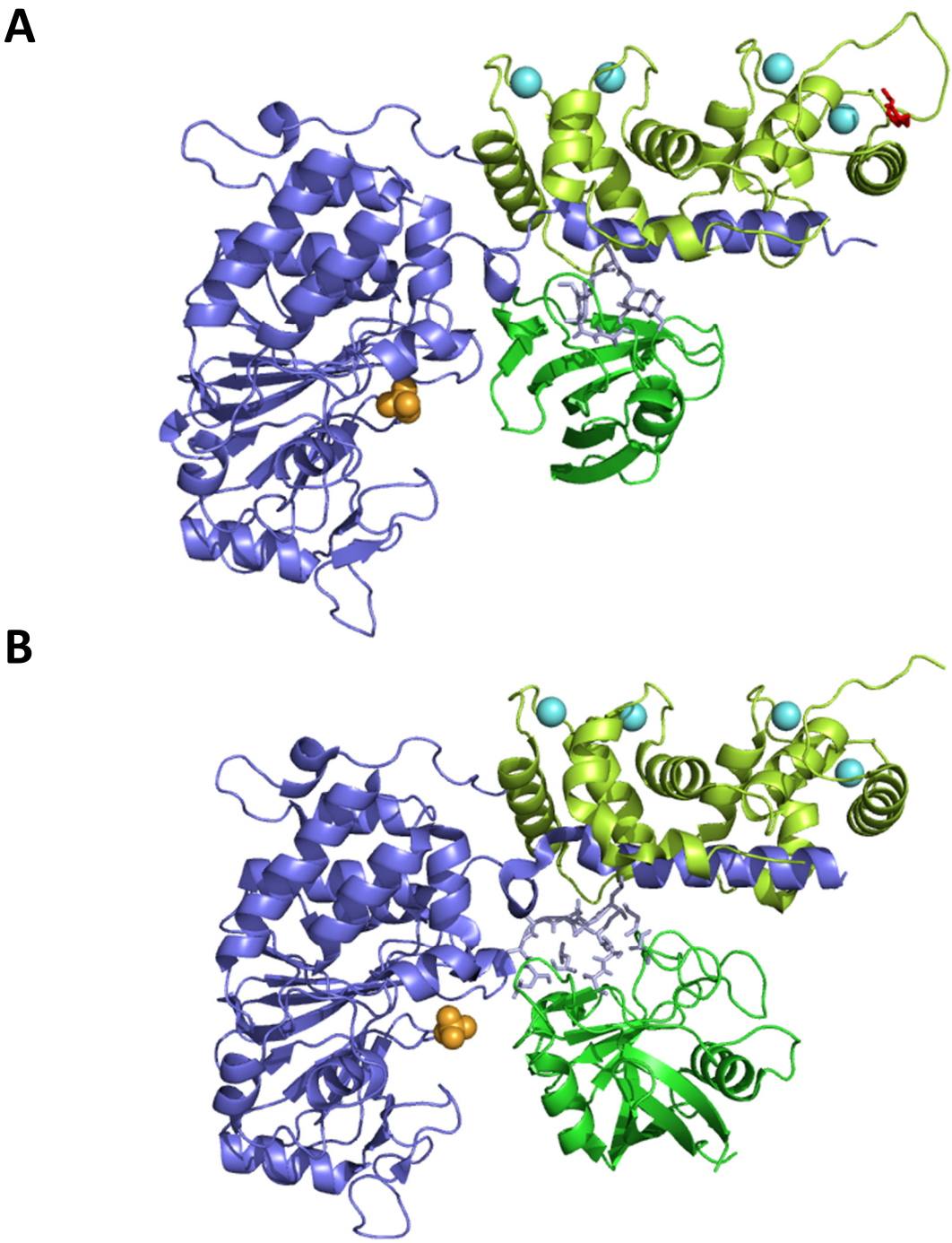



**Figure 1.10: Immunosuppressive drugs of calcineurin block access to its active site**. The figure depicts the CaN-A subunit (slate color) with a $PO_4^{3-}$ molecule (orange sphere) bound at the active site of the enzyme. CaN-B regulatory subunit is shown in limon color with four bound $Ca^{2+}$ atoms (aquamarine spheres). Post-translational myristoylation covalent modification of CaN-B is shown as red stick myristoyl group. FK506 binding protein-12 (FKBP12) immunophilin is shown in green while FK506 is shown in stick model in light blue color (panel A). Similarly, cyclophilin A immunophilin is shown in green, and cyclosporine A is shown in light blue stick model (panel B).[7, 126, 166]

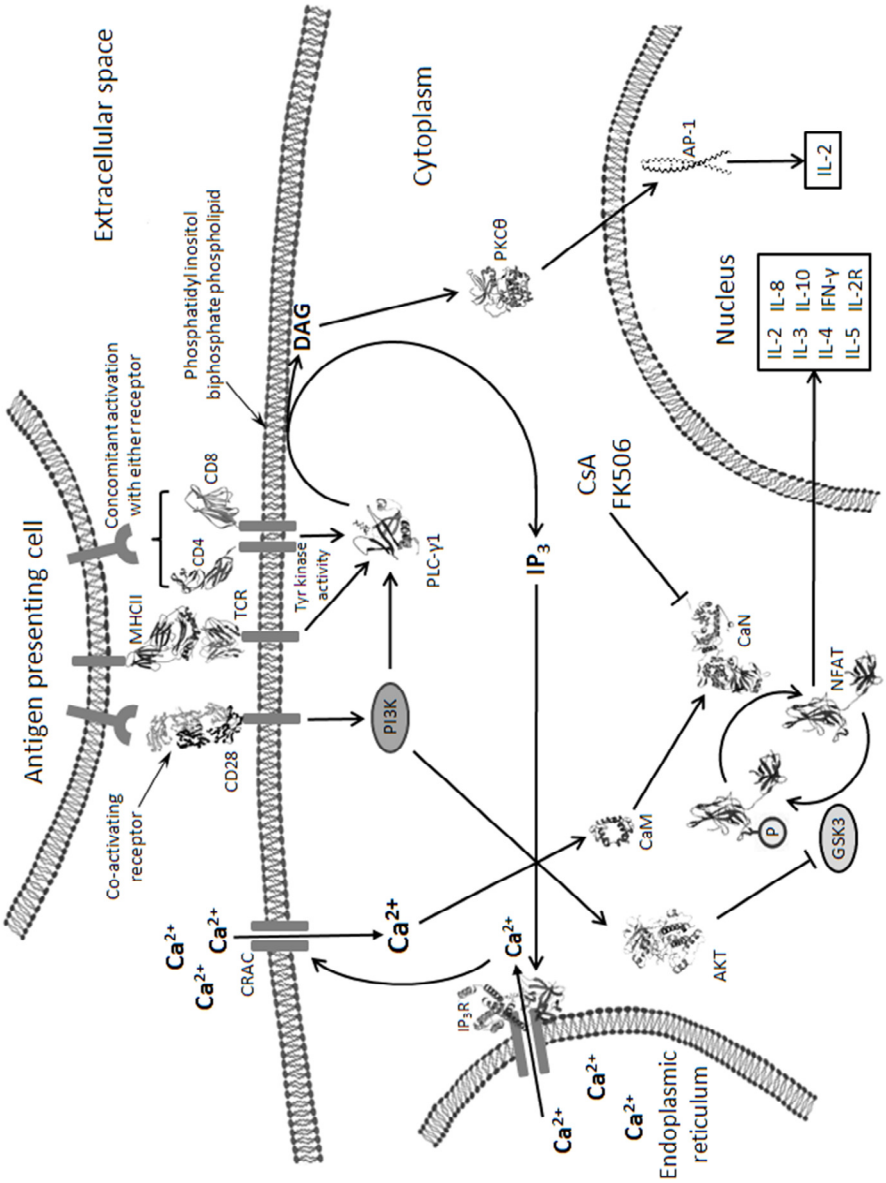



**Figure 1.11: Signal transduction pathways involved in T-cell activation.** Illustrated are the different signal transduction pathways that lead to the altered state of genetic expression, and T-cell activation during the immune response. CaN is shown regulated by exogenous inhibitors, cyclosporine A (CsA) and FK506. Open-end arrows (→) indicate stimulatory action; closed-end arrows (→►) show calcium transport; blunt-end lines (—┤) designate process of inhibition. Black rim boxes list products of genes with altered transcriptional expression. AKT = protein kinase B, AP-1 = activator protein-1, CaM = calmodulin, CRAC = $Ca^{2+}$-release-activated $Ca^{2+}$ channel, CD = cluster of differentiation, GSK-3 = glycogen synthase kinase-3, IFN-γ = interferon-γ, IL = interleukin, $IP_3$ = inositol 1,4,5-trisphosphate, $IP_3R$ = $IP_3$ receptor, MHCII = major histocompatibility complex class II, PI3K = phosphatidylinositol 3'-kinase, PKC = protein kinase C, PLC = phospholipase C, TCR = T-cell receptor. [22, 101, 109, 119, 141, 164, 175, 190, 206, 233, 256]

nuclear factor of activated T-cells (NFAT), leading to its nuclear translocation.[141, 206, 233] NFAT transcription factor leads to synthesis of cytokines. Cytokines are a category of immunomodulating proteins, such as interleukins and interferons, that are required for proliferation and differentiation of immune cells. CsA and FK506 prevent this gene transcription by inhibition of CaN enzyme activity, aborting the immune response (see Figures 1.11, p.30, and 1.12, p. 32).[22, 175, 206, 256]

### 1.4.3 Calcineurin immunosuppressants: associated problems

The major drawback of CsA/FK506 usage is the numerous physiological complications they produce. The most common symptoms include nephrotoxicity, hepatic toxicity, neurotoxicity, and increased risk of lymphomas and other cancers. In extremely rare cases, administration of CsA/FK506 has resulted in fatalities.[145, 175] One of the major reasons why patients need to be treated with a combination of both drugs is due to the many toxic side effects exhibited in patients undergoing the treatment. The combinatorial use of both drugs is utilized to minimize the adverse effects of one of the drugs, since toxicity is drug dosage dependent.[145] The phenotypes resulting from the toxicity of these drugs are summarized in Appendix A (p. 279). In addition, it appears that treatment of non-invasive cancer cells with CsA leads to increased production of transforming growth factor-β which can make the cells invasive.[18] The plethora of complications seen with the current inhibitors of CaN argues strongly for a renewed search for novel drugs effective against CaN.

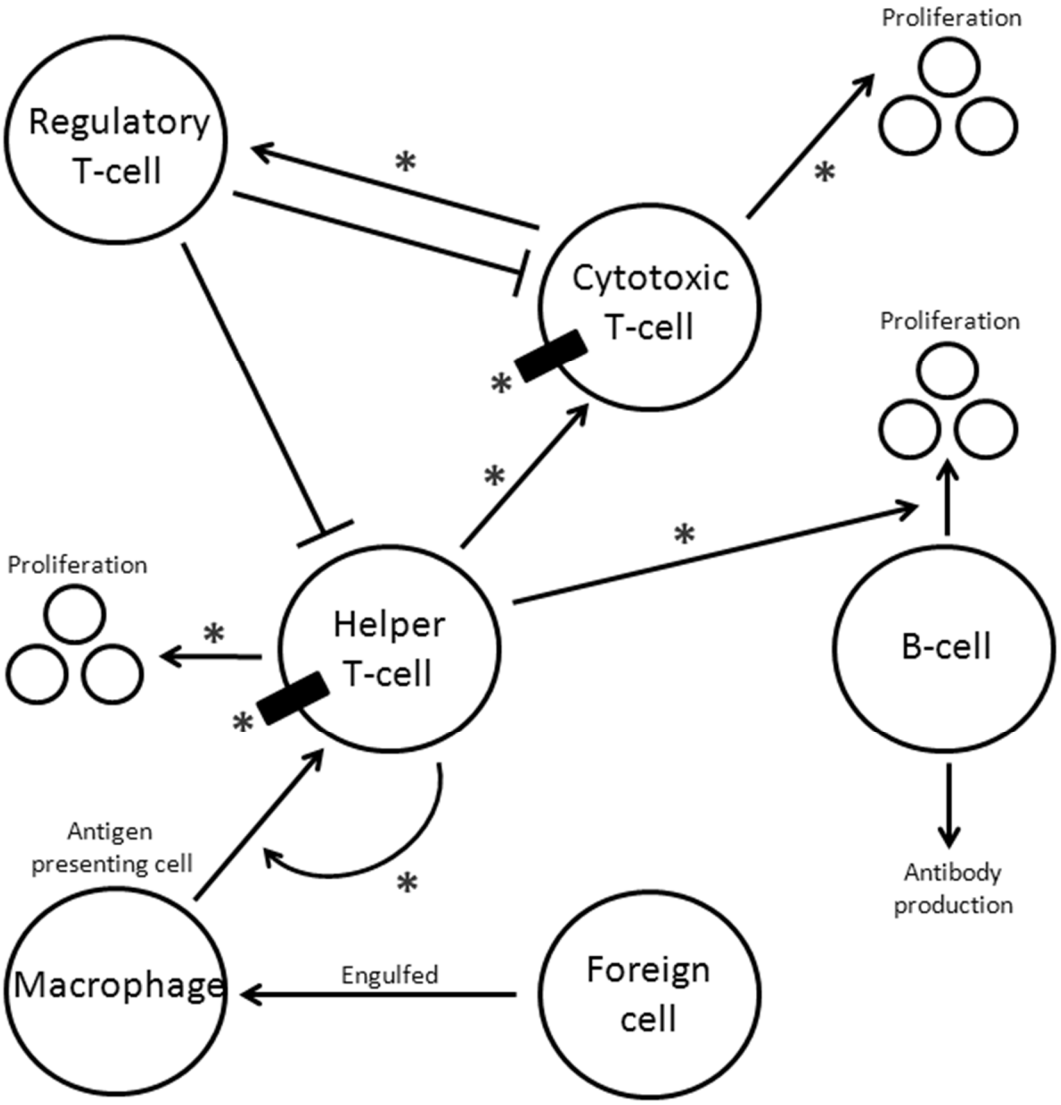



**Figure 1.12: Effects of calcineurin inhibition drugs on suppression of the immune system.** Illustrated is a schematic of the immune system response to foreign cell insult. Arrows (→) indicate stimulatory action by cytokines; blunt-end lines (⊣) designate the process of inhibition. Black rectangles represent cytokine receptors. Phagocytosis of a foreign cell by a macrophage allows the macrophage to activate helper T-cells. Activated helper T-cells can undergo proliferation as well as further regulate the immune response against the foreign cell by stimulating the activation of cytotoxic T-cells and proliferation of B-cells. Cytotoxic T-cells can also activate regulatory T-cells that inactivate the immune response in a negative-feedback mechanism. The processes that are inhibited by CaN inhibitors, cyclosporine A and FK506, are marked with asterisks. [41, 45, 101, 128, 134, 179, 235, 236, 281]

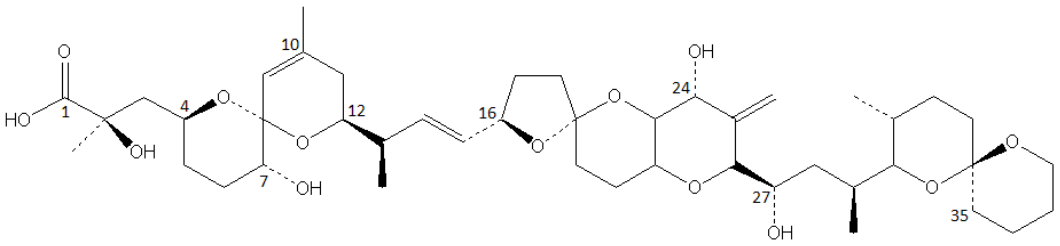
1.4.4 Okadaic acid: a natural toxin that can pose health danger to mammals

Okadaic acid (OA) is a polyether fatty acid that was first isolated from the marine sponge *Halichondria okadaii*, from which it derives its name. It was later discovered that OA was actually produced by unicellular marine dinoflagellates, specifically *Procentrum lima* and *Dinophysis spp*.[70, 91]

Ingestion of OA contaminated mussels, which can collect OA or its most common congeners when feeding on phytoplankton, can cause diarrhetic shellfish poisoning (DSP). DSP is frequent in countries where mussels are a common part of the diet, especially in northwestern Europe and Japan.[86, 108] Symptoms include gastrointestinal problems, such as diarrhea, nausea, vomiting and abdominal pain.[86] DSP has also had a tremendous economic impact, with billions of dollars in lost revenue by the shellfish industries.[66]

OA comprises 38 contiguous carbons. $C_2$ is the α-carbon of the α-hydroxy carboxylic acid as well as an α-methyl group. There are three additional secondary alcohols at $C_7$, $C_{24}$ and $C_{27}$, one *trans* alkene at $C_{14}$–$C_{15}$, a 1,1-disubstituted exocyclic alkene at $C_{25}$, and three spiroketal moieties with central carbons at $C_8$, $C_{19}$ and $C_{34}$ (see Figure 1.13A, p. 34). OA forms a pseudomacrolide by formation of an intramolecular hydrogen bond between the carboxylate moiety of $C_1$ and the hydroxyl group of $C_{24}$. This is further reinforced by a hydrogen bond between the $C_2$ hydroxyl group and the $C_4$ pyranyl oxygen, the *trans* alkene at $C_{14}$–$C_{15}$, the configuration of the spiroketals at $C_8$ and $C_{19}$ and the chair conformation of the

**A**

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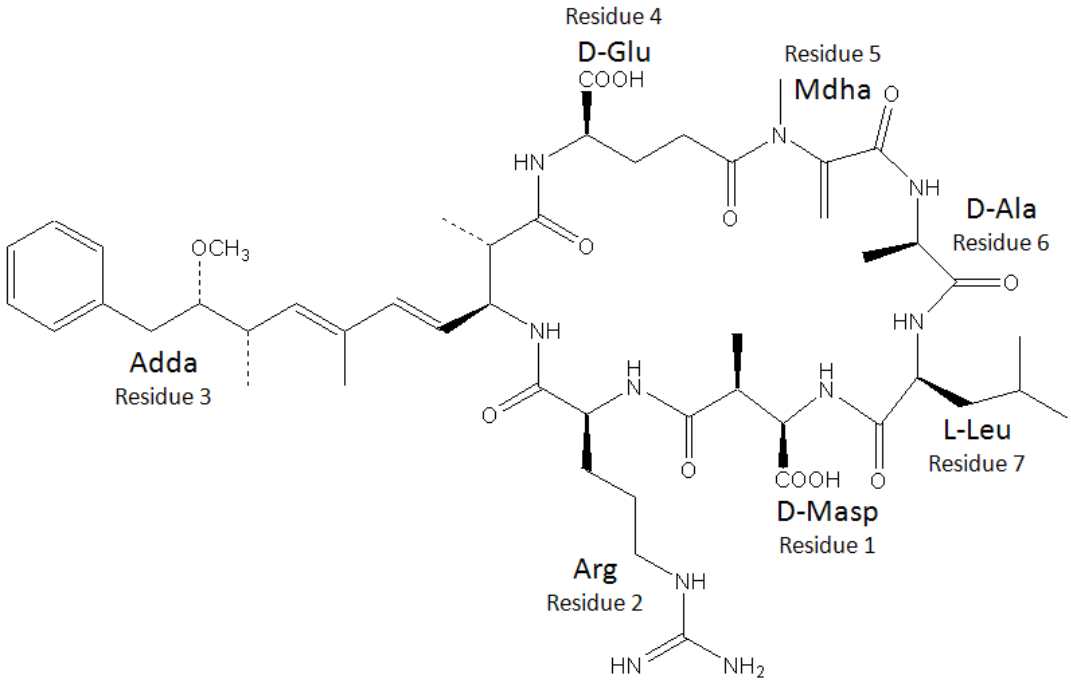



**Figure 1.13: Chemical structures of the natural toxins okadaic acid and microcystin-LR.** Okadaic acid (panel A) is shown in linear form, with the carbon atoms numbered. Microcystin-LR (MCLR, panel B) is shown with its amino acids labeled. Microcystins have a cyclo-peptide structure (-D-Masp-X-Adda-D-Glu-Mdha-D-Ala-Y-) with X and Y indicating variable amino acids. In MCLR residues 2 and 7 are the variable amino acids. Adda = 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid; D-Masp = D-erythro-β-methyl aspartic acid; Mdha = *N*-methyldehydroalanine.

oxane rings (also termed tetrahydropyran ring) of the ketals. Projecting from the pseudomacrolide core is a hydrophobic $C_{28}$–$C_{38}$ domain with a spiroketal moiety at the end.[91, 217]

The consensus $IC_{50}$ value for OA inhibition of PP-1c is 30 nM.[147, 239, 304] PP-2Ac, on the other hand, is inhibited more potently, with an accepted $IC_{50}$ value near 0.1 nM.[147] Other members of the PPP family of phosphatases can also be targeted by OA, and these are listed in Table 1.1 (p. 5).

1.4.5 Molecular enzymology underlying okadaic acid binding to Ser/Thr protein phosphatases

Chinese hamster ovary cells cultured in the presence of OA produced two strains that were less sensitive to OA. The phosphatase affected was PP-2Ac, with a C269G mutation accounting for the increased resistance to OA.[263] Identification of these mutant strains provided the first clue to where OA binds the PPP phosphatases. The location of the mutation (the β12–13 loop) also provided a potential rationale for the variation in OA sensitivity seen between different protein phosphatases, as the corresponding residue in PP-1c is Phe-276, and that of CaN-A is Tyr-315 (see Figures 1.4, p. 17, and 1.6, p. 21).

Thorough mutagenesis of PP-1c and PP-2Ac established the critical importance of the β12–13 loop in interactions between these enzymes and OA toxin. Mutagenesis of individual amino acids of the PP-2Ac segment

265-YCY-267 and Cys-269 of the loop, negatively impacted the OA sensitivity of the phosphatase with 40–100-fold increases in $IC_{50}$s.[169] Mutagenesis of individual PP-1c residues 268-SAPNYCGEFD-277 in the loop, recognized Ser-268, Tyr-272 and Phe-276 as important in OA interaction.[318]

1.4.6 Structures of okadaic acid toxins bound to Ser/Thr protein phosphatases

The structure of PP-1c bound to OA was first solved in 2001 in a collaboration between Dr. Holmes and Dr. James laboratories.[217] The overall structure is similar to two previous structures of PP-1c bound to tungstate and MCLR.[94, 124] OA does not change conformation from the free structure when binding PP-1c. The double ring spiroketal moiety of OA (carbons $C_{28}$ to $C_{38}$) binds the hydrophobic groove, interacting with Trp-206 and Ile-130. Within the active site, OA hydrogen bonds via its $C_1$ carboxylic acid with Arg-221 of PP-1c. The hydroxyl groups of $C_2$ and $C_{24}$ hydrogen bond with Arg-96 and Arg-221, respectively. The $C_{24}$ hydroxyl group also makes an intramolecular hydrogen bond with the C1 acid to form a cyclic structure of the inhibitor. Additional interactions include hydrophobic interactions between $C_4$ and the $C_{16}$ region of OA with the Phe-276 and Val-250 of PP-1c (see Figures 1.14A, p. 37, and 2.1, p. 73).

The structure of PP-2Ac bound to OA has been solved more recently.[59, 305] The toxin was found to bind the enzyme in a similar manner to PP-1c, despite some amino acid dissimilarities between the two phosphatases in the vicinity of

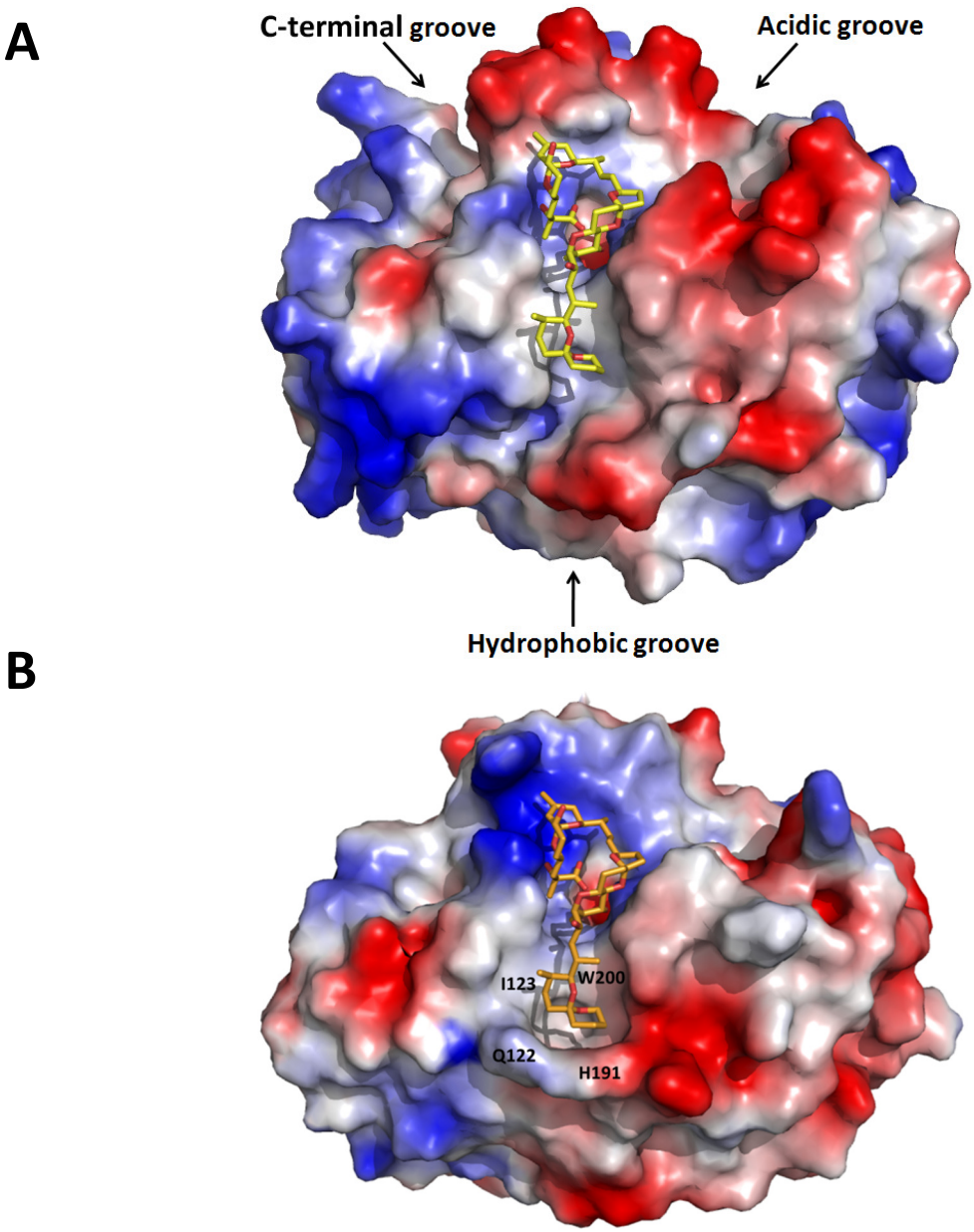



**Figure 1.14: Structures of PPP phosphatases bound to natural toxin okadaic acid.** (A) This panel depicts the structure of human PP-1cγ bound to okadaic acid (OA). OA occludes the active site and is represented by a stick structure (yellow color). The PP-1c structure is shown as a representation of the electrostatic potential of its surface (red represents negatively charged regions; blue represents positively charged regions; white represents uncharged regions). (B) This panel depicts human PP-2Acα as an electrostatic surface representation. OA is shown in stick model (gold color). Note the altered architecture of the hydrophobic groove in PP-2Ac compared with PP-1c, where the hydrophobic pocket formed by Gln-122, Ile-123, His-191 and Trp-200 in PP-2Ac is not present in PP-1c. These residues are highlighted in a single letter amino acid code.[217, 305]

the OA binding site. A significant difference observed was the presence of a hydrophobic pocket in PP-2Ac formed by Gln-122, Ile-123, His-191 and Trp-200 that accommodated the hydrophobic tail of OA. This pocket is not present in PP-1c, and Xing et al. (2006) speculated this structural difference could explain the difference in OA sensitivity seen between PP-2Ac and PP-1c (see Figure 1.14B, p. 37). No mutagenesis studies have been undertaken to support this hypothesis. Thus, only the role of the β12–13 loop has been clearly established to account for the difference in OA sensitivity between PP-1c and PP-2Ac.[74, 169, 318, 320]

### 1.4.7 Microcystins: a large group of natural toxins

The blue-green algae are cyanobacteria that are responsible for many toxic effects on domestic animals and humans who drink contaminated water.[78, 86] Cyanobacteria blooms occur in waters when nutrients, such as nitrogen and phosphorus, are rich. The source of nutrients most frequently is from agricultural and industrial effluents.[78] The toxicity of water is predominantly linked to the presence of microcystins, first isolated from cyanobacteria belonging to the *Microcystis* genera.[78, 86] Microcystins are also potent inhibitors of PP-1c and PP-2Ac protein phosphatases.[147]

Consumption of cyanobacteria cells or microcystin contaminated water can lead to exposure to potentially dangerous levels of the toxin.[78] Microcystin contaminated waters pose a substantial health hazard to humans and livestock, as

it is estimated that blue-green algae blooms occur in 70% of water reservoirs worldwide.[66] The most publicized case of human casualties from microcystin exposure occurred in Brazil, where 126 patients underwent hemodialysis with toxin contaminated water, with 60 patients subsequently dying.[78]

When ingested, microcystins target the liver via the enterohepatic circulation of bile salts, and cause reorganization of the hepatocyte cytoskeleton, resulting in hepatic lesions. These lesions cause hemorrhage, organ enlargement and tissue necrosis. In extreme cases, entire hepatocyte cells can be dislodged into the bloodstream.[78, 86]

Microcystins have a cyclo-peptide structure, with two residues being variable amino acids (see Figure 1.13B, p. 34). Sixty-four different microcystins have been isolated. The most common differences between them are seen in the degree of methylation, configuration of Adda, and at the variable amino acids.[51] The toxicity of microcystins differs between the congeners, correlating with the potency of inhibition of PP-1c and PP-2Ac.[78] MCLR and MCLA appear to be the most toxic forms.[78, 261] MCLR is the most common isolated form of the toxin.[78, 86] It inhibits both PP-1c and PP-2Ac with an $IC_{50}$ of 0.1 nM.[147] The extent of inhibition of other PPP phosphatases by MCLR is listed in Table 1.1 (p. 5).

1.4.8 Molecular enzymology underlying microcystin binding to Ser/Thr protein phosphatases

The importance of the β12–13 loop in interactions between PP-1c and microcystin toxin has also been clearly established. Mutagenesis of individual PP-1c residues 268-SAPNYCGEFD-277 in the loop, recognized the Tyr-272 as well as Phe-276 to be important in interaction with MCLR.[318] The β12–13 loop's role in toxin interaction was further confirmed when PP-1c with deleted sequence beyond residue 270 was no longer inhibited by MCLR.[74, 76]

1.4.9 Structures of microcystin toxins bound to Ser/Thr protein phosphatases

Elucidation of the crystal structure of MCLR bound to PP-1c showed that MCLR interacts with PP-1c at three principal sites: the metal binding site, the hydrophobic groove, and the edge of the C-terminal groove containing the β12–β13 loop near the active site (see Figure 1.15A, p. 41). The carboxylate group of the γ-linked D-Glu (residue 4 of MCLR, see Figure 1.13B, p. 34), as well as the adjacent carbonyl oxygen, form hydrogen bonds with water molecules. The carboxylate oxygen of Masp (residue 1) hydrogen bonds with Arg-96 and Tyr-134 of PP-1c. The hydrophobic Adda group (residue 3) packs into the hydrophobic groove. The variable amino acid, most commonly occupied by arginine (residue 2) points directly away from the active site.[124]

Determination of the structure of PP-2Ac bound to MCLR shows a nearly identical interaction between the toxin and the enzyme to that described for PP-1c

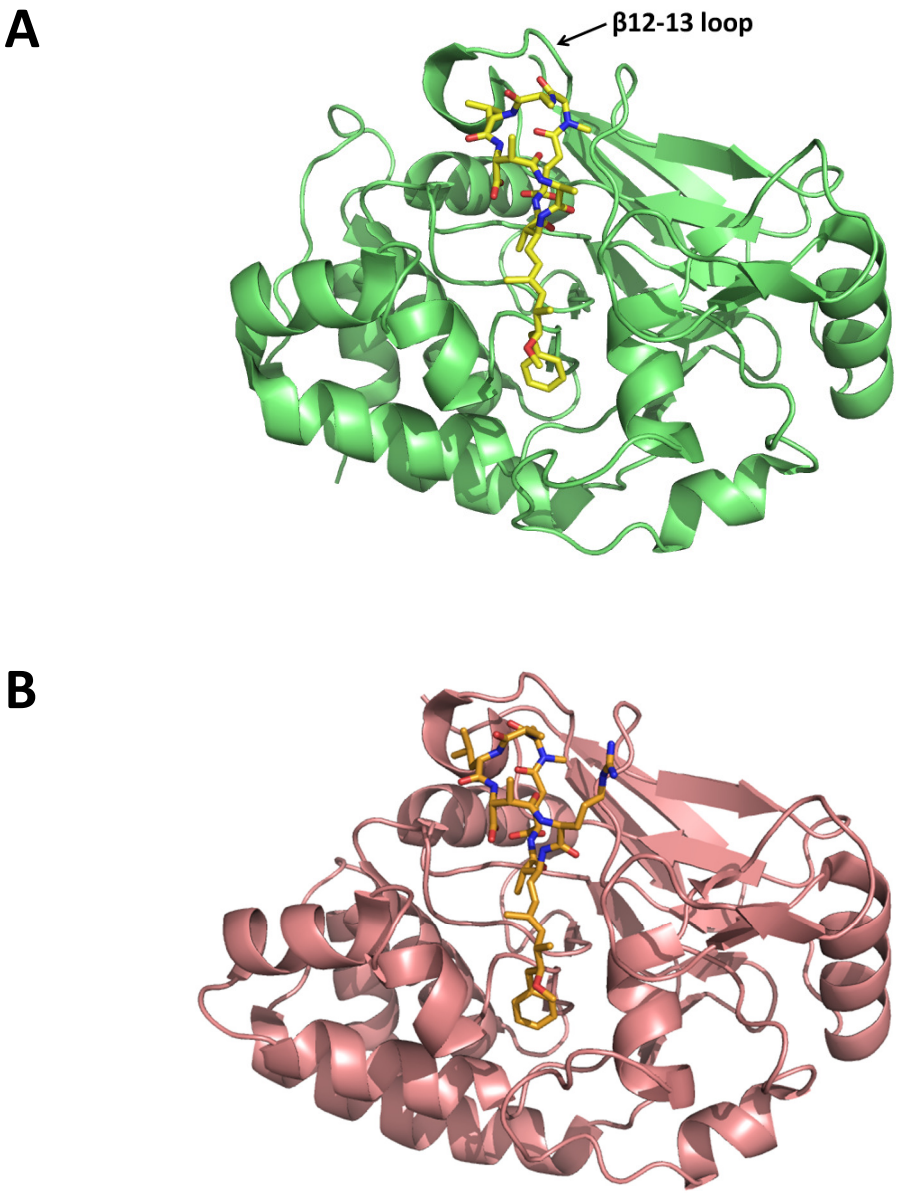



**Figure 1.15: Structures of PPP phosphatases bound to a natural toxin microcystin.** (A) This panel depicts human PP-1cγ (lime color cartoon representation) bound to dihydromicrocystin-LA (MCLA-2H, yellow color in stick representation), a microcystin analog that does not bind covalently to the protein phosphatase. The β12–13 loop, a structural feature important for interaction with natural toxins, is annotated. (B) This panel shows the structure of PP-2Acα, shown in a cartoon representation (salmon color), bound to microcystin-LR (MCLR) at the active site (stick representation, gold color).[218, 305]

(see Figure 1.15B, p. 41).[124, 305]

The inhibition of PP-1c and PP-2Ac by microcystin proceeds in two phases: an initial fast binding and inhibition of the phosphatase enzymatic activity, followed by slower covalent attachment of the toxin to the enzyme between the Mdha residue of the toxin and Cys-273 of PP-1c (equivalent residue Cys-266 in PP-2Ac).[78, 79, 249] Covalent bond formation leads to shifting of the β12–13 loop in PP-1c. This shift was confirmed crystallographically when the structure of dihydromicrocystin-LA (MCLA-2H) bound to PP-1c was solved. MCLA-2H has a hydrogenated Mdha residue preventing the side chain from participating in covalent bond formation with Cys-273 of PP-1c. The inability to form a covalent link prevented a shift of the β12–13 loop (see Figure 1.16, p. 43).[124, 217, 218]

## 1.5 The role of protein sequence binding motifs in mediating the interaction of calcineurin with its substrate proteins

The discovery that CsA and FK506 inhibited CaN stimulated elucidation of the many cellular roles of this phosphatase.[12] One of the most well studied functions of CaN is the critical role it plays in activation of the immune system.[12, 233] The principal mediators involved in activation and proliferation of T-cells are nuclear factor of activated T-cells (NFAT) proteins. These transcription factors are activated when dephosphorylated by CaN.[175, 233] Suppression of the immune system is required during and after mammalian organ transplantation, and this is

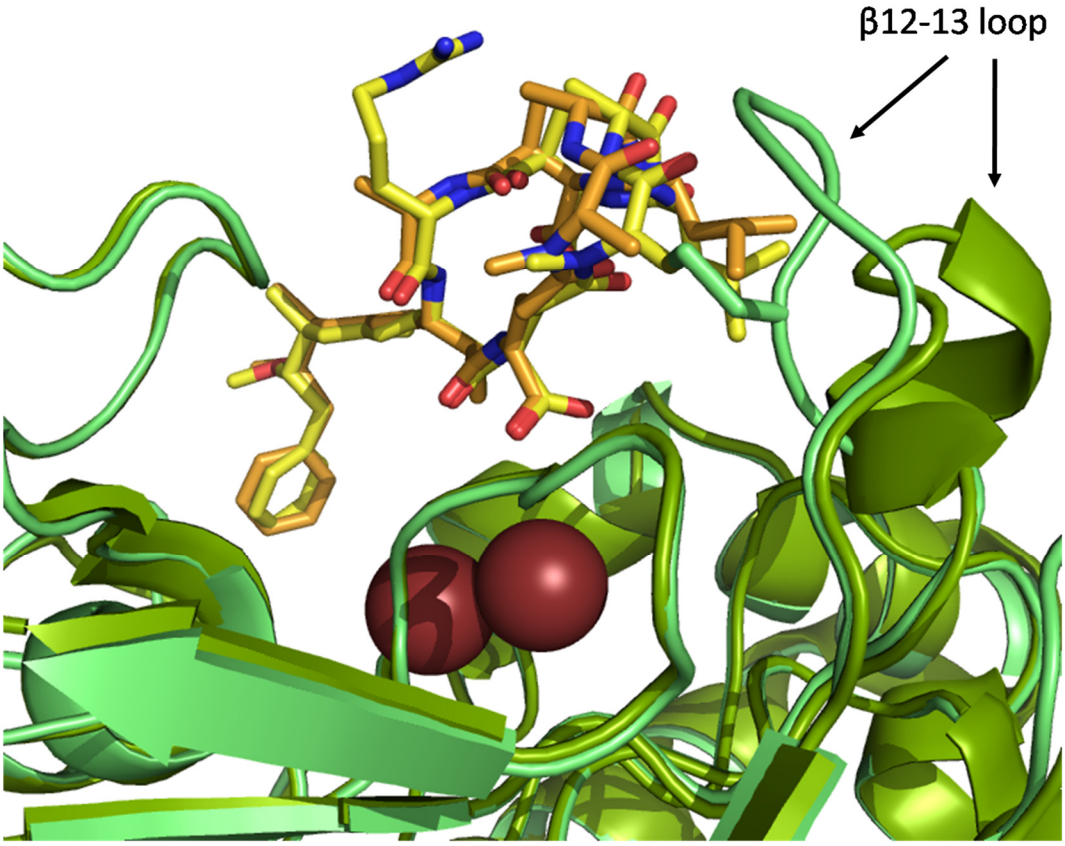



**Figure 1.16: Covalent bonding of microcystin-LR changes the orientation of the β12–13 loop of PP-1c.** This figure depicts the structures of PP-1c bound to either microcystin-LR (yellow) or dihydromicrocystin-LA (MCLA-2H, orange). Cys-273 that participates in the formation of the covalent bond between PP-1cα (lime color) and microcystin-LR is shown, resulting in the shift of the β12–13 loop. The hydrogenated *N*-methyldihydroalanine residue of MCLA-2H prevents the formation of a covalent bond. PP-1cγ interacting with MCLA-2H is shown in splitpea color. $Mn^{2+}$ metal ions are shown as ruby spheres.[124, 218]

nearly always achieved via inhibition of CaN-mediated signal transduction pathways.[129]

1.5.1 NFAT family overview

The NFAT family consists of five homologs: NFAT1-5 (see Table 1.3, p. 45), with all but one (NFAT5) being substrates of CaN. All NFAT proteins, with the exception of NFAT3, are predominantly expressed in the immune system.[12, 206, 215] All NFAT family members have a related DNA binding domain that is termed Rel homology region (RHR). This region, however, is also necessary for dimer formation. NFAT-homology region (NHR) is a highly conserved region also found in all NFAT proteins, and contains DNA binding regions, as well as a regulatory domain. The regulatory domain contains the docking sites of CaN (see Figure 1.17, p. 46).[141, 175, 206] The regulatory domain also contains many serine residues located within five serine containing motifs. Thirteen of these serine residues are known to be phosphorylated by different kinases, with all but one dephosphorylated by CaN. These sites of phosphorylation differ between NFAT proteins: thus, it is believed that different NFATs are subject to specific regulation.[141, 175, 201, 206] The *in vivo* protein kinases that phosphorylate NFAT proteins have not been conclusively identified.[141, 175, 190]

Dephosphorylation of serine residues exposes the nuclear localization signal which allows rapid NFAT targeting to the nucleus (see Figure 1.17, p. 46).[256] How NFAT remains dephosphorylated in the nucleus is a source of debate,

**Table 1.3: List of nuclear factor of activated T-cells (NFAT) proteins.**[141]

| Protein | Other names | Regulation |
|---|---|---|
| NFAT1 | NFATp/NFATc2 | $Ca^{2+}$/calcineurin |
| NFAT2 | NFATc/NFATc1 | $Ca^{2+}$/calcineurin |
| NFAT3 | NFATc4 | $Ca^{2+}$/calcineurin |
| NFAT4 | NFATx/NFATc3 | $Ca^{2+}$/calcineurin |
| NFAT5 | Tonicity enhancer binding protein (TonEBP) | Osmotic stress |

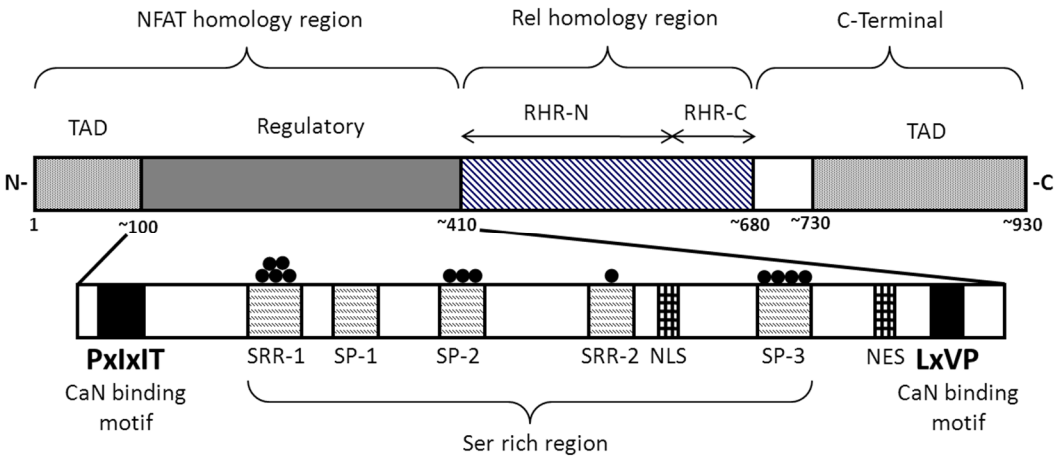



**Figure 1.17: Schematic representation of different domains of nuclear factor of activated T-cells proteins.** Nuclear factor of activated T-cells (NFAT) proteins can be divided into three main regions: the NFAT homology region (NHR), Rel homology region (RHR), and C-terminal region. Both NHR and C-terminal regions contain trans-activation domains (TAD) that are necessary for NFAT transcriptional activity. NHR also contains a regulatory domain which includes the phosphorylation sites (designated by black circles) within different serine containing motifs: serine rich regions (SRR) and SPXX motifs (SP), marked with boxes with horizontal lines of stripes. CaN binding sites are designated by black squares, while the nuclear localization sequence (NLS) is shown as checkered box. NLS is exposed upon CaN dephosphorylation. RHR (designated by striped lines) can be subdivided into N-terminal (RHR-N) and C-terminal (RHR-C) domains which are involved in DNA binding, and transcription factor interaction, respectively. The schematic representation is based on NFAT1 protein.[11, 120, 141, 175, 190, 200, 201, 203, 206, 237]

and even though the presence of CaN in the nuclei of lymphocytes has never been demonstrated, it is believed that CaN is required to reside inside the nucleus to keep NFAT in a dephosphorylated state.[109, 141, 175] The presence of a nuclear export sequence, as well as rephosphorylation of NFAT, allows the transcription factor to return to the cytoplasm.[182]

NFAT proteins are responsible for the activation of transcription of genes coding for numerous cytokines, cell surface receptors, as well as other transcription factors, primarily in cells involved in the immune system (see Figure 1.11, p. 30). The individual roles of each CaN NFAT substrate have been difficult to ascertain, due to the apparent redundancy of their function.[141, 175, 206]

### 1.5.2 NFAT proteins as potential drug targets

Currently, medical treatments available for targeting CaN function in the immune system activation stem from inhibition of CaN enzymatic activity. Although successful, this results in ubiquitous inhibition of CaN function throughout the body, resulting in a series of complex side effects (see Appendix A, p. 279). A successful search for novel CaN drugs should thus aim for inhibitors that would target NFAT activity. NFAT interaction with CaN, or the transcriptional activity of NFAT, have recently been a focus of studies for identifying novel drugs. Compounds studied include: synthetic and natural products, RNA aptamers, peptides and V7.1 antibody (see Appendix B, p. 281). In last two years alone, eleven such novel inhibitors of NFAT function have been

described.[50, 104, 225, 245, 264] The search for novel inhibitors of CaN is the focus of chapter four of this thesis. In our approach, inhibitors were sought in extracts of marine organisms using methods that could lead to identification of compounds that prohibit NFAT interaction with CaN.

### 1.5.3 The interaction of NFAT proteins with calcineurin

The molecular mechanisms underlying the binding of NFAT proteins to CaN are complex and await full elucidation. The interaction of NFAT with CaN is facilitated by several binding sites:

1) Sequence analyses of NFAT proteins have identified a conserved consensus sequence, PXIXIT, that is found in all NFAT proteins (residues 110–116 in NFAT1, see Figure 1.17, p. 46). The PXIXIT motif is necessary for NFAT binding to CaN;[11, 109, 120, 175, 215, 237]

2) In NFAT1, the C-terminal 40 residues flanking the PXIXIT sequence also contribute to CaN binding;[120]

3) Peptides designed to specifically bind to the active site of CaN-A reduce binding of CaN-A to NFAT1, suggesting that part of NFAT binds at or near the active site;[120]

4) Another site of CaN interaction has been found in all NFAT proteins. Termed the LXVP motif, it may be an even stronger binding partner of CaN than the PXIXIT motif (see Fig 1.17, p. 46). The location on CaN that binds the LXVP motif is distinct from that involved in binding the PXIXIT motif. LXVP motif binding site has been mapped to a small hydrophobic pocket composed of

residues contributed from both CaN-A and CaN-B subunits. Not surprisingly peptides generated from either the LXVP motif or the PXIXIT motif do not compete with one another.[120, 141, 198, 200, 201, 215, 237, 245]

1.5.4 The PXIXIT motif facilitates the interaction of calcineurin with multiple binding partners

Several CaN binding proteins contain PXIXIT motifs, suggesting binding to CaN in a manner similar to NFAT proteins. The ability of CaN to interact with multiple substrates or regulatory proteins highlights the importance of the PXIXIT motif in binding CaN, as it is the only evolutionarily conserved motif found amongst different CaN binding partners.

The site on CaN-A that binds the PXIXIT motif from NFAT has been identified. NMR and X-ray crystallography was used to determine the site of interaction of VIVIT (a fourteen residue-long peptide) bound to CaN-A.[193, 195, 277] The VIVIT peptide comprises an altered PXIXIT motif, and can compete with NFAT for CaN binding (see Figure 1.18, p. 50). VIVIT was found to be 25-fold more effective in displacing NFAT1 from CaN than a peptide based solely on the PXIXIT sequence itself. VIVIT greatly reduced the rate of NFAT1–4 dephosphorylation by CaN, but did not affect the dephosphorylation of substrates that lacked the PXIXIT motif.[11, 13]


**Figure 1.18: The amino acid sequence of the VIVIT peptide, an inhibitor of nuclear factor of activated T-cells protein interaction with calcineurin.** The amino acid sequence of the peptide, termed VIVIT is shown with corresponding residue numbers shown above the sequence. Location of the PXIXIT motif is marked underneath the peptide, where X designates the variable residues.[277]

The VIVIT peptide binds on the side of CaN-A 18 Å away from the active site. The VIVIT peptide binds CaN-A along the β14 strand of the phosphatase; VIVIT itself forms a short β strand of four residues (see Figure 1.19A, p. 52). The following residues in the backbones of CaN-A and VIVIT β-strands participate in hydrogen bonding: Met-329 of CaN-A with Val-7 of VIVIT peptide, Ile-331 with Val-7, and Ile-331 with Thr-9. The groove into which VIVIT binds appears to be divided into miniature hydrophobic pockets, with Phe-195, Tyr-324, Met-329, and Ile-331 of CaN-A forming the base of the groove. Leu-275 and Pro-300 form the walls of the groove and define its shape. Tyr-288, Met-290 and Phe-299 complete the groove, and divide it into tiny hydrophobic pockets. All of these residues are evolutionarily conserved in CaN.

Pro-4 of the VIVIT peptide fits into the pocket formed by Leu-275 and Phe-299. Ile-6 lies against Phe-299 and Met-290, while Ile-8 inserts into the pocket formed by Met-290 and Tyr-288 (see Figure 1.19B, p.52). Val-5 and Val-7, which correspond to the two variable residues in the PXIXIT motif, are exposed to the solvent which explains the ability to accommodate many different residues in these positions. Thr-9 is also exposed, but in 30% of determined NMR structures, Thr-9 participated in hydrogen bond formation with Asn-330, and in 40% of structures it also hydrogen bonded with Arg-332 of CaN.[277]

X-ray crystallography data further confirmed the interactions between the VIVIT peptide and CaN-A, but the network of hydrogen bonds was more

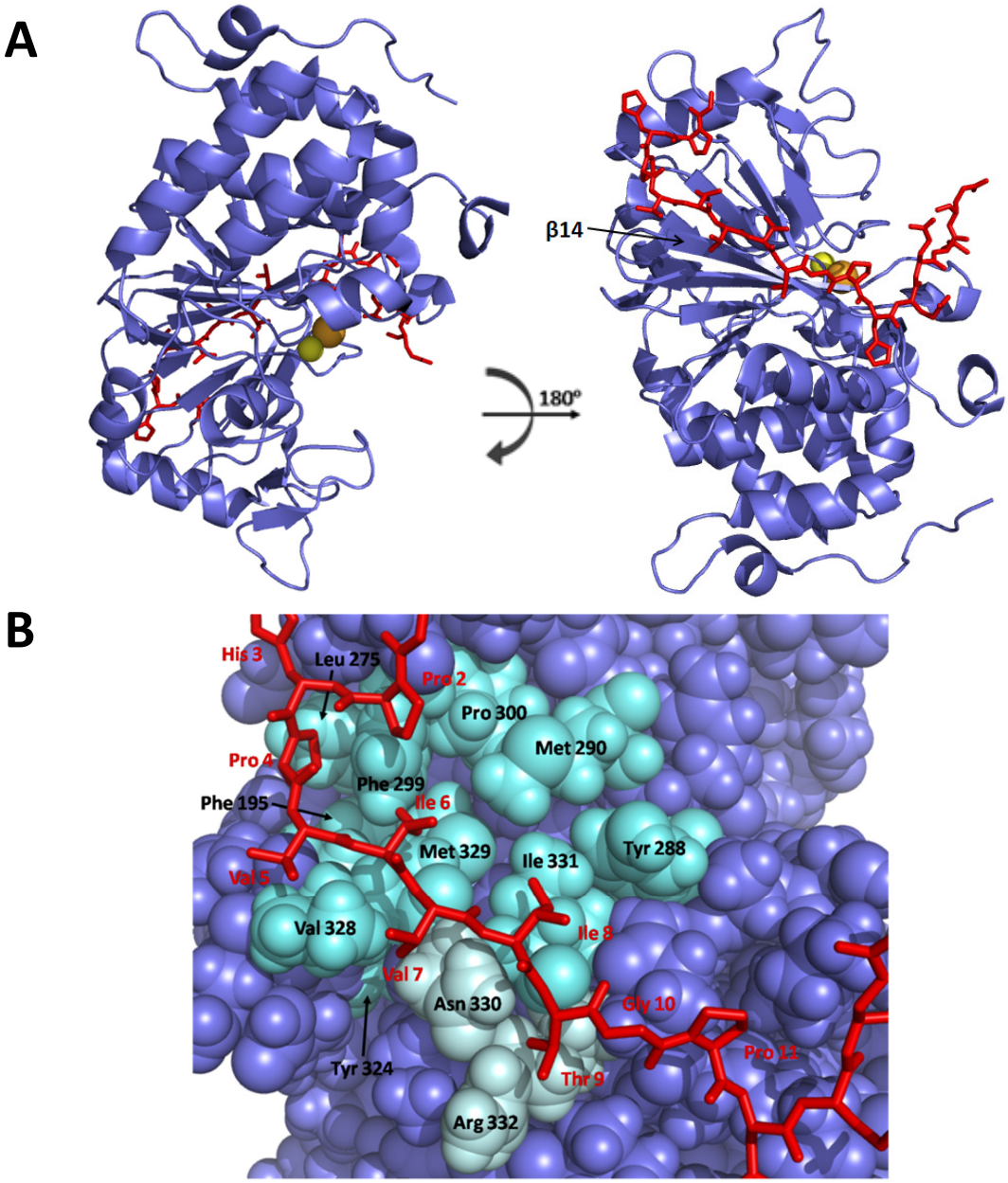



**Figure 1.19: The VIVIT peptide binding to calcineurin.** (A) This panel depicts a VIVIT peptide (red stick model) shown binding to CaN-A (slate) at the PXIXIT motif binding groove, as determined by NMR techniques. The left panel shows accessible active site, denoted by the presence of $Fe^{3+}$ and $Zn^{2+}$ ions (orange and yellow spheres, respectively) with the VIVIT peptide interacting with CaN on the opposite side of the phosphatase. The right panel shows the reverse view of the protein, away from the active site. The β14 strand along which the VIVIT peptide binds is highlighted. (B) This panel depicts the close up view of the interaction of the VIVIT peptide with CaN. The orientation is the same as in the right side of panel A. CaN residues forming the PXIXIT motif binding groove are shown in aquamarine color. Two CaN residues, Asn-330 and Arg-332, that can interact with Thr-9 amino acid of the VIVIT peptide, are shown in pale cyan color. CaN residues are labeled in black; the VIVIT peptide residues are labeled in red.[277]

extensive. In addition, Val-5 participated in backbone hydrogen bonding with the β14 strand of CaN-A. The sidechain of Asn-330 hydrogen bonded with Val-7 and Thr-9 as well as Arg-332. Hydrogen bond interaction between Thr-9 of the VIVIT peptide and Arg-332 was not seen.[195]

1.5.5 Related protein-binding grooves found in calcineurin-A and PP-1c

PP-1c possesses a structural feature termed the RVXF motif binding groove that binds to protein sequences containing the RVXF motif. RVXF motifs are found on many regulatory proteins that bind PP-1c.[54, 95, 155, 280] The PXIXIT motif binding groove on CaN-A closely resembles the RVXF motif binding groove on PP-1c (see Figure 1.20, p. 54). Structural comparison between the two phosphatases shows that relatively few mutations result in significant changes that may prevent these phosphatases from sharing common binding partners.[193, 277]

Comparison of the related protein-binding grooves of CaN-A and PP-1c reveals that the grooves look similar and are lined with hydrophobic residues. However, the PXIXIT motif binding groove is obstructed by an exposed Phe-299, which is present due to insertion of six additional amino acids in the CaN-A β11–12 loop, not found in PP-1c. In CaN-A, the PXIXIT motif binding groove contains a hydrophobic pocket that accommodates the Pro-4 residue of the VIVIT peptide. Such a pocket does not exist in the RVXF motif binding groove of PP-1c; instead of a proline pocket, negatively charged residues, absent in CaN, are found in PP-1c These negatively charged residues are important for binding the arginine

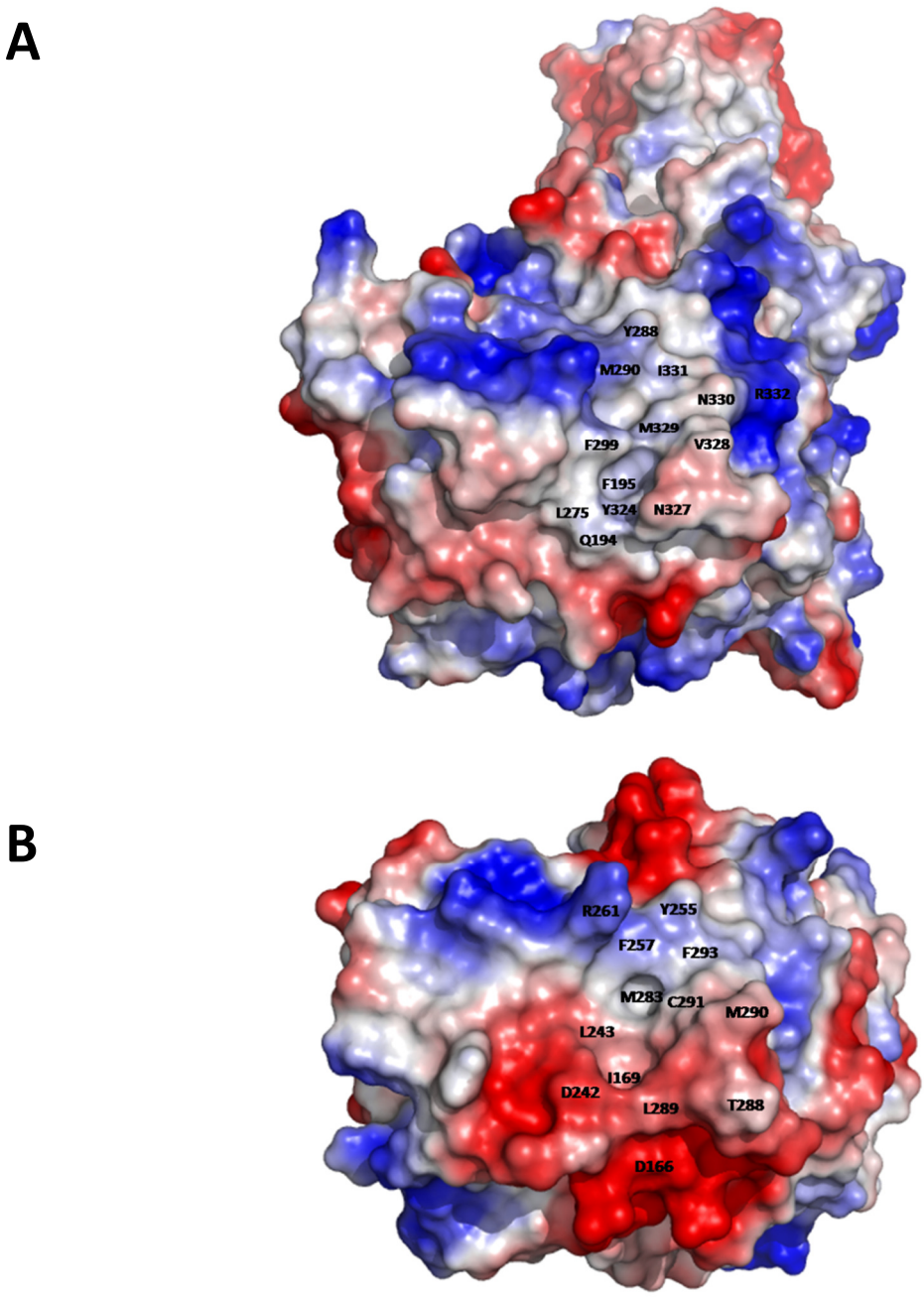



**Figure 1.20: Calcineurin and PP-1c contain related protein-binding grooves.** The electrostatic surface areas of CaN (panel A) and PP-1c (panel B) are shown. Positive charges are denoted in blue color, negative charges are highlighted in red while white surface represents uncharged residues. Catalytic subunits of each phosphatase are oriented to expose related protein-binding grooves, termed the PXIXIT motif binding groove in CaN, and the equivalent RVXF motif binding groove in PP-1c. The residues that are involved in protein-protein interaction are highlighted in a single letter amino acid code. CaN is shown with bound CaN-B subunit in the background. The active site of either enzyme is located on the opposite side of the phosphatase.[54, 95, 155, 180, 193, 217, 277, 280]

residue in the RVXF motif (see Figure 1.20, p. 54).[95, 155, 280]

1.5.6 Protein phosphatase inhibitor-1: a regulatory protein inhibitor of PP-1c is also a calcineurin substrate

Protein phosphatase inhibitor-1 (I-1) was first identified in 1975 and isolated soon after by Nimmo and Cohen.[150, 151, 231] It was immediately recognized that I-1 is an unusual protein in that it has very little tertiary structure, forming only four small helical regions at residues 24–28, 81–86, 103–109 and 127–131.[5, 62, 100, 103, 205, 231, 260] It is widely distributed in mammalian tissues, and is highly conserved, especially in the N-terminus (97% identity in the first 58 residues between human, rat and rabbit species).[65, 100, 103, 205, 258, 260]

I-1 is a specific inhibitor of PP-1c. In order to be an active PP-1c inhibitor ($IC_{50}$ 1–7 nM), I-1 must first be phosphorylated by protein kinase A (PKA).[159, 274, 275] Phosphorylated I-1 (I-1-P) is also one of the most well characterized substrates of CaN; however, how CaN and I-1 interact is unknown.[159, 181, 274] The research described in chapter 2 and 3 contributes to the understanding of how such interactions might occur.

I-1 is one of the proteins that bind to PP-1c via a modified RVXF motif (9-KIQF-12).[5, 231, 260] Mutation or deletion of KIQF prevents I-1 interaction with PP-1c, or PP-1c inhibition by I-1-P.[6, 102, 103, 258, 260] Furthermore, truncation of the I-1 protein has revealed that this inhibitory activity is confined to the first 54

residues.[5, 6, 102, 103, 259, 260] Another site of interaction of PP-1c with I-1 has been established by mutagenesis studies. These studies have unequivocally demonstrated that the β12–13 loop of PP-1c plays a critical role in both binding and inhibition of PP-1c by phosphorylated I-1.[76, 229]

1.5.7 Inhibitor-1 involvement in heart failure development in response to β-adrenergic signaling

I-1 is an important hormonal regulator of PP-1c activity in skeletal muscle. In response to adrenaline or the β-adrenergic agonist, isoproterenol, cellular cAMP levels rise, resulting in increased levels of phosphorylated I-1 (I-1-P) with increased inhibition of PP-1c.[66, 114, 174, 278] Conversely, treatment with propranolol, a blocker of β-adrenergic receptors, potently inhibited I-1 phosphorylation.[113, 114, 174] Thus, it appears that hormones control PP-1c activity via its inhibition through I-1 phosphorylation by PKA.

A similar β-adrenergic response effect on increased levels of I-1-P and inhibition of PP-1c activity was demonstrated in cardiac muscle.[4, 228, 229] However, these studies have also shown that the effect of cAMP level agonists could be antagonized by treatment with acetylcholine.[4] Acetylcholine is a ligand for muscarinic receptors. Activation of muscarinic receptors leads to activation of phospholipase C-$\beta_1$ which in turn leads to degradation of phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate ($IP_3$).[42] Thus, these studies have highlighted a link between the level of PP-1c

inhibition and $Ca^{2+}$ release (see Figure 1.21, p. 58).

It is perhaps surprising that the effect of the β-adrenergic pathway on I-1 phosphorylation levels has not received more attention until recently when the link between heart failure and PP-1c activity was recognized. Heart failure is caused by prolonged $Ca^{2+}$ signaling with abnormal sequestration of $Ca^{2+}$ into the sarcoplasmic reticulum during the diastole.[197] The link between heart failure and PP-1c followed from studies of mice with overexpressed PP-1cα in cardiomyocytes. The PP-1c overexpressing hearts showed diminished contractile response and expanded left ventricles, resulting in premature death of transgenic mice, compared with wild type mice.[52]

The role of I-1 in the development of heart failure was demonstrated by I-1 gene knock-out (KO) experiments.[52] *Ex vivo* perfused hearts treated with isoproterenol showed that hearts from KO mice exhibited decreased cardiac contractile response, and this correlated with increased PP-1c activity (23% higher). KO mice hearts also had significantly reduced levels of PKA phosphorylated phospholamban. The function of phospholamban is to inhibit sarcoplasmic/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) $Ca^{2+}$ pump. PKA phosphorylated phospholamban can no longer inhibit SERCA, allowing SERCA to pump $Ca^{2+}$ ions back to the sarcoplasmic reticulum, priming the cells for further muscle contraction (see Figure 1.21, p. 58).[54, 216]

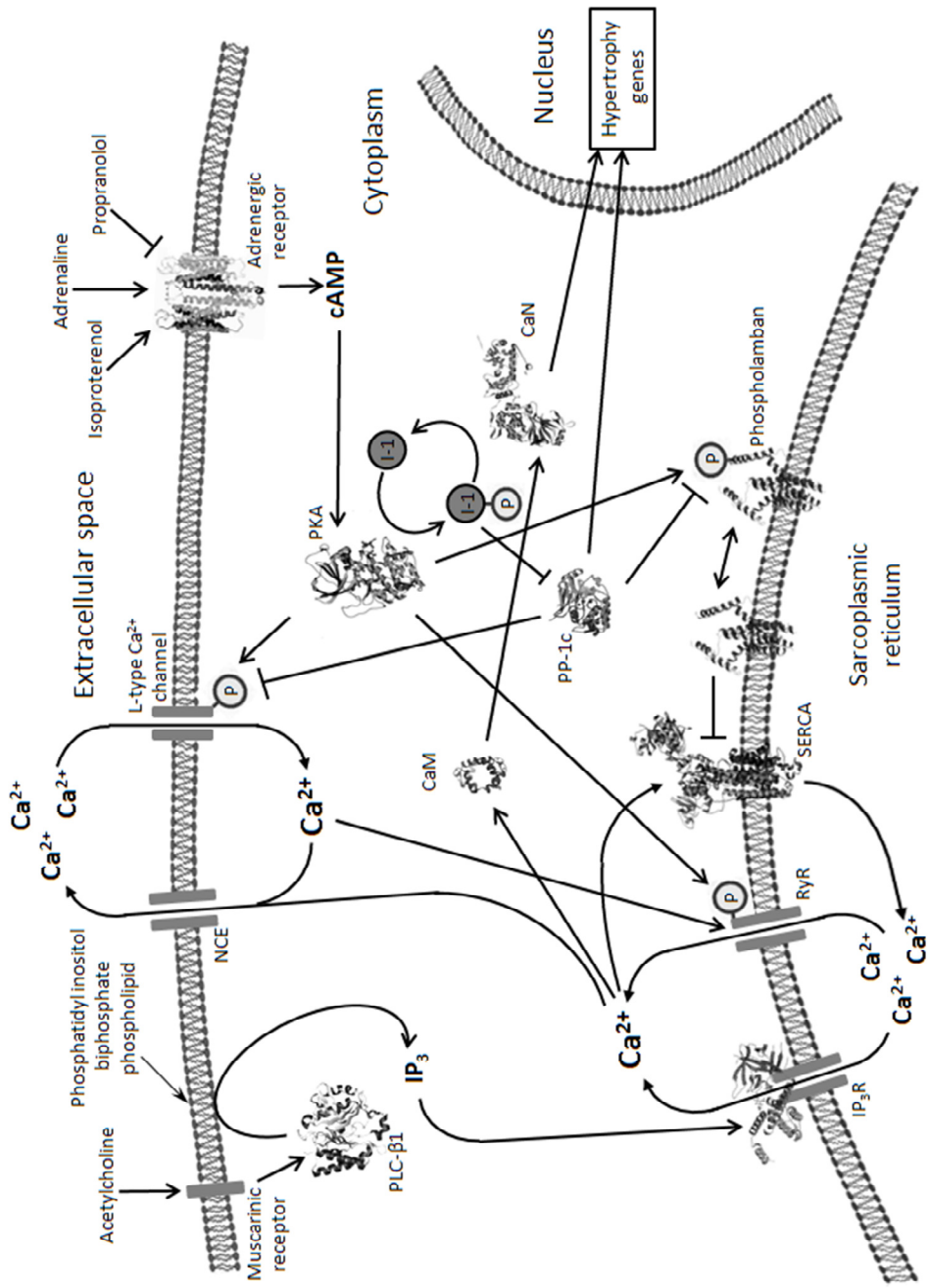



**Figure 1.21: The effect of β-adrenergic signaling pathways in cardiomyocyte cells.** Signaling pathways upon hormonal or artificial stimulation of the β-adrenergic response that can lead to hypertrophy development are illustrated. The counteracting effects of acetylcholine are also highlighted. Open-end arrows (⟶) indicate stimulatory action; closed-end arrows (⟶►) show calcium transport; blunt-end lines (⟶┤) designate process of inhibition. CaM = calmodulin, cAMP = cyclic AMP, I-1 = protein phosphatase inhibitor-1, $IP_3$ = inositol 1,4,5-trisphosphate, $IP_3R$ = $IP_3$ receptor, NCE = $Na^+/Ca^{2+}$ ion exchanger channel, PKA = protein kinase A, PLC = phospholipase C, RyR = ryanodine receptor $Ca^{2+}$ channel, SERCA = sarcoplasmic/endoplasmic reticulum $Ca^{2+}$ ATPase.[4, 25, 52, 54, 65, 69, 99, 100, 216, 229]

To further test I-1's role in heart failure development, levels of I-1-P were examined in either healthy hearts or failing hearts with dilated cardiomyopathy in various animal models. Failing hearts showed ~60% reduction in phosphorylated I-1, as well as corresponding mRNA and protein levels.[52, 98, 99, 127, 229] Failing hearts also showed decreased levels of SERCA (by over 50%), as well as reduced levels of phospholamban phosphorylation.[52] Therefore, the link between $Ca^{2+}$ levels and PP-1c function is again revisited.

Since CaN is activated by prolonged releases of $Ca^{2+}$ concentrations, the level of CaN activity was measured in human failed hearts. Failed hearts on average displayed 400% activation of CaN as compared with the healthy hearts control group.[197] Therefore, I-1-P levels could be affected either due to dephosphorylation by CaN or due to decreased sensitivity of cardiomyocytes to β-adrenergic signaling. However, one study has implicated CaN and PP-2Ac in lowering protein levels of both phosphorylated I-1 and phospholamban, respectively, in I-1 overexpressing cardiomyocytes.[97] Although the role of CaN in the development of hypertrophy and heart failure has been clearly established, regulation of PP-1 by CaN in heart failure is the first demonstration of a phosphatase cascade (see Figure 1.21, p. 58).[25, 97, 110, 187, 191, 216, 222]

Therefore, I-1 plays an important role in mediating the β-adrenergic stimulation effects on the heart. I-1 thus could be a potential therapeutic target in failing hearts, where overexpression of I-1 in human failing hearts could rescue

the reduced contractility by inhibition of PP-1c. The rescuing ability of I-1 was demonstrated in cardiomyocytes from failing hearts treated with the β-adrenergic agonist, isoproterenol; overexpression of I-1 resulted in increased sensitivity to β-adrenergic signaling, with a corresponding increased contraction cell shortening, as compared to control cells.[99] This has been further corroborated by recent studies showing that an increase in I-1 expression in adult mice augmented basal cardiac performance in hearts subjected to ischemic injury.[229, 230] The ability to uptake $Ca^{2+}$ by the sarcoplasmic reticulum of cardiomyocytes was enhanced by the treatment with I-1 in presence of PKA.[172]

### 1.5.8 Alternative physiological roles for inhibitor-1

I-1 is also involved in long term depression (LTD). LTD is an activity-dependent decrease in postsynaptic potential which occurs primarily in the hippocampus and cortex. It is a form of a synaptic plasticity that is believed to be involved in learning and memory formation. It has been demonstrated that LTD formation in hippocampal cells is dependent on CaN dephosphorylation of I-1-P, which in turn leads to relief of PP-1c inhibition (see Table 1.2, p. 14).[54, 65, 226]

## 1.6 Thesis research objectives

Despite the close evolutionary sequence conservation between CaN and other Ser/Thr protein phosphatases, CaN rarely overlaps its functions with those of PP-1c or PP-2Ac. CaN plays an important role in numerous eukaryotic signal transduction pathways; activation of the immune response by CaN is the best

characterized signal transduction pathway involving CaN.[12] Clinically, CaN is a target for immunosuppression which is vital for patient survival during and after organ transplantation.[12, 171, 262] Use of current CaN drugs, CsA and FK506, can result in many toxic side effects, and the search for novel inhibitors of CaN or that of immune system activation has continued for the last fifteen years (see Appendix B, p. 281).

The primary focus of this thesis work was to investigate new approaches to the development of CaN inhibitors. This was achieved in two ways. First, the existing structural and mutagenesis data on how okadaic acid (OA) and microcystin bind to PP-1c and PP-2Ac were used to investigate why CaN is more resistant to these natural toxins. Understanding the underlying causes of CaN resistance to OA and microcystin may aid in the generation of CaN specific inhibitors from existing Ser/Thr protein phosphatase inhibitors.

In a second approach, the isolation of novel inhibitors of CaN was sought directly, from extracts of invertebrate marine organisms. A bioassay-guided method was developed that allowed the monitoring of the isolation and purification of novel CaN inhibiting compounds from marine extracts.

The substrate used in the bioassay-guided isolation of novel CaN inhibitors was I-1. The demonstration that I-1 interacts with CaN using the PXIXIT motif in an analogous manner to NFAT proteins provided the possibility

for a search of inhibitors of CaN:NFAT interaction.

CaN is closely related to PP-1c and PP-2Ac but, in contrast to these phosphatases, is poorly inhibited by the natural toxins, OA and MCLR.[94, 126, 147, 180] The molecular mechanisms underlying the resistance of CaN towards these natural toxins is not understood.

*The first objective of this thesis work was to determine whether calcineurin can be mutated to generate a form more sensitive to the exogenous protein phosphatase inhibitors, okadaic acid and microcystin-LR.*

Structural information delineating why the CaN enzyme is less potently inhibited by natural toxins can be the basis of a rationale for how OA or MCLR could be modified to become CaN specific inhibitors. Such novel analogs could have a potential to act as immunosuppressants. In addition, new inhibitors of CaN that are not dependent on binding endogenous proteins for inhibition would be valuable tools in deciphering cellular functions of CaN. An inhibitor that targets the active site of CaN-A but does not need immunophilin binding should block cellular CaN activity, without affecting the function of endogenous immunophilins, allowing the study of CaN function in different cell types or tissues. This is further underscored by mounting evidence of immunophilins role in the development of hypertension and heart problems.[44, 77]

I-1 is one of the best substrates of CaN and the most widely used full length protein substrate for *in vitro* assays.[159, 181, 274] However, there is no structural or molecular understanding of how I-1 and CaN interact. Study of the I-1 sequence suggests a presence of a PXIXIT sequence motif at the N-terminal end of the protein.[11] The PXIXIT motif is the only known structural element found in many CaN binding partners, including NFAT proteins.[109]

*The second objective was to determine whether mutagenesis of the inhibitor-1 PXIXIT motif, or mutagenesis of the PXIXIT motif binding groove in calcineurin will reduce dephosphorylation of inhibitor-1 by calcineurin.*

Reduction in I-1 dephosphorylation would indirectly show that I-1 contains the PXIXIT motif that is used to bind CaN. The knowledge that I-1 contains the PXIXIT motif and therefore could bind CaN in a similar manner to NFAT proteins, identifies I-1 as an excellent *in vitro* substrate for a search of novel immunosuppressants. A CaN enzyme assay using I-1 as a substrate could identify inhibitors that either occlude the active site, or occlude the PXIXIT motif binding groove on CaN-A, disrupting binding of I-1 to CaN. Either type of inhibitor would be expected to reduce I-1 dephosphorylation by CaN. Inhibitors that disrupt binding of I-1 to CaN via the PXIXIT motif should inhibit the interaction between CaN and any binding partner that contains the PXIXIT motif. Such compounds could have the potential to be less toxic as clinical drugs than the currently used regimen of choice for CaN immunosuppression.

I-1 and CaN could be mutated to strengthen the binding between the two proteins. For example, the PXIXIT motif of I-1 could be mutated to result in a stronger interaction with CaN. The effectiveness of such mutagenesis was previously successfully demonstrated in NFAT proteins.[11] Strengthened binding between I-1 and CaN could be an advantage in obtaining crystals of CaN bound to I-1 for structural determination of the complex.

In addition, I-1 appears to play a role in the development of heart hypertrophy and eventual heart failure. Heart failure could be dependent on CaN dephosphorylation of the I-1-P substrate.[98, 99, 127] Dephosphorylation of I-1-P results in loss of inhibition of PP-1 by I-1; PP-1 is an important regulator of transcriptional function that can lead to heart hypertrophy.[52] Therefore, regulation of CaN dephosphorylation of I-1-P by disrupting their interaction via the PXIXIT motif could have important consequences in the treatment of hypertrophic hearts.

The ability of I-1 to bind CaN via the PXIXIT motif can contribute to the search for novel inhibitors of CaN that could potentially target CaN:NFAT interaction. Such novel inhibitors could be isolated from the natural environment. Natural products have a long history of medicinal use, with half of current drugs estimated to be derived from natural sources, or their derivatives.[8, 63, 160] One of the sources of natural compounds that has not been well explored is the marine environment.[63] The enormous concentration of life in the fringes of oceans and seas leaves many marine organisms in a fierce competition for food and living

space. This has resulted in many organisms adopting chemical means of defense with many potent and structurally unique compounds. For these reasons the marine environment has been gaining increasing attention, with novel compounds being uncovered at an increasing pace.[8, 63, 160] However, exploring the marine environment for novel inhibitors of CaN has never been attempted.

*The third objective was to determine whether novel inhibitors of calcineurin could be identified, isolated and purified from extracts of marine invertebrate organisms, guided by an in vitro calcineurin assay using inhibitor-1 as a substrate.*

The *in vitro* assay allows the testing of large quantities of marine organism extracts for CaN inhibitory activity. The same assay can also be used to guide the fractionation, isolation and purification of novel inhibitory compounds from the candidate inhibitory extracts. The technique of monitoring bioassay-guided purification could be used to follow purification of an inhibitory compound from any marine organism.

Furthermore, the *in vitro* assay used to identify extracts of marine organisms that inhibit CaN activity could be adapted for use directly in the field where organisms are collected. Use of such an assay would have the advantage of identifying organisms of interest at their environmental source. Organisms of interest could then be collected in large quantities for future studies.

The objectives of this thesis were designed to further the research in finding novel CaN-specific inhibitors, either directly, such as from marine organisms, or indirectly by providing valuable information that could aid in the construction of novel CaN inhibitors. Furthermore, the CaN assay method used in the search for novel inhibitors could result in finding inhibitors that, not only obstruct the active site of CaN, but also inhibitors that target interaction of NFAT proteins with CaN. Such advances could greatly improve the chances of identification and isolation of novel CaN interacting drugs.

# Chapter 2: Molecular mechanisms underlying the resistance of calcineurin to the exogenous protein phosphatase inhibitors okadaic acid and microcystin-LR

The finding that the phosphoprotein phosphatases (PPP) PP-1c and PP-2Ac are inhibited by the natural toxins okadaic acid (OA, found in shellfish) and microcystin-LR (MCLR, derived from cyanobacteria), provided impetus for extensive research into these protein phosphatases and allowed the complexities of their regulation to be unraveled. Due to the impact of these natural toxins on human and animal health, and their deleterious effects on the international seafood economy, microcystins and OA have been the most studied natural products from both biological and molecular points of view.[86] These compounds have been studied extensively for more than fifteen years with respect to their molecular mechanisms of action; however, an important question that has not been answered concerns their comparative lack of activity against calcineurin (CaN, comprised of the catalytic subunit CaN-A and the regulatory subunit CaN-B), another member of the PPP family of protein phosphatases. Why CaN, a phosphatase that is structurally closely related to PP-1c and PP-2Ac (see Figure 1.4, p. 17), is resistant to OA and MCLR was poorly understood at the outset of this research project.

*The hypothesis I set out to test in this chapter was whether calcineurin can be mutated to generate a form more sensitive to the exogenous protein phosphatase inhibitors, okadaic acid and microcystin-LR.*

## 2.1 Calcineurin-natural toxin interaction assessed from comparative mutagenesis studies of PP-1c and PP-2Ac

Numerous studies have indicated the importance of the β12–13 loop (a flexible structural element that contributes in the formation of the active site) in natural toxin interaction. One residue in particular appears to account in large measure for the difference in toxin sensitivity observed between the PPP phosphatases. In PP-2Ac, the Ser/Thr protein phosphatase most sensitive to natural toxins (see Table 1.1, p. 5), it is the Cys-269 residue, with Phe-276 being the corresponding residue in PP-1c, and Tyr-315 in CaN:

1) Chinese hamster ovary cells cultured in the presence of OA produced two strains that were less sensitive to OA. The phosphatase affected was PP-2Ac, with a C269G mutation accounting for the increased resistance to OA (4-fold). This finding provided the first clue to where OA binds the PPP phosphatases;[263]

2) In further studies of the β12–13 loop, the residues 274-GEFD-277 of PP-1c were substituted for the cognate region of PP-2Ac (267-YRCG-270). The PP-1c mutant showed 10-fold increase in sensitivity towards OA, and a 4-fold increase in sensitivity towards MCLR.[320] Cys-276 of the 274-YRCG-277 PP-1c mutant was subsequently mutated to glycine, mimicking the original mutation observed in PP-2Ac by Shima et al. (1994); the C276G substitution resulted in 10-fold

decrease in sensitivity towards OA[318];

3) Mutagenesis of individual amino acids of the PP-2Ac segment 265-YCY-267 and Cys-269 of the β12–13 loop, negatively impacted the OA sensitivity with 40–100-fold increases in $IC_{50}$s. Substitution of Cys-269 for tyrosine (the corresponding residue found in CaN) resulted in 40-fold decrease in PP-2Ac sensitivity towards OA;[169]

4) Mutation of individual PP-1c β12–13 loop residues 268-SAPNYCGEFD-277 recognized the Ser-268 amino acid as important in OA interaction, while Tyr-272 as well as Phe-276 to be important in interaction with both OA and MCLR. F276C substitution resulted in 40-fold increase in sensitivity towards OA and 2-fold decrease in sensitivity towards MCLR.[318]

The effects of these mutations are also summarized in Table 2.1 (p. 70).

In a first attempt to understand CaN resistance to natural toxins, a region of PP-1c comprising the β12–13 loop (273-CGEFD-277) was substituted by the corresponding residues of CaN-A (312-LDVYN-316).[87] The loop substitution resulted in a 3-fold increase (10 nM to 30 nM) in the $IC_{50}$ of OA for PP-1c. This result was later confirmed by Perreault et al. (2004) who reported an $IC_{50}$ change from 32 nM to 97 nM.[239] In comparison, CaN has been reported to be inhibited by OA with an $IC_{50}$ of 5μM.[26] These results suggested that apart from the β12–13 loop, additional structural elements must also account for the higher sensitivity of PP-1c to OA, as compared to CaN. Similar, but more pronounced effect was observed with 6-fold decrease in sensitivity of the loop mutant PP-1c to MCLR.[239]

**Table 2.1: Summary of PP-1c and PP-2Ac mutagenesis impacting potency of inhibition by natural toxins.**

| | Mutant | $IC_{50}$ Effect | | Ref |
|---|---|---|---|---|
| | | OA | MC | |
| PP-1c | L D V Y N | 3↑ | 6↑ | 239 |
| | Y R C G | 10↓ | 4↓ | 320 |
| | S A P N Y C G E F D → A | 8↓ | | 318 |
| | S A P N Y C G E F D → F L | 10↑ | 3↑ | 318 |
| | | 4↑ | 6↑ | 239 |
| | S A P N Y C G E F D → C G | 40↓ | 2↑ | 318 |
| | | 10↓ | | 318 |
| PP-2Ac | Y C Y R C → F G G | 42↑ | | 169 |
| | | 100↑ | | 169 |
| | | 83↑ | | 169 |
| | Y C Y R C → G Y | 4↑ | | 263 |
| | | 42↑ | | 169 |

Mutagenesis of PP-1c sequence 265-SAPNYCGEFD-277, or PP-2Ac 265-YCYRC-269, either as point mutants or a sequence of amino acids, is shown in boxes. The effect on $IC_{50}$ is listed to the right of the boxed mutant, either for okadaic acid (OA) or microcystin (MC). The $IC_{50}$ effect refers to the fold increase (↑), or decrease (↓), for the $IC_{50}$ of a mutant as compared to the WT enzyme.

In addition, Dawson et al. (2000) showed that Tyr-134, a residue known through X-ray crystallography to be important for the interaction of PP-1c with microcystin, when substituted for a corresponding CaN-A residue (Phe-160), had no effect on OA interaction, while resulting in a 7-fold decrease in sensitivity to MCLR.[87] Perreault et al. (2004) showed that a single residue change in the β12–13 loop of PP-1c to a corresponding amino acid found in CaN-A (C273L) resulted in a 4-fold decrease in sensitivity of PP-1c to OA and a 6-fold decrease in sensitivity of PP-1c to MCLR (see Table 2.1, p. 70).[239]

Thus the past mutagenesis data suggest that at least two residues in the β12–13 loop, and one within the active site that differ between PP-1c, PP-2Ac and CaN-A, can contribute to CaN's reduced sensitivity to toxins MCLR and OA.

## 2.2 Calcineurin mutagenesis rationale

In an effort to understand the molecular basis of differential toxin sensitivities among PP-1c, PP-2Ac, and CaN, structural biology studies were used to predict CaN-A residues that might alter sensitivity to toxins. Several residues were selected for mutation by:

1) Examining crystal structure of PP-1c bound to OA which showed OA contacts (within 4 Å) with at least thirteen residues of the catalytic core;[217]

2) Examining crystal structure of PP-1c bound to MCLR which showed the heptapeptide contacting at least thirteen residues of the catalytic core. Later structure determination of PP-1c bound to dihydromicrocystin-LA (MCLA-2H), a

microcystin analog that does not covalently bind to PP-1c and therefore does not lead to conformational change of the protein phosphatase (see Figure 1.16, p. 43), allowed additional study of the unaltered β12–13 loop interaction with the natural toxin;[218]

3) Examining past data of PP-1c and PP-2Ac mutagenesis that impacted potency of inhibition by natural toxins (see Table 2.1, p. 70).[87, 239]

Subsequently, the structures of PP-2Ac bound to either OA or MCLR have been published.[305] When binding PP-1c or PP-2Ac, OA and microcystin inhibitors contact nearly all of the same residues in the active site and in a hydrophobic groove, despite the fact that not all the amino acids involved in these interactions are conserved between the two phosphatases.

Thus a comparative analysis of differing residues between PP-1c and CaN-A was performed by juxtaposing the crystal structures of PP-1c bound to OA (PDB code 1JK7, Figure 2.1, p. 73) or MCLA-2H (PDB code 2BDX, Figure 2.2, p. 74) with that of the free CaN-A catalytic subunit (PDB code 1AUI).[180] The residues of PP-2Ac involved in contacting the natural toxins are also compared with those of PP-1c and free CaN-A. Two residues in the L7 loop (Leu-312 and Tyr-315) and two in the catalytic center of the active site (Tyr-159 and Phe-160) of CaN-A were selected for mutagenesis.[143] Site specific mutants of CaN-A were expressed and purified to homogeneity, their activities were measured and their susceptibilities to inhibition by OA and MCLR toxins were determined.

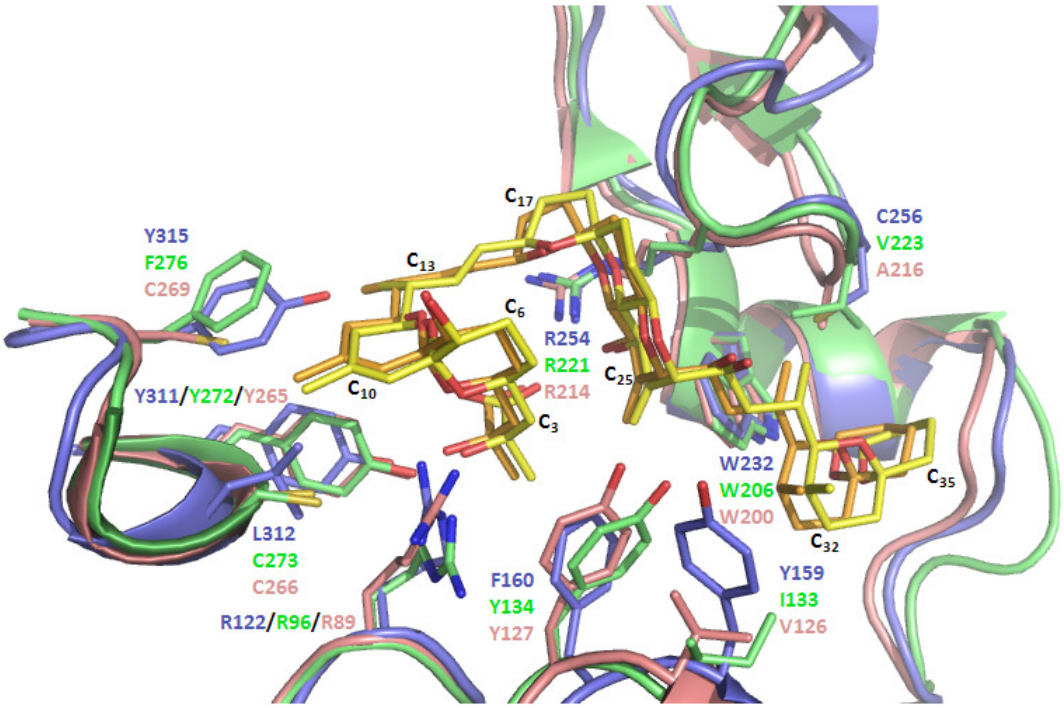



**Figure 2.1: Comparison of residues involved in okadaic acid natural toxin binding between PP-1c, PP-2Ac and calcineurin.** Superimposition of human PP-1cγ (lime color):okadaic acid (OA, yellow stick model) complex, human PP-2Acα (salmon color):OA (orange stick model) structure, and free human CaN-Aα catalytic subunit (slate color). Key residues involved in the binding of PP-1c and PP-2Ac to toxins are shown in stick model, overlaid with corresponding amino acids in CaN, highlighted in a single letter amino acid code. The contiguous carbon atoms of OA are indicated in black.[180, 217, 305]

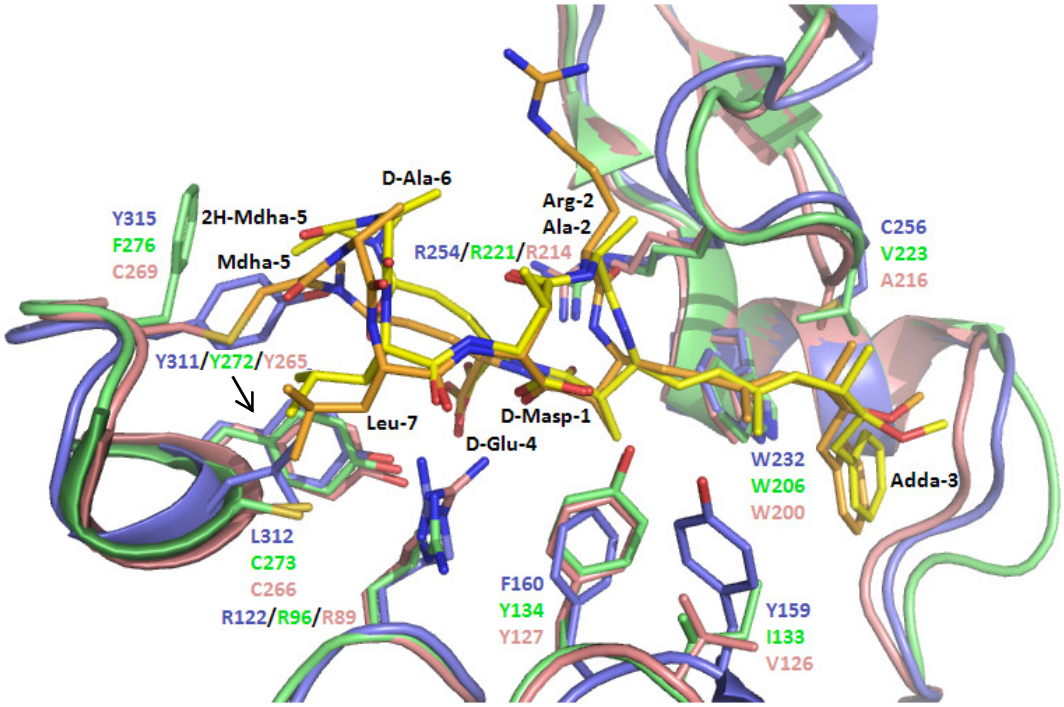



**Figure 2.2: Comparison of residues involved in microcystin natural toxin binding between PP-1c, PP-2Ac and calcineurin.** Superimposition of human PP-1cγ (lime color) bound to dihydromicrocystin-LA (yellow stick model), human PP-2Acα (salmon color):microcystin-LR (orange stick model) complex, and human CaN-Aα catalytic subunit (slate color). Key residues involved in the binding of PP-1c and PP-2Ac to toxins are shown in stick model, overlaid with corresponding amino acids in CaN, highlighted in a single letter amino acid code. The heptapeptide toxin residues are labeled in black. Adda = 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-deca-4,6-dienoic acid; Mdha = *N*-methyldehydroalanine; 2H-Mdha = hydrogenated *N*-methyldehydroalanine; D-Masp = D-erythro-β-methyl aspartic acid.[180, 218, 305]

The fact that the closely related CaN is more resistant to OA and MCLR than other members of the PPP family, could be an important advantage for the synthesis of CaN-specific inhibitors. OA or MCLR could serve as templates for rational design of such inhibitors. Structural understanding, achieved through mutagenesis, of how OA and MCLR interact with CaN-A, can provide the insight how these toxins should be chemically manipulated to generate forms that inhibit only CaN. This provides a new approach to obtain CaN inhibitors.

## 2.3 Experimental Procedures

*Materials.*

Rat CaN-Aαδ and rat CaN-B clones were generously provided by Dr. T.R. Soderling (Vollum Institute, Portland, OR). Calmodulin (CaM) was obtained from Sigma-Aldrich (Oakville, ON). Human PP-1cγ was provided by Kathleen Perreault (University of Alberta, Edmonton, AB).[239] Bovine protein kinase A (PKA) was provided by Andrea Fong (University of Alberta, Edmonton, AB) or purchased from Sigma-Aldrich. A human protein phosphatase inhibitor-1 (I-1) clone was donated by Dr. Marcia Craig (University of Alberta, Edmonton, AB). MCLR was purified by Dr. Craig.[80] OA was obtained from Moana Bioproducts (Honolulu, HI) or purified from *Prorocentrum lima*. DNA restriction enzymes and transcription polymerase were obtained from Invitrogen (Burlington, ON). CaM-Sepharose 4B was purchased from GE Healthcare (Baie d'Urfé, PQ). *para*-nitrophenyl phosphate (*p*NPP) was obtained from Sigma-Aldrich. All other

reagents were purchased from Sigma-Aldrich, Fisher Scientific (Ottawa, ON) or EMD Chemicals Inc. (Gibbstown, NJ), unless otherwise stated.

*Construction of vectors.*

Genes encoding CaN-A and CaN-B were cloned as described previously.[295] Rat CaN-Aαδ in pET21a(+) expression vector (Novagen, Madison, WI) was transformed into HMS174 (DE3) *E. coli* cells (Novagen) as outlined in the Novagen competent cells transformation protocol. Rat CaN-B in pET21a(+) vector was transformed into BL21 (DE3) pLysS *E. coli* cells (Stratagene, La Jolla, CA) as outlined in the Stratagene competent cells transformation protocol.

*Construction of calcineurin mutant isoforms.*

Mutagenesis primers used in this work are listed in Table 2.2, p. 77. Template DNA was isolated using a QIAprep Spin Miniprep Kit (Qiagen Inc., Mississauga, ON). Mutations were generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene). PCR reactions included 10 × reaction buffer (100 mM KCl, 6 mM $(NH_4)_2SO_4$, 200 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 1% Triton X-100, 100 μg/mL bovine serum albumin [BSA]), DNA template (5–50 ng), oligonucleotide mutagenesis primers (125 ng), 0.2 mM dNTP mix and $H_2O$ for a total volume of 50 μL. The reaction mixture was covered with one drop of mineral oil. The reaction was initiated by addition of 2.5 U of *Pfu* Turbo DNA polymerase. PCR cycling parameters included 1 cycle of 95 °C for 30 s, followed by 16 cycles of 95 °C for 30 s, 45 °C for 1 min and 72 °C for 14 min. PCR

**Table 2.2: Primers used in calcineurin-A catalytic subunit mutant generation.**

| CaN mutant | Temp-late used | Mutagenesis primer | Novel restri-ction site |
|---|---|---|---|
| Y315F | WT[a] | CGGCACCAAATTATCTAGATGTGTTCAATAATAAAGC | XbaI |
| Y315C | WT[a] | CGGCACCAAATTATCTAGATGTGTGCAATAATAAAGC | XbaI |
| Y315L | WT[a] | CGGCACCAAATTATCTAGATGTGCTCAATAATAAAGC | XbaI |
| L312C | WT[a] | CCAAATTACTGTGATGTGCTCAATAATAAAGCGGCAGTGTTGAAGTACG | Removes PstI |
| L312C: Y315L | Y315L | GCACCAAATTACTGTGATGTCTACAATAATAAAGCTGCAGTG | AccI |
| Y159I: F160Y: Y315L | Y315L | GGCACCTAACAGAGATCTACACGTTTAAACAAG | BglII |
| Y159I: F160Y: L312C: Y315L | Y159I: F160Y: Y315L | GCACCAAATTATGTGATGTCTACAATAATAAAGCTGCAGTG | AccI |
| | Y159I: F160Y: L312C | CCAAATTACTGTGATGTGCTCAATAATAAAGCCGCAGTGTTGAAGCTACG | Removes PstI |
| 1–460[b] Y159I: F160Y: L312C: Y315L | Y159I: F160Y: L312C: Y315L | CCCCCCATATGTCCGAGCCCAAGGCGATTGATCCC | NdeI and HindIII |
| | | GCCCTTAAGCTTTCAGAAGCTGGTAATCTTATGTTGTTGTGAG | |
| 26–344[b] WT[a] | WT[a] | AAAAACATATGAGTCACCGGCTGACAGCAAAGG | NdeI and HindIII |
| | | AAAAAAAGCTTTCATGGGAGCCAGTACGGATGGGGGG | |

[a]WT refers to full length wild type CaN-A; [b]Numbers refer to residues of the truncated protein. All mutants show only one of the two complementary primers, from 5' to 3'. Truncation mutants show two primers used to amplify the DNA fragment of interest to be later used for vector ligation. The quadruple mutant shows the two step procedure used to generate the final mutant.

reactions were cooled to 4 °C, and subsequently restricted with 5 U of Dpn I restriction enzyme for a minimum of 1 h at 37 ºC. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen Inc.).

Primers were designed to alter the DNA restriction map of resulting mutants in order to verify successful mutagenesis (see Table 2.2, p. 77). To generate the Y159I:F160Y:L312C:Y315L quadruple mutant, the Y159I:F160Y:Y315L triple mutant CaN-A DNA was used to generate the Y159I:F160Y:L312C triple mutant first. The Y159I:F160Y:L312C triple mutant CaN-A DNA was then used to generate the quadruple mutant. Attempts to generate a quadruple mutant using the Y159I:F160Y:Y315L triple mutant CaN-A DNA template directly were not successful, necessitating the two step procedure. Truncation mutants of CaN-A were amplified using a PCR method identical to that used for point mutation insertion. Amplified DNA was purified from a 1% agarose gels using a QIAquick Gel Extraction Kit (Qiagen Inc.), and ligated into the pET21a(+) vector using a T4 DNA ligase (Invitrogen), as directed in the protocol provided. To generate a 26–344 residues-long CaN-A, wild type (WT) CaN-A DNA template was used. Truncated WT CaN-A was subsequently mutated to a quadruple mutant. All mutants were verified with DNA sequencing performed by the Department of Biochemistry DNA Core Laboratory (University of Alberta, Edmonton, AB).

*Transformation.*

PCR products of mutant CaN were transformed into XL-1 Blue *E. coli* cells (Stratagene) according to the QuikChange Site-Directed Mutagenesis Kit (Stratagene) or into MAX Efficiency DH5α *E. coli* competent cells (Invitrogen), as described in the protocol provided with the cells, except that half volumes of cells and media were used. Miniprep DNA mutants that were verified with sequencing were transformed into HMS174 (DE3) *E. coli* as outlined in the Novagen competent cells transformation protocol.

*DNA restriction digests.*

DNA restrictions were performed according to the Invitrogen protocol provided with each restriction enzyme; reactions were stopped by heating for 10 min at 95 °C. DNA was visualized on 1% agarose gels containing ethidium bromide, as described in Sambrook and Russel.[252]

*Inhibitor-1 purification.*

BL21 (DE3) *E. coli* cells expressing histidine-tagged inhibitor-1 (6×His-I-1) in the pET28a vector (Novagen) were provided by Dr. Marcia Craig (University of Alberta, Edmonton, AB). The 6×His -I-1 protein was purified as described previously with the following changes.[75] Cells cultured in Luria broth media (5 mL, 50 mg/L kanamycin) were grown overnight at 37 °C, and used to inoculate 2 L of the same media that was subsequently incubated at 37 °C for 4.5 h. Cultured cells were centrifuged at 3000 × g at 4 °C for 15 min. The bacterial

pellet was resuspended in 20 mL of lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.625 mg/mL lysozyme, 2 µg/mL DNase, 2 µg/mL leupeptin, 2 µg/mL pepstatin, and 2 µg/mL aprotinin) and frozen overnight at -70 °C. The following day, an additional 20 mL of lysis buffer was added to the frozen cells, the mixture was resuspended, and sonified (Branson Sonifier 450, VWR Scientific, Edmonton, AB, output control 6, duty cycle 30%, 7 × 20–30 s with 2 min cooling on ice between treatments). Sonified cells were centrifuged at 16 300 × g at 4 °C for 20 min. Trichloroacetic acid (TCA) precipitation was carried out as described in the Connor et al. (1998) protocol except that the 5% TCA precipitation step was omitted. In brief, the supernatant was precipitated on ice with 1% TCA, the mixture was centrifuged as above and the pellet was discarded; the remaining supernatant was further precipitated on ice with 15% TCA and centrifuged; the precipitate was resuspended in 24 mL of 500 mM Tris-HCl, pH 7.5 by trituration with a mortar and pestle and dialyzed four times against 1.5 L dialysis buffer (5 mM Tris-HCl, pH 7.5) for 2–3 h each time. The dialysate was centrifuged at 16 300 × g at 4 °C for 20 min and the supernatant was dialyzed into 1.5 L of equilibrating buffer (50 mM Hepes, pH 7.5, 10 mM imidazole, 0.1 mM $MnCl_2$, 100 mM NaCl). The 6×His-I-1 preparation was loaded on a $Ni^{2+}$-charged 1 mL HiTrap Chelating HP column (GE Healthcare), washed with equilibrating buffer, and eluted with 7–8 column volumes of 50 mM Hepes, pH 7.5, 500 mM imidazole, 100 mM NaCl. The eluate was dialyzed into 5 mM Tris-HCl, pH 7.5. The dialysate was lyophilized and stored at -20 °C. Protein purity was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis

(SDS-PAGE) by running 30 μL samples of each fraction on a 12% polyacrylamide gel. Preparations yielded 5 to 10 mg of 6×His-I-1 protein near homogeneity.

*Inhibitor-1 protein substrate phosphorylation.*

The phosphorylation reaction was carried out as previously described with the following modifications.[75] 6×His-I-1 substrate protein (5–8 mg) was phosphorylated with either 250 U of Sigma-Aldrich bovine PKA or 16 μg of human PKA in 50 mM Tris-HCl, pH 7.5 reaction buffer (1 mL) that contained 200 μM ATP, 1 mM $MgCl_2$ (Caledon Laboratories Ltd., Georgetown, ON), and 0.5–1 mCi $^{32}$P-γ-ATP (GE Healthcare). Phosphorylation was allowed to proceed to completion overnight at 30 °C and the progress of phosphate incorporation was monitored by taking 10 μL sample aliquots at timed intervals and precipitating them with 25% TCA (100 μL) and 10 mg/mL BSA (100 μL). The resulting pellets were resuspended in 1 mL scintillation fluid and radioactivity was measured using a Wallac 1209 Rackbeta scintillation counter (Pharmacia). When no further increase in radioactivity of the pelleted sample was seen, the phosphorylation reaction was loaded on an analytical Vydac $C_{18}$ column (218TP54) equilibrated in solution A ($H_2O$, 0.1% trifluoroacetic acid [TFA]) using a System Gold HPLC (Beckman, Mississauga, ON). The column was washed with 100% solution A for 60 min to remove unbound $^{32}$P-γ-ATP, then developed at a flow rate of 1 mL/min with a 120 min gradient program: 0–20% solution B ($CH_3CN$, 0.1% TFA) in 10 min, 20–60% solution B in 40 min,

60–100% solution B in 5 min, 100% solution B for 2 min, and 100% solution A in 3 min. Protein elution was monitored by absorption at a wavelength of 206 nm. Fractions (1 mL) were collected in the 60–120 min interval. Fractions were Cherenkov counted (measured for radioactivity without using the scintillation cocktail) and fractions exceeding 100 000 cpm were pooled. Pooled fractions were lyophilized, resuspended in $H_2O$, lyophilized again, and resuspended in $H_2O$.

*Okadaic acid purification*.

OA was purified as previously described with the following changes.[33] Extraction buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% 2-mercapto-ethanol [BME, BioShop Canada Inc., Burlinton, ON.], 0.01% Brij-35, 0.625 mg/mL lysozyme, 2 µg/mL DNase, 2 µg/mL leupeptin, 2 µg/mL pepstatin, and 2 µg/mL aprotinin) was used to resuspend *P. lima* cells (3 mL/g of wet weight). Cell extract was sonified (Branson Sonifier 450, output control 6, duty cycle 30%, $3 \times 45$ s with 2 min cooling on ice between sonications). Sonified cells were centrifuged at 16 300 × g at 4 °C for 20 min. The supernatant was dialyzed with twelve volumes of extract buffer using 10 000 MWCO Amicon Ultra Centrifugal Filter Devices (Millipore, Bedford, MA.). After dialysis, samples were lyophilized, resuspended in 50% MeOH, $H_2O$, 0.05% TFA and purified by System Gold HPLC using the low pH step only, as described previously.[32] In brief, the sample (2 mL/HPLC run) was loaded on an HPLC semiprep Vydac $C_{18}$ column (218TP510) equilibrated in solution A ($H_2O$, 0.05%

TFA); the column was developed by applying a gradient comprising 0–100% of solution B ($CH_3CN$, 0.05% TFA) in 40 min at 2.5 mL/min flow rate. OA elution was monitored by absorption at 214 nm. Fractions (1 mL) were collected and assessed for inhibition of PP-1c activity using *p*NPP substrate. Pooled fractions were lyophilized and resuspended in MeOH.

OA was quantified by measuring inhibition of PP-1c activity using phosphorylase a (Phos a) substrate (n = 6). Commercially available OA (Moana Bioproducts) was used as a certified reference standard ($IC_{50}$ = 32 nM against PP-1c, n = 5).

*Calmodulin-Sepharose 4B resin preparation.*

CaM-Sepharose 4B affinity resin was prepared and regenerated according to the manufacturer's four step protocol (GE Healthcare). In brief, resin slurry was washed with three volumes of $H_2O$, followed by three volumes of the following buffers: 1) 0.1 M $Na_2CO_3$, pH 8.6, 2 mM EGTA; 2) 1 M NaCl, 2 mM $CaCl_2$; 3) 0.1 M $CH_3COONH_4$, pH 4.4, 2 mM $CaCl_2$; 4) 20 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT), 1 mM $CaCl_2$.

*Calcineurin-A catalytic subunit purification.*

CaN-A subunit purification was performed as described previously with the following changes.[295] 100 mL of Terrific media (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 17 mM $KH_2PO_4$, 72 mM $K_2HPO_4$, 50 μg/mL ampicillin)

was inoculated with HMS174 (DE3) *E. coli* cells containing CaN gene vector, and grown overnight at 37 °C. 100 mL subculture was used to inoculate Terrific media (2 L) and grown at 37 °C in an Omni Culture Plus Benchtop Fermenter System (Virtis, Gardiner, NY) until the optical density at 600 nm reached 0.7 absorbance units. Cultured cells were then induced with 0.5 mM isopropyl β-D-thiogalacto pyranoside (IPTG, Rose Scientific, Edmonton, AB) and grown at 37 °C for an additional 7 h. Cultured *E. coli* cells (2 L) were harvested by centrifuging at 3000 × g at 4 °C for 20 min and freezing the cell pellet overnight at -70°C. The cell pellet was resuspended in 100 mL of buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride [PMSF, Bioshop Canada Inc., Burlington, ON]). Lysozyme and protease inhibitors were added in the same concentrations as described for 6×His-I-1 lysis buffer. The cell suspension was passed twice through an EmulsiFlex-C3 cell homogenizer (Avestin, Inc., Ottawa, ON). The cell extract was centrifuged at 16 300 × g for 1 h or at 41 700 × g for 20 min at 4 °C. The resulting supernatant was made up to 50% $(NH_4)_2SO_4$ and stirred gently overnight at 4 °C. The precipitated protein pellet was collected by centrifugation at 3000 × g at 4 °C for 20 min. The pellet was resuspended in buffer A (15 mL) and dialyzed against 1.5 L of buffer A containing 1 mM PMSF and 5 mM benzamidine three times for 4 h each. The dialysate was centrifuged at 16 300 × g at 4 °C for 1.5–1.75 h to pellet particulate matter. The resulting clear supernatant was mixed with 10 mL of CaM-Sepharose 4B resin slurry overnight at 4 °C by

gentle rotation. The resin was packed into a 25 mm diameter column and washed with equilibrating buffer (20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM $CaCl_2$).

Protein was eluted with 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EGTA in 60 fractions (1 mL) using gravitational force. Fractions were assayed for phosphatase activity using *p*NPP substrate. Active fractions were pooled and concentrated to 150–250 µL volume using 10 000 MWCO Amicon Ultra Centrifugal Filter Devices (Millipore). The CaN-A concentrate was loaded on a gel filtration Hi-Load Superdex 75 HR26/60 column (GE Healthcare) equilibrated in buffer A containing 200 mM NaCl, and eluted overnight at a flow rate of 0.2 mL/min with the same buffer; 1 mL fractions were collected. Protein purity was assessed by SDS-PAGE by running 30 µL samples of each fraction on a 12% polyacrylamide gel. Protein fractions of the highest purity were pooled and concentrated to 150–250 µL. This concentrate was loaded on a Hi-Load Superdex 200 HR16/60 column (GE Healthcare) and eluted in a manner similar to elution of the Superdex 75 column. Protein content was determined using SDS-PAGE; the purest fractions were pooled and concentrated to 1 mL. The final concentrate was dialyzed against 50% glycerol buffer A (1.5 L) for 3–5 h. Purified protein was stored at -20 °C. A typical CaN-A protein expression yield was 0.3–1 mg of protein. CaN-A was purified to homogeneity or near homogeneity. The specific activities of purified CaN proteins are described in Table 2.3, p. 86.

**Table 2.3: Summary of calcineurin mutant proteins prepared in the current study and $IC_{50}$ inhibition values for microcystin-LR and okadaic acid natural toxins**

| Protein type | Specific activity (U/mg) | MCLR inhibition (nM) | | OA inhibition (μM) | |
|---|---|---|---|---|---|
| | | Average[a] | +/-[b] | Average[a] | +/-[b] |
| WT[c] | 7 | 1248 (n=19) | 641 | 12.06 (n=15) | 4.78 |
| 26–344[d] WT[c] | 242 | 1161 (n=4) | 188 | 10.66 (n=4) | 3.53 |
| Y315F | 14 | 1013 (n=5) | 343 | 19.21 (n=4) | 1.98 |
| Y315C | 6.5 | 177 (n=4) | 39 | 4.87 (n=4) | 0.88 |
| Y315L | 5.3 | 113 (n=8) | 15 | 2.4 (n=10) | 0.71 |
| L312C | 14 | 413 (n=3) | 81 | 1.55 (n=3) | 0.43 |
| L312C: Y315L | 20 | 23 (n=6) | 6.7 | 0.33 (n=3) | 0.04 |
| Y159I: F160Y: Y315L | 11.5 | 15 (n=11) | 5.8 | 4.03 (n=10) | 0.55 |
| Y159I: F160Y: L312C: Y315L | 32.9 | 2.1 (n=8) | 1 | 0.58 (n=6) | 0.07 |
| 1–460[d] Y159I: F160Y: L312C: Y315L | 3.9 | 1.8 (n=4) | 0.3 | 0.42 (n=4) | 0.13 |
| 26–344[d] Y159I: F160Y: L312C: Y315L | ND[e] | 2.2 (n=3) | 0.6 | 0.32 (n=5) | 0.13 |

[a]Average column shows the average $IC_{50}$ values, the concentration of inhibitor which inhibits 50% of enzyme activity. [b]"+/-" indicates the variation seen in $IC_{50}$ values summarized; [c]WT refers to full length wild type CaN-A; [d]Numbers refer to residues of the truncated protein; [e]Not determined. All assays contained CaN-B and CaM except assays that included 26–344 residue-long truncated proteins. Unit of activity is defined as nmol of inhibitor-1 substrate dephosphorylated per minute. WT activity value is an average of 60 assays. Mutant activities are relative to WT enzyme used at the time a given mutant was tested.

*Catalytic core calcineurin-A purification.*

A truncated variant of CaN that comprised residues 26–344 from CaN-A was isolated as described above, except that the CaM-Sepharose 4B column was substituted with a Heparin Sepharose 6 XK26/20 column (GE Healthcare). CaN was loaded onto the Heparin column equilibrated with buffer A. Protein was eluted using 0–100% gradient of buffer A containing 500 mM NaCl in 80 min at 5 mL/min flow rate. The truncated CaN-A protein expression yield was between 0.3–1 mg. The WT truncated CaN protein was purified to near homogeneity; 26–344 residues-long Y159I:F160Y:L312C:Y315L quadruple matant CaN could not be purified to homogeneity. The specific activity of purified catalytic core CaN-A enzyme is described in Table 2.3, p. 86. Truncated CaN, residues 26–344, was nearly 35-fold more active than full length CaN-A. Removal of regulatory domains from the CaN-A subunit renders the protein enzymatically hyperactive (see chapter one, Figure 1.5, p. 19).

*Calcineurin-B purification.*

The CaN-B subunit was purified as previously described with the following changes.[295] Cultured BL21 (DE3) plysS *E. coli* cells (2L) were grown as described for CaN-A subunit purification, except that Terrific media contained 0.6% glycerol. Lysates of cells were prepared as for CaN-A. Crude lysate was heated in a 100 °C water bath for 30 min. The cell extract was centrifuged at 16 300 × g at 4 °C for 30–45 min. The supernatant was loaded on a Phenyl Superose HR6/6 column (former Pharmacia Biotech Inc., Piscataway, NJ) equilibrated in

20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM $CaCl_2$. The column was washed with the same buffer and eluted with 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EGTA in 10 fractions (1 mL) using a 1 mL/min flow rate. The CaN-B protein expression yield was 20 mg of protein and was purified to homogeneity.

*Calcineurin phosphatase activity assay using para-nitrophenyl phosphate substrate*.

CaN activity was measured as described previously with the following changes.[295] In brief, dephosphorylation reaction buffer (50 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 0.2 mM $CaCl_2$, 0.5 mM $MnCl_2$, 0.2 mg/mL BSA, 0.3 μM CaM, 170 μL) with addition of CaN from fractions collected during the purification procedure (10 μL), was pre-incubated at 30 °C for 10 min, followed by addition of 200 mM *p*NPP (20 μL). The mixture was incubated at 30 °C for either 20, 40, or 60 min depending on enzyme activity. CaN preparations that exhibited lower activity (determined by visual inspection of the colorimetric assay) required longer incubation times. Reactions were terminated by addition of stopping solution (2 M $Na_2CO_3$, 100 mM EDTA, 100 μL). Absorbance was measured at 405 nm using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

*Calcineurin phosphatases activity assay using inhibitor-1 substrate*.

The I-1 assay was performed as described previously with the following changes.[75] The assay buffer used was the same as for the *p*NPP assay. Buffer containing CaN and CaM was pre-incubated at 30 °C for 20 min prior to addition

of [$^{32}$P]-labeled I-1 to a final volume of 100 μL. The reaction was incubated for 20 min and stopped by addition of 25% TCA (100 μL) and 10 mg/mL BSA (100 μL). Reaction mixtures were centrifuged at 16 000 × g for 2 min and measured for radioactivity in scintillation fluid (1 mL) using a Wallac 1209 Rackbeta scintillation counter (Pharmacia). Reactions contained 0.5–10 pmol of CaN-A (with typically 2–5-fold excess of CaN-B and CaM, see Table 2.3, p. 86, for enzyme activities of CaN preparations) and 25–90 pmol of I-1, depending on CaN activity and $^{32}$P release. The volume of the substrate did not exceed 10% of the total reaction volume. There was a direct correlation between the amount of substrate used and the amount of radioactivity measured. This linear correlation was seen up to a certain amount of added substrate, and the limit of this linear correlation varied between the different enzyme preparations. The amount of enzyme and substrate used in the CaN inhibition assays was determined to be in the linear range of radioactive release of the dephosphorylation product.

*PP-1c phosphatase assay.*

The activity of PP-1c was measured by its ability to dephosphorylate [$^{32}$P]-labeled phosphorylase a (Phos a) substrate as described previously.[207, 239] In brief, 15 μL of reaction buffer (50 mM Tris-HCl, pH 7.0, 0.1 mM EDTA, 1 mg/mL BSA, 1 mM $MnCl_2$, 0.2% BME) containing PP-1cγ (7.5 fmol) and 5 μL toxin (various dilutions of MCLR or OA in 50% MeOH) were preincubated at 30 °C for 10 min. The reaction was initiated by addition of 10 μL of [$^{32}$P]-Phos a (300 pmol), and allowed to incubate at 30 °C for an additional 10 min. The reaction

was stopped by addition of ice-cold 20% TCA (200 μL), cooling on ice for 2 min, and centrifugation at 16 000 × g for 2 min. Radioactivity of the entire reaction was measured in scintillation fluid (1 mL) using a Wallac 1209 Rackbeta scintillation counter (Pharmacia).

*Microcystin-Sepharose binding*.

Binding of the CaN quadruple mutant to microcystin-Sepharose resin was performed as described previously with following changes.[283] Microcystin-Sepharose and control Tris-Sepharose resins were treated equally. Resin was washed two times with forty volumes of buffer A (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.5 mM $MnCl_2$, 0.1% BME), followed by forty volumes of buffer A with 0.3M NaCl. Protein phosphatase (140 pmol of PP-1c or 430 pmol of CaN/20 μL of resin) was diluted in forty volumes of buffer B (buffer A containing 0.3 M NaCl, 0.2 mM $CaCl_2$, 1% Tween-20) and mixed with resin overnight end-over-end at 4 °C. The protein/matrix mixture was washed four times with forty volumes of buffer A containing 0.5 M NaCl. Resin was collected by brief centrifugation at 16 000 × g. Binding of proteins to the resin was ascertained by loading the resin directly on a SDS-PAGE gel. For the study of CaN binding to microcystin-Sepharose, molar quantities of CaN-B and CaM were 3 × greater than CaN-A.

*SDS-PAGE.*

Gel electrophoresis was performed as previously described.[185]

*Protein determinations.*

Protein concentrations were measured using the protein assay dye binding procedure (Bio-Rad, Mississauga, ON) based on the Bradford method.[40]

*Molecular docking.*

The crystal structure of human CaN (PDB code 1AUI) was selected to construct a predictive model for the binding of MCLA-2H and OA.[180] The coordinates of CaN were prepared using the molecular modeling software Sybyl 7.1 (Tripos Inc., St. Louis, MO). Water molecules were removed from the PDB file; essential hydrogen atoms and Kollman united atom charges were added to the protein. Three-dimensional structures of MCLA-2H and OA were obtained from the crystal structures of PP-1c complexed with MCLA-2H (PDB code 2BDX) and OA (PDB code 1JK7), respectively.[217, 218] The hydrogen atoms were added to MCLA-2H and OA; partial atomic charges were calculated using the Gasteiger-Marsili method.[224] The docking program AutoDock 3.0.5 was used to perform automated molecular docking.[224] A grid map with $60 \times 60 \times 60$ points spaced at 0.375 Å was generated using the Autogrid program to evaluate binding energies. The docked complexes of CaN with MCLA-2H or OA were evaluated according to the predicted binding energies and the fitness of the geometry in the complex models.

*Contributions to this work.*

All of the work described was performed by the author with the following exceptions by members of Dr. Holmes laboratory. WT CaN-A and CaN-B cell stocks were generated by Hue-Anh Luu. Hue-Anh Luu also generated the 1–460 and 26–344 residue truncated versions of WT CaN-A clones. Mackenzie Sawyer established the CaN-B purification protocol. Micheal Shopik contributed to the radiolabeling of substrates and to the purification of CaN. Hue-Anh Luu performed the microcystin-Sepharose binding experiments. Molecular docking images were generously provided by Chunying Niu of Dr. Michael James laboratory.

## 2.4 Results

*Substitution of residue 315 to leucine enhanced calcineurin sensitivity to microcystin-LR*.

It would appear that very few residues play a key role in specifying inhibitor selectivity between CaN, PP-1c, and PP-2Ac. The crystal structure of free CaN was juxtaposed on structures of PP-1c and PP-2Ac, both bound to OA (Figure 2.1, p. 73), and on structures of PP-1c bound to MCLA-2H and PP-2Ac bound to MCLR (Figure 2.2, p. 74).[180] These comparisons suggested candidate residues important in OA and microcystin binding that are different in CaN. In particular, Tyr-315 which corresponds to Phe-276 in PP-1c and Cys-269 in PP-2Ac, appeared to be a strong candidate for several reasons:

1) The proximity of CaN Tyr-315 to the toxins (see Figures 2.1 and 2.2,

p. 73–74); [59, 217, 218, 305]

2) Previous mutagenesis evidence showed that amino acid at Tyr-315 position in the β12–13 loop of PPP phosphatases is one of the most important residues for natural toxin interaction (see Table 2.1, p. 70);[169, 239, 263, 318]

3) CaN Tyr-315 is one of the most variable residues in the β12–13 loop among the PPP family of phosphatases (see Figure 2.3A, p. 94). The difference in amino acid composition at this position is known to play a role in the OA sensitivity difference observed between PP-2Ac and PP-1c.[169, 239, 263, 318]

Therefore, three point mutants of CaN Tyr-315 were constructed:

1) Y315F, a mutation to the corresponding Phe-276 in PP-1c;

2) Y315C, a mutation to the corresponding Cys-269 in PP-2Ac;

3) Y315L, a mutation to a nonpolar residue whose intermediate size between cysteine and phenylalanine was hypothesized to be more favorable for forming nonpolar interactions with OA.[143]

Sensitivity to OA and microcystin was measured for all mutants. MCLR was used in inhibition assays since this is the most common form of the toxin.[86] $IC_{50}$ values for MCLR inhibition of CaN mutants were compared to the value against WT CaN (Table 2.3, p. 86, and Figure 2.4A, p. 95): WT = 1248 nM, Y315F = 1013 nM, Y315C = 177 nM, and Y315L = 113 nM. Substitution of Tyr-315 to the corresponding PP-2Ac Cys-269 (Y315C) resulted in a 7-fold increase in MCLR sensitivity, further underscoring the importance of Cys-269 in

**A**

| | |
|---|---|
| CaN | 306 - F S A P N Y L D V Y N N – 317 |
| PP-1c | 267 - F S A P N Y C G E F D N – 278 |
| PP-2Ac | 260 - F S A P N Y C Y R C G N – 271 |

**B**

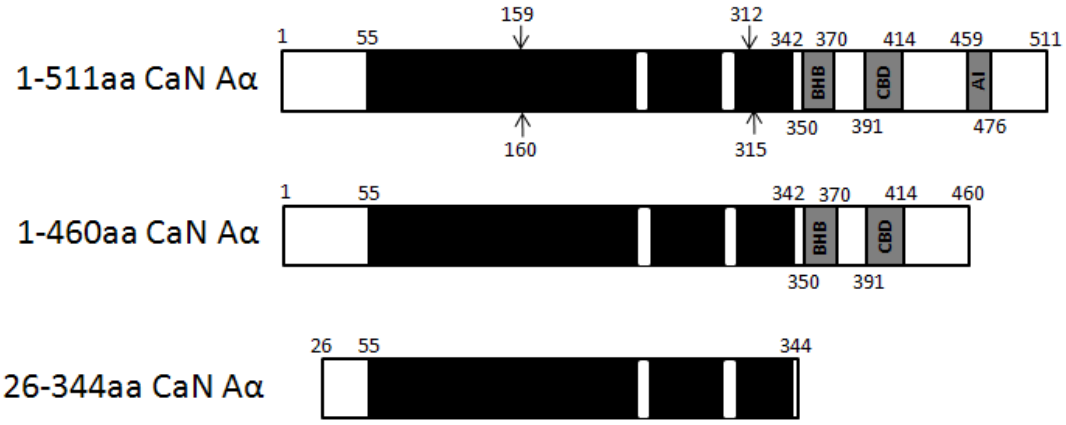



**Figure 2.3: Mutants of calcineurin used in this study based on differences found between PPP phosphatases.** (A) This panel depicts the alignment of the β12–13 loops between the CaN, PP-1c, and PP-2Ac phosphatases. Two of the four residues mutated in CaN are found in the β12–13 loop. (B) This panel depicts a schematic of mutant and truncated forms of CaN proteins prepared for the current study. Arrows point to the four different residues within CaN that were mutated. Residue numbers outline the different domains of CaN. The catalytic domain, as defined by Barton et al. (1994), is indicated in black.[20]

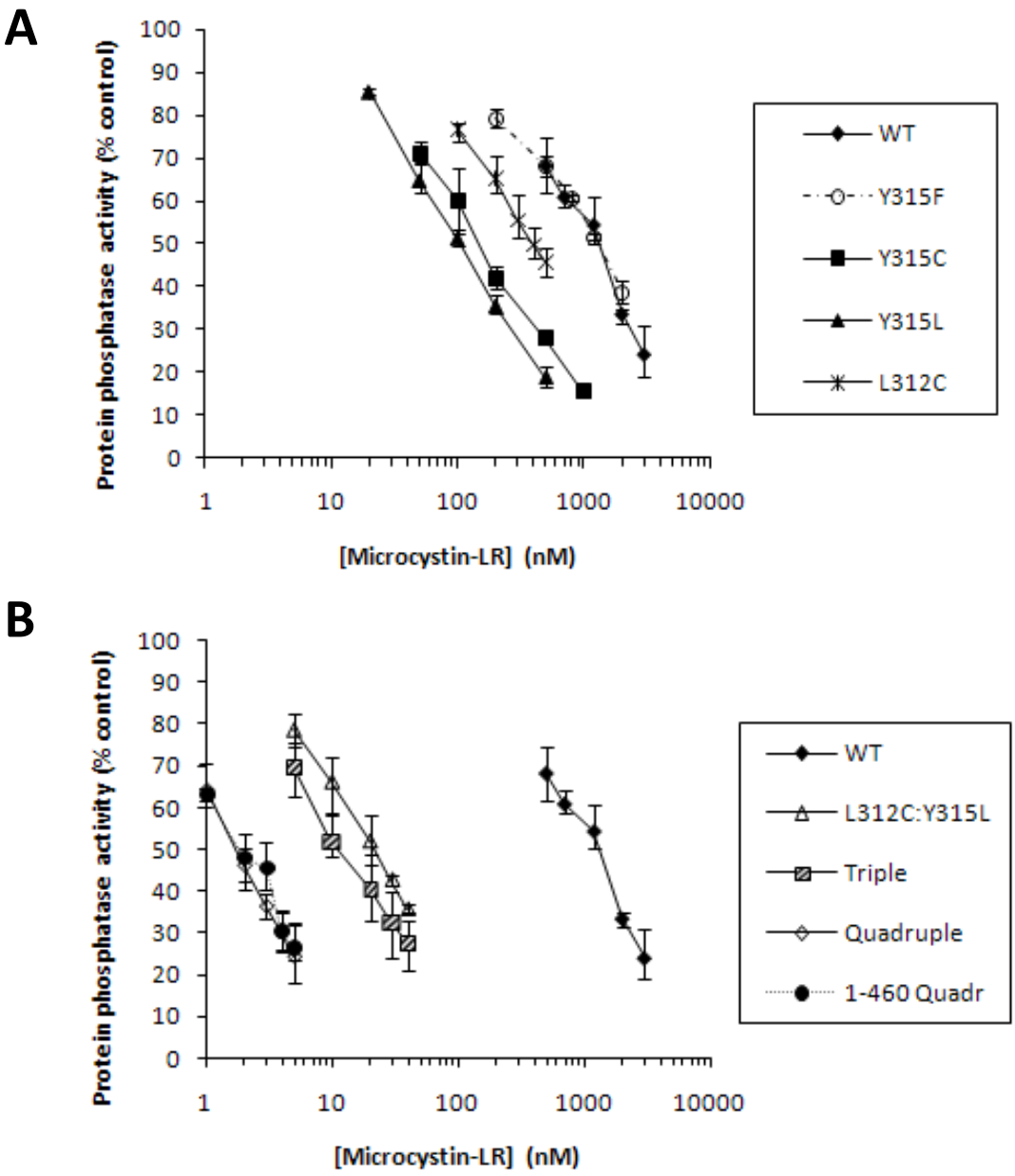



**Figure 2.4: Mutagenesis of calcineurin results in increased sensitivity to microcystin-LR natural toxin.** Enzyme activities of wild type (WT) CaN (—◆—) and CaN mutants Y315F (···⊖···), Y315C (—■—), Y315L (—▲—), L312C (—✳—), L312C:Y315L double mutant (—△—), Y159I:F160Y:Y315L triple mutant (—▨—), Y159I:F160Y:L312C:Y315L quadruple mutant (—◇—), and 1–460 quadruple mutant (···●···) were measured using [$^{32}$P]-I-1 as substrate in the presence of increasing amounts of microcystin-LR. Data is represented as single point mutants (panel A), or multiply mutated CaN enzymes (panel B) with WT CaN represented in both panels for clarity. All values were measured in duplicate with an average value of 3 assays shown except Y315F (n = 2). Vertical bars represent percent variation seen in the represented assays.

MCLR binding to PP-2Ac. The Y315L mutant was the most sensitive to MCLR, an 11-fold increase in sensitivity compared with WT. This suggests that residues other than Tyr-315 must be involved in CaN resistance to microcystin and that further mutagenesis was needed to reveal the source of toxin resistance in CaN.

*Triple mutation of calcineurin further increased the sensitivity to microcystin-LR*.

Although single point mutations of CaN residues displayed increased sensitivity to microcystin, further mutagenesis was performed to enhance toxin sensitivity to a level comparable to PP-1c or PP-2Ac. Two additional candidate residues were Tyr-159 and Phe-160.

Tyr-134 of PP-1c and Tyr-127 of PP-2Ac are equivalent to Phe-160 in CaN and have been shown to make hydrogen bonds with microcystin.[87, 124] A F160Y mutation would result in tandem tyrosine residues (native CaN Tyr-159 and substituted F160Y) that might pose steric problems for MCLR binding. Therefore it was decided that both residues 159 and 160 would be mutated to the corresponding residues in PP-1c. Since the CaN Y315L mutant showed the highest sensitivity to MCLR, it was chosen for further mutagenesis of Y159I and F160Y. The MCLR $IC_{50}$ for the CaN Y159I:F160Y:Y315L triple mutant inhibition was 15 nM (Table 2.3, p. 86, and Figure 2.4B, p. 95), an 80-fold decrease in toxin resistance. This large increase in sensitivity to the toxin is likely due to hydrogen bonding of mutant residue Tyr-160 with MCLR.

*Quadruple mutant calcineurin displays the greatest sensitivity to microcystin-LR.*

In previous work, a block of PP-1c β12–13 loop residues (273-CGEFD-277) was changed to five corresponding CaN residues (312-LDVYN-316). This chimeric PP-1c showed ~6-fold decreased sensitivity to MCLR and ~4-fold decreased sensitivity to OA. Interestingly, this difference was attributed to a single amino acid change, C273L, since this PP-1c point mutant exhibited the same increased toxin resistance as the block mutant.[239] These results suggested that the corresponding residue in CaN was important in reducing sensitivity to toxins. We decided to investigate this by substituting Leu-312 of CaN for cysteine, the corresponding residue in the PP-1c and PP-2Ac β12–13 loop.

Three mutants containing the L312C substitution were generated. The L312C point mutant resulted in a 3-fold increase in CaN sensitivity to MCLR, closely matching the reverse result seen in PP-1c (see Table 2.3, p.86, and Figure 2.4A, p. 95). MCLR inhibited a L312C:Y315L double mutant with an $IC_{50}$ of 23 nM, representing a 54-fold increase in CaN sensitivity to MCLR, closely matching the effect of the triple CaN mutant. Since the triple mutant was still more sensitive, we generated a quadruple mutant Y159I:F160Y:L312C:Y315L (see Figure 2.3B, p. 94). The quadruple mutant was nearly 600-fold more sensitive to MCLR than WT CaN-A; MCLR inhibited the quadruple mutant with an $IC_{50}$ of 2.1 nM (Table 2.3, p.86, and Figure 2.4B, p. 95). The quadruple mutant of CaN was only ~20-fold more resistant to MCLR than PP-1c.[79, 239]

*Truncation of C-terminal regulatory domains of calcineurin does not affect microcystin-LR inhibition.*

Domains other than the catalytic site and the β12–13 loop were investigated for their effects on toxin sensitivity. Two truncation mutants were generated: 1) residues 1–460 lacking the autoinhibitory (AI) domain of CaN, and 2) residues 26–344 lacking the variable terminal tail of CaN, mimicking the size of PP-1c (see Figure 2.3B, p. 94). This removed all regulatory domains of CaN leaving the phosphatase no longer dependent on CaN-B and CaM activation as previously observed.[300, 302, 309] No significant effect was seen with truncation of the CaN C-terminal tail on the inhibition by MCLR, whether tested with WT or quadruple mutant truncated constructs (Table 2.3, p. 86).

*Residue 312 is more critical in calcineurin for okadaic acid interaction than 315.*

OA inhibited WT CaN with an $IC_{50}$ of 12.06 μM. In comparison, point mutants tested against OA had the following $IC_{50}$ values (Table 2.3, p. 86, and Figure 2.5A, p. 99): Y315F = 19.2 μM, Y315C = 4.9 μM, Y315L = 2.4 μM, and L312C = 1.55 μM. Similarly to that seen for MCLR inhibition, a single amino acid change at residue 315 resulted in an increased sensitivity of CaN to OA; a 2.5-fold increase for the Y315C mutant and a 5-fold increase for the Y315L mutant. Of the two residues mutated in the β12–13 loop, however, L312C was the most sensitive to OA—nearly 7-fold more sensitive than WT CaN.

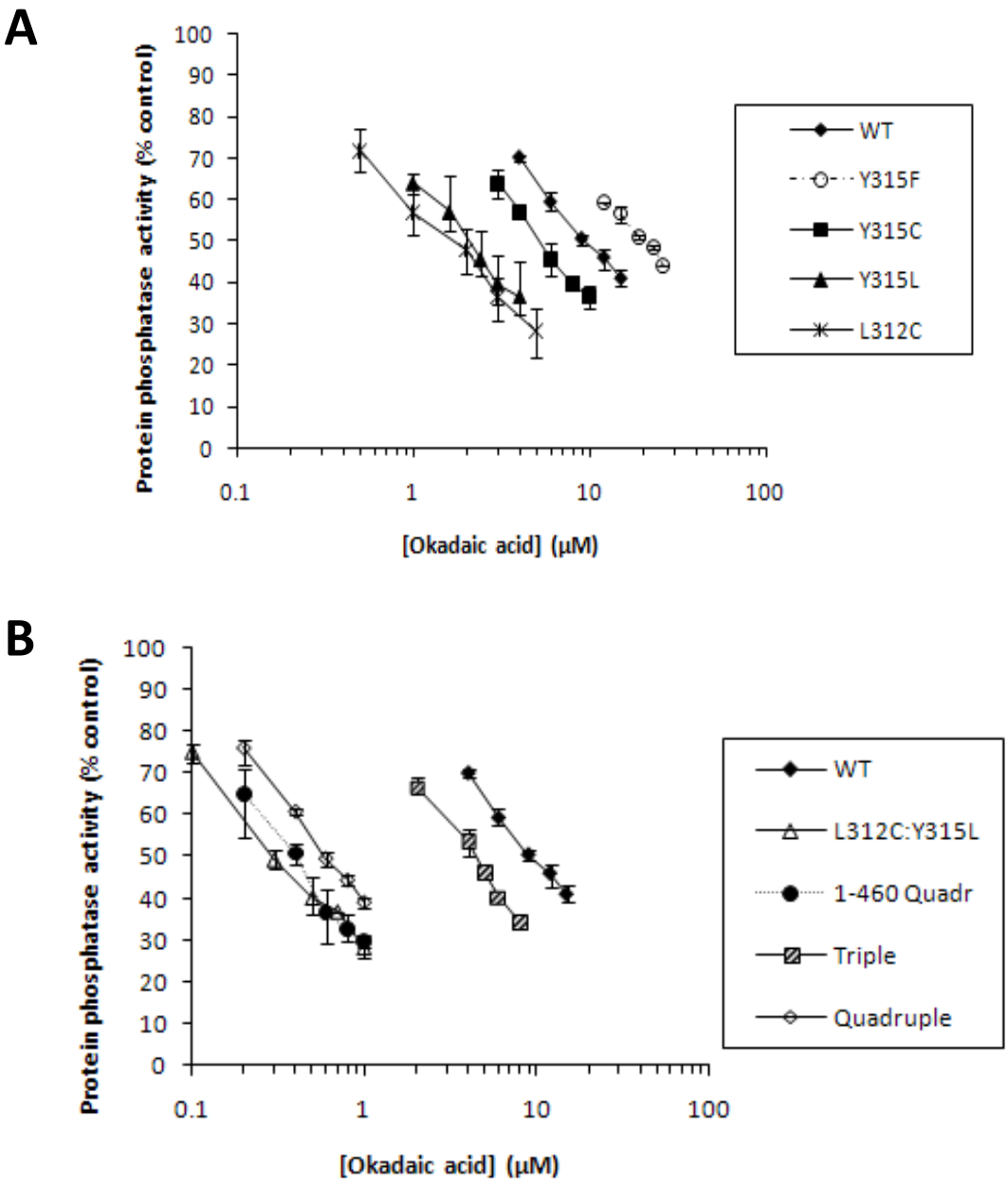



**Figure 2.5: Mutagenesis of calcineurin results in increased sensitivity to okadaic acid natural toxin.** Enzyme activities of wild type (WT) CaN (—◆—) or single point mutants forms of CaN (panel A), Y315F (···Θ···), Y315C (—■—), Y315L (—▲—), L312C (—✳—), or multiply mutated phosphatases (panel B), L312C:Y315L double mutant (—△—), Y159I:F160Y:Y315L triple mutant (—▨—), Y159I:F160Y:L312C:Y315L quadruple mutant (—◇—), and 1–460aa quadruple mutant (···●···) were assayed using [$^{32}$P]-I-1 as substrate in the presence of increasing amounts of okadaic acid. WT CaN is included in both panels for clarity. All values were measured in duplicate with an average of 3 assays shown, except Y315F, Y315C, and L312C:Y315L (n = 2). Vertical bars represent percent variation seen in the represented assays.

*Triple mutant calcineurin does not further enhance the sensitivity to okadaic acid.*

Considering only a limited number of residues involved in OA binding are different among PP-1c, PP-2Ac, and CaN, it was expected that the CaN triple mutant, harboring two additional mutations to corresponding PP-1c/PP-2Ac residues, would result in a further increase in OA sensitivity compared with Y315L. However, when sensitivity to OA of the triple mutant was measured, an $IC_{50}$ of 4.03 μM was obtained (Table 2.3, p. 86, and Figure 2.5B, p. 99). Although this value is 3-fold lower than the $IC_{50}$ of OA for WT CaN, the mutant was less sensitive than the single point mutants Y315L and L312C.

*The L7 loop calcineurin residues are the most critical in affecting okadaic acid sensitivity.*

Since a previous report showed the importance of Cys-273 of PP-1c in OA binding, this residue was incorporated in the corresponding position of CaN.[239] A quadruple mutant of CaN (Table 2.3, p. 86, and Figure 2.5B, p. 99) showed a drastic increase in sensitivity to OA, $IC_{50}$ = 0.58 (22-fold difference), compared with WT CaN, rescuing the negative effects of the Y159I:F160Y mutation on OA sensitivity that was seen in the triple mutant. The triple mutant was less sensitive to OA than the single point mutants tested, but the quadruple mutant was more sensitive to OA inhibition than the point mutants. A double mutant L312C:Y315L was constructed to combine the two most effective point mutations. The L312C:Y315L double mutant was inhibited by OA with an $IC_{50}$ of 0.33 μM (see Table 2.3, p. 86, and Figure 2.5B, p. 99).

*Calcineurin regulatory domains play a role in okadaic acid inhibition.*

In contrast to the results found for MCLR inhibition, truncation of the regulatory domains of CaN had an impact on CaN sensitivity to OA. WT CaN (26–344), $IC_{50}$ = 10.7 μM, showed a small increase in sensitivity. Truncated CaN quadruple mutants Y159I:F160Y:L312C:Y315L also showed significant increases in sensitivity, with $IC_{50}$s of 0.42 μM for amino acids 1–460 and 0.32 μM for amino acids 26–344 (Table 2.3, p. 86). The latter mutant was the most OA sensitive construct, resulting in a 39-fold increase in sensitivity; that is, the $IC_{50}$ was only ~10-fold higher than the $IC_{50}$ for OA inhibition of PP-1c as measured in the Holmes laboratory.[239]

*Structural model of calcineurin bound to natural toxins.*

From published structures of PP-1c bound to OA, it is known that the OA structure does not change when it is bound to WT PP-1c or chimeric PP-1c.[217, 239, 305] Thus OA was modeled as the native molecule into the putative structure of the CaN quadruple mutant (Figure 2.6, p. 102).

The β12–13 loop accommodates covalent bond formation between MCLR and Cys-273 of PP-1c.[124] It is known that such covalent bond formation is a slow process that follows rapid inhibition of enzyme activity, and it is unlikely covalent bond formation could advance significantly during activity assays of the CaN quadruple mutant.[78, 79, 249] However, to remove uncertainty about covalent bond formation, MCLA-2H was used to model the bound CaN quadruple

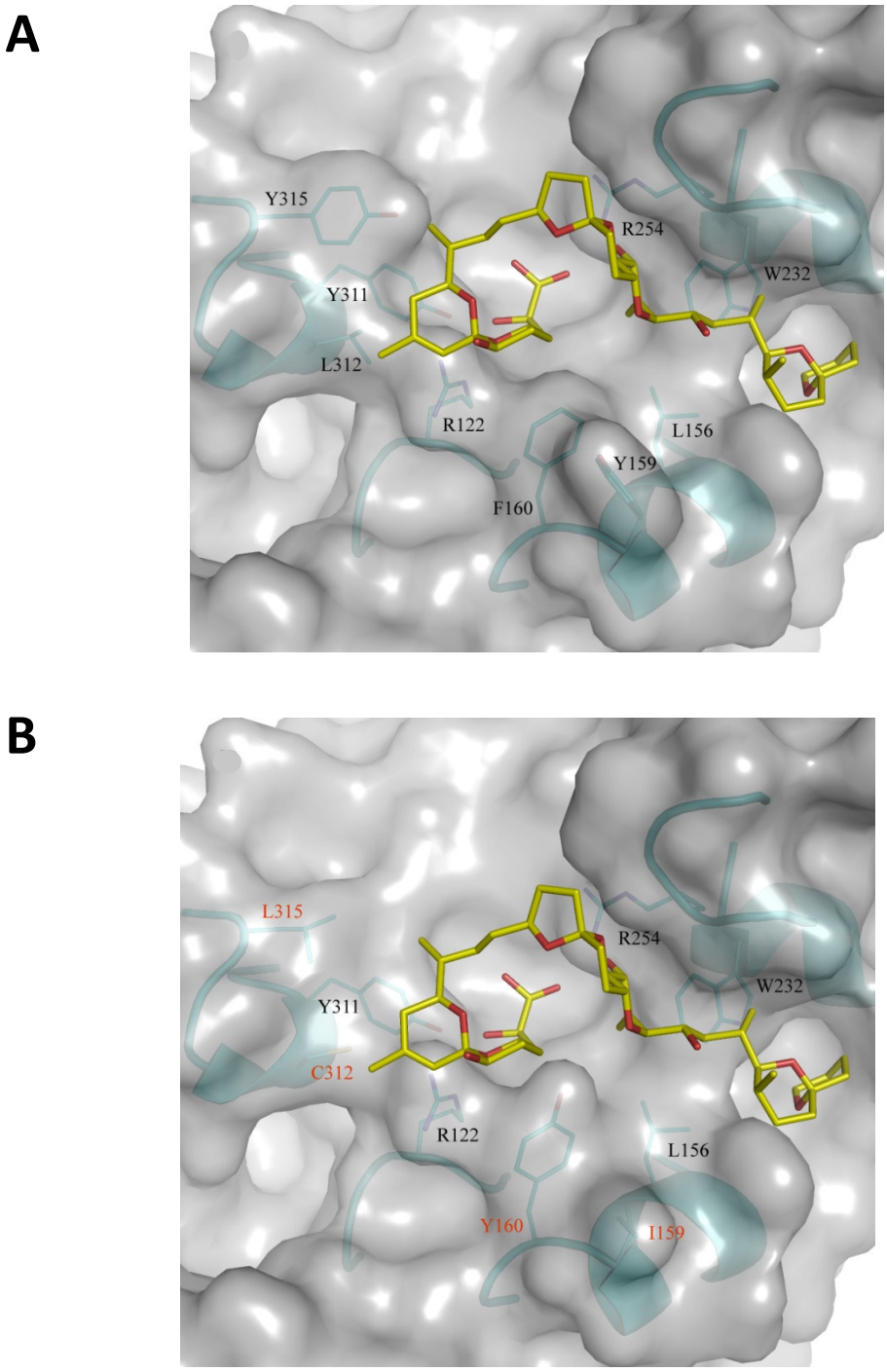



**Figure 2.6: Model depicting the Y159I:F160Y:L312C:Y315L quadruple mutant calcineurin bound to okadaic acid.** (A) This panel depicts the molecular docking of okadaic acid (OA) bound to the solvent-accessible surfaces of wild type CaN. (B) This panel depicts the molecular docking of OA bound to the quadruple mutant CaN with the four mutated residues labeled in red.

mutant:microcystin structure (Figure 2.7, p. 104), as MCLA-2H does not form a covalent bond with Cys-273 of PP-1c.

## 2.5 Conclusions

CaN, PP-1c, and PP-2Ac are highly homologous enzymes of the PPP family of phosphatases. In spite of homology, they exhibit differing substrate specificities and differing sensitivities to natural toxins such as MCLR and OA. Although structurally similar to PP-1c and PP-2Ac in the catalytic region, CaN is much more resistant to these toxins in comparison with PP-1c and PP-2Ac.[26, 143, 147, 208, 217, 305] Numerous studies have probed individual residues and blocks of residues of PP-1c and PP-2Ac to explain inhibition by OA and MCLR. These studies provide insights into CaN resistance to these natural toxins.[74, 87, 152, 169, 249, 263, 318-320] However, very little study of CaN has been undertaken to investigate which residues are responsible for this resistance. Wei and Lee (1997) generated mutants of CaN in the L7 loop, including substitution of the CaN 313-DVYN-316 amino acid sequence with 274-GEFD-277 of PP-1c or 267-YRCG-270 of PP-2Ac. Wei and Lee, (1997) also constructed a CaN L312C mutant.[296] These authors reported that none of these mutants had altered sensitivity towards OA, in contrast to the data presented in this study.[74] Reported here is the first mutagenesis evidence delineating specific amino acids responsible for the difference in sensitivity to OA and MCLR between CaN and its closely related cousins, PP-1c and PP-2Ac.

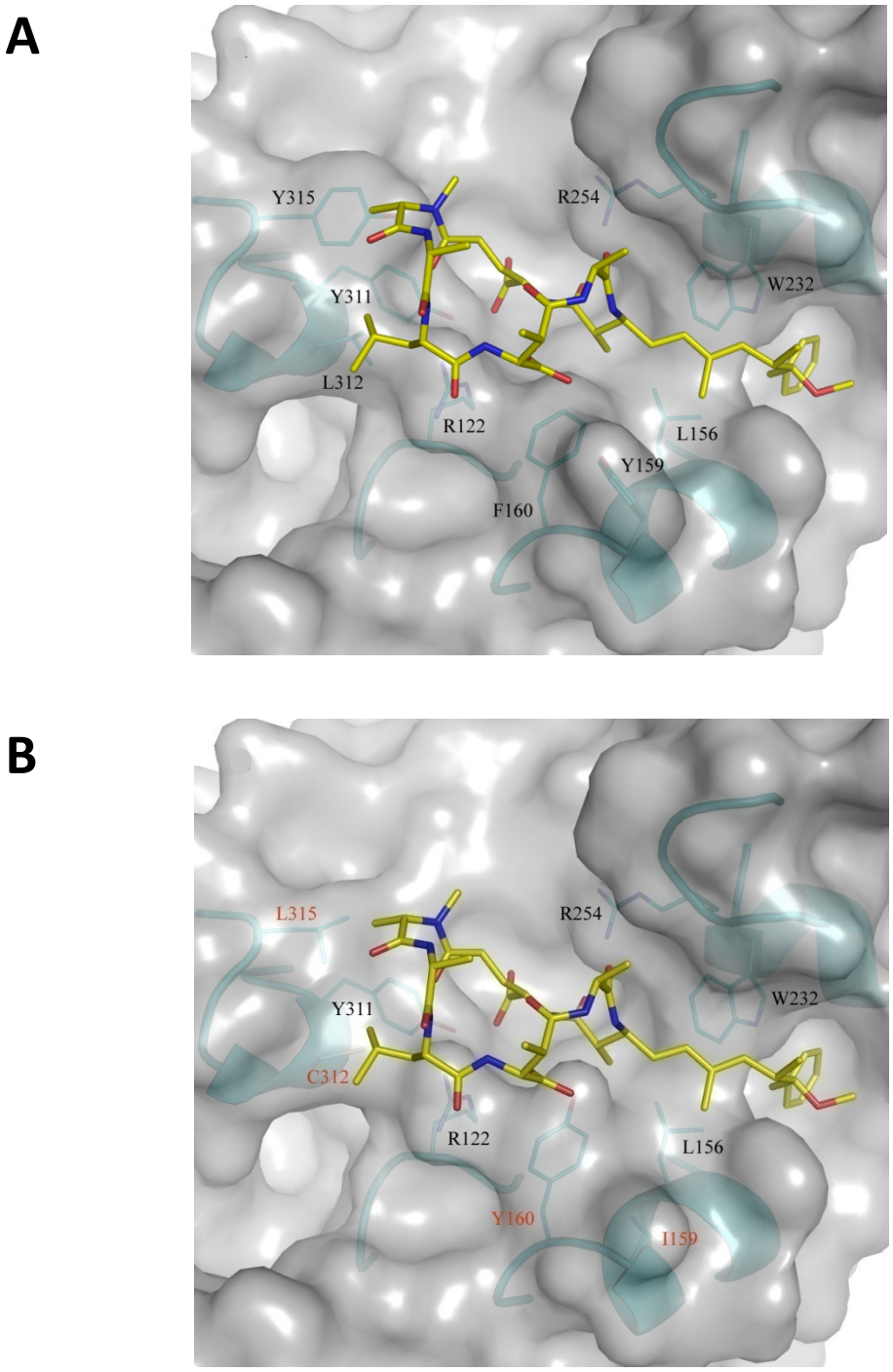



**Figure 2.7: Model depicting the Y159I:F160Y:L312C:Y315L quadruple mutant calcineurin bound to microcystin.** Molecular docking of dihydromicrocystin-LA (MCLA-2H) is shown bound to either the solvent-accessible surfaces of wild type CaN (panels A), or the quadruple mutant CaN (panels B). The four mutated residues are labeled in red.

A change of four amino acid residues (Y159I:F160Y:L312C:Y315L) was necessary to generate a form of CaN only 20-fold less sensitive than PP-1c to either MCLR or OA. These changes included two residues in the L7 loop (L312C:Y315L) and two residues in the active site of CaN (Y159I:F160Y). Mutants were still able to dephosphorylate CaN's physiological substrate I-1 in a $Ca^{2+}$/CaM/CaN-B dependent manner (see Table 2.3, p. 86).

The $IC_{50}$ values reported here for WT CaN are different from those previously reported. The only published values for inhibition of CaN are ~5μM for OA and ~0.2 μM for MCLR.[26, 70, 208] These $IC_{50}$ values have gone unchallenged in the scientific community for over 20 years. One review of toxin inhibition of PPP phosphatases by Honkanen and Golden (2002) reported a value of ~1μM for microcystin inhibition of CaN, which agrees more closely with the one obtained in the current study, albeit this value was not substantiated with data.[147]

With the exception of the Y315F and the triple mutant Y159I:F160Y:Y315L, both measured against OA, all mutants generated showed increased sensitivity to natural toxins, with the multiple mutants showing additive effects of increased toxin sensitivity. Mutagenesis of Tyr-315 to cysteine and leucine, conferred an increased sensitivity to OA by 2.5 and 5-fold, respectively. The effect of the Y315C mutation was expected, based on a previous observation that a corresponding reverse mutation in PP-2Ac, C269Y, displayed a 40-fold

decrease in OA sensitivity.[169] PP-2Ac structure suggests that Cys-269 (the corresponding residue to Tyr-315) does not make significant interactions with OA; its primary benefit appears to be that it no longer provides the steric disadvantage of the larger residues present in PP-1c (Phe-276) or CaN-A (Tyr-315).[59, 305] However, a leucine in the same position in CaN-A, apart from removing the steric hindrance of the bulkier tyrosine or phenylalanine residue, might also provide a more hydrophobic interaction with the $C_{10}$ methyl group of OA. This could explain why the Y315L mutant was over 5-fold more sensitive than WT CaN to OA inhibition (see Figure 2.8A, p. 107).

The Y315F substitution in turn, resulted in an even more toxin resistant CaN enzyme against OA. The structural basis for this is difficult to understand, since phenylalanine, lacking the hydroxyl group of tyrosine, is less bulky and was expected to be more accommodating to toxin binding. The PP-1c structure bound to OA suggests that Phe-276 could impede PP-1c inhibition by blocking entry of the toxins to the active site.[217, 218] One possibility is that the CaN-A Tyr-315 residue is more flexible than phenylalanine; that is, tyrosine can move more than phenylalanine to accommodate toxin binding (see Figure 2.8B, p. 107). In support of this theory, a crystal structure of chimeric PP-1c (containing CaN's L7 loop 312-LDVYN-316 amino acids substituted for PP-1c β12–13 loop residues 273-CGEFD-277) bound to OA showed no electron density at the F276Y substitution indicating that the tyrosine was highly flexible.[239] The structure of WT PP-1c bound to OA shows a clearly defined electron density map for Phe-276

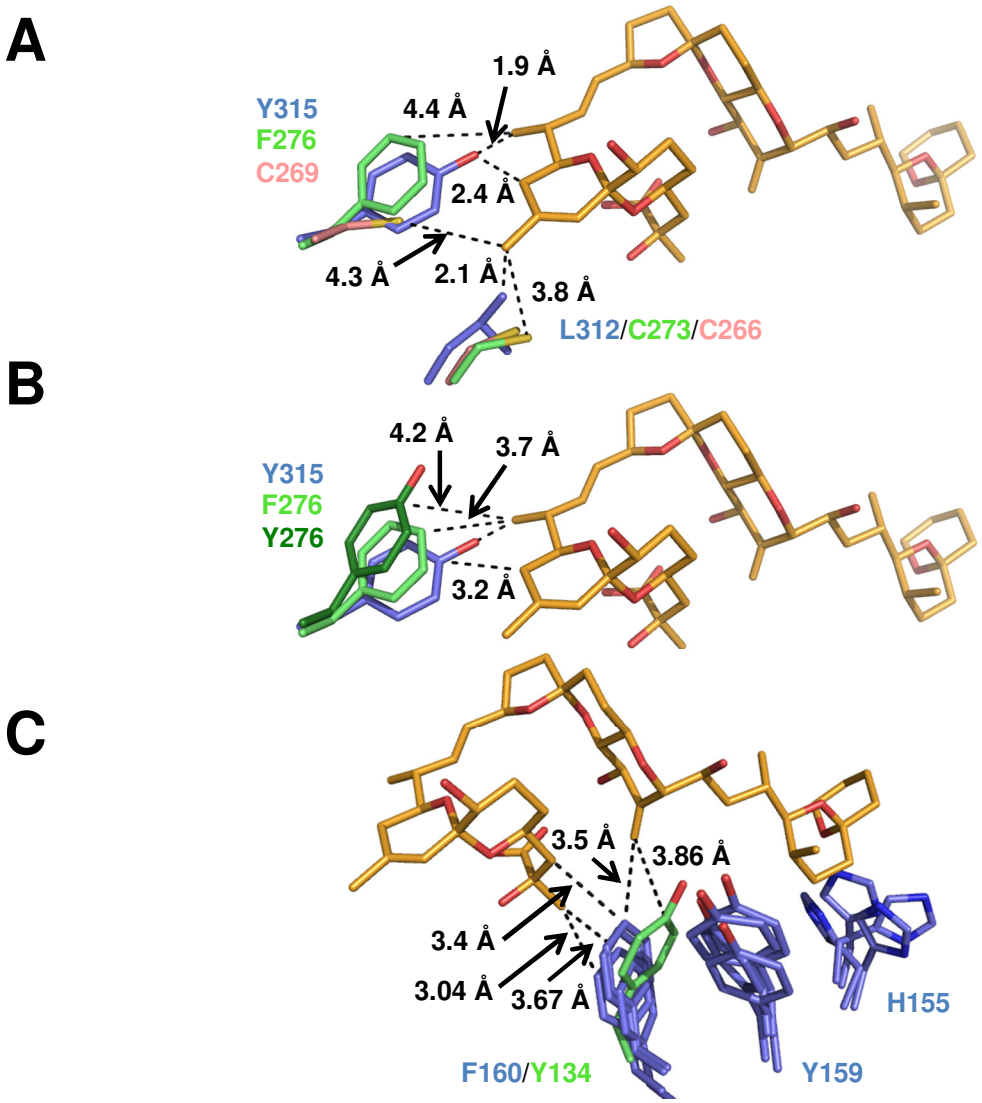



**Figure 2.8: Steric hindrance of key amino acids impacts calcineurin sensitivity to okadaic acid.** (A) Smaller amino acids are more advantageous in place of the native CaN-Aα residues (slate color) in the point mutants most sensitive to the toxin (L312C and Y315L). These substitutions afford greater distance from okadaic acid (OA) that is more favourable for electrostatic interactions. Leu-315 (not shown) would be at a 4 Å proximity to OA, as judged by distance of the Phe-276 of PP-1cγ (lime color), similar to the distance adopted by Cys-269 of PP-2Acα (salmon color). (B) Tyr-276 (dark green color) of a PP-1cγ mutant bound to OA is further removed than the native Phe-276 residue. This suggests that a tyrosine residue in this position is more flexible. The equivalent amino acid in CaN-A, Tyr-315 is shown for comparison. (C) This panel depicts the positioning of Tyr-159 and Phe-160 residues from four different structures of CaN-A in relation to Tyr-134 of PP-1cγ. Phe-160 could participate in more numerous van der Waal interactions with OA than Tyr-134. Tyr-159, in right conformation, could be involved in hydrogen bonding with His-155, removing His-155 from obstructing proper positioning of okadaic acid in the hydrophobic groove.[126, 149, 166, 180, 217, 305]

indicating that the phenylalanine residue is more rigid in its interaction with the toxin.[217]

Of the single point CaN mutants, L312C was the most sensitive to inhibition by OA, further corroborating the importance for toxin binding of having cysteine in this position.[239] Once again, the residue size appears to be the determining factor responsible for the improvement in enzyme's sensitivity.

With three PP-1c residues in place of three CaN residues, the triple mutant was expected to be more sensitive to OA than each of the point mutants. Instead, the triple mutant Y159I:F160Y:Y315L was ~1.5-fold less sensitive to OA than the Y315L mutant. The behavior of this phenotype is difficult to understand. Based on previous work in the Holmes laboratory, it was expected that inhibition by OA of the triple mutant would be similar to or greater than inhibition of Y315L CaN, as the reciprocal mutations in PP-1c, I133Y and Y134F, had less or no sensitivity to OA, respectively.[87] It is possible that the Y159I:F160Y mutations resulted in steric problems not seen in PP-1c.

Phe-160 could perhaps be more advantageous by being able to participate in more van der Waal interaction than a tyrosine. The aromatic ring of phenylalanine with its π electrons could provide stronger dipole interactions than tyrosine residue whose electron cloud distribution would be affected by presence of the hydroxyl group. Tyr-134 of PP-1c (the corresponding residue to Phe-160 of

CaN) is removed from the natural product, and in such configuration provides only one possible van der Waal contact. Presence of Phe-160 as modeled from different available CaN structures, suggest that the amino acid would be in close proximity to provide multiple van der Waal interactions (see Figure 2.8C, p. 107). Such proximity would have to be achieved in the context of CaN landscape only as Y134F mutation in PP-1c does not improve enzyme sensitivity.[87]

Tyr-159 could also be an important contributor to OA sensitivity in CaN. Different CaN-A structures show that His-155 could impede proper placement of OA in the hydrophobic groove. In fact, position of His-155 from one of such structures would directly clash with the natural product. Tyr-159, in the right conformation could perhaps aid in removing such steric hindrance by forming a hydrogen bond with His-155 that would move His-155 out of the way of OA (see Figure 2.8C, p. 107). However, this second hypothesis is not supported by the crystallographic data, with Tyr-159 always appearing to point away from His-155, and thus not available for such hydrogen bond formation. Individual mutagenesis of Tyr-159 and Phe-160 could help determine if the triple mutant phenotype is a product of one or both of the amino acid changes.

All of the single point mutants were more sensitive to MCLR than WT CaN; however, the Y315F mutant was only marginally more sensitive to MCLR. This was not unexpected as the reverse mutation in PP-1c, F276Y, had very little effect on toxin sensitivity.[239] The observed increase in inhibition in the Y315C

mutant is in contrast to the decrease in MCLR inhibition in PP-1c with the same mutation, F276C.[318] This highlights the notion that despite the similarity of the active sites of the PPP phosphatases, it is the microenvironment of these enzymes that determines their individual sensitivities to the natural toxins.

Of the single point mutants, Y315L was the most sensitive to MCLR. This shows that an intermediate size hydrophobic residue (compared with phenylalanine or cysteine, the corresponding residues found in PP-1c and PP-2Ac, respectively) at this position of the β12–13 loop is conducive to toxin interaction (see Figure 2.9A, p. 111). The effect of the PP-2Ac Cys-269 residue contribution to MCLR interaction cannot be readily ascertained as it participates in covalent bond formation with the toxin.[59, 79, 249, 305]

MCLR had increased potency against the phosphatase activity of the triple mutant Y159I:F160Y:Y315L. This is in contrast to what has been observed with OA, and it is hypothesized that the steric problems imposed by the Y159I:F160Y mutations are overcome by hydrogen bond formation between Tyr-160 and MCLR which does not occur with OA (see Figure 2.9B, p. 111). The triple mutant was 83 times more sensitive to MCLR than WT CaN. Surprisingly, previous data obtained in the Holmes laboratory showed that a reverse mutation, Y134F, in PP-1c had only a 7-fold decreased sensitivity to MCLR, with much more pronounced reduction seen with Y134A and Y134D mutations.[87] Perhaps an

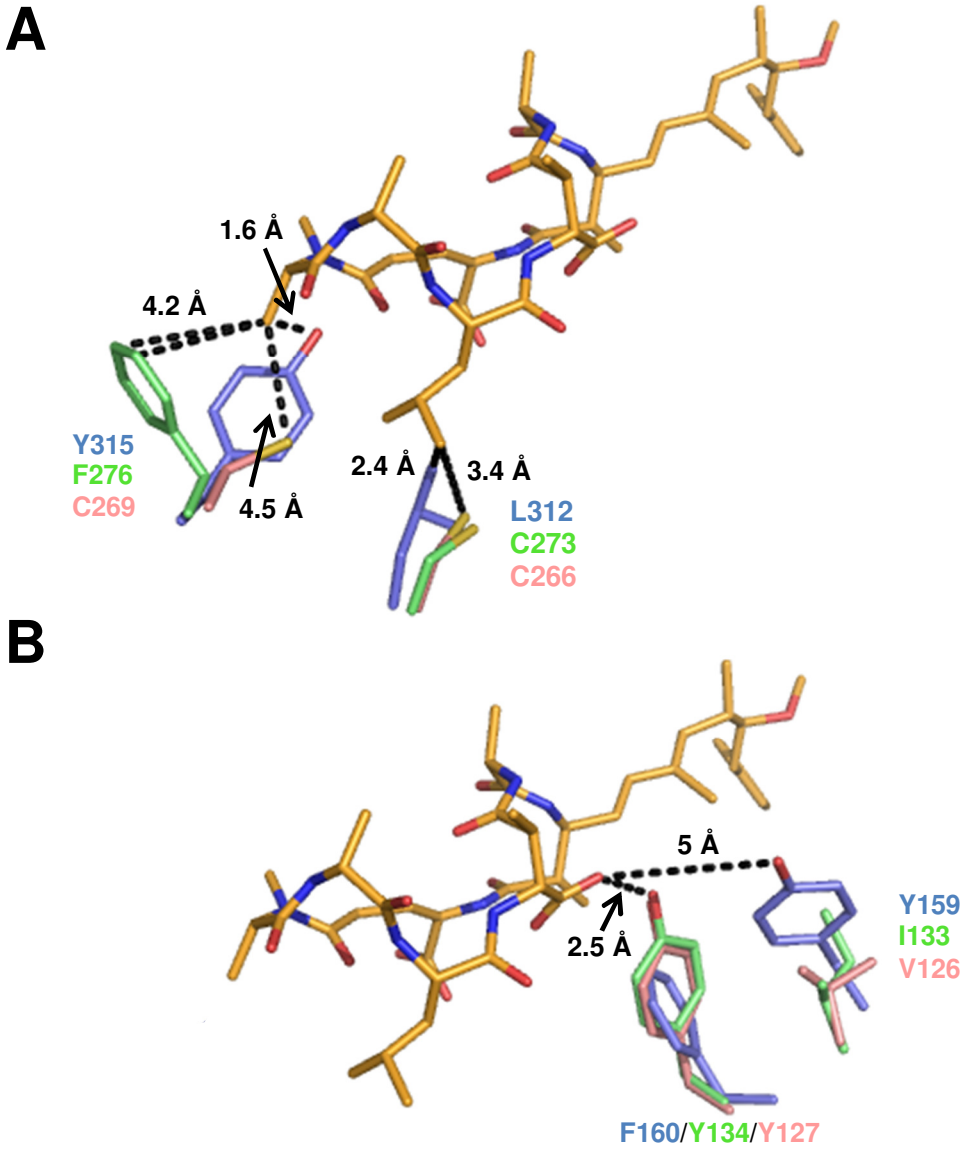



**Figure 2.9: Steric hindrance of key amino acids impacts calcineurin sensitivity to microcystin.** (A) Smaller amino acids are more advantageous in place of the native CaN-Aα Tyr-315 (slate color). Leu-315 (not shown) would be at a 4 Å proximity to microcystin, as judged by distance of the Phe-276 of PP-1cγ (lime color), similar to the distance adopted by Cys-269 of PP-2Acα (salmon color). Tyr-315 is also disadvantageous due to its hydroxyl group being in close proximity to negatively charged microenvironment presented by the natural product. (B) Substitution of CaN-A Phe-160 for tyrosine, the corresponding residue of both PP-1c and PP-2Ac, likely provides the hydrogen bond that accounts for the significant increase in CaN mutant sensitivity to the natural product. The adjacent WT CaN-A amino acid, Tyr-159, is too far removed to participate in hydrogen bonding.[124, 180, 305]

aromatic ring in the PP-1c environment aids in tighter packing of MCLR to a larger extent than the bond formation itself.

The quadruple mutant showed a 600-fold increase in sensitivity to MCLR and a 40-fold increase in sensitivity to OA compared to WT CaN. $IC_{50}$ values of 320 nM (OA) and 1.8 nM (MCLR) were obtained with a truncated form of the CaN quadruple mutant (see Table 2.3, p. 86); these numbers approach values seen for PP-1c inhibition by these toxins.[26, 208]

Since truncation of the CaN regulatory domains affected the $IC_{50}$ of OA, it is likely that the regulatory domains influence the shape of the active site. Two truncated forms of quadruple mutant CaN were used in this study: 1–460 or 26–344 residues-long. In the case of 1–460 quadruple mutant CaN, the increased sensitivity to OA can be directly attributed to deletion of the AI domain. In the case of 26–344 quadruple mutant CaN, however, the further increase in OA sensitivity could be accounted by further deletion of the regulatory domain of CaN-A, or by removal of the N-terminal section of CaN. There is evidence to support both possibilities.

Crystal structures of CaN show that the L7 loop interacts with the linker region (residues 335–347) with hydrogen bonding between Asp-313 and His-339.[126, 180] If this interaction is important for maintaining the L7 loop position, then perhaps removal of the regulatory domains produced a conformation change

of the L7 loop which could result in altered sensitivity to OA. Alterations in the L7 loop shape have previously been shown to have a drastic effect on the catalytic activity, with loss of dependence on the regulatory domains of CaN and a concomitant change in protein conformation.[202, 296, 303, 306] The architecture of the catalytic core could also be shaped by interactions of CaN-A with CaM regulatory protein.[140] Such interactions with CaM would not be possible for residues 26–344 truncated CaN-A. In addition, recent evidence emerged documenting the impact that variable N-terminal tails of PP-1c and CaN-A have on substrate dephosphorylation and OA sensitivity.[304]

Alternatively, our findings suggest that MCLR and OA are able to effectively compete with the autoinhibitory region of CaN to cause phosphatase inactivation, provided favorable amino acid substitutions (such as those delineated in the CaN quadruple mutant) are present to facilitate interaction with the active site of the enzyme. This is reminiscent of the ability of these toxins to displace the tetratricopeptide repeat region of the PPP family phosphatase PP-5.[147, 276] Truncation of the C-terminal tail increased CaN sensitivity to OA but not to MCLR; these results suggest that MCLR may compete more successfully than OA with the AI region of CaN. The abundant hydrogen bond and electrostatic interactions of MCLR may draw it into the active site more easily than the more hydrophobic OA. Figure 2.10, p. 114, illustrates the superposition of MCLA-2H, OA, and the Glu-481 residue from CaN AI domain in the active site of CaN. The model shows how all three inhibitors rely on a similar mode of blocking access to

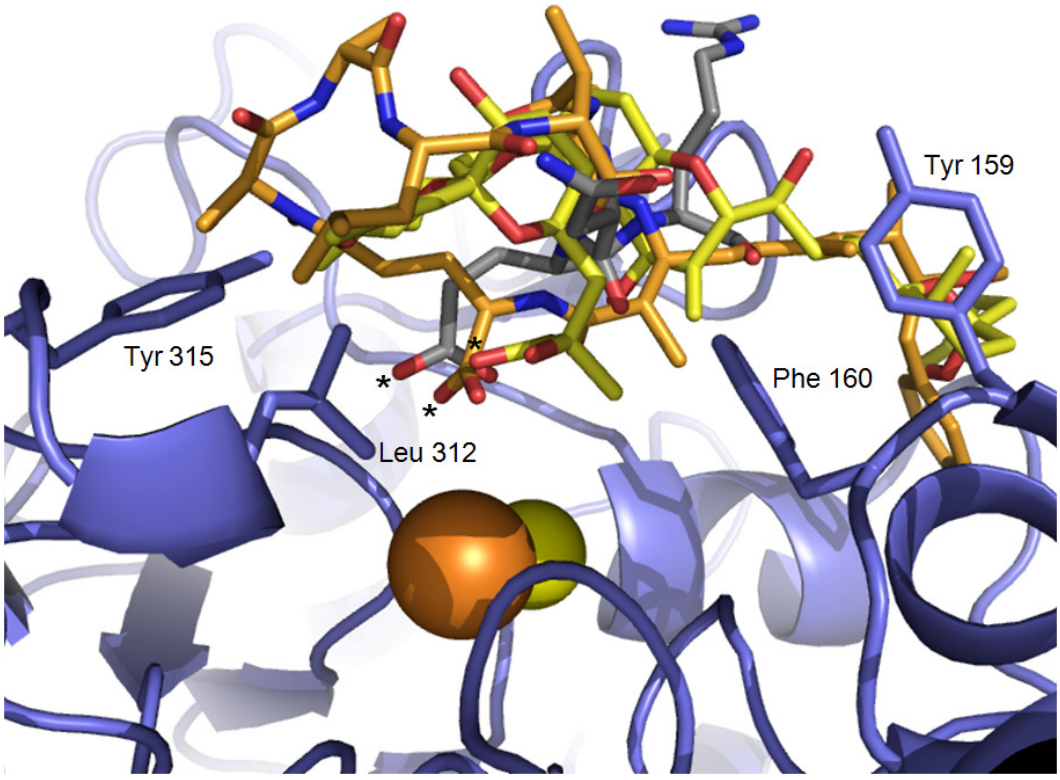



**Figure 2.10: Mode of inhibition of calcineurin by toxins and the autoinhibitory domain.** Superimposition of OA (yellow), MCLA-2H (orange), and the three residues of AI domain (480-NER-482, grey) is shown in stick mode in the active site of CaN. The catalytic metals are shown as dark orange and yellow spheres, representing $Fe^{3+}$ and $Zn^{2+}$, respectively. The carbonyl group of OA, γ-linked D-Glu residue of MCLA-2H, and the carboxyl group of Glu-481 of AI at the active site are highlighted with asterisks. The close proximity of these groups to one another suggests that natural toxins and AI domain effect CaN inhibition in a same fashion. The four residues that were subject of mutagenesis in the current study are indicated.[180, 217, 218]

the active site—by placing a negatively charged moiety against the metal ions, as first pointed out by Barford (1996). Therefore additional interactions determine binding strength and thus potency of inhibition of OA and MCLR. These interactions also govern to what extent the AI domain can compete with each toxin.

Sensitivity of the quadruple mutant CaN construct could be taken advantage to study native protein interactions of CaN. Recently, it was demonstrated in Dr. Holmes laboratory that MCLR-sensitive CaN-A ($IC_{50}$ = 1.8–2 nM), along with CaN-B and CaM regulatory proteins, could be trapped by microcystin-Sepharose resin (see Figure 2.11, p. 116). Such resin interaction could be used to sequester native CaN binding partners from cell extracts or other media. This method has been successful in identifying novel binding partners of PP-1c, and it could be expected that novel CaN binding proteins will be found with this powerful technique.[283]

The ability of CaN-A to interact with CaN-B and CaM while trapped on a microcystin resin is an important result regarding the folded state of CaN-A. Assembly of CaN holoenzyme supports the notion that CaN-A mutants used in this study are properly folded. Fully retained activity of the mutants is also in agreement with that interpretation. Structural integrity of the mutants could also be examined by a western blot analysis.

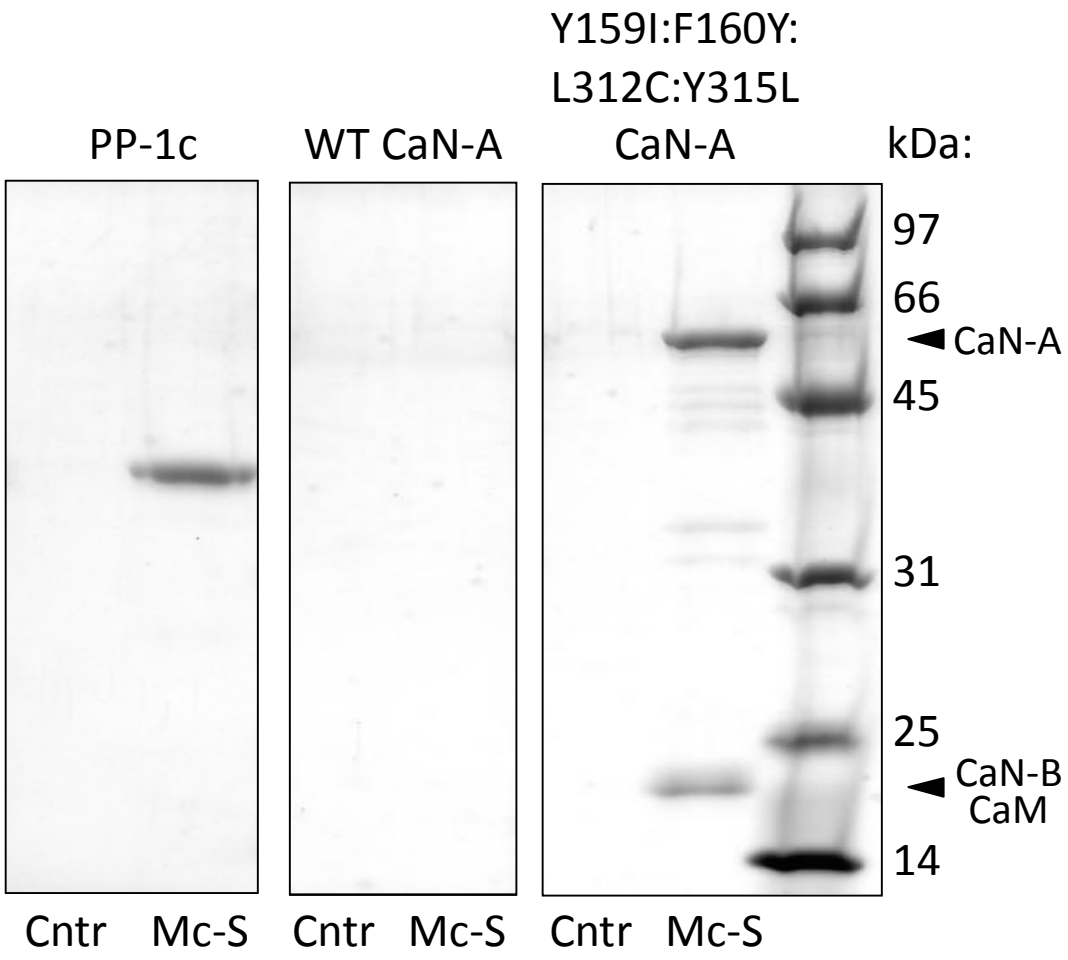



**Figure 2.11: Binding of calcineurin to microcystin-Sepharose facilitates study of calcineurin interaction with its binding partners.** Human PP-1cγ (140 pmol) and rat quadruple mutant (Y159I:F160Y:L312C:Y315L) CaN-Aαδ (430 pmol), but not WT CaN, are shown binding to microcystin-Sepharose (Mc-S). No proteins bound to the control Tris-Sepharose resin (Cntr). Proteins were mixed with resin over night and the mixture run on the gel in its entirety. Binding of CaN-A to microcystin-Sepharose was performed in presence of CaN-B and CaM (3 × the molar amount of CaN-A).

The recent structures of PP-2Ac bound to MCLR and OA shed further light on the differential toxin sensitivities among members of the PPP family.[305] Surprisingly, the authors' sole explanation for the different toxin sensitivities of PP-2Ac and PP-1c is a hydrophobic pocket in PP-2Ac, formed by Gln-122, Ile-123, His-191, and Trp-200, that accommodates the hydrophobic Adda side chain of MCLR and the hydrophobic tail of OA (see Figure 1.15, p. 41). The hydrophobic pocket is not as fully formed in PP-1c and the authors suggest that this is the reason for the different $IC_{50}$s seen for PP-1c and PP-2Ac. Asp-197 in PP-1c is farther away from OA than the corresponding His-191 in PP-2Ac. Also, van der Waal interactions would not be expected to be as strong with Ser-129 (PP-1c) compared to Gln-122 (PP-2Ac). These authors did not mention the role of the β12–13 loop in toxin binding.

Nevertheless, work done in the Holmes laboratory supports the above hypothesis of the importance of the hydrophobic pocket. Mutation of PP-1c Ser-129 to the corresponding residue found in CaN, S129H, resulted in 3- and 4-fold decreases in sensitivity to OA and MCLR, respectively.[87] This suggests that a reverse mutation in the corresponding CaN residue (His-155) to an amino acid found in PP-2Ac (Gln-122), H155Q, might further elucidate CaN's toxin resistance. Mutation of a CaN residue that corresponds to PP-2A's His-191, A223H, would also be a good future experiment. These mutations would shape CaN's toxin binding hydrophobic pocket more closely to PP-2Ac.

Only one other structure in the PPP family of phosphatases has been solved—that of PP-5.[276] This protein is also highly sensitive to OA and MCLR and its toxin binding site is remarkably similar to those of PP-1c and PP-2Ac (see Table 1.1, p. 5).[56, 147, 305] Only one residue involved in PP-5 toxin binding is markedly different from PP-1c or PP-2Ac. It is Met-455, a residue which corresponds to Tyr-315 in the L7 loop of CaN. Since this position is important for toxin binding, it would be interesting to test the sensitivity of a Y315M mutation. Another candidate for further study is the variable residue Val-314 of CaN. The corresponding residues found in PP-1c, PP-2Ac, and PP-5 are Glu-275, Arg-268, and Gln-454, respectively.

Given the medical importance of CaN in organ transplantation and cardiac hypertrophy, novel inhibitors of CaN are eagerly pursued. The modeling of our quadruple mutant bound to toxins could be useful in the modification of such inhibitors to CaN-specific forms. The modification of microcystin to generate a congener 7-fold more sensitive to PP-1c than to PP-2Ac has already been demonstrated.[3] Chemically engineered CaN-specific compounds could be utilized to study cellular CaN functions without compromising the role of immunophilins. In addition, such compounds could have the potential to act as novel immunosuppressants.

# Chapter 3: Interaction of calcineurin with its substrate protein inhibitor-1 is mediated via the PXIXIT binding motif.

## 3.1 Inhibitor-1 as a calcineurin substrate

Protein phosphatase inhibitor-1 (I-1) has been demonstrated to regulate PP-1c function in several mammalian tissues.[4, 114, 174, 226, 228] When phosphorylated, I-1 becomes a potent and specific inhibitor of PP-1c activity.[5, 102, 231, 260] This role of I-1 has been best studied in the β-adrenergic signaling pathway.[4, 113, 228] Deregulation of this pathway has been linked to cardiomyopathy, and failed human hearts have been shown to have reduced I-1 levels with concomitant increase in PP-1c activity.[52, 98, 99, 229] Phosphorylated I-1 (I-1-P) is also an excellent substrate of CaN.[159, 274, 275] CaN can relieve I-1-P inhibition of PP-1c by dephosphorylating I-1-P, thus increasing the cellular pool of active PP-1c. Examples of this phosphatase cascade have been well documented in both cardiac muscle and brain (see Figure 1.21, p. 58).[65, 127, 226]

Binding of I-1 to PP-1c is mediated via a KIQF sequence, a form of the RVXF motif found in many PP-1c binding partners.[54, 95, 102, 103, 155, 258, 260, 280] The binding groove on PP-1c that interacts with the RVXF motif is structurally related to a similar but altered groove found in CaN (see Figures 1.19, p. 52, and 1.20, p. 54). The CaN groove accommodates a PXIXIT amino acid sequence, first found

in NFAT proteins which interact via this sequence with CaN; therefore, PP-1c and CaN do not share any known binding partners.[193, 277]

The notion that a binding groove in PP-1c that can interact with I-1 has a structurally related counterpart in CaN, and the fact that both CaN and PP-1c bind I-1, raised the question of whether I-1 could interact with both of these phosphatases via this related binding groove. This prompted a closer examination of the I-1 sequence to see if I-1 harbours a PXIXIT motif that could explain its interaction with CaN.

Relatively few proteins are known to contain a PXIXIT motif. However, comparison of these proteins shows that the PXIXIT motif is not canonical and can accommodate degeneracy (see Table 3.1, p. 121). The I-1 amino acid sequence was compared with other currently known motifs and shown to be a very close fit. Of the eleven residues analyzed around the PXIXIT sites of other proteins, I-1 shows nine amino acids that have been previously observed, including five residues within I-1's putative PXIXIT motif itself.

The sequence of the first 54 amino acids of I-1 is shown below. When phosphorylated on Thr-35, this same peptide is known to inhibit PP-1c to the same degree as full length I-1 protein.[5, 6, 102, 103]

[1]MEQDNSPRKIQFTVPLLEPHLDPEAAEQIRRRRPT*PATLVLTSDQSSPEVDEDR[54]

**Table 3.1: Comparison of the putative inhibitor-1 PXIXIT motif with published PXIXIT motifs found in other proteins.**

| Protein | Motif | | | | | | | | | | | | Refs |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | -3 | -2 | -1 | 1 | 2 | - | 3 | 4 | 5 | 6 | +1 | +2 | |
| Human NFAT1 | G | L | S | P | R | | I | E | I | T | P | S | [11] |
| Human NFAT2 | L | E | S | P | R | | I | E | I | T | S | C | [11] |
| Human NFAT3 | L | E | C | P | S | | I | R | I | T | S | I | [11] |
| Human NFAT4 | F | E | C | P | S | | I | Q | I | T | S | I | [11] |
| A238L Protein | K | K | K | P | K | | I | I | I | T | G | C | [109] |
| $p12^I$ protein | L | L | S | P | S | | L | P | L | T | M | R | [109] |
| Human cabin1/cain | K | F | P | P | E | | I | T | V | T | P | P | [109, 186] |
| AKAP79 | R | M | E | P | I | A | I | I | I | T | D | T | [64, 109] |
| Human RCAN1 | D | T | T | P | S | | V | V | V | H | V | C | [136, 213, 220, 225] |
| Yeast Rcn1 | S | K | V | G | A | | I | T | I | D | R | C | [194] |
| Yeast Rcn2 | P | K | S | P | S | | I | T | V | N | E | F | [194] |
| Murine TRESK | E | A | I | P | Q | | I | V | I | D | A | G | [83] |
| Human TRESK | E | A | V | P | Q | | I | I | I | S | A | E | [195] |
| Yeast Slm1 | D | Q | V | P | N | | I | Y | I | Q | T | P | [43] |
| Yeast Slm2 | N | R | V | P | E | | F | Y | I | E | N | V | [43] |
| Yeast Crz1p | P | V | T | P | I | | I | S | I | Q | E | F | [39, 109] |
| Yeast Hph1 | S | R | L | P | V | | I | A | V | N | D | N | [131] |
| VIVIT peptide | G | P | H | P | V | | I | V | I | T | G | P | [13] |
| Human Elk-1 | V | H | I | P | S | | I | Q | I | T | G | L | [176] |
| Putative I-1 motif | **D** | N | **S** | **P** | **R** | K | **I** | **Q** | F | **T** | **V** | **P** | |

Residues of a putative CaN binding motif highlighted in bold font designate amino acids that have previously been found in other PXIXIT motifs. Residues of the PXIXIT motif are designated 1-6, with the upstream and downstream residues marked with "-" and "+" prefixes, respectively.

In the amino acid sequence above, the KIQF sequence important for PP-1c binding is underlined. The box outlines a sequence that resembles a PXIXIT motif, in this case PXXIXXT; that is, the motif has an additional amino acid insertion and the last isoleucine of the motif is replaced with a phenylalanine residue. The threonine residue that is phosphorylated to form the CaN substrate is marked with an asterisk.

*The hypothesis that I set out to test in this chapter of the thesis was that mutagenesis of the inhibitor-1 PXIXIT motif, or mutagenesis of the PXIXIT motif binding groove in calcineurin will reduce dephosphorylation of inhibitor-1 by calcineurin.*

The notion that I-1 contains a motif that allows I-1 to bind CaN in the same fashion as NFAT proteins is supported by the following evidence:
1) The CaN substrate AKAP79 contains a PXIXIT motif and interacts with CaN via this motif. AKAP79 has an amino acid insertion in the PXIXIT sequence.[109, 192] This suggests that there is flexibility with respect to how many residues can be accommodated by a PXIXIT motif that can bind to CaN; thus the additional amino acid in the I-1 putative PXIXIT motif could be acceptable;
2) NFAT1 and 2 sequences contain arginine in the second position of the PXIXIT motif; arginine is also found in the PRKIQFT sequence of I-1;[11]
3) The NFAT4 sequence contains a glutamine residue in the fourth position of the PXIXIT motif as does I-1 (see Table 3.1, p. 121);[11]

4) Several NFAT sequences contain serine one residue upstream of the PXIXIT motif; serine is found in I-1 in the same position;[11]

5) In an effort to create an inhibitor of NFAT binding to CaN, mutagenesis of a 16 residue peptide derived from the NFAT sequence containing the PXIXIT motif has been undertaken to generate an inhibitor containing a VIVIT sequence. In the process of the VIVIT peptide generation, it was found that peptides with proline two residues downstream of a PXIXIT motif were the most effective inhibitors; proline is also found two residues downstream from the putative PXIXIT motif of I-1 (see Table 3.1, p. 121);[13]

6) The most conspicuous difference between the putative I-1 PXIXIT motif and PXIXIT motifs in other proteins is a phenylalanine residue in place of the typical isoleucine in the fifth position of the motif (see Table 3.1, p. 121). This residue has not been previously observed in any of the motifs, with isoleucine being the most common amino acid seen in these motifs. Nevertheless, some variation has been observed which could suggest that a more bulky residue such as phenylalanine could be accommodated. In addition, Li et al. (2007) have shown that CaN can also bind threonine in this position.[195]

Furthermore, previous structural data has shown that a residue in position five does not undergo as extensive binding as the preceding isoleucine residue of the motif in the third position of the PXIXIT sequence. Since the isoleucine residue in the third position of the motif has been shown to accommodate phenylalanine, valine or leucine in previous mutagenesis studies, it could be

speculated that the even less buried fifth residue could allow more flexibility in accepting another hydrophobic residue such as phenylalanine.[13, 109, 280]

The sequence comparison of I-1 with the binding partners of CaN that also contain PXIXIT motifs suggests that I-1 contains a degenerate form of this motif, PXXIXXT. This would make I-1 the only protein known to interact with both CaN and PP-1c via structurally similar binding grooves—the PXIXIT motif binding groove in CaN, and the RVXF motif binding groove in PP-1c—allowing the downstream Thr-35 residue to reach the active site of each phosphatase.

The VIVIT peptide was designed from NFAT's own PXIXIT motif as a competitive inhibitor of the interaction of NFAT with CaN. This was accomplished through selective mutagenesis of the PXIXIT motif amino acids that would increase binding to CaN beyond that of the native motif. Introduction of the VIVIT sequence in NFAT improved its binding to CaN to a point that even unstimulated T-cells demonstrated NFAT dephosphorylation.[13]

It has been established that VIVIT peptide inhibits NFAT binding to CaN at an $IC_{50}$ of ~0.5 μM by directly competing for the PXIXIT motif binding groove of CaN.[11, 13] Another CaN substrate analyzed for inhibition by VIVIT was the $R_{II}$ peptide. The $R_{II}$ peptide is the minimal protein kinase A (PKA) sequence that is dephosphorylated by CaN as efficiently as the full length PKA, making the $R_{II}$ peptide one of the most common substrates of CaN used in *in vitro* assays.[30, 31]

However, since the $R_{II}$ peptide does not interact with the PXIXIT motif binding groove of CaN, the VIVIT peptide does not impact the dephosphorylation of $R_{II}$ peptide by CaN. In this way it was demonstrated that the VIVIT peptide is a specific inhibitor of proteins that interact with CaN by means of PXIXIT motif binding.[13] Thus VIVIT can be used in the same manner to test for I-1 interactions with CaN.

In the present study, the interaction of CaN with the potential PXIXIT motif of I-1 was investigated either through mutagenesis of the PXIXIT motif binding groove of CaN, or by mutation of the motif itself in I-1. The putative PXIXIT motif in full length I-1 was mutated to a series of alanine residues. The effect of deletion of the motif was also studied using I-1 peptides. The interaction between I-1 and CaN was further analyzed by generating two different mutants of the PXIXIT motif binding groove in CaN and testing interactions of I-1 with the mutant CaN. One mutant, termed NIR CaN, had three tandem residues altered: 330-NIR-332→ 330-AAA-332. The NIR mutant was chosen for further mutagenesis to generate a sextuple mutant Y288A:M290A:F299A:330-NIR-332→330-AAA-332 (termed F299A for simplicity). All of the residues chosen for mutagenesis were shown to be important in the formation of the binding groove shape, and all are involved in VIVIT binding (see Figure 1.19, p. 52).[193, 195, 277]

## 3.2 Experimental Procedures

*Materials.*

CaN, CaM, CaM-Sepharose 4B resin, *p*NPP, PKA and I-1 protein were obtained as described in chapter two Experimental Procedures (p. 75). I-1 peptides were obtained from the Alberta Peptide Institute (Edmonton, AB). VIVIT peptide was purchased from Calbiochem (San Diego, CA). $R_{II}$ peptide was donated by Dr. Shairaz Baksh (University of Alberta, Edmonton, AB). All other reagents were purchased from Sigma-Aldrich (Oakville, ON), Fisher Scientific (Ottawa, ON) and EMD Chemicals Inc. (Gibbstown, NJ) unless otherwise stated.

*Construction of mutant isoforms.*

The primers used in this work for mutagenesis of CaN and I-1 are listed in Table 3.2, p. 127. DNA isolation and mutagenesis was performed as described in chapter two Experimental Procedures (p. 76–78).

*Transformation*.

PCR products were transformed into MAX Efficiency DH5α competent cells (Invitrogen) as described in the protocol provided with the cells, except that half volumes of cells and media were used. Miniprep DNA of I-1 mutants verified with sequencing were transformed into BL21α *E. coli* (Stratagene) in the same manner used with DH5α cells. Miniprep DNA of CaN-A mutants were transformed as described in chapter two Experimental Procedures (p. 79).

**Table 3.2: Primers used in the generation of inhibitor-1 and calcineurin mutants.**

| I-1 mutant | Template used | Mutagenesis primer | Novel restri-ction site |
|---|---|---|---|
| P7A | WT[a] | GGTCGCGGATCGATGGAGCAAGACAACA GCGCCCGAAAGATCCAGTTCACGGTCCC | ClaI |
| I10A | P7A | CGATGGAGCAAGACAACAGCGCCCGAAA GGCCCAGTTCACGGTCCCG | BglI |
| S6A: R8A | I10A | CGGATCGATGGAGCAAGACAACGCGGCC GCGAAGGCCCAGTTCACGGTCCC | SfiI |
| K9A | S6A: R8A | GCGGCCGCGGCGGCGCAGTTCACGGTCCC GCTGCTGG | SacII |
| F12A | K9A | GCGGCGGCGCAGGCCACGGTACCGCTGCT GGAGCC | RsaI |
| Q11A | F12A | CAACGCGGCCGCGGCAGCGGCGGCCACG GTACCGCTGC | --- |

| CaN mutant | Template used | Mutagenesis primer | Novel restri-ction site |
|---|---|---|---|
| NIR[b] | WT[a] | CGAGAACAACGTGATGGCCGCCGCGCAG TTCAACTGCTCCCCCC | BstUI |
| Y288A: M290A | NIR[b] | GCCCAGGACGCAGGGGCCCGCGCGTACA GGAAAAGCC | ApaI |
| F299A | Y288A: M290A | GCCAAACAACTGGCGCCCCGTCTCTAATT ACG | NarI |

[a]WT refers to full length wild type I-1 or CaN; [b]NIR refers to the 330-NIR-332→ 330-AAA-332 CaN mutant. Each mutant shows only one of the two complimentary primers, from 5' to 3'.

*DNA restriction digests.*

Restriction digests were performed according to the Invitrogen protocol provided with each restriction enzyme; reactions were stopped for 10 min at 95 °C and visualized on 1% agarose gel with ethidium bromide as described in Sambrook and Russel.[252]

*Inhibitor-1 purification and phosphorylation.*

Described in chapter two, Experimental Procedures (p. 79–82).

*Calmodulin-Sepharose 4B column preparation.*

CaM-Sepharose 4B affinity resin was prepared and regenerated as described in chapter two, Experimental Procedures (p. 83), except that the final buffer 4 consisted of 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM DTT, 0.4 mM PMSF.

*Calcineurin-A catalytic subunit purification.*

Mutagenesis of the PXIXIT motif binding groove of CaN-A was undertaken to generate following mutants 330-NIR-332→330-AAA-332 (termed NIR), and Y288A:M290A:F299A:330-NIR-332→330-AAA-332 (termed F299A, see Figure 3.1, p. 129). Wild type (WT) CaN and mutant forms of CaN were expressed and purified as previously described with following changes.[303] Transformed *E. coli* cells were cultured in terrific media and induced with IPTG as described for CaN-A subunit purification in chapter two, Experimental

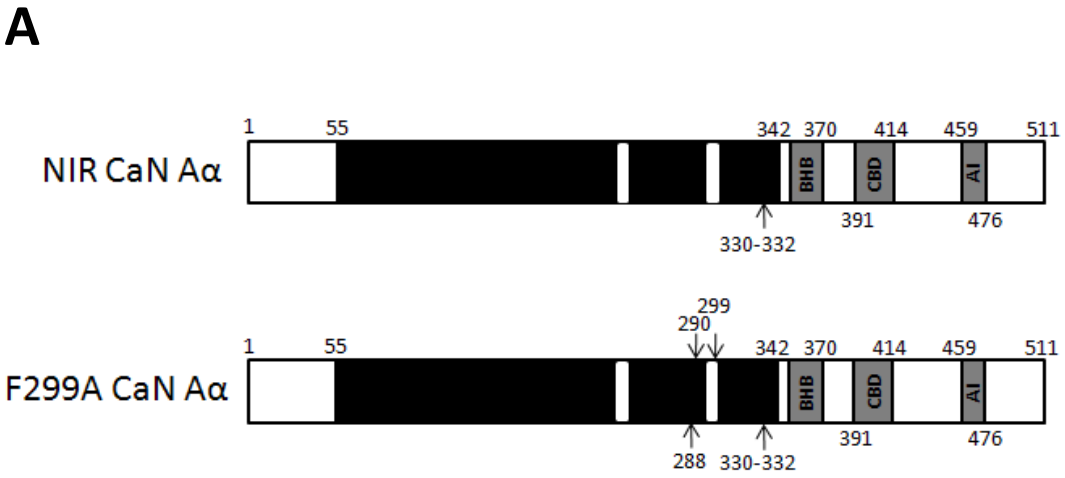



**Figure 3.1: Calcineurin and inhibitor-1 mutants that abrogate interaction with the PXIXIT motif binding groove.** (A) This panel depicts a schematic showing the mutants of CaN used in the study. Arrows point to residues in CaN that were mutated. Residue numbers outline the different domains of CaN; the catalytic domain is indicated in black, as defined by Barton et al. (1994). NIR = 330-NIR-332→330-AAA-332; F299A = Y288A:M290A:F299A:330-NIR-332→ 330-AAA-332. (B) This panel depicts the alignment of the first 25 amino acids of wild type (WT) and mutant protein phosphatase inhibitor-1 (I-1) proteins used in the study. Six residues covering the putative PXIXIT motif plus one upstream residue were substituted for Ala amino acids. The I-1 mutant was termed F12A for simplicity.

Procedures (p. 83–85), except that cells were grown for 4–7 h. Pelleted cells were resuspended in buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 2 mM BME, 0.4 PMSF) and disrupted as described for I-1 purification, chapter two, Experimental Procedures (p. 79). The subsequent steps were all done at 4 °C. Sonified cells were centrifuged at 16 300 × g, for 20 min. Supernatant was made up to 45% in $(NH_4)_2SO_4$, stirred for 10 min and incubated on ice for minimum of 40 min. The precipitate was centrifuged at 11 000 × g for 30 min. The pellet was resuspended in 2 mL of buffer A, combined with 10 mL of CaM-Sepharose 4B resin, and mixed end-over-end for 20 min. $CaCl_2$ (2 mM according to total volume) was added and the mixture was mixed for additional 30 min. The resin was packed into a 25 mm diameter column and washed with 10 volumes of buffer B (50 mM Tris-HCl, pH 7.5, 0.5 mM $CaCl_2$, 2 mM BME, 0.4 mM PMSF). Proteins were eluted from CaM-Sepharose resin by gravity using buffer C (50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1 mM DTT, 0.4 mM PMSF) in 1 mL fractions. The protein content was determined by SDS-PAGE and CaN activity was measured against *p*NPP substrate. Purest fraction that also exhibited CaN activity were pooled and concentrated to 1 mL. The final concentrate was dialyzed against 50 % glycerol buffer A (1.5 L) for 3–5 h. Purified protein was stored at -20 °C. Proteins were purified to near homogeneity with the following yields and specific activities observed: WT = 1.5 mg, 7 U/mg; NIR = 5 mg, 4.7 U/mg; F299A = 0.5 mg, 3.5 U/mg.

*Calcineurin-B purification.*

Described in chapter two, Experimental Procedures (p. 87–88).

*Inhibitor-1 protein and pNPP calcineurin phosphatase assay.*

See chapter two, Experimental Procedures for details (p. 88–89).

*Inhibitor-1 peptide substrate calcineurin assay*.

It has been previously demonstrated that deletion of the RVXF motif in I-1-P attenuates its ability to bind and inhibit PP-1c.[5, 6, 102, 103, 258, 260] Since the putative PXIXIT motif in I-1 actually overlaps the RVXF motif, deletions of the sequence containing the RVXF motif should also have an impact in CaN binding, and this could be monitored by altered substrate dephosphorylation. A peptide consisting of the first 54 residues of I-1 (termed protein phosphatase inhibitor peptide of 54 residue length, PPIP54) when phosphorylated at Thr-35 (PPIP54-P) inhibits PP-1c with the same potency as full length I-1-P.[5, 6, 102, 103] The shortest phosphorylated fragment of I-1 that inhibits PP-1c with the same potency as full length I-1 is a peptide consisting of residues 9–54 (PPIP46-P) of the I-I protein (see Table 3.3, p. 132). An assay was developed to monitor the dephosphorylation of I-1-P peptides. PPIP54-P, PPIP46-P, and a 12–41-mer, PPIP30-P (all phosphorylated on Thr-35), were tested as substrates for CaN. In PPIP46-P, the first two residues of the putative PXIXIT motif have been deleted. In PPIP30-P, all but the last two residues of the putative PXIXIT motif have been deleted (see Table 3.3, p. 132).

**Table 3.3: Protein phosphatase inhibitor-1 peptides used in the calcineurin dephosphorylation assays.**

| Peptide name and sequence |
| --- |
| PPIP54: $^{1}$MEQDNSPRKIQFTVPLLEPHLDPEAAEQIRRRRPT*PATLVLTSDQSSPEVDEDR$^{54}$ |
| PPIP46: $^{9}$KIQFTVPLLEPHLDPEAAEQIRRRRPT*PATLVLTSDQSSPEVDEDR$^{54}$ |
| PPIP30: $^{12}$FTVPLLEPHLDPEAAEQIRRRRPT*PATLVL$^{41}$ |

Thr-35, the site of protein phosphatase inhibitor-1 phosphorylation by protein kinase A and required for PP-1c inhibition, is marked with an asterisk. The putative PXIXIT motif is underlined.[6, 103]

Dephosphorylation assays were performed as described for CaN *p*NPP assay in chapter two, Experimental Procedures (p. 88) with the following changes. I-1 peptide substrates were used instead of *p*NPP. Prior to addition of substrate to the assay, the assay buffer containing 0.3 pmol of CaN-A (with minimum 5-fold excess of CaN-B and CaM) was pre-incubated at 30 °C for 10 min. Phosphorylated peptide (400–600 pmol) was added and the mixture was further incubated for 1–5 min at 30 °C. Reactions were stopped with 0.1% TFA which inactivates the enzyme by lowering the pH. The dephosphorylation rate was measured using a Hewlett-Packard Series 1100 HPLC by directly monitoring the amount of both phosphorylated and dephosphorylated forms of the peptide.

Products of the reaction were loaded onto a narrowbore Vydac $C_{18}$ column (218TP52) equilibrated in solution A (10 mM $NH_4C_2H_3O_2$) and eluted using a protocol that allowed for separation between phosphorylated and dephosphorylated peptides. The column was developed in a 75 min gradient program: 0–20% solution B (50% 20 mM $NH_4C_2H_3O_2$:50% $CH_3CN$) in 5 min, 20–80% solution B in 60 min, 80–100% solution B in 5 min, 100% solution B for 2 min, and 100% solution A for 3 min. Absorbance was monitored at a wavelength of 206 nm. Phosphorylated and dephosphorylated peptides were observed as separate peaks on the chromatogram. Known quantity of peptide eluted from the column was considered to be consistent with the intensity of absorbance at the characteristic retention time, as measured by absorbance peak heights. Therefore, enzymatic dephosphorylation rates were estimated by

measuring respective peak heights.

*$R_{II}$ peptide labeling and dephosphorylation assay.*

$R_{II}$ peptide preparation and dephosphorylation by CaN was performed as described previously, except that no okadaic acid was used in the assay.[116] In brief, buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM $CaCl_2$, 0.5 mM DTT, 0.1 mg/mL BSA, 0.5 mM $MnCl_2$, 0.3 μM CaM, CaN-A (0.3 pmol) and 5-fold excess of CaN-B was mixed with various dilutions of VIVIT peptide for a total volume of 40 μL. Reaction was initiated by addition of 20 μL of $R_{II}$ substrate (15 μM). Samples were incubated at 30 °C for 15 min and reaction was stopped by addition of 500 μL of buffer containing 1 M $K_2HPO_4/KH_2PO_4$, pH 7.0, 25% TCA. Stopped samples could be stored indefinitely at room temperature. Samples were separated over Dowex cation exchange columns.

Columns were prepared as follows. 20 grams of resin was washed in 500 mL of $H_2O$, followed by washing in 50 mL of 1M NaOH, 100 mL of 1N HCl, and 200 mL of $H_2O$. Washed resin was resuspended in 23 mL of $H_2O$, and 0.5 mL of resin slurry was placed in polyprep columns (BioRad). $H_2O$ was allowed to flow through and the 560 μL CaN dephosphorylation reaction samples were applied to the resin and washed with 0.5 mL of $H_2O$. The flow through was collected, mixed with 5 mL of scintillation fluid, and measured for radioactivity using a Wallac 1209 Rackbeta scintillation counter (Pharmacia).

*SDS-PAGE gel electrophoresis.*

Purity of the protein preparations was assessed using SDS-PAGE as described in chapter two, Experimental Procedures (p. 90).

*Protein determinations.*

Purified proteins were quantitated as described in chapter two, Experimental Procedures (p. 91).

*Contributions to this work.*

All of the work described was produced by the author with exception of preparation of the $R_{II}$ substrate, and $R_{II}$ peptide dephosphorylation by CaN, performed by Dr. Shairaz Baksh.

## 3.3 Results

*VIVIT peptide inhibits inhibitor-1 dephosphorylation.*

The hypothesis that I-1 contains a PXIXIT motif was tested by measuring VIVIT peptide competition for CaN binding against I-1-P. If I-1-P uses the PXIXIT motif to interact with CaN, then VIVIT would be expected to abrogate this interaction and a reduced dephosphorylation of I-1-P substrate by CaN should be observed. It was shown that VIVIT inhibited I-1-P dephosphorylation by CaN in a dose dependent manner, with an $IC_{50}$ of 8.79 μM. In contrast, no inhibition was seen when the $R_{II}$ peptide was used as a substrate (see Figure 3.2, p. 136).

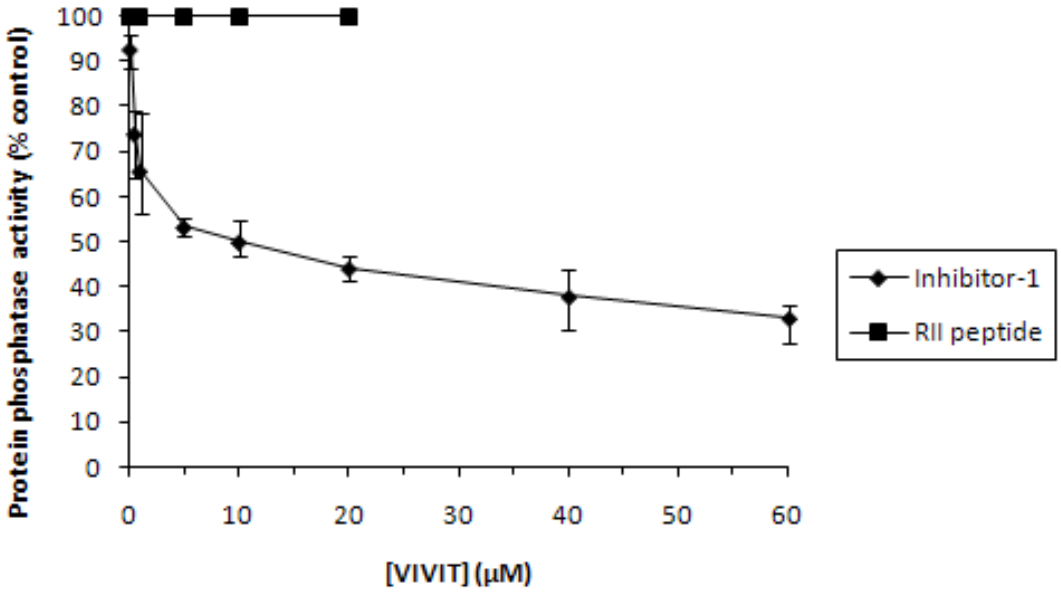



**Figure 3.2: VIVIT peptide inhibits inhibitor-1 substrate dephosphorylation.** The VIVIT peptide was shown to reduce CaN enzyme activity against phosphorylated inhibitor-1 (I-1-P) substrate with an $IC_{50}$ of 8.79 ± 2.95 µM, but not against $R_{II}$ peptide substrate. Summarized on the figure are five different experiments with two separate enzyme and substrate preparations; each point was performed in duplicate. The $R_{II}$ peptide control was tested in a single assay, with each point performed in duplicate. All values of $R_{II}$ dephosphorylation are shown as 100% control for simplicity; activity higher than control was observed in some points.

*Deletion of the PXIXIT motif reduces peptide substrate dephosphorylation.*

To study the effect that deletion of the PXIXIT motif would have on I-1 dephosphorylation, I-1 peptides containing the full length motif or a compromised motif (see Table 3.3, p. 132) were used as substrates for CaN. The peptide comprising the amino acid sequence of first 54 residues of I-1 (PPIP-54), encompassed the PXIXIT motif (residues 7–13) and the I-1 phosphorylation site (Thr-35). Peptides with the compromised PXIXIT motif exhibited truncation of the motif to varying degree (PPIP46 and PPIP30). Peptide dephosphorylation was monitored using HPLC, as described in Experimental Procedures (p. 133).

It was noted that 410 pmol PPIP54-P is dephosphorylated more efficiently than 410 pmol PPIP30-P peptide (see Figures 3.3 and 3.4, p. 138–139). Most of the PXIXIT motif is missing in PPIP30-P. A peak of dephosphorylated PPIP54 appeared about 2-fold faster than the dephosphorylated peak of PPIP30 (see Figure 3.5, p. 140).

Interestingly, the PPIP46-P peptide—which is missing the first 8 residues of the I-1 protein sequence and the first two residues of the PXIXIT motif—appeared to be dephosphorylated at an efficiency half way between that of PPIP54-P and PPIP30-P (see Figure 3.5, p. 140). Similar results were also observed for all of the above peptides when 580 pmol of peptide was used as substrate.

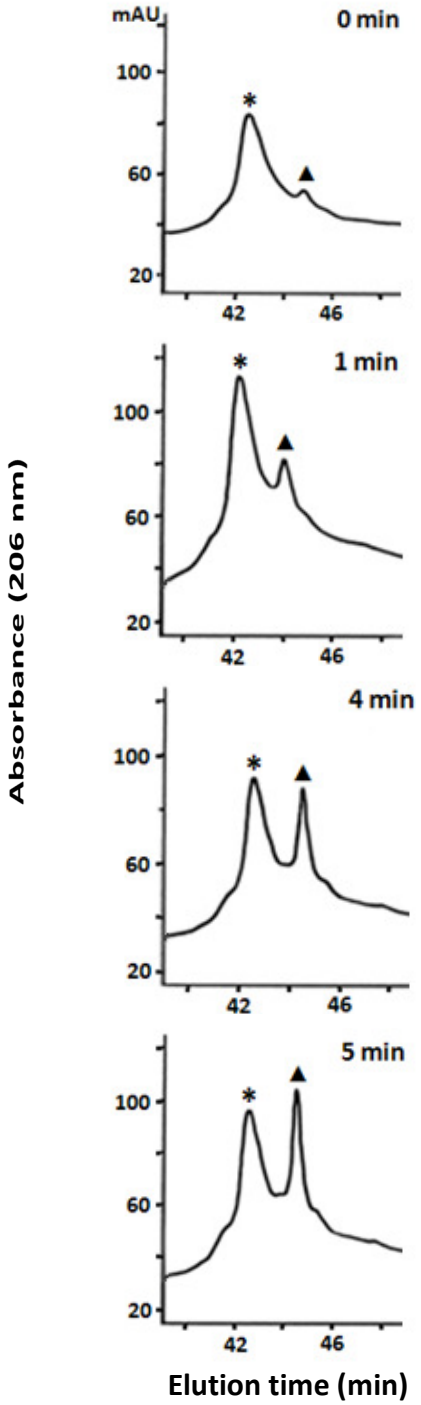



**Figure 3.3: The PXIXIT motif facilitates rapid substrate dephosphorylation by calcineurin.** HPLC chromatographs of CaN dephosphorylation of 410 pmol PPIP54-P, a protein phosphatase inhibitor-1 peptide that contains the entire PXIXIT motif, are shown in increasing assay time length. The upper right hand corner of each chromatogram indicates the duration of the assay. Asterisks (*) denote the phosphorylated substrate; triangles (▲) designate the dephosphorylated form of the peptide.

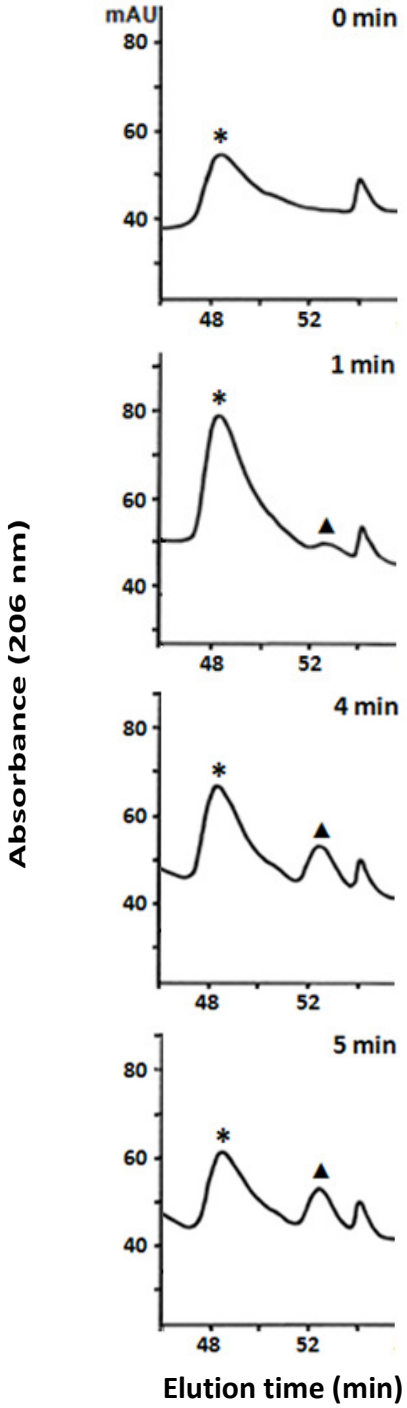



**Figure 3.4: Deletion of the PXIXIT motif decreases substrate dephosphorylation by calcineurin.** HPLC chromatographs of CaN dephosphorylation of 410 pmol PPIP30-P, a protein phosphatase inhibitor-1 peptide that is missing most of the putative PXIXIT motif, are shown in increasing assay time length. The upper right hand corner of each chromatogram indicates the duration of the assay. Asterisks (*) denote the phosphorylated substrate; triangles (▲) designate the dephosphorylated form of the peptide. Peptides were identified using mass spectrometry.

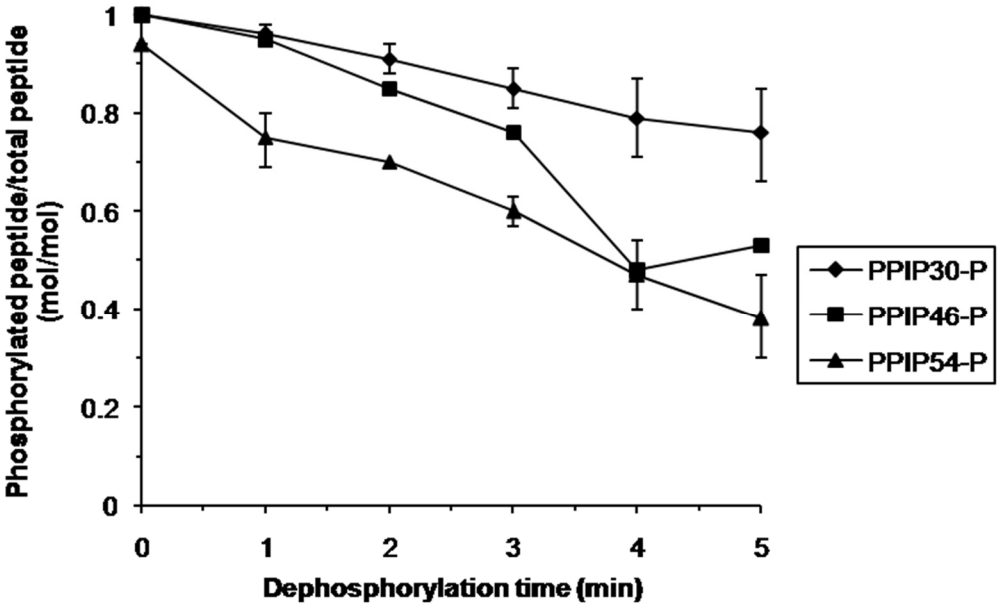



**Figure 3.5: The PPIP54-P peptide is dephosphorylated more rapidly than the peptides with truncated PXIXIT motif (PPIP46-P and PPIP30-P).** 410 pmol of given peptide was dephosphorylated by CaN for a specified duration of time, and the amount of dephosphorylation was measured using HPLC. Assays were performed in duplicate, except for the PPIP46-P peptide.

*Mutation of the PXIXIT motif binding groove alters calcineurin's enzymatic activity.*

Mutagenesis of CaN-A's PXIXIT motif binding groove was undertaken to study its effect on I-1 dephosphorylation. Figure 3.6 (p. 142) shows that both NIR and F299A CaN mutants showed decreased rates of dephosphorylation of WT I-1-P protein with more pronounced effect seen for the F299A mutant of CaN. Both mutants also showed reduced activities against *p*NPP and $R_{II}$ substrates, indicating that the mutant enzyme preparations have reduced enzymatic activity in general. Additional preparations of the enzymes showed similar behavior. The influence of mutagenesis of the PXIXIT motif binding groove of CaN-A on I-1 binding was to be determined indirectly by monitoring the effect of such mutagenesis on I-1 dephosphorylation by CaN. Since mutagenesis of the PXIXIT motif binding groove altered the catalytic behavior of CaN, the impact of such mutagenesis on I-1 binding could no longer be assessed.

*Targeting the PXIXIT motif interaction by mutagenesis of inhibitor-1 and calcineurin-A shows the greatest impact on substrate dephosphorylation by calcineurin.*

To elucidate the interaction of I-1 with CaN, nearly the entire PXIXIT motif of I-1 was mutated to a series of alanine residues: 6-SPRKIQF-12→ 6-AAAAAAA-12 (termed F12A for simplicity). Ser-6 was chosen for mutation as this residue is also commonly found to precede the PXIXIT motif in NFAT proteins. Dephosphorylation of F12A versus WT I-1-P substrate by WT CaN, the

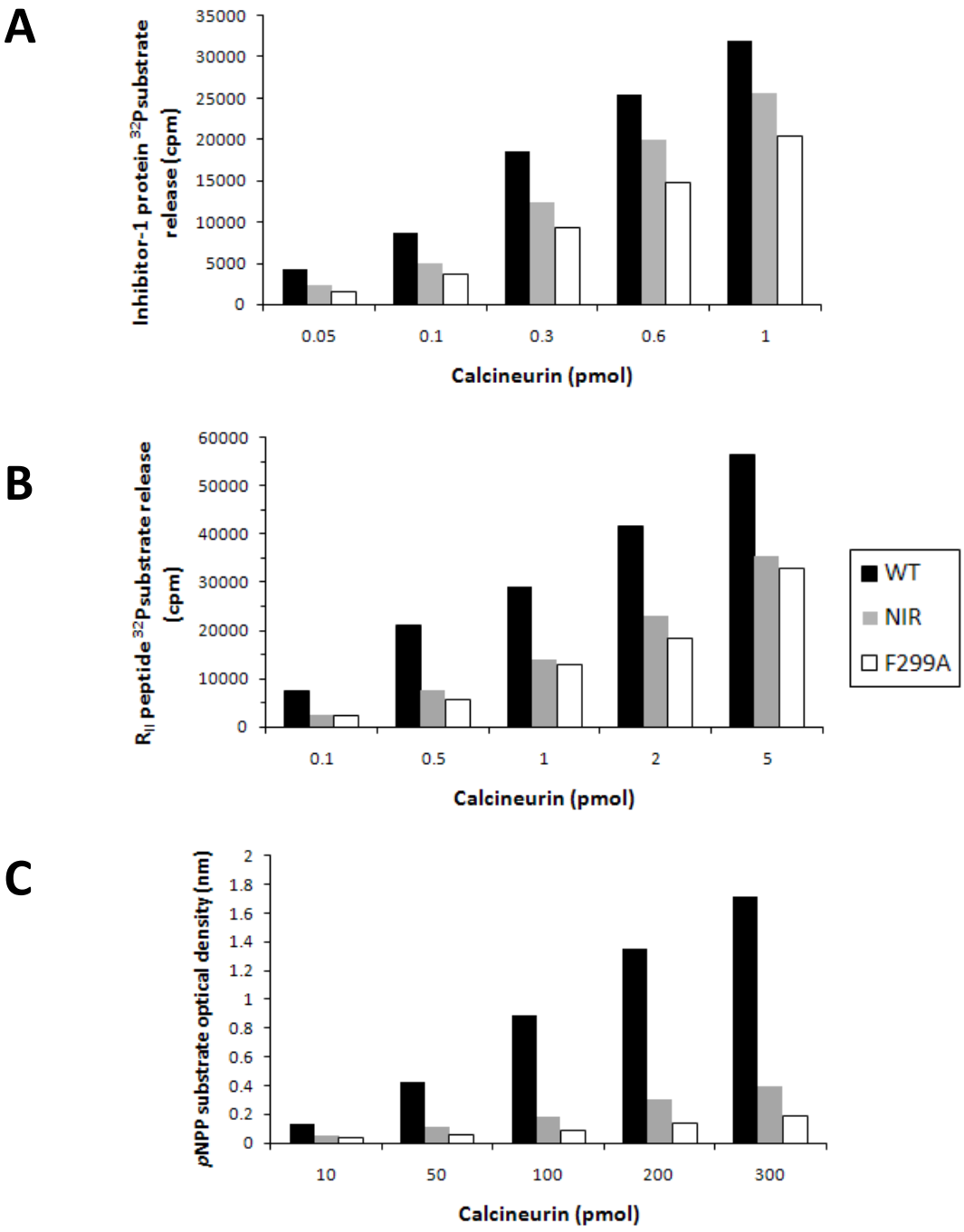



**Figure 3.6: Mutagenesis of calcineurin's PXIXIT motif binding groove alters enzymatic activity.** Comparison of activities of wild type (WT) CaN (black bars), NIR CaN mutant (light grey), and F299A CaN mutant (white) against different substrates: full length protein phosphatase inhibitor-1 protein (panel A), $R_{II}$ peptide (panel B), and *para*-nitrophenylphosphate (*p*NPP, panel C). All assays were done in duplicate. NIR = 330-NIR-332→330-AAA-332; F299A = Y288A:M290A:F299A:330-NIR-332→330-AAA-332.

NIR CaN mutant, and the F299A CaN mutant were compared. Experiments were performed by increasing the amount of CaN enzyme in the assay, or by increasing the amount of substrate.

Mutagenesis of I-1's putative PXIXIT motif resulted in 1.1–1.4-fold decrease in dephosphorylation rate by 0.1–1 pmol WT CaN compared to the rate of dephosphorylation of WT I-1 substrate (see Figure 3.7A, p. 144). Higher fold difference was observed when ≤0.3 pmol enzyme was used. This suggested that mutagenesis of I-1's PXIXIT motif could have a modest effect on its interaction with CaN. Similar trend was seen when the WT and mutant I-1 substrates were dephosphorylated with the NIR CaN mutant (see Figure 3.7B, p. 144).

Comparison of the dephosphorylation of mutated I-1 with WT I-1 by F299A CaN, however, showed a 1.6–2-fold decrease in dephosphorylation rate, depending on the amount of phosphatase present in the assay (see Figure 3.7C, p. 144). Lower amounts of enzyme (≤0.3 pmol) once again had greater impact. This result indicates that mutation of the PXIXIT motif binding groove in CaN had a negative effect on the dephosphorylation of I-1.

Assays were performed in which the amount of enzyme was kept constant, and the amount of substrate was varied. Above experiments showed CaN mutagenesis effect on the dephosphorylation of I-1 was more evident at lower levels of CaN. Thus a constant 0.1 pmol of CaN was used while varying the

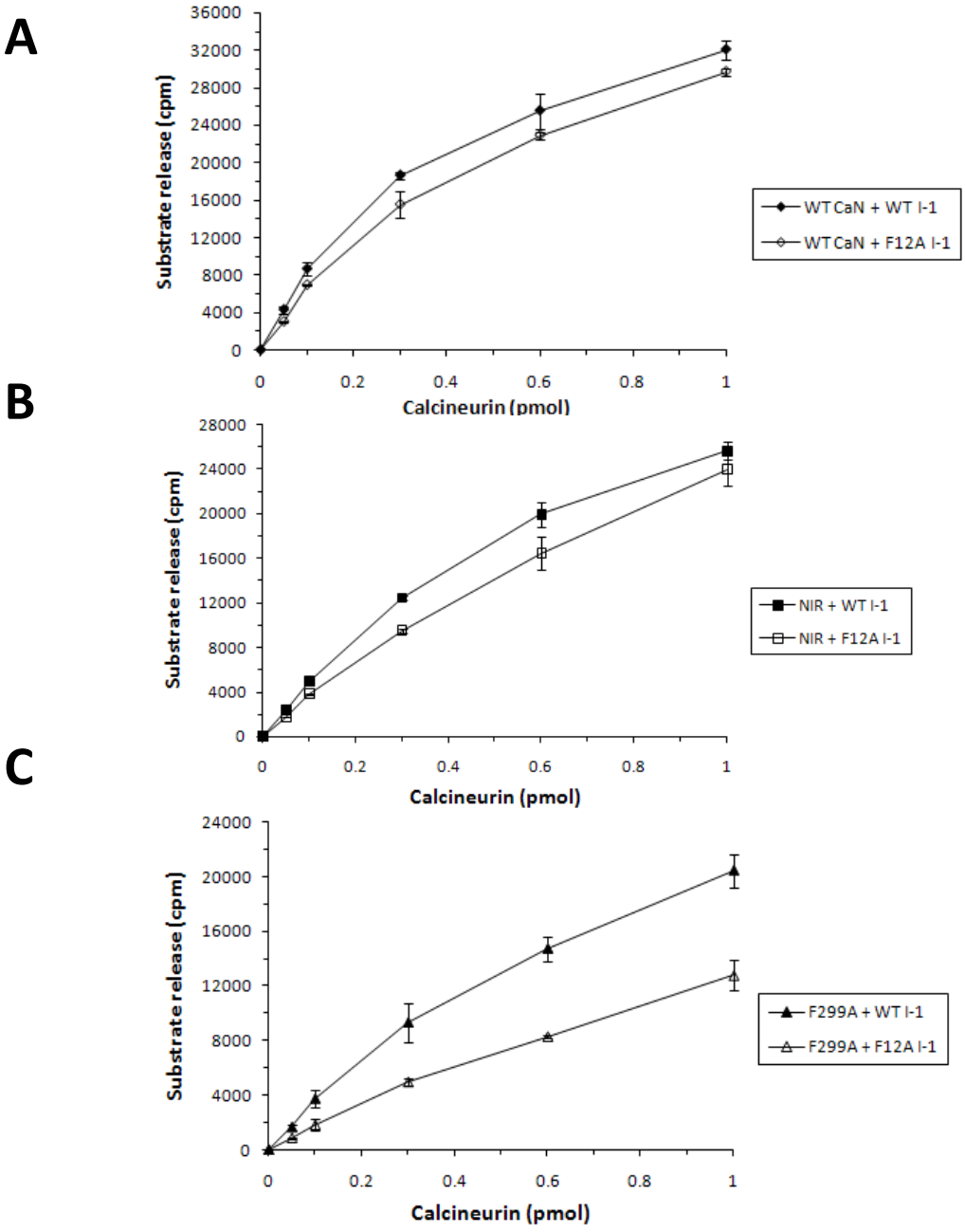



**Figure 3.7: Mutation of the PXIXIT motif of inhibitor-1 or its binding groove in calcineurin decreases inhibitor-1 dephosphorylation rates as assayed with increasing amounts of enzyme.** The effect of increasing amounts of enzyme is shown on the dephosphorylation of 5 pmol of protein phosphatase inhbitor-1 (I-1) substrate. Wild type (WT) CaN (diamond, panel A) is compared with 330-NIR-332→330-AAA-332 CaN (NIR, square, panel B) or Y288A:M290A:F299A:330-NIR-332→330-AAA-332 CaN (F299A, triangle, panel C) for dephosphorylation of either WT I-1 (filled symbols) or 6-SPRKIQF-12→6-AAAAAAA-12 I-1 (F12A, open symbols). All assays were done in duplicate, with an average of two assays shown.

amount of WT or mutant I-1 substrate. In these assays, mutants of I-1 were dephosphorylated ~1.5–2-fold slower than WT I-1 with nearly identical results observed for both WT and NIR CaN (see Figure 3.8A and B, respectively, p. 146). Higher fold difference was seen when lower amounts of substrate were used. A more pronounced effect was observed once again when the F299A mutant CaN phosphatase was used, with 2–3-fold difference observed in the dephosphorylation rate, demonstrating a modest effect of the mutagenesis of CaN on its interaction with I-1 (see Figure 3.8C, p. 146).

Considering that the amount of phosphatase used in the I-1 dephosphorylation assays had a bearing on the rate of dephosphorylation of WT and mutant I-1 substrates, the level of enzyme activity may have an impact. Since NIR and F299A CaN mutants were less active than WT CaN, it was decided to calibrate the three enzymes to obtain equal activities. Each enzyme was quantitated with respect to an equivalent rate of dephosphorylation of WT I-1 substrate.

Based on the results shown in Figure 3.7 (p. 144), with WT I-1 as a substrate, 0.1 pmol of WT CaN, 0.2 pmol of NIR CaN, and 0.4 pmol of F299A CaN were the quantities chosen to provide equivalent phosphatase activities. Nearly equivalent dephosphorylation rates were achieved for all three enzymes, with a marginally lower rate seen for F299A CaN. It was observed that WT CaN, NIR and F299A CaN mutants dephosphorylated PXIXIT motif mutant I-1-P to a

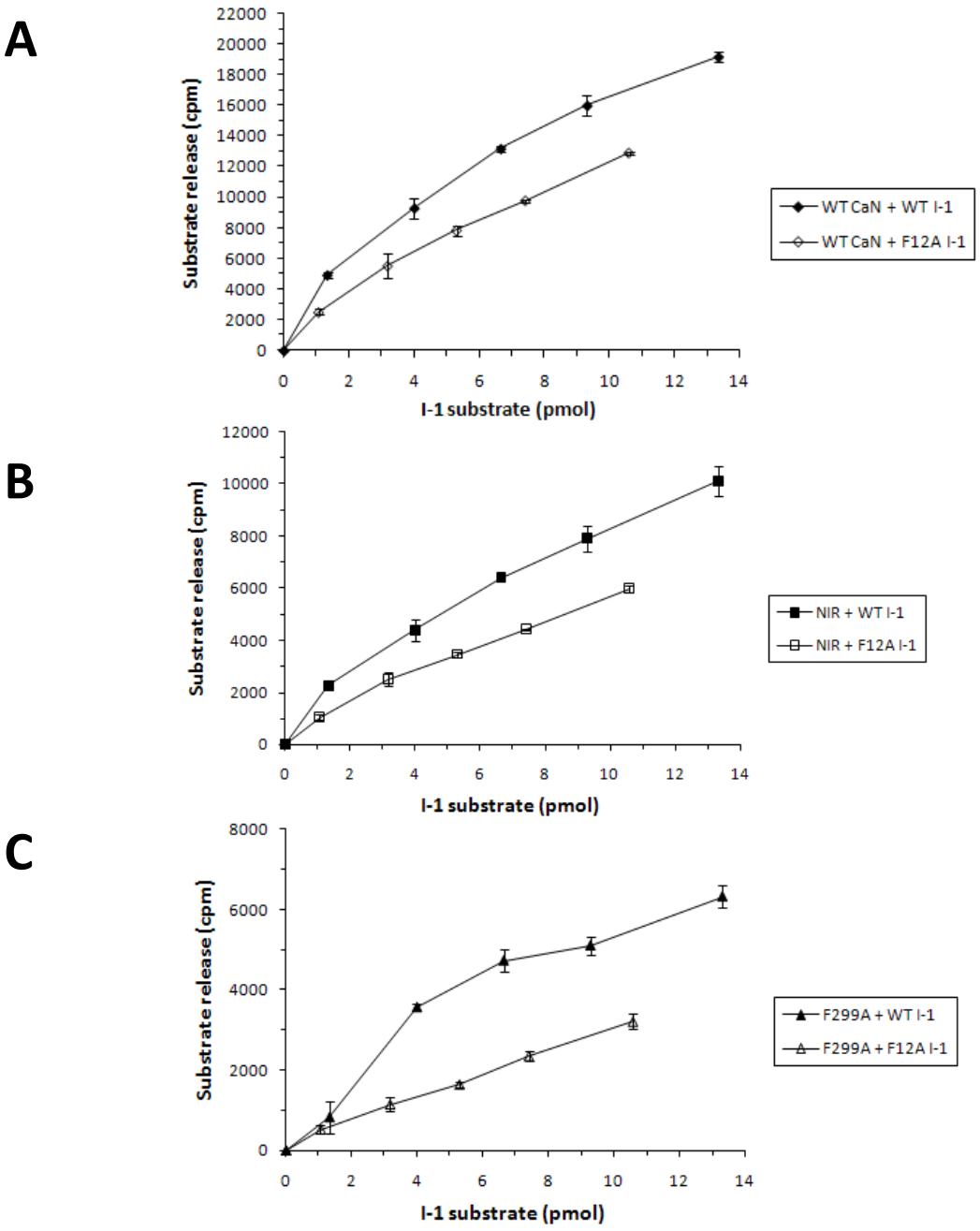



**Figure 3.8: Mutation of the PXIXIT motif or its binding groove decreases inhibitor-1 dephosphorylation as assayed with increasing amounts of substrate.** A constant amount of enzyme (0.1 pmol) was assayed against increasing amounts of wild type (WT) protein phosphatase inhibitor-1 (I-1, filled symbols) or mutant PXIXIT motif I-1 (F12A, open symbols) for three different forms of CaN: WT (diamond, panel A), triple mutant of the PXIXIT motif binding groove (NIR, square, panel B) or sextuple mutant of the same binding groove (F299A, triangle, panel C). Mutants are the same as described in Figure 3.6 (p. 142). All assays were performed in duplicate, with an average of two assays shown.

lesser degree than WT I-1-P, with ~1–1.3, ~1.4–1.8, and 1.5–2.1-fold differences observed, respectively (see Figure 3.9, p. 148).

## 3.4 Conclusions

NFAT proteins are crucial players in the activation of the immune system; they need to be dephosphorylated by CaN in order to translocate to the nucleus where they can carry out their transcriptional functions.[12, 190, 206] Structural data for the interaction of CaN with NFAT protein is not currently available, however, the binding of NFAT's PXIXIT motif with CaN is well established.[11, 13, 277] Since the first description of the PIXIT motif, it has come to light that many other proteins rely on a similar mechanism to interact with CaN (see Table 3.1, p. 121).[109] Studies of these proteins reveal that the PXIXIT motif can accommodate large degeneracy, with only the proline residue being invariable; an effective PXIXIT motif can also contain amino acid insertions (see Table 3.1, p. 121). The CaN binding groove for the PXIXIT motif is analogous to a groove used by PP-1c to bind an RVXF sequence found on many proteins.[193, 277] A potent inhibitor of PP-1c, I-1, is known to require an altered form of the RVXF motif to bind PP-1c.[6, 102, 103, 258, 260] However, phosphorylated I-1 that inhibits PP-1c, is also an excellent substrate for CaN.[159, 274, 275] This prompted us to examine the I-1 sequence, and led to the discovery of a potential form of the PXIXIT motif in the first 12 residues of I-1. Within that sequence, the RVXF motif is present itself, possibly helping elude earlier detection of the PXIXIT motif. This would place I-1 in an

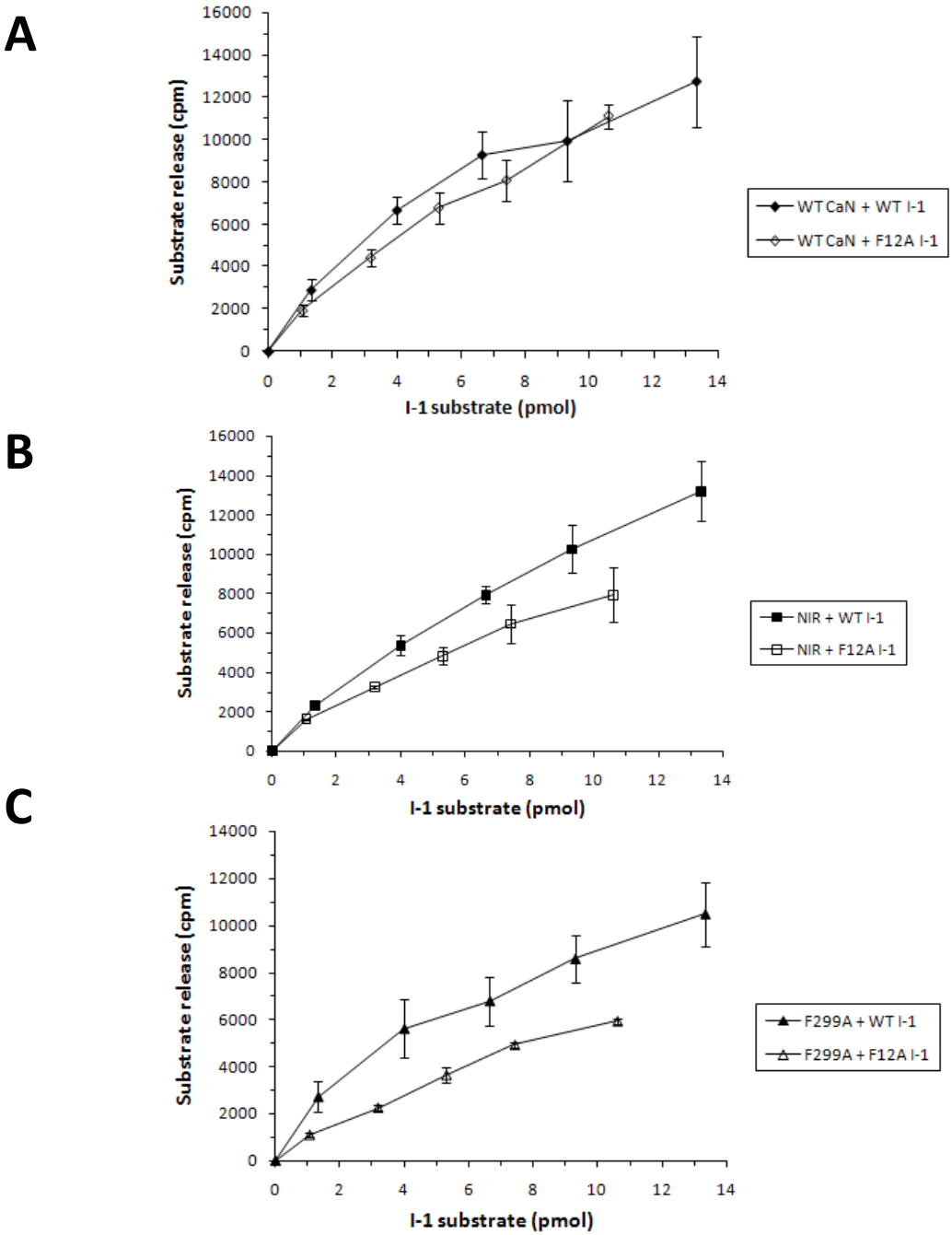



**Figure 3.9: Mutation of the PXIXIT motif or its binding groove decreases inhibitor-1 dephosphorylation as assayed with enzymes of equivalent activity.** To determine if a difference in enzymatic activity of the CaN triple mutant (NIR, square, panel B) or the CaN sextuple mutant (F299A, triangle, panel C) masked the effects of mutagenesis compared with wild type (WT) CaN (diamond, panel A), different amounts of substrate were dephosphorylated by the three enzymes which were applied at similar activities. Mutants are as described in Figure 3.6 (p. 142). All assays were done in duplicate, with an average of two assays shown.

exclusive group of proteins that bind CaN via a PXIXIT motif, including the NFAT proteins. I-1 would also become the only known protein that binds PP-1c and CaN phosphatases using structurally related binding grooves. Several *in vitro* experiments were carried out to test this hypothesis.

VIVIT is a 16-residue peptide based on the NFAT PXIXIT motif but mutated to make it a specific inhibitor of NFAT interactions with CaN.[13] Our work shows that VIVIT can also inhibit I-1 dephosphorylation by CaN with an $IC_{50}$ of 8.79 μM (see Figure 3.2, p. 136). Since VIVIT binds to CaN's PXIXIT motif binding groove and blocks access of proteins that contain PXIXIT sequences, this strongly suggests that I-1's putative PXIXIT motif interacts with CaN.

The work presented in this chapter shows that a peptide corresponding to the first 54 residues of the I-1 protein (PPIP54) when phosphorylated (PPIP54-P), is a better substrate for CaN than a 30 residue phosphorylated I-1 peptide (PPIP30-P) that is missing nearly the entire purported PXIXIT sequence (see Table 3.3, p. 132, Figures 3.3–3.5, p. 138–140). This once again suggests that I-1 contains the PXIXIT motif and uses it to interact with CaN, as deletion of this sequence results in a decreased dephosphorylation rate.

Interestingly, the dephosphorylation rate of a peptide that had only a partial deletion of the putative PXIXIT motif (PPIP46-P) was between that of

PPIP54-P and PPIP30-P. This suggests that even a portion of the PXIXIT sequence aids in interaction of the substrate with CaN.

It is possible that the slower dephosphorylation rate of PPIP30-P by CaN is not due to an absence of the PXIXIT motif but rather:

1) The deletion of a different binding sequence, since PPIP30-P is truncated at both N- and C-termini (see Table 3.3, p. 132);

2) Interaction with CaN does not require binding to the PXIXIT motif binding groove of CaN-A at all, but rather proper conformational fit into the active site which could have been distorted in PPIP30-P due to the severe truncation.

Using PPIP46-P as a substrate eliminated the first argument since it contains same residues at the C-terminal end as PPIP54-P. Therefore if these residues were the determining factor in CaN dephosphorylation, PPIP46-P and PPIP54-P dephosphorylation would be expected to be the same. As for the second possibility, it is known that I-1 is fairly unstructured, containing only few α-helices and no tertiary structure, so it is unlikely that specific tertiary shape affects the dephosphorylation rate.[5, 62, 231, 260] In addition, it is known that both PPIP46-P and PPIP54-P inhibit PP-1c to the same extent as full length I-1, strongly suggesting that they retain the necessary basic structure to inhibit PP-1c equipotently, thus any difference seen between these two substrates by CaN is expected to be a direct result of PXIXIT motif alteration.[6]

Experiments involving PPIP54-P dephosphorylation by CaN in the presence of VIVIT peptide inhibitor would be expected to show results similar to the PPIP30-P peptide which lacks most of the PXIXIT sequence. This would further confirm that CaN interacts with PPIP54-P via the putative PXIXIT sequence.

Mutagenesis of the CaN PXIXIT motif binding groove as well as the putative PXIXIT motif in I-1 was performed to abrogate the interaction between these two proteins. Rates of dephosphorylation of I-1 and I-1 mutants by WT CaN and CaN mutants were compared to see if the changes in sequence modified the interactions between these proteins. Mutation of the CaN-A PXIXIT motif binding groove was done as suggested previously by Li et al. (2004); that is 330-NIR-332 residues were mutated to alanine amino acids (330-NIR-332→ 330-AAA-332, termed NIR). In addition, NIR CaN was used to introduce further mutations Y288A:M290A:F299A to generate a sextuple CaN mutant termed F299A.

The effect of the CaN mutagenesis could not be studied directly against the WT I-1-P as the mutant phosphatases showed decreased activities not only when dephosphorylating I-1-P but also with other substrates (see Figure 3.6, p. 142). This suggests that mutagenesis of the PXIXIT motif binding groove affects the conformation of the active site of CaN. 330-NIR-332 mutant CaN has been shown to have reduced activity against these same substrates by other

researchers .[193, 213]

It was possible to assess the effects of mutagenesis of CaN's PXIXIT motif binding pocket by comparing the difference in dephosphorylation rate of WT I-1 versus I-1 with a mutated PXIXIT motif. For this purpose, six residues of the PXIXIT motif were mutated to alanine amino acids.

Mutagenesis of I-1 resulted in a modest decrease in its dephosphorylation compared to WT I-1, with a maximal 1.4-fold difference. Similar results were observed with the NIR CaN mutant, showing that this series of mutations did not have significant impact on interactions with the I-1 protein (see Figure 3.7, p. 144).

Structures of the VIVIT and AKAP79 peptides binding to CaN can help explain this surprising result. Takeuchi et al. (2007) reported that Asn-330 and Arg-332 show enough flexibility to only partially engage Thr-9 of the VIVIT peptide.[277] Difference in the position of these residues between the CaN structures binding either the VIVIT or the AKAP79 peptides confirms the range of flexibility Asn-330 and Arg-332. Ile-331 on other hand forms the base of the binding cleft, thus different hydrophobic residues could be accommodated in its place (see Figure 1.19, p. 52).[192, 277] Although, based on previous VIVIT work, some effects of 330-NIR-332→330-AAA-332 mutagenesis should have been apparent; perhaps additional I-1 binding sites on CaN help to mask the effects of

this mutation.[76]

The dephosphorylation rate of the mutant substrate was observed to be more significant in F299A CaN—there was a 2–3-fold difference in dephosphorylation of mutant I-1 substrate as compared to WT I-1 substrate. This suggests that mutagenesis of the CaN PXIXIT motif binding groove had an impact on dephosphorylation of the I-1 substrate (see Figures 3.6–3.8, p. 142, 144 and 146, respectively). This further helped establish that I-1 and CaN proteins interact in a way analogous to that of CaN and NFAT.

The experiments described above clearly establish that the I-1 protein binds to CaN using the PXIXIT motif binding groove of CaN-A. Although a few binding partners of CaN are known to interact with this phosphatase via the same PXIXIT motif binding groove, the implication of I-1 using the same mode of interaction with CaN is particularly interesting. These experiments established I-1 as the only protein that interacts with PP-1c and CaN via structurally related binding pockets. It is expected that a closely related protein to I-1, DARPP-32, that shows over 60% homology with I-1 over the first 50 residues, also binds CaN via a modified form of the PXIXIT motif.[65, 260, 299]

Our discovery of the PXIXIT motif in I-1 raises new questions, and interesting possibilities for further research. I-1 plays an essential role in the only phosphatase cascade known both in brain and cardiac muscle (see Figure 1.21, p.

58). The key event is dephosphorylation of I-1-P by CaN so that I-1-P can no longer inhibit PP-1c.[65, 127, 226]

There are a couple of potential ways of how CaN could aid in reduction of PP-1c inhibition by I-1-P:

1) CaN directly competes with PP-1c for binding of I-1 to the structurally related groove. If CaN were to have more affinity to I-1-P than PP-1c, it would be able to regulate I-1-P inhibition of PP-1;

2) CaN does not outcompete PP-1c for I-1-P binding and dephosphorylation occurs only during equilibrium changes from PP-1c bound by I-1-P to unbound state.

This raises the question whether the PXIXIT motif of I-1-P is a stronger binding partner for CaN than the KIQF motif is for PP-1c, thus allowing CaN to outcompete PP-1c for binding of I-1-P.

Given the crucial role I-1 plays in mediating development of cardiac hypertrophy through its involvement in the phosphatase cascade, understanding I-1's role in this pathway will hopefully allow to abrogate its negative effects during hypertrophy development.[229] In hypertrophic hearts it appears that I-1-P levels are reduced, resulting in increased PP-1 activity.[52, 97-99, 127, 228] Learning and memory formation also involves PP-1c function regulation by CaN. Modulating the PP-1c activity with I-1-P could aid in brain injury recovery by enhancing

memory capacity and improved learning.[219, 226, 262] Therefore inhibitors targeting I-1-P interactions with CaN would be of high therapeutic interest, especially if CaN actively competes with PP-1c for I-1-P binding.

Currently there are no crystal structures of PPP family phosphatases bound to substrate proteins. Results of this research provide valuable information for obtaining an interaction of increased strength between CaN and I-1, and therefore stabilizing such binding. A unique truncated form of CaN (residues 26–346) that results in removal of many flexible components of the CaN catalytic subunit—thus making the protein more similar in structure to PP-1c—has been generated (see chapter two, p. 87). Mutagenesis of CaN's active site described in chapter two resulted in a more active form of enzyme against I-1-P; i.e., mutagenesis apparently resulted in an improved interaction with the substrate. Therefore, a thiophosphorylated form of I-1, a form that is not dephosphorylated by CaN, if bound to CaN could not be dephosphorylated and may not be released from CaN. A stable CaN-thiophosphorylated I-1 complex could perhaps be crystallized for structure determination. Information presented in this chapter argues that I-1 could be mutated to contain the VIVIT peptide sequence instead of the PXIXIT motif. This could lead to improved binding to CaN, forming another possibility for crystallization.

Structure of I-1 bound to CaN could verify whether CaN/CaM holoenzyme exist as a single complex or as a homodimer complex, as has been

recently suggested. Structure of CaM bound to a peptide spanning the CaM-binding domain of CaN-A suggested that such interaction requires dimerization of CaN.[312] This novel theory is supported by structural findings of Li et al. (2007) who reported CaN dimerization upon binding of a single VIVIT peptide.[195] Formation of the holoenzyme as a dimer of CaN/CaM complexes would require spatial access to the active site of either/both of CaN-A molecules. Determination of the CaN structure interacting with I-1 would provide an opportunity to test the CaN dimerization theory.

Knowledge that I-1-P binds to CaN via the PXIXIT motif allows for development of an *in vitro* assay to screen potential CaN inhibitors that interact with CaN-A via the PXIXIT motif binding groove. Such inhibitors would decrease the rate of CaN dephosphorylation of I-1-P. Finding novel drugs that target CaN in such way would be of great value in the field of immunology (see chapter four), cardiomyopathy and learning/memory formation. Structure of I-1 bound to CaN could help in the development of drugs that disrupt the interaction with CaN via the PXIXIT motif. Knowledge of how I-1 interacts with CaN could also allow for potential mutagenesis studies that could abrogate its interaction with CaN but still allow it to bind to PP-1c, resulting in a potential therapeutic tool in the fight against heart failure.

# Chapter 4: Search for novel calcineurin inhibitors in extracts of invertebrate marine organisms.

## 4.1 Rationale for the search for novel immunosuppressive drugs

The CaN enzyme is a key element responsible for immune system activation.[12, 233] It is involved in a myriad of high profile disorders, thus control of the enzymatic function of this protein through drug manipulation is of high interest.[22, 23, 145, 167] Currently the immunosuppressants used clinically, CsA and FK506, appear to inhibit CaN indiscriminately throughout the body, resulting in the many adverse effects upon their usage (see Appendix A, p. 279).[22, 23, 145, 167, 267]

With the various problems associated with use of the current clinical CsA/FK506 drugs, there is a strong impetus to discover novel immunosuppressants, especially drugs that would not target all functions of CaN. Possible ways to achieve this goal are:[175]

1) Inhibition of the capacitative $Ca^{2+}$ entry at the plasma membrane which is responsible for the majority of cellular $Ca^{2+}$ influx when lymphocytes are activated;

2) Inhibition of NFAT binding to CaN;

3) Inhibition of NFAT binding to DNA;

4) Preventing interaction of NFATs with other transcription factors.

One of the best ways to diminish the plethora of toxic side effects associated with immunosuppressive treatments is by targeting the function of NFAT proteins, the principal executors of CaN activation of the immune response.[12, 109, 164, 175, 190, 256] The role of NFAT as a potential target of immunosuppression is exemplified by patients who do not express functional NFATs, and thus suffer from severe immunodeficiency with corresponding lack of cytokine production. Such individuals do not show the same phenotype as CsA/FK506 associated toxic side effects.[175]

The search for novel drugs against target proteins is not only the task of multi-million dollar drug companies; the academic world is also in pursuit of novel CaN inhibitors. The search for novel immunosuppressants dating from 1990 is summarized in Appendix B (p. 281). Over 60 molecular targets have been developed during this time span, underscoring the great weight placed on finding novel immunosuppressants that can alleviate problems associated with current clinical applications. Seven of these drugs are either in clinical trials or have been approved for medicinal use as immunosuppressants. Four other drugs have been approved as treatments of other nonimmune as well as autoimmune conditions. Of these, the most promising is the ISA247 (Voclosporin) developed by Isotechnika (Edmonton, AB) which is currently in phase II and III trials.[162] ISA247 was derived from CsA and thus targets CaN function directly. ISA247 is more potent than CsA against CaN, resulting in longer graft survival in animal models with diminished toxicity. Although the reasons for this are currently not understood, it

demonstrates that novel inhibitors of CaN with reduced side effects can be found.

At least 30 compounds have been found via screening of existing chemical libraries—a current trend in the search for novel drugs (Appendix B, p. 281).[50, 104, 225, 264] Although this has been successful in identification of clinical candidates, such libraries have limitations; chemical libraries are typically designed based on previously known biologically active structures, and generated in a cheap and readily made manner.[160] Nevertheless, this provides a clear example of the usefulness of chemical libraries when screening for novel drugs.

## 4.2 Marine environment as a source of novel drugs

Marine wildlife is a relatively untapped source for novel drugs; its diversity presents a form of a chemical library of natural products. Indeed, the marine environment is the source of one of the most widely used phosphatase inhibitors: okadaic acid (OA) from dinoflagellates.[70] To our knowledge, inhibitors of CaN have never been searched for in the marine environment.

The vast majority of marine species live in the ocean fringe, the land-sea interface, one of the most biologically diverse environments in the world. The resulting concentration of biological diversity places extreme competitiveness in such environment for food source or a living space. This results in natural adaptation of simple marine invertebrates with primitive immune systems to use chemical means for defense. Because these compounds are necessarily diluted by

large volumes of water, they need to be extremely potent to have an effect. Besides having evolved under intense competition for resources, marine derived compounds are unique in that the biological adaptations needed for marine microorganisms are vastly different than those of organisms found on land. Therefore, the marine environment places at science's disposal a wealth of natural products of vast diversity.[8, 266]

As potential leads for target drugs, natural compounds have certain advantages over synthetic products:[160]

1) Natural compounds have evolved in nature to serve specific purposes and hence it is highly probable that they are recognized by specific targets;

2) Natural compounds are often readily bioavailable;

3) Many natural compounds are structurally unique.

In the past few years, large advances have been made in the development of marine derived potential drugs (many are now in clinical trials) with over 2000 compounds being identified from the marine environment. Although marine ecosystems represent 95% of the biosphere, drugs derived from marine sources are largely under-represented in natural source drugs libraries.[8, 266] Thus it is reasonable to think that potential targets of CaN can be discovered considering how well conserved CaN is throughout evolution.[20]

*The hypothesis I set out to test in this chapter of the thesis was whether novel inhibitors of calcineurin could be identified, isolated and purified from extracts of marine invertebrate organisms, guided by an in vitro calcineurin assay using inhibitor-1 as a substrate.*

The Holmes laboratory has had a long term collaboration with Dr. Raymond Andersen in Vancouver, B.C. Dr. Andersen, a leading marine natural product chemist, is skilled in collecting marine samples worldwide, and in purification and isolation of chemical compounds from these samples. Dr. Andersen is also adept at chemically altering these compounds in order to change their properties. Concerted efforts with the partners in Vancouver have yielded a novel inhibitor of PP-1c, motuporin, a compound related to the microcystin family. Motuporin is one of the most potent known inhibitors of PP-1c, with an $IC_{50}$ of <1 nM.[88] Drs. Holmes and Andersen were also responsible for the first identification of microcystins in the marine environment.[55] However, the marine extracts collected by the colleagues in Vancouver have never been tested against CaN.

There is a need for novel immunosuppressants, especially those involved in inhibition of NFAT regulation by CaN. The abundance of drugs derived from natural products, and the fact that the marine environment provides a very rich and diverse source of compounds, strongly suggest that marine organisms could harbor CaN inhibitors. This is underscored by the previous discoveries of potent

protein phosphatase inhibitors from marine samples.

In our search for nonvertebrate marine organisms that could yield CaN inhibitors, we had several advantages:

1) An access to Dr. Andersen's immense bank of marine organism extracts;

2) A sensitive CaN assay developed in our laboratory to efficiently screen many samples for inhibition;

3) An access to instrumentation that can perform bioassay-guided fractionation and isolation of compounds that prove inhibitory to CaN;

4) The use of protein phosphatase inhibitor-1 (I-1) as a substrate in the screening assay allowing identification of compounds that block the active site of CaN, and compounds that could prevent interactions with CaN via the PXIXIT motif (see chapter three).

Novel drugs that target CaN, especially those that could target NFAT interactions with CaN, could potentially revolutionize the field of organ transplantation, just as the discovery of CsA did 20 years ago.

## 4.3 Experimental Procedures

*Materials.*

CaM, CaM-Sepharose 4B resin, PKA, PP-1cγ and I-1 substrates are described in the Experimental Procedures of chapter two (p. 75). Dried extracts of marine organisms were obtained from Dr. R. Andersen (University of British

Columbia, Vancouver, BC). Bioassay-guided purified compounds from marine extracts, and their analogs were also obtained from Dr. Andersen. All other reagents were purchased from Sigma-Aldrich, Fisher Scientific (Ottawa, ON) and EMD Chemicals Inc. (Gibbstown, NJ) unless otherwise stated.

*Marine sample collection.*

Marine invertebrates were collected using scuba diving, classified and identified by the Andersen research group in Indonesia and Papua New Guinea. Samples were frozen immediately and transported frozen to Vancouver, BC. Thawed tissues were extracted with MeOH, filtered, and concentrated *in vacuo*. These dried samples are referred to as crude extracts.

*Crude extracts bioactive analysis.*

Indonesian or Papua New Guinea extracts were resuspended in MeOH to 10 mg/mL. Resuspended extracts were tested for inhibition of either CaN using [$^{32}$P]-I-1 substrate assays, or inhibition of PP-1c using [$^{32}$P]-Phos a substrate. The final extract concentration in the assay was 1 mg/mL.

*High performance liquid chromatography fractionation of Indonesian extracts.*

Indonesian extracts (4.7–4.8 mg of 10 mg/mL) were loaded individually onto a $C_{18}$ Vydac (218TP54) analytical column equilibrated with solution A ($H_2O$/0.1% TFA), using the System Gold HPLC (Beckman, Mississauga, ON). Extracts were fractionated by elution at a flow rate of 1 mL/min using solution A

as a starting solution and developed with a 50 min gradient program: 0–95% of solution B ($CH_3CN$/0.1% TFA) in 40 min, 95–100% solution B in 5 min, 100% solution B for 2 min, and 100% solution A for 3 min. Absorbance was measured at 220 nm. Fractions (1 mL) were assayed for activity in the same way as crude extracts.

*Marine extract compound purification.*

Vancouver team fractionated the marine extract of choice into different organic phases which were then bioassayed in Edmonton, AB, to select fractions that harbored activity against CaN. Active fractions were sent back to Vancouver for isolation of the active compounds via silica gels and LH-20 columns, and to determine their structures using NMR.[53] This novel approach to identification of potential inhibitory compounds of CaN is summarized in Figure 4.1, p. 165.

*Calcineurin purification.*

CaN-A and CaN-B subunits were purified as described in chapter two, Experimental Procedures, p. 83–85 and 87–88, respectively)

*PP2-Ac purification.*

PP-2Ac was purified from bovine hearts as previously described with the following modifications.[68] Four fresh bovine hearts were cut into 1 inch cubes, fat was removed and tissue was minced and homogenized in solution containing 2 mM EGTA, 3 mM EDTA, pH 7.0 (2.5 L/kg of heart tissue). All of the buffers

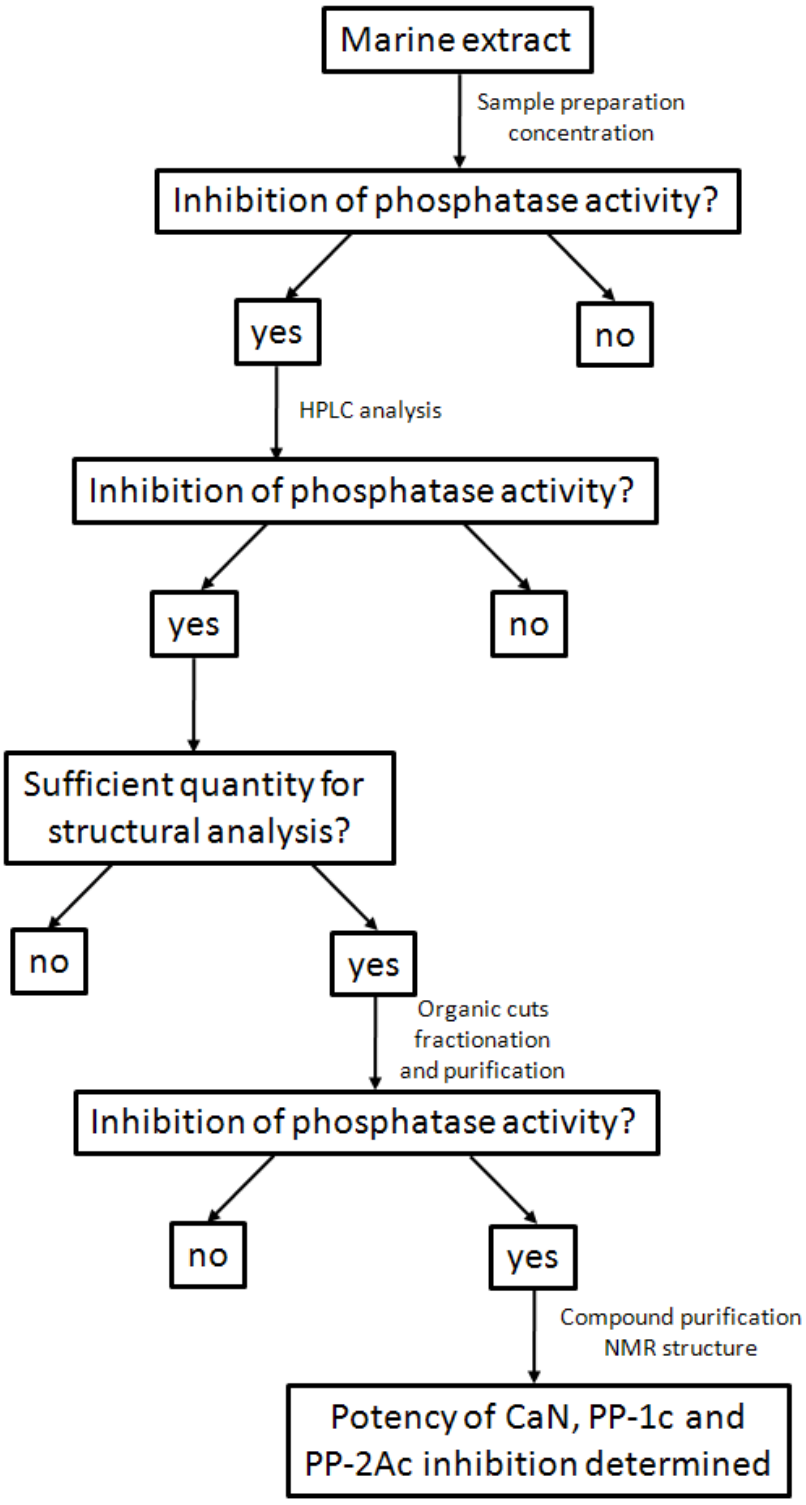



**Figure 4.1: Diagram outlining a procedure for identification of novel calcineurin inhibitors.** Purification of compounds active against CaN was guided by a bioactive assay for inhibition of either wild type CaN or PP-1c. Purified compounds were also tested for PP-2Ac inhibition.

prior to dialysis (see below) contained following protease inhibitors: antipain dihydrochloride (120 µg/L), 4-amidinobenzylsulfonyl fluoride hydrochloride (120 µg/L), aprotinin (120 µg/L), leupeptin (60 µg/L), phosphoramidon (60 µg/L), bestatin (120 µg/L), pepstatin (300 µg/L), chymostatin (60 µg/L), E-64 (120 µg/L). The final amount of muscle tissue was ~5 kg. Crude extract was centrifuged at 3000 × g for 30 min at 4 °C, and the supernatant filtered by passing through glass wool. Filtered supernatant was brought to pH 7.2 with $NH_4OH$ (Anachemia Canada Inc., Montreal, QC) while stirring on ice, made up to 55% in $(NH_4)_2SO_4$, and centrifuged at 3000 × g for 40 min at 4 °C. The pellet was resuspended in 1/10 × the volume of original crude extract of buffer A (20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 10% glycerol, 1 mM PMSF, 1 mM benzamidine, and 0.1% BME). Protein was precipitated by addition of 5 × the volume of 95% EtOH at room temperature, and centrifuged immediately at 3000 × g for 5 min at 4 °C. The pellet was resuspended in buffer A (1.5 L/kg of heart tissue), and subsequently centrifuged at 15 000 × g for 15 min at 4 °C. The pellet was resuspended in buffer B (20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 1 mM PMSF, 1 mM benzamidine, 0.1% BME, 0.75L/kg of heart tissue) and centrifuged as last time. Supernatant was brought to pH 7.5 with $NH_4OH$. Supernatant was made up to 65% in $(NH_4)_2SO_4$ and left overnight at 4 °C. The precipitate was centrifuged 15 000 × g for 40 min at 4 °C, and the pellet was resuspended in buffer A (30 mL/kg of heart tissue). The supernatant was dialyzed five times in 2 L of buffer C (20 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 10% glycerol, 0.1 mM PMSF, 0.1% BME). The dialysis buffer and subsequent buffers no longer

contained the protease inhibitors. The dialysate was centrifuged at 2000 × g for 10 min at 4 °C and the supernatant was diluted 2-fold with buffer C. The supernatant was loaded onto Diethylaminoethyl (DEAE)-Sepharose (GE Healthcare) column (16 × 5 cm) that was previously equilibrated in buffer C at 2 mL/min flow rate, and washed overnight with buffer C containing 80 mM NaCl at 1 mL/min flow rate. The column was developed in a 264 min program at 3 mL/min flow rate with a linear gradient of 0–80% buffer C containing 500 mM NaCl, collecting 3 mL fractions.

DEAE-Sepharose fractions were assayed for PP-2Ac activity using *p*NPP substrate, and active fractions were pooled and concentrated to 1.5 mL using 10 000 MWCO Amicon Ultra Centrifugal Filter Devices (Millipore, Bedford, MA). The concentrated sample was loaded onto Hi-load Superdex 75 HR 26/60 resin. Protein was eluted with 200 mM NaCl buffer A as described in CaN-A purification (see chapter two, Experimental Procedures, p. 83–85), collecting 1 mL fractions. Active fractions were pooled, concentrated to 2 mL and loaded onto a UNO-Q1 ion exchange column (Bio-Rad Laboratories Ltd., Mississauga, ON) previously equilibrated with buffer C containing 100 mM NaCl. Protein was eluted at 1 mL/min using 0–100% gradient of buffer C containing 700 mM NaCl in 160 min. Active fractions were pooled, concentrated down to 0.5 mL, loaded onto Hi-Load Superdex 200 HR 16/60 resin, and eluted as described above for the Hi-Load Superdex 75 HR 26/60 column. Active fractions were pooled, concentrated to 0.4 mL and stored in 50% glycerol at -20 °C. PP-2Ac could not be

purified to homogeneity.

*Inhibitor-1 purification and phosphorylation.*

See chapter two, Experimental Procedures for details, p. 79–82.

*PP-1c and PP-2Ac phosphatase assays using phosphorylase a substrate*.

PP-1c and PP-2Ac activity against [$^{32}$P]-labeled Phos a was measured as described in chapter two, Experimental Procedures, p. 89–90.

*PP-2Ac phosphatase assays using para-nitrophenyl phosphate substrate.*

PP-2Ac activity against *p*NPP was measured as described previously with following changes.[265] Phosphatase sample (10 µL) was added to 40 µL of buffer containing 50 mM Tris (Invitrogen, Burlington, ON), pH 8.3, 1 mM EGTA (Caledon Laboratories Ltd.), 30 mM $Mg(CH_3COO)_2$, 0.5 mM $MnCl_2$, 1 mg/mL bovine serum albumin, 0.2% 2-mercaptoethanol (BioShop Canada Inc.), and incubated at 37 °C for 15 min. After preincubation, the reaction was initiated by addition of 10 µL of 30 mM *p*NPP substrate. The mixture was incubated between 10 min to 1 h at 37 °C; incubation times were depending on the activity of the samples, as the color intensity of the *p*NPP dephosphorylated product, *p*-nitrophenolate, is time dependent. The reaction was stopped by addition of 4 µL of 2M $Na_2CO_3$. The release of *p*-nitrophenolate was measured by absorbance at 405 nm using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA).

*Calcineurin phosphatase assay.*

See chapter two, Experimental Procedures for details, p. 88–89.

*Interleukin-2 production by T-cells.*

Quantification of Jurkat T-cell production of IL-2 was performed according to instructions in the Cytokine Sandwich ELISA assay protocol, BD Biosciences (San Jose, CA).

*Contributions to this work.*

All of the work described was produced by the author with following exceptions by members of Dr. Holmes laboratory. The I-1 protein used in this study was prepared by Micheal Shopik. Micheal Shopik also contributed to radiolabeling substrate, purification of CaN, solely done the screen of 281crude extract samples from Papua New Guinea, and three CaN $IC_{50}$ assays against hipposulfate C and halisulfate-7 inhibitory compounds. Inhibition of PP-1c by select Papua New Guinea extracts was performed by Cheryl McKormick. Inhibition of IL-2 production by T-cells by sesterterpene sulfate compounds was analyzed by a summer student, Peter Holmes in the laboratories of Dr. Holmes and Dr. Chris Bleakley (University of Alberta, Edmonton, AB).

The following work was generated in Dr. Andersen's laboratory (University of British Columbia, Vancouver, BC). Purification of RJA-03-193 extract, biofractionation and pure compound structure determination and chemical

modification was done by Gavin Carr. Handling of the biofractions of the RJA-03-269 extract was done by Justin Parker. Biofractions of RJA-03-58, RJA-03-124 and RJA-03-287 were prepared by Dr. Chris Gray. Purification of the RJA-03-111 compounds was initially undertaken by Harry Brastianos, and later continued by Gavin Carr.

## 4.4 Results

*Extracts of marine organisms inhibit calcineurin activity.*

A novel approach was undertaken where the identification of potential inhibitors of CaN were to be assessed directly by their impact on CaN enzymatic activity. To determine whether use of marine samples in search of novel regulatory compounds of CaN is indeed applicable, a preliminary test was done of 205 Indonesian marine extracts from the Dr. Andersen sample bank. Crude extracts (100 µg) were tested at a concentration of 1 µg/µL. At least 20 extracts potently inhibited CaN (see Figure 4.2, p. 171)

Serial dilutions of the 20 extracts were performed to assess the potency of the diluted samples. Although all extracts showed nearly full inhibition of CaN activity at 100 µg of extract, the effectiveness was greatly reduced with 10-fold dilution (10 µg of extract, see Figure 4.3, p. 172). Fewer samples showed inhibition of CaN at 100-fold dilution (1 µg of extract); only two of these samples inhibited CaN activity by more than 20% (see Figure 4.3, p. 172). It was decided to pursue only 10 µg extracts that showed at least 30% inhibition of protein

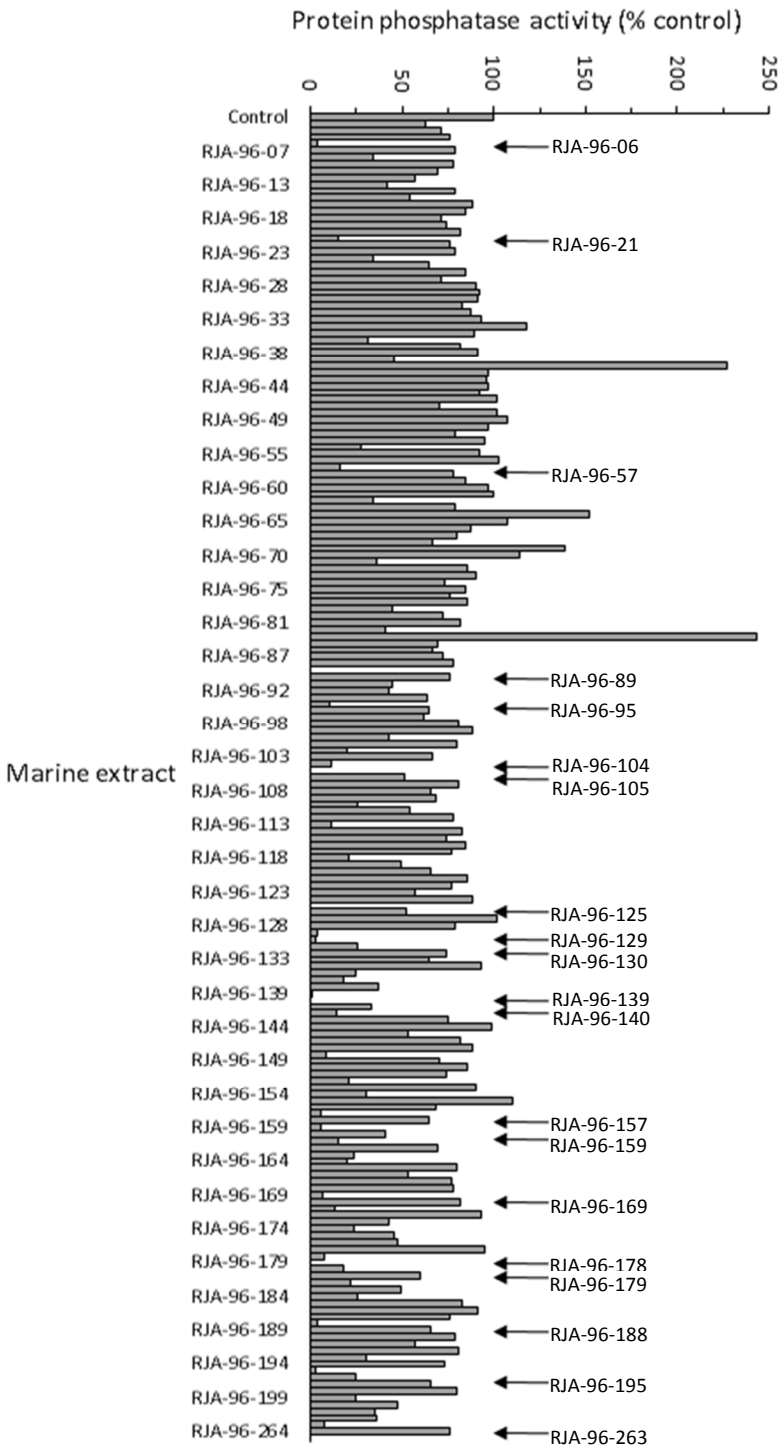



**Figure 4.2: Calcineurin inhibition profile with Indonesia marine extracts.** 205 marine samples were homogenized, extracted with MeOH, and dried to completion. Extracts were resuspended in MeOH at 10 mg/mL and 100 µg were tested for activity against CaN using [$^{32}$P]-inhibitor-1 substrate. Extracts that were further analyzed are indicated with arrows. For clarity, only every fifth extract identification number is listed.

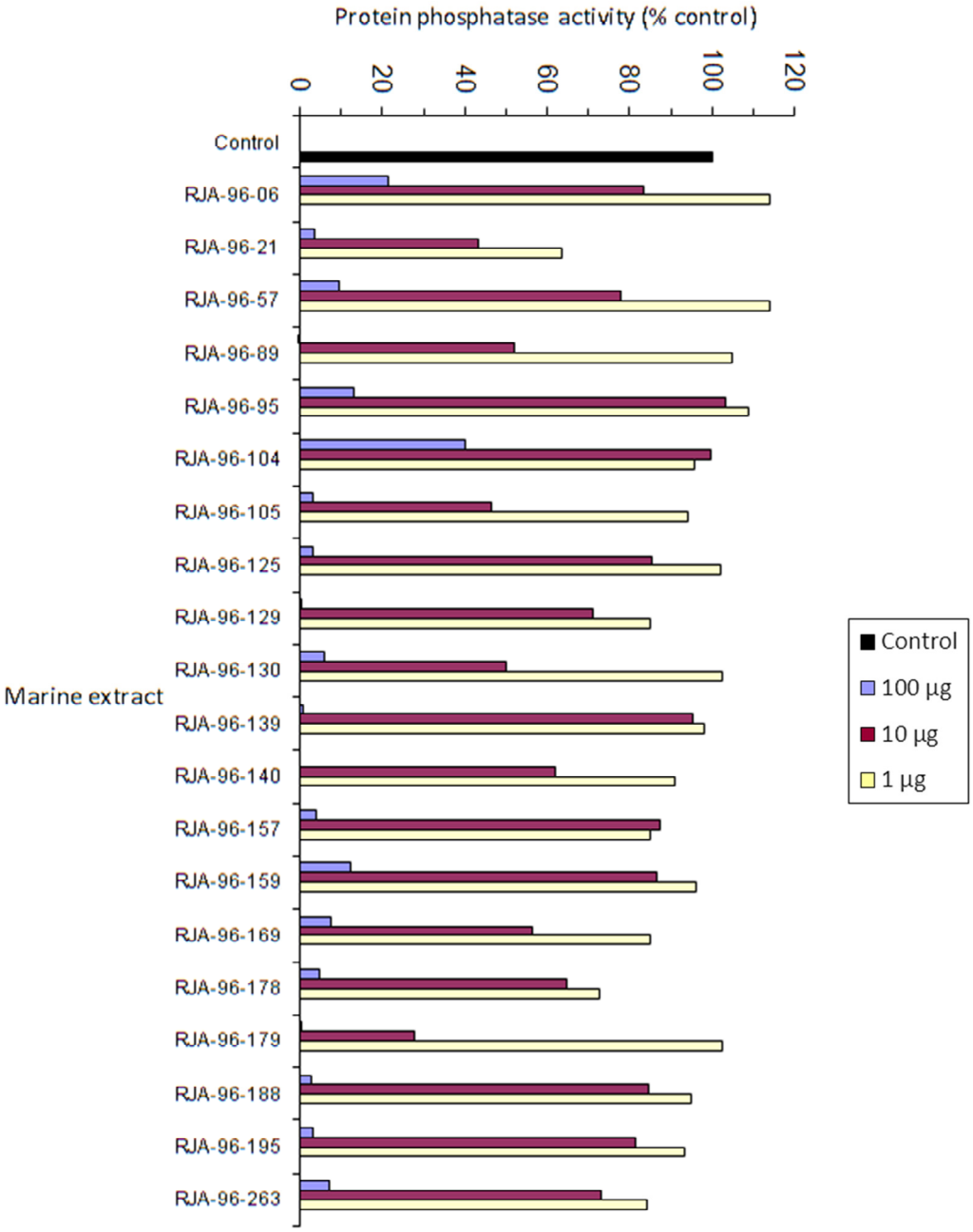



**Figure 4.3: Serial dilutions of the twenty most potent Indonesian marine extract samples.** Best candidate environmental extracts were chosen based on their ability to inhibit CaN enzyme activity at 100 µg, and tested at 10- and 100-fold dilutions for inhibition of CaN phosphatase activity against [$^{32}$P]-inhibitor-1 substrate.

phosphatase activity. Thus from the original bioprospect screen of 205 samples, 9 lead extracts were to be studied further: RJA-96-21, RJA-96-89, RJA-96-105, RJA-96-129, RJA-96-130, RJA-96-140, RJA-96-169, RJA-96-178, and RJA-96-179.

*Novel bioassay-guided fractionation procedure for identifying calcineurin specific inhibitors in marine organisms.*

To determine the specificity and potency of the 9 lead samples, extracts were further analyzed by HPLC chromatography. HPLC allows for a significant purification of samples as different components elute from the column at different concentrations of the organic solvent. Nearly 5 mg of each of the 9 samples of interest was purified by HPLC; 10 μL of each 1 mL fraction was analyzed for PP-1c and CaN inhibition. Figure 4.4 (p. 174) shows the inhibitory fractions of the HPLC profiles as determined with the bioassay. In all but one case, CaN inhibition and PP-1c inhibition overlapped. In case of extract RJA-96-178, a CaN specific inhibitory peak was observed that suggested the presence of a novel chemical entity that targeted CaN only. These pilot studies showed that bioassay-guided fractionation can be used to identify and isolate compounds that inhibit CaN. The Indonesian extracts were of insufficient quantity to further isolate the active compounds. However, the CaN assay was very effective in testing purified components of marine extracts, thus guiding their purification. This scheme is outlined in Figure 4.1 (p. 165).

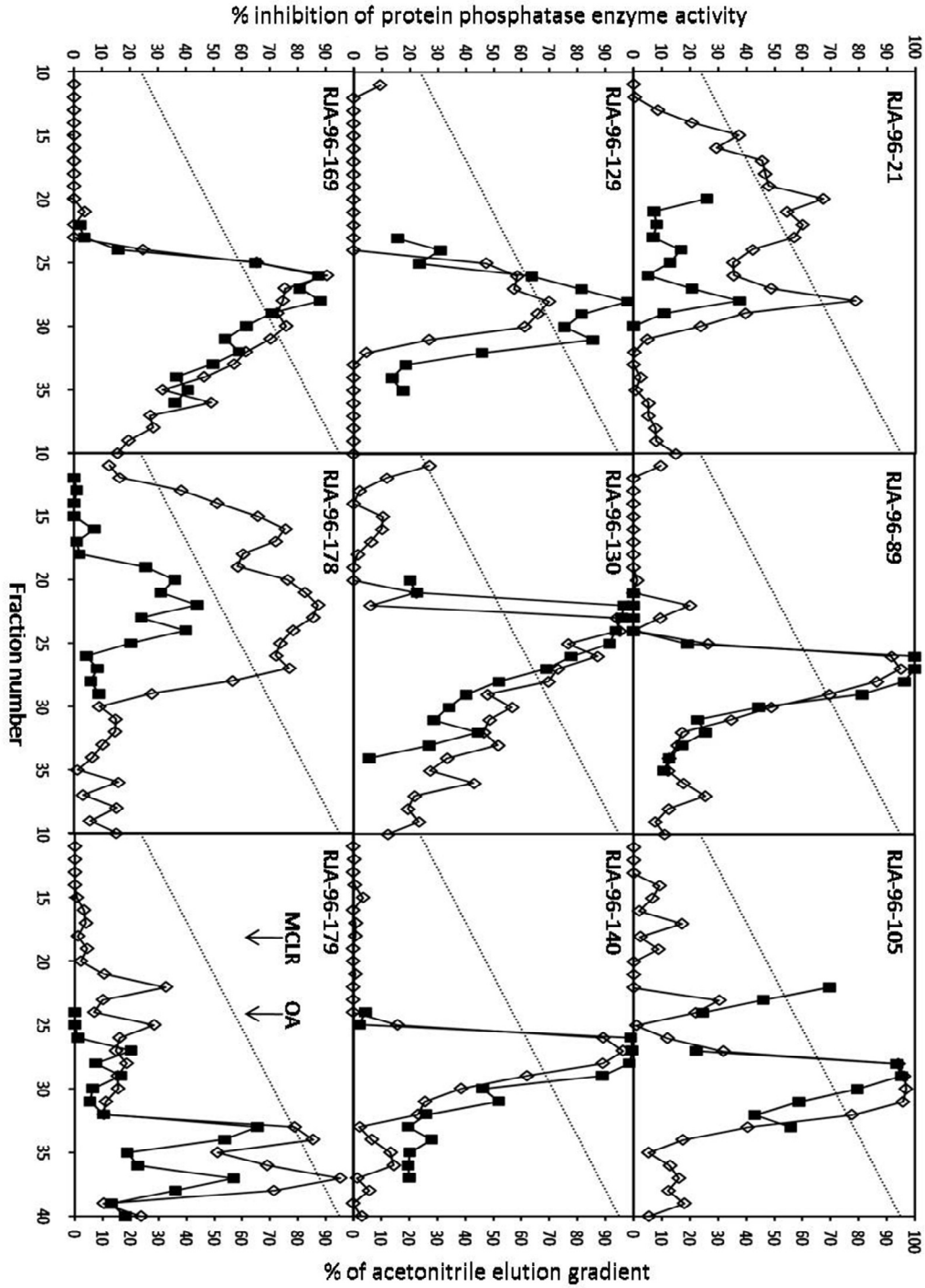



**Figure 4.4: Inhibition profiles of calcineurin and PP-1c phosphatases by the nine most potent Indonesian extracts.** Extracts were chromatographed on HPLC and individual fractions tested for inhibition of CaN against [$^{32}$P]-inhibitor-1 (◇), and inhibition of PP-1c against [$^{32}$P]-Phosphorylase a substrates (■). The acetonitrile gradient is indicated with dotted lines. The name of each extract is shown in top left corner. For comparison, peak elution of microcystin-LR (MCLR) and okadaic acid (OA) are indicated in the RJA-96-179 panel.

*Identification of inhibitory extracts from Papua New Guinea marine samples.*

After establishing that marine samples can be a source of inhibitory compounds against CaN, 281 marine samples from Papua New Guinea collected in 2003 were obtained from Dr. R. Andersen for examination. Analysis of the Papua New Guinea samples (1 mg/mL) using the CaN bioassay identified nearly 50 extracts that exhibited potent inhibition of CaN activity (see Figure 4.5, p. 176). To determine the specificity of these extracts, inhibition of PP-1c was monitored at the same concentration of extract (see Figure 4.6, p. 177). Several extracts showed greater inhibition of CaN than PP-1c. These included extracts RJA-03-58, RJA-03- 111, RJA-124, RJA-125, RJA-03-132, RJA-03-153, RJA-03-169, and RJA-03-229. None of the extracts that inhibited CaN showed complete lack of activity against PP-1c. Nevertheless, extract RJA-03-193 was pursued first, since this marine sample was available in the largest amount.

*Identification and characterization of novel calcineurin inhibitors of the sesterterpene sulfate family.*

Further fractionation and purification of extract RJA-03-193 by the collaborators in Vancouver, guided by activity-based bioassays performed in our laboratory, has produced three separate inhibitors within this single extract: halisulfate-7, hipposulfate C, relatively unknown compounds, and a novel compound termed irregularsulfate.[82, 117, 242]

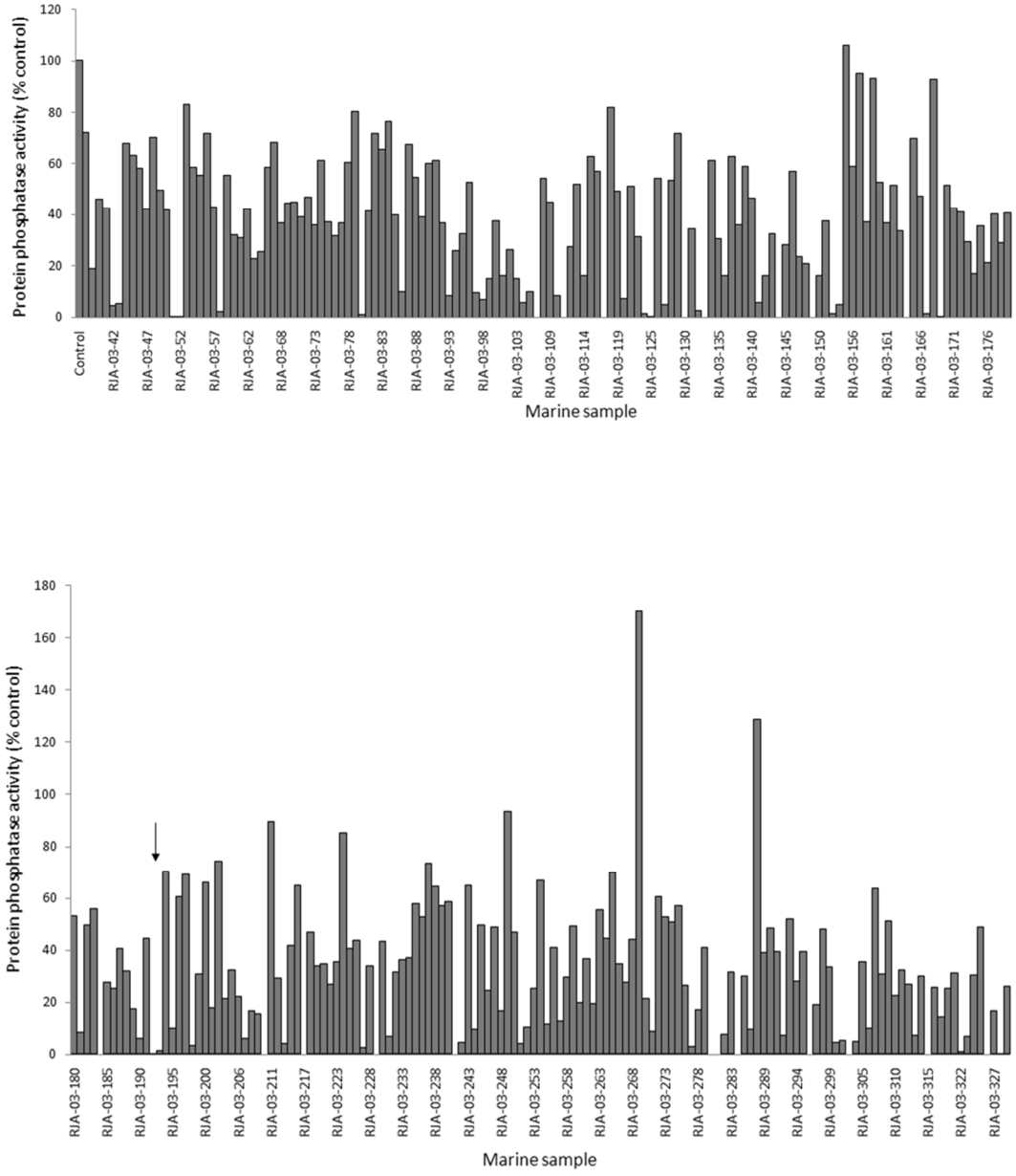



**Figure 4.5: Calcineurin inhibition profile with Papua New Guinea marine extracts.** 281 marine samples were homogenized, extracted with MeOH, and dried to completion. Extracts (100 µg) were resuspended in methanol at 10 mg/mL and tested against activity of CaN using [$^{32}$P]-inhibitor-1 substrate. Extract RJA-03-193 was further analyzed and is indicated with an arrow. For clarity, only every fifth extract identification number is listed.

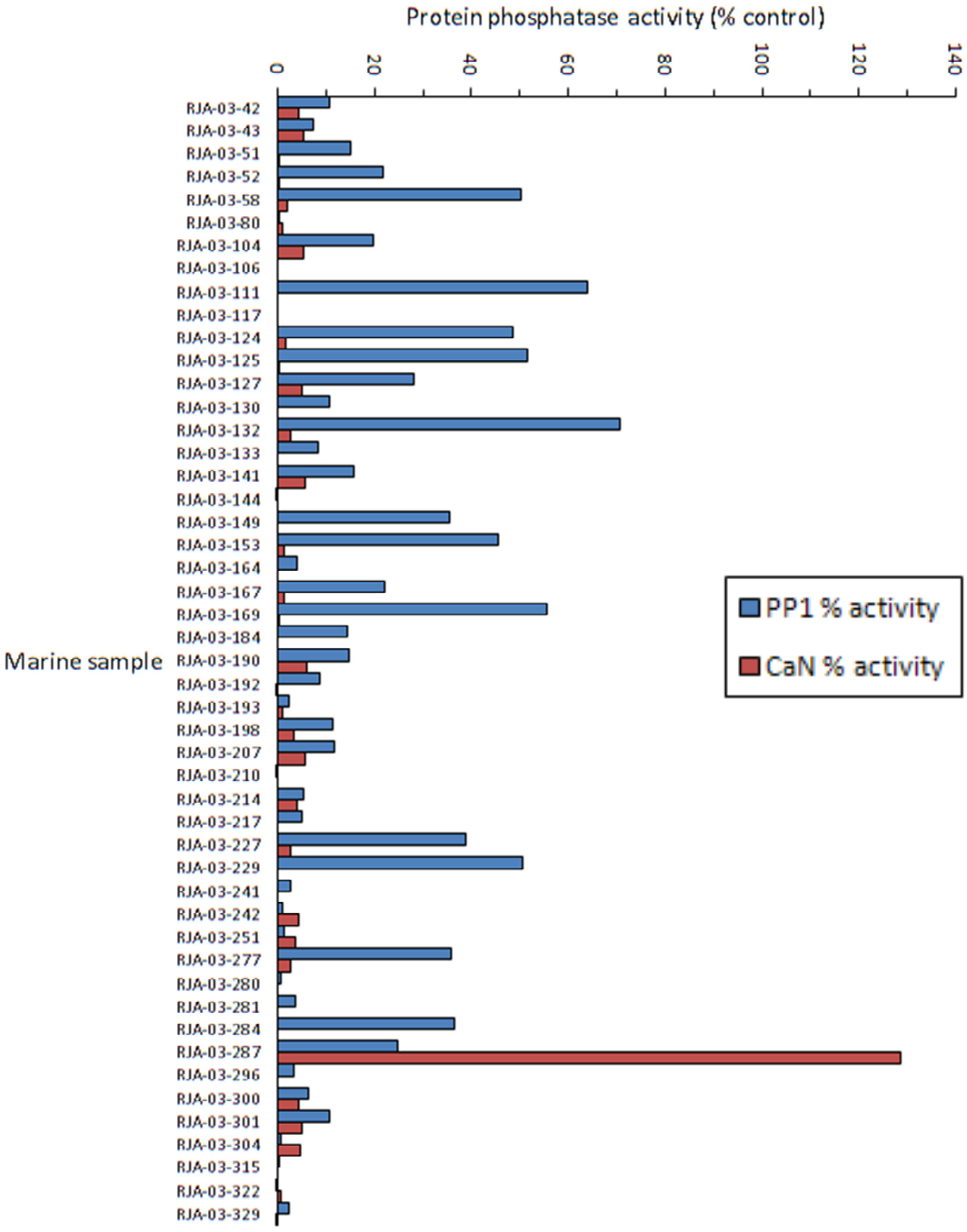



**Figure 4.6: Inhibition of PP-1c and calcineurin phosphatase activities by selected Papua New Guinea marine extracts.** Comparison of the most potent CaN inhibiting extracts (with exception of one activating extract RJA-03-287), is compared with PP-1 inhibition by the same extracts at the same concentration (1 mg/mL).

Figure 4.7 (p. 179) shows how bioassay-guided fractionation of the marine extract led to purification of the sesterterpene compounds. Chemical structures of the compounds as solved by nuclear magnetic resonance (NMR) are shown in Figure 4.8 (p. 180). These compounds belong to the sesterterpene sulfate family of compounds, a rare group of hydrocarbons characterized by 25 contiguous carbon skeleton, all of which have been derived from marine sponge sources. However, the role of these compounds in their native environment is not known.

The activity of CaN, PP-1c and PP-2Ac against their respective substrates in the presence of halisulfate-7, hipposulfate C or irregularsulfate was tested. Halisulfate-7 and hipposulfate C modestly and equipotently inhibited CaN at an $IC_{50}$ of ~70 µM. Irregularsulfate inhibited CaN at nearly the same potency, $IC_{50}$ of 57 µM (see Figure 4.9, p. 181). Halisulfate-7 and hipposulfate C inhibited PP-1c and CaN with similar potency, $IC_{50}$ ~70 µM. Inhibition of PP-1c by irregularsulfate was 2-fold more potent, $IC_{50}$ of 36 µM (see Figure 4.10, p. 182). PP-2Ac appeared more resistant to halisulfate-7 and hipposulfate C with $IC_{50}$ ~130–140 µM (see Figure 4.11, p. 183); however, these compounds were tested against only one enzyme preparation due to the difficulty of purification of the native PP-2Ac. Effect of irregularsulfate on PP-2Ac activity was not tested.

*Chemical modification of novel calcineurin inhibitors improved the sensitivity and specificity of the derivatives.*

The three inhibitors identified belong to the sesterterpene sulfate family of

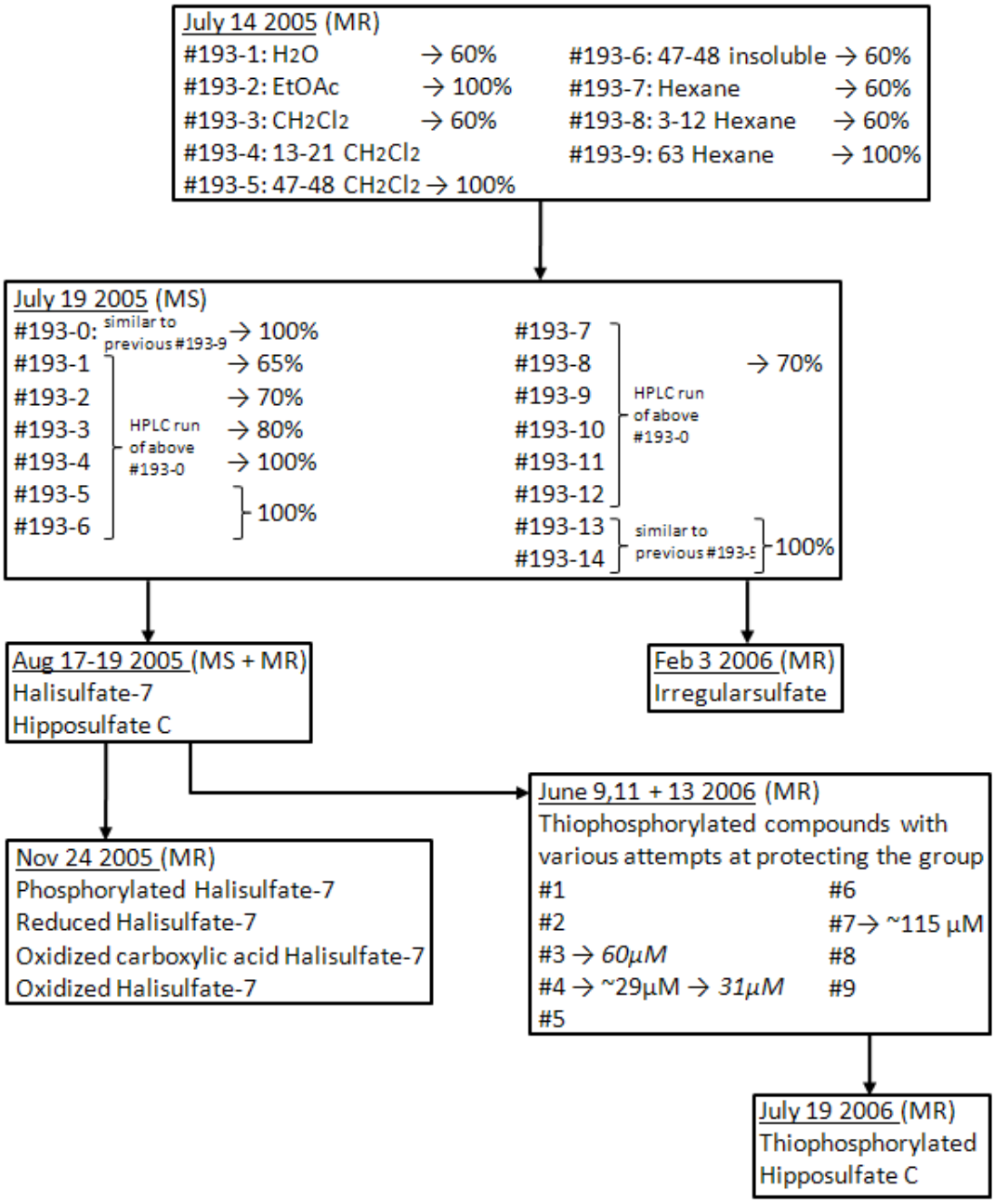



**Figure 4.7: Schematic chronological representation of calcineurin bioassay-guided isolation of inhibitory compounds from the RJA-03-193 marine extract.** Each box designates a batch of samples that were bioassayed for inhibitory activity; date of assay is shown. Initials in brackets indicate who performed the bioassays. Organic fractions or impure chemical compounds received from Vancouver for analysis are designated with the "#" symbol. Names of pure compounds tested are listed. Open ended arrows (→) show the amount of inhibition of CaN activity or the estimated $IC_{50}$—if followed by a second arrow, it indicates inhibition of PP-1c. MR = Mikolaj Raszek; MS = Micheal Shopik.

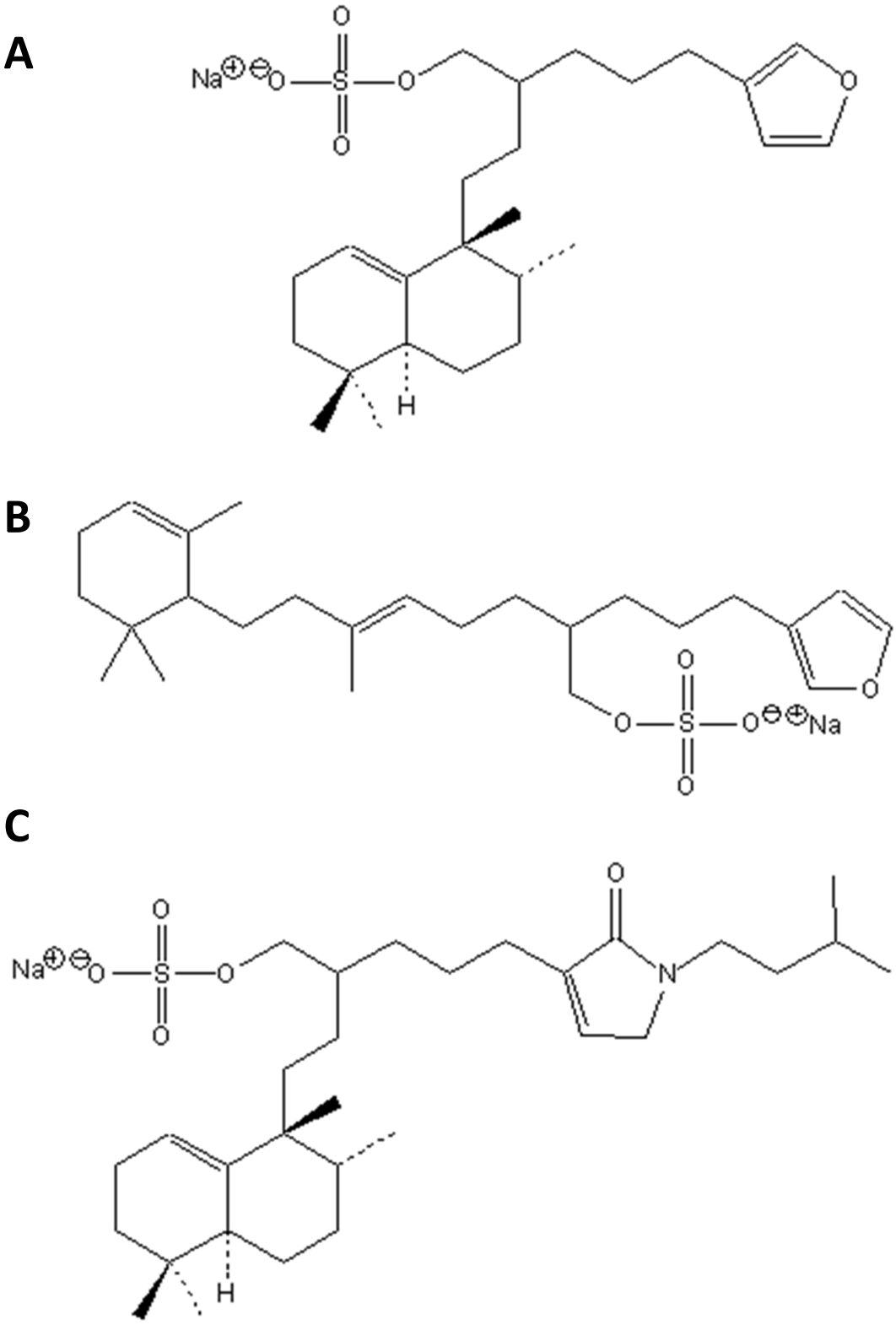
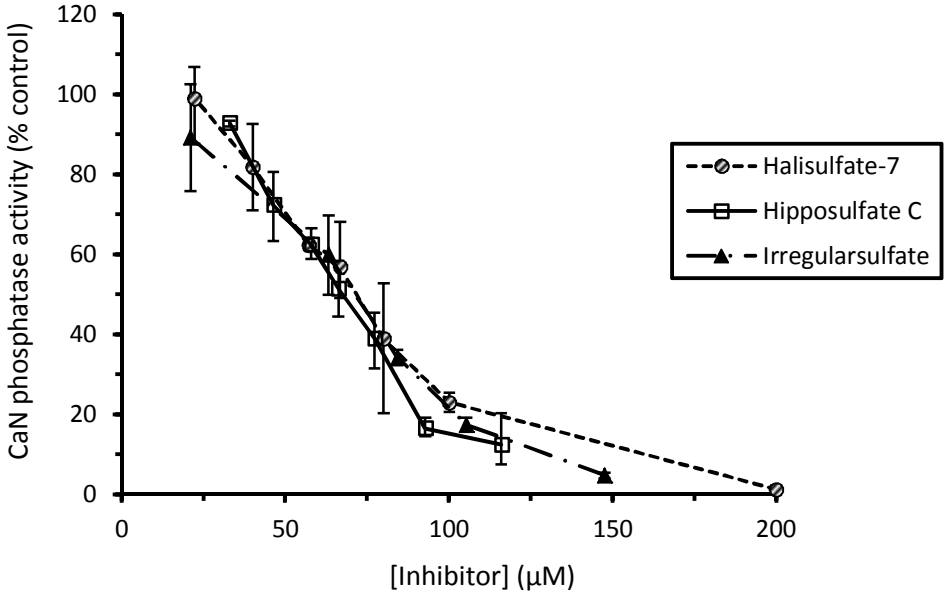



**Figure 4.8: Chemical structures of inhibitory compounds isolated from marine extract RJA-03-193.** Extract RJA-03-193 belonging to *Spongia irregularis* yielded three compounds showing moderate inhibition against Ser/Thr protein phosphatases of the PPP family: halisulfate-7 (panel A), hipposulfate C (panel B), and the novel irregularsulfate (panel C).

**A**

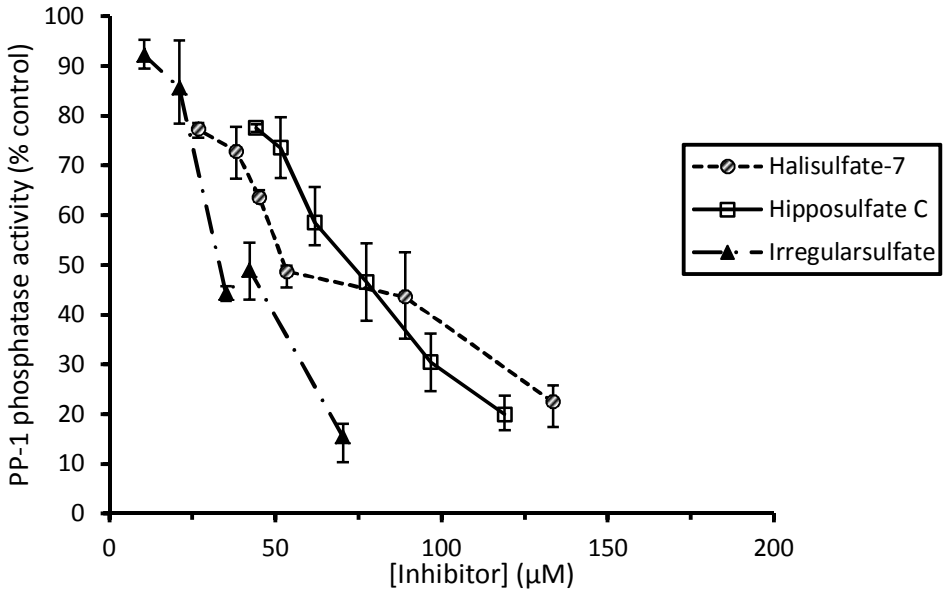



**B**

| Inhibitor | $IC_{50}$ (μM) | +/- |
|---|---|---|
| Halisulfate-7 | 69 | 10 |
| Hipposulfate C | 66 | 5 |
| Irregularsulfate | 57 | 8 |

**Figure 4.9: Sesterterpene sulfate compounds from extract RJA-03-193 inhibit calcineurin.** The effects of halisulfate-7 (--⊘--), hipposulfate C (—⊟—) and irregularsulfate (—▲· -) on CaN activity is shown in the inhibition profile plot (panel A). The corresponding $IC_{50}$ values are summarized in the table (panel B). Note that large error bars of the irregularsulfate inhibition of CaN skew the $IC_{50}$ value despite an apparent equipotency between the compounds. Assays were done in duplicate (n = 2–5). "+/-" indicates the variation seen in $IC_{50}$ values.

**A**

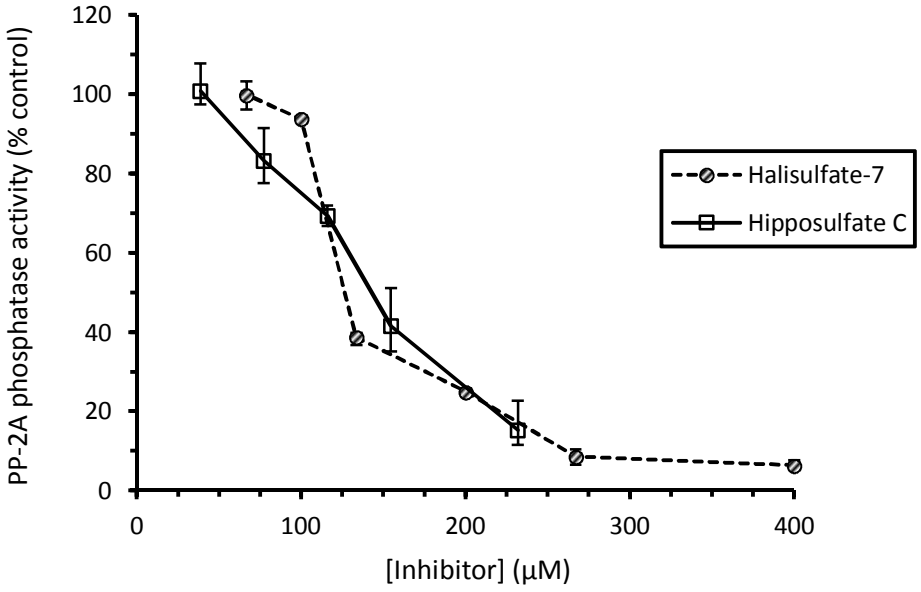



**B**

| Inhibitor | $IC_{50}$ (μM) | +/- |
|---|---|---|
| Halisulfate-7 | 64 | 8 |
| Hipposulfate C | 71 | 3.5 |
| Irregularsulfate | 36 | 2 |

**Figure 4.10: PP-1c inhibition by sesterterpene sulfate compounds from extract RJA-03-193 resembles that of calcineurin.** The effects of halisulfate-7 (--⊘--), hipposulfate C (—□—), and irregularsulfate (—▲· -) on PP-1c activity is shown in the inhibition profile plot (panel A). The corresponding $IC_{50}$ values are summarized in the table (panel B). Assays were done in duplicate (n = 3–4). "+/-" indicates the variation seen in $IC_{50}$ values.

**A**




**B**

| Inhibitor | $IC_{50}$ (µM) | +/- |
|---|---|---|
| Halisulfate-7 | 143 | 10 |
| Hipposulfate C | 132 | 19 |

**Figure 4.11: Sesterterpene sulfate compounds from extract RJA-03-193 inhibit PP-2Ac less potently than PP-1c and calcineurin.** The effects of halisulfate-7 (--⊘--) and hipposulfate C (—⊟—) on PP-2Ac activity is shown in the inhibition profile plot (panel A). The corresponding $IC_{50}$ values are summarized in the table (panel B). Assays were done in duplicate (n = 3). "+/-" indicates the variation seen in $IC_{50}$ values.

compounds, all of which contain a sulfate moiety. It was considered whether the mode of inhibition by these compounds is to block the active site of CaN with the sulfate group in the same way that a phosphate group of the substrate would fit. To test this hypothesis, chemical alterations to the sulfate group were undertaken in both halisulfate-7 and hipposulfate C (see Figures 4.12 and 4.13, p. 185–186).

The phosphorylated form of the halisulfate-7 compound resulted in worsening of inhibition of CaN activity about 3-fold, but improved its potency against PP-1c nearly 2-fold to an $IC_{50}$ of 36 μM (see Figure 4.12, p. 185).

Thiophosphorylated forms of halisulfate-7 and hipposulfate C were generated. Thiophosphorylated molecules cannot be dephosphorylated by the phosphatases; therefore this could test the hypothesis if phosphorylated halisulfate-7 could act as a substrate. Thiophosphorylated analogs resulted in lower sensitivity toward the phosphatases (see Figure 4.13, p. 186).

Substitution of the sulfate group with a carboxyl group in the sesterterpene sulfate compounds resulted in generation of oxidized form of carboxylic acid halisulfate-7. This modification also did not have a positive impact on the potency of the compound toward any of the phosphatases. Additional byproducts of these modifications were reduced and oxidized forms of halisulfate-7. Neither compound improved sensitivity or potency toward CaN or PP-1c. Oxidized halisulfate-7 was nearly 2-fold more sensitive toward PP-2Ac, with 75 μM $IC_{50}$.

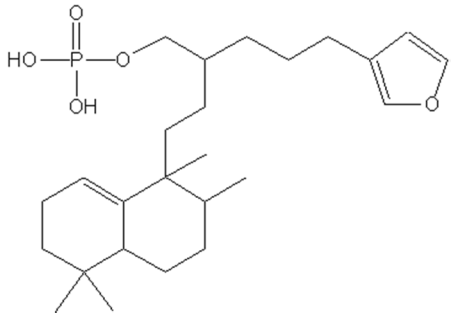
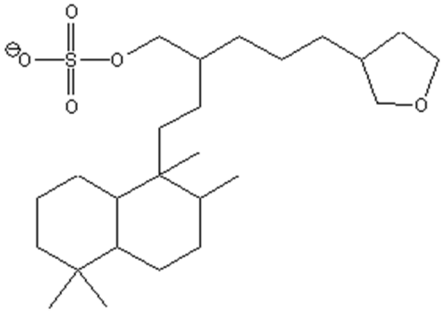
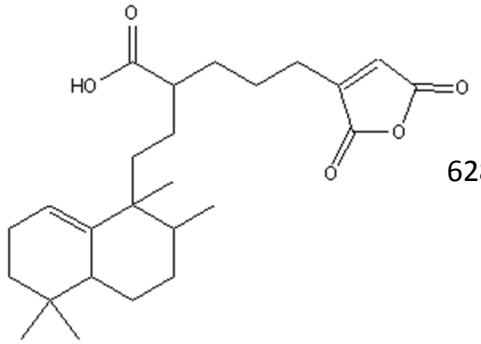
| | CaN | PP-1c | PP-2Ac |
|---|---|---|---|
| Phosphorylated Halisulfate-7  | 198 (±27) µM | 36 (±10) µM | Not tested |
| Reduced Halisulfate-7  | 93 (±7.5) µM | 68.5 (±2) µM | 189 (±30) µM |
| Oxidized carboxylic acid Halisulfate-7  | 628.5 (±33) µM | 106 (±35) µM | 170 (±25) µM |

**Figure 4.12: Inhibition of principal members of the PPP family of protein phosphatases by analogs of halisulfate-7.** $IC_{50}$ values against PP-1c, CaN and PP-2Ac are shown. Assays were done in duplicate (n = 2–5). "+/-" indicates the average variation in the reported $IC_{50}$. Each row corresponds to the chemical compound shown.

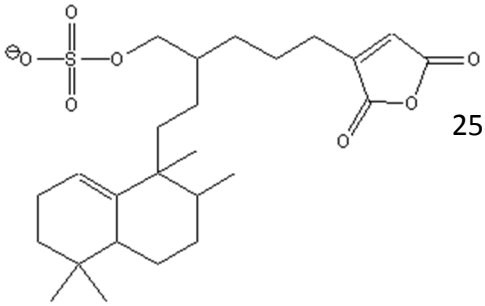
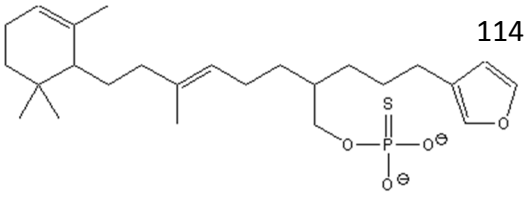
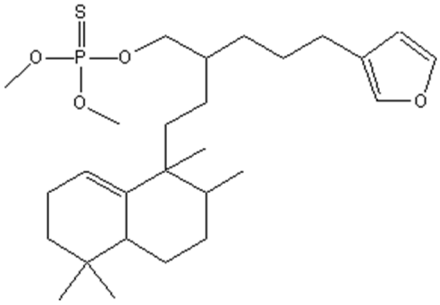
| | CaN | PP-1c | PP-2Ac |
|---|---|---|---|
| Oxidized Halisulfate-7  | 254.5 (±17) µM | 131 (±16.5) µM | 75 (±13) µM |
| Thiophosphorylated Hipposulfate C  | 114 (±46) µM | 172.5 (±30) µM | 355 (±98) µM |
| Protected thiophosphorylated Halisulfate-7  | No inhibition | Not tested | Not tested |

**Figure 4.13: Inhibition of principal members of the PPP family of protein phosphatases by analogs of halisulfate-7 and hipposulfate C.** $IC_{50}$ values against PP-1c, CaN and PP-2Ac are shown. Assays were done in duplicate (n = 3–6). "+/-" indicates the average variation in the reported $IC_{50}$. Each row corresponds to the chemical compound shown.

*Novel inhibitors of calcineurin inhibit T-cell production of interleukin-2.*

To test the bioavailability of the sesterterpene sulfate compounds, their effectiveness in inhibiting IL-2 production by T-cells was tested. Hipposulfate C inhibited IL-2 production in a dose dependent manner. Halisulfate-7 was also shown to significantly inhibit IL-2 production, but did not show concentration dependence (see Figure 4.14, p. 188). This confirmed the *in vitro* results, and established a way to monitor the ability of novel inhibitors to inhibit CaN against NFAT substrates. Effects of irregularsulfate were not studied.

*Analysis of additional marine extracts.*

Other extracts that inhibited CaN activity were investigated: RJA-03-58, RJA-03-111, RJA-03-124, RJA-03-149, RJA-03-214, RJA-03-269, and RJA-03-287. Appendix C (p. 287) summarizes bioassay-guided fractionation and purification of these extracts. Of these, RJA-03-269 and RJA-03-287 appeared to harbor CaN activators (see Figure 4.5, p. 176), while RJA-03-111 extract appeared to be CaN specific for inhibition (see Figure 4.6, p. 177). Extract RJA-03-149 was also pursued extensively for potential inhibitors. Unfortunately, while the bioassay allowed the inhibiting compounds to be followed during fractionation of the extract, the fractions did not contain enough material to obtain structures of the inhibitors.

## 4.5 Conclusions

CaN inhibition in T-cells during organ transplantation is necessary to

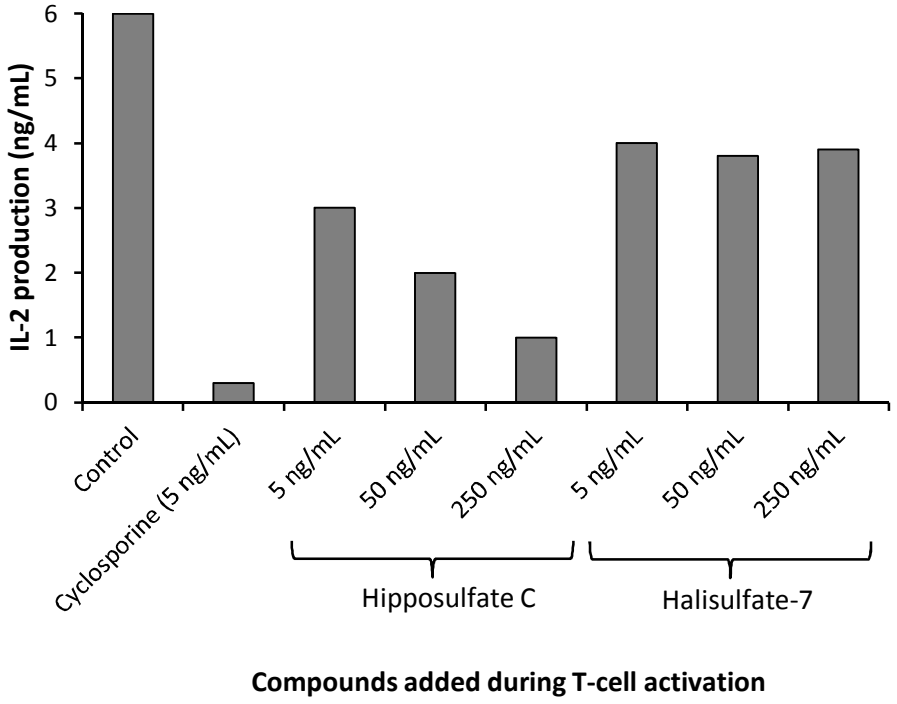



**Figure 4.14: Sesterterpene sulfate compounds from extract RJA-03-193 inhibit Jurkat T-cell activation.** T-cell activation was indirectly detected by measuring the interleukin-2 (IL-2) cytokine release by lymphocytes. The control demonstrates the amount IL-2 produced by uninhibited T-cells.

improve chances of successful tolerance. However, use of the clinical drugs for this purpose is marred by many toxic side effects experienced by the patients.[23, 145, 175] It is believed that the main reason for the toxicity of these drugs is global inhibition of CaN throughout the body.[23, 145, 167, 267] In recent years the search for novel immunosuppressants has generated much interest. Although several candidates have been obtained from chemical libraries, no inhibitors have been sought in organisms from a marine environment. With an estimated half of current drugs based on natural product compounds, surprisingly, only a small fraction of such drugs are derived from marine environment source.[8, 160]

This chapter outlines bioassay-guided fractionation and purification of CaN inhibitors from extracts of marine organisms. This technique led to isolation and identification of three compounds from extract RJA-03-193: halisulfate-7, hipposulfate C, and a novel compound, irregularsulfate, all members of the sesterterpene sulfate family of compounds (see Figure 4.8, p. 180).

Extract RJA-03-193 belonged to the marine sponge *Spongia irregularis*, however, halisulfate-7 was previously isolated from *Coscinoderma mathewsi, Stoeba extensa* and *Fasciospongia cavernosa* sponges, while *Hippospongia* sp. sponge was a described source for hipposulfate C.[82, 117, 242, 311] *Spongia irregularis* is the first reported source of irregularsulfate. Although its name is derived from the taxonomic name of the sponge in which it was identified, irregularsulfate, as its name suggests, is not a typical member of the sesterterpene family of

compounds. Unlike halisfulfate-7 or hipposulfate C, and many other sulfated sesterterpenes, it does not contain a furan ring. Instead, irregularsulfate contains a pyrrole ring. At the time of its discovery, to our knowledge, it was the only known sulfated sesterterpene alkaloid compound. However, during the course of publication submission two other sulfated sesterterpene alkaloids were reported, termed fasciospongine A and B.[310] Currently irregularsulfate belongs to a very unique class of compounds that includes only five members.[53, 310, 311] Other than the furan ring portion at position $C_{17}$, irregularsulfate is identical to halisulfate-7 (see Figure 4.8, p. 180).

Sesterterpene sulfates have been tested for numerous biological effects, and were shown to exhibit antimicrobial activity, cytotoxicity against various cancer cell lines, as well as inhibition of phospholipase $A_2$, serine proteases, lypoxygenases, isocitrate lyase, proteasome, inflammation and cell division of fertilized sea urchin eggs.[9, 82, 173, 177, 189, 211, 298, 310, 311] It has been demonstrated that halisulfate-7 is cytotoxic to cervical carcinoma human epithelial HeLa cells with an $IC_{50}$ of 16 µg/mL.[242] A separate study did not show halisulfate-7 to be cytotoxic to cancerous human prostate, breast and lung cells at 20 µg/mL. Halisulfate-7 and hipposulfate C have also been reported to be inhibitors of human lypoxygenases.[82] To our knowledge this is the first report of sesterterpene sulfate inhibition against protein phosphatases. Manoalide and its analog, luffariellolide, which closely resembles hipposulfate C, have been reported to inhibit Cdc25 phosphatase with $IC_{50}$ of 1mM.[29, 93] However, the sulfate group is

absent from these compounds.

Halisulfate-7 and hipposulfate C were shown to inhibit both CaN and PP-1c at an $IC_{50}$ of ~70 μM (see Figures 4.9 and 4.10, p. 181–182). Inhibition of CaN by irregularsulfate had a calculated $IC_{50}$ of 57 μM. However, this value is skewed due to the variation seen in the assays. A plot of the average values of the different assays indicated inhibition similar to that seen with other sesterterpene sulfates (see Figure 4.9, p. 181). In another notable exception irregularsulfate inhibition of PP-1c appeared to be nearly 2-fold more potent than inhibition with other sulfated sesterterpenes against either PP-1c or CaN (see Figure 4.10, p. 182). PP-2Ac was more resistant to halisulfate-7 and hipposulfate C, with reported $IC_{50}$ values of ~130–140 μM. Currently we have no explanation to account for these observations. The effect of irregularsulfate on PP-2Ac was not studied.

A typical goal of high-throughput screening of a chemical library is actually not to discover a novel drug of nM range, which would be a rare incident, but rather a compound of μM or sub-μM activity that can be used as a starting compound to generate more active substances via chemical manipulation of the parent compound.[160] Since the compounds identified in this research belonged to the sesterterpene sulfate family, it was thought that they might inhibit the protein phosphatases as substrate inhibitors, the sulfate group mimicking the phosphate group. Therefore the sulfate groups of the sesterterpene compounds were altered

and effects of these changes were measured.

First, a phosphate group was substituted for the sulfate to theoretically make the compound more potent by incorporating a better fit into the active site of the enzyme. This change did not have the expected impact on sensitivity to CaN, but increased the sensitivity 2-fold towards PP-1c. This turned out to be the most potent inhibition observed against any of the three phosphatases tested, with an $IC_{50}$ of 36 μM (see Figure 4.12, p. 185).

It was considered that the increased inhibition of PP-1c by the phosphate derivative of halisulfate-7 and the decreased potency against CaN, could be explained if the phosphorylated analog acted as a substrate of CaN but not of PP-1c. To test this hypothesis, thiophosphorylated hipposulfate C, and methyl protected thiophosphate halisulfate-7 analogs were generated and tested against the phosphatases (see Figure 4.13, p. 186). However, the thiophosphate form of hipposulfate C resulted in compromised potency with at least a 2-fold higher $IC_{50}$ against the phosphatases than the sulfated parent compound. The methyl protected form of halisulfate-7 thiophosphate was even less potent, showing only 20% inhibition against CaN at 210 μM, and thus was not tested further against any of the phosphatases (data not shown). Therefore it could be concluded that the phosphorylated form of halisulfate-7 did not behave as a substrate for CaN.

In an effort to render the sesterterpene sulfates more potent or more CaN specific, other chemical alterations were made to the starting compounds. They include the following derivatives of halisulfate-7: reduced form, oxidized form, and oxidized analog containing carboxylic acid in place of the sulfate group. Reduced and oxidized forms of halisulfate-7 either have double bonds removed, or have altered furan ring structures, respectively. Improvements in $IC_{50}$s were seen only for PP-2Ac with the oxidized halisulfate-7 analog, a 2-fold reduction in $IC_{50}$ to 75 μM (see Figure 4.13, p. 186). Why certain analogs had altered sensitivity to specific phosphatases is currently not understood.

Bioavailability of the sesterterpene sulfate compounds was also investigated using human T-cells. One of the main cytokines produced by activated T-cells is IL-2 which is necessary for further proliferation and differentiation of the T-cells. IL-2 production itself requires activation of the CaN signaling pathway (see Figure 1.10, p. 29).[206] Thus, inhibition of IL-2 production by sesterterpene compounds not only provides the means to confirm *in vitro* results in live cells, it also establishes bioavailability of the compounds against the cell-line that would be most important to be targeted by novel inhibitors of CaN.

Hipposulfate C showed potent inhibition of IL-2 production in a dose dependent manner but required at least 50-fold higher doses than CsA to obtain an equivalent effect. Halisulfate-7 also inhibited IL-2 production but did not exhibit the concentration dependence seen with hipposulfate C (see Figure 4.14, p. 188).

It is difficult to interpret this data. One possible explanation is that the halisulfate-7 compound is being sequestered away by an unknown molecule before it can act on CaN. However, this demonstrates that both compounds are readily bioavailable with immunosuppressive activity. Role of sesterterpene sulfates as immunosuppressants deserves further and thorough investigation. Manoalide, the parent compound of the sesterterpene class of metabolites, has reached phase II clinical trials as a topical antipsoriatic.[93] ISA247 (Voclosporin), the drug developed by Isotechnika, Edmonton, a CaN-specific immunosuppressant, is also tested for such treatment, currently in phase III clinical trials.

Additional marine extracts were investigated for CaN inhibition. These included RJA-03-58, RJA-03-111, RJA-03-124, RJA-03-149, RJA-214, RJA-03-269, and RJA-03-287. The bioassay procedures that guided the fractionation of these extracts are summarized in Appendix C (p. 287). Of particular interest was RJA-03-111 which showed specificity toward CaN. Unfortunately, with this, as with the other extracts, there was insufficient material to characterize the inhibiting compounds. This highlights one of the main problems in searching for novel compounds from natural sources. Either a large amount of extract is necessary to pursue isolation of a novel inhibitor, or the targeting compound must be produced in a large quantity by the organism.

The above methods have good potential in the search for novel drugs and the procedures do not have to be limited to a search for a regulator of one specific

enzyme. This approach can also be fast if the components required for compound identification are available and the marine source produces a sufficient amount of natural product. In fact, when these conditions were met, purification of sesterterpene compounds was very fast as can be seen from the chronological schematic of Figure 4.7 (p. 179), allowing for further chemical modifications of the lead compounds.

The principle of a successful bioassay-guided fractionation of marine extracts leading to isolation of novel CaN inhibitory compounds is clearly established. This is an important achievement as it can readily be applied toward any extract of natural source. Furthermore, the quantity of natural product limitation could be overcome if testing of extracts was performed on the site where the organism is actually collected.

On-site screening could be achieved using a *p*NPP substrate. Dephosphorylation of *p*NPP is easily detected as the products of the reaction change color. Another added advantage of such an assay is that it would not even require any instrumentation for detection, as colorimetric assay lends itself to easy visual inspection to determine inhibition of dephosphorylation.

In a further simplification for on-site testing, the dephosphorylation assay could use a truncated form of CaN that is not dependent on regulation by CaM or CaN-B. Chapter two of this thesis describes how such form of CaN was generated

and expressed. Finally, extracts could be tested on site for inhibition of PP-1c, as all Ser/Thr phosphatases dephosphorylate the *p*NPP substrate. These modifications would allow more specific selection of marine organisms whose extracts inhibit CaN in quantities sufficient for characterization. A diagram summarizing this novel procedure is shown in Figure 4.15 (p. 197). Collected marine samples could then undergo the bioassay-guided fractionation and purification as demonstrated in this chapter, using the more sensitive approach of radiolabeled I-1 as a substrate.

Use of I-1 as a substrate in the bioassay-guided fractionation of samples of interest has another important advantage. It was shown in chapter three that I-1 binds to CaN in a manner analogous to NFAT before being dephosphorylated; thus the bioactive screen is potentially doubly useful:

1) It identifies novel potential inhibitors of CaN;

2) It allows for identification of inhibitors that abrogate CaN function by blocking the PXIXIT motif binding groove of CaN-A on which I-1 depends for interaction with CaN.

The I-1 substrate can be used to find inhibitors of CaN that would target only the dephosphorylation of CaN substrates that require the PXIXIT motif binding groove to dock onto the phosphatase. Therefore only a small subset of all CaN substrates would be affected by such inhibitors. An inhibitor that would prevent dephosphorylation of NFAT, and only a few more substrates (I-1

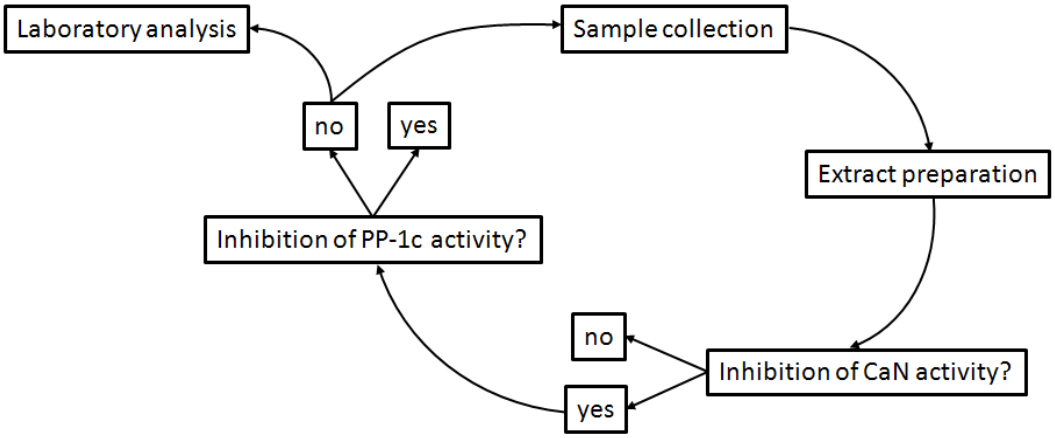



**Figure 4.15: Diagram outlining a procedure for on-site identification of marine organisms as a potential source of calcineurin inhibitors.** Phosphatase activity would be assessed using *para*-nitrophenylphosphate substrate. For CaN assays, a truncated form of the enzyme would be used that is no longer dependent on CaM and CaN-B subunit regulation.

included), could be expected to provide fewer of the toxic side effects currently seen with clinical CaN drugs.

Whether inhibitors of CaN found using our bioassay-guided method are targeting the PXIXIT motif binding groove could be determined in a 2-fold procedure:

1) Purified inhibitors are tested against WT CaN using substrate directed only to the active site such as $R_{II}$ peptide or *p*NPP. This will differentiate between inhibitors that bind to the active site and hence diminish $R_{II}$ peptide substrate dephosphorylation, and those that bind elsewhere on the CaN protein, which ought to have no effect on the $R_{II}$ peptide dephosphorylation;

2) Inhibitors that do not affect $R_{II}$ dephosphorylation, and hence possibly bind at CaN sites other than the active site, would then be further tested to see if they can obstruct the PXIXIT motif binding groove of CaN. This method, first developed by Roehrl et al. (2004), relies on displacement of fluorescently labeled VIVIT peptide from CaN by the inhibitor. The authors utilized this method in their identification of novel inhibitors of CaN that targeted NFAT dephosphorylation specifically.[246, 247] Recently such approach was also undertaken by Mulero et al. (2010) using a peptide spanning the PXIXIT motif of regulators of CaN (RCANs) to find four small molecule candidates that disrupted the peptide interaction with CaN.[50, 225]

Screening marine organisms for novel inhibitors of CaN represents a new approach in the search for CaN drugs. Due to the numerous problems encountered with the use of CsA/FK506, and the variety of common disorders in which CaN plays a role, finding regulators of CaN that are more specific to one or a few of its many bodily functions is of obvious significant importance. Information included in this chapter allows streamlining of such projects, with the added bonus of finding drugs that could be NFAT specific.

The marine environment has proven to be a prolific and diverse source of novel compounds.[8, 266] The number of CaN-inhibiting candidates seen in our assays of marine organism extracts strongly argue in support of using the marine environment as a source of natural chemicals. Halisulfate-7, hipposulfate C and irregularsulfate sesterterpene sulfates act as examples of compounds that could target many biological functions, including CaN inhibition. More specific compounds could also be expected. Further search for CaN inhibitors of marine origin is prudent. Faced with the increasing marine habitat loss under ever increasing environmental duress to which many inhabitants cannot adapt, the time to exploit the marine environment as a source of potential therapeutic drugs is running out.[138, 139, 294]

# Chapter 5: Conclusion

The study of protein phosphatases is a look at the mechanics of eukaryotic cell life itself, as a number of these enzymes are essential for viability.[223] Protein phosphorylation and its reversal is one of the most important methods for regulating the function of proteins in myriad signal transduction pathways. Ser/Thr phosphatases perform the bulk of protein dephosphorylation.[66, 147, 223, 257] New members of the Ser/Thr family of protein phosphatases continue to be uncovered, but historical evidence suggests that CaN, PP-1c and PP-2Ac are the most prolific phosphatases in eukaryotic cells, all belonging in the PPP family of phosphatases.[219]

CaN is unique among the Ser/Thr protein phosphatases in that its activation is regulated by cellular $Ca^{2+}$ levels. This dependence on $Ca^{2+}$ ions for activation restricts the number of cellular functions CaN performs compared to the diversity of roles ascribed to PP-1c and PP-2Ac. Important roles of CaN in mammals include cardiac valve development, skeletal muscle type switching and, most notably, activation of the immune system. The involvement of CaN in cardiac hypertrophy development has also been convincingly established.[12, 250]

Despite the structural similarity and high amino acid homology among members of the PPP family of phosphatases, CaN shares very little in common with PP-1c and PP-2Ac.[143, 180] For instance, CaN is resistant to OA, microcystin,

and various other natural toxins that potently inhibit PP-1c and PP-2Ac.[26, 147, 208, 261] Uniquely, CaN is inhibited by drugs that require concomitant binding of endogenous immunophilin proteins, which do not interact with other Ser/Thr protein phosphatases.[199] CaN shares no common binding partners such as regulatory proteins or substrates with other protein phosphatases; protein phosphatase inhibitor-1 (I-1) is a notable exception, however. Phosphorylated I-1 is a substrate of both CaN and PP-2Ac, and is a potent and specific inhibitor of PP-1c.[261] This results in the only known Ser/Thr phosphatase cascade, where one phosphatase is responsible for regulation of another phosphatase. Inhibition of PP-1c by I-1 can thus be regulated upstream by CaN or PP-2Ac in either the brain or cardiac muscle.[65, 127, 226]

The marine environment is a rich source of chemicals that have the potential to treat human disease. This thesis contributes to the search for and development of such chemicals by discovering and characterizing inhibitory compounds of CaN. In addition, the molecular basis of CaN inhibition was investigated in the interest of developing inhibitors that regulate CaN functions more specifically than the present clinical immunosuppressants. First, the molecular basis of the resistance of CaN to the natural toxins OA and microcystin was investigated. Understanding of interactions between phosphatases and toxins would be expected to aid chemical synthesis of CaN specific analogs. In second approach, a search for novel CaN inhibitors was undertaken directly using marine extracts, and a practical method was developed to screen samples and separate

compounds that inhibit CaN. Third, the hypothesis that I-1 interacts with CaN in a same manner as NFAT proteins was investigated. Compounds that disrupt substrate binding to CaN via the PXIXIT motif, as observed for NFAT proteins, would be expected to target a smaller subset of CaN reactions, thereby providing more specific regulation of CaN.

## 5.1 Mutagenesis of calcineurin to generate a form of enzyme sensitive to okadaic acid and microcystin

The starting point of this study was a comparison of the crystal structures of CaN holoenzyme with that of PP-1c bound to either OA or microcystin natural toxins. Residues in the vicinity of 4 Å from the toxin binding sites on PP-1c were examined in an effort to find physical or chemical reasons for the difference in sensitivity between CaN and PP-1c/PP-2Ac.[180, 217, 218] Four CaN residues, Tyr-159, Phe-160, Leu-312, and Tyr-315 were mutated to the corresponding residues of PP-1c or PP-2Ac. One additional residue was considered for mutagenesis that was not present in either PP-1c or PP-2Ac. The choice of this amino acid was directed by its size compared to the residues found in the other phosphatases. This thesis describes the first study of CaN mutagenesis of this kind, and facilitated the first study of OA and MCLR binding to a form of CaN with increased sensitivity to these toxins.

A quadruple mutant of CaN (Y159I:F160Y:L312C:Y315L) resulted in the largest increase in sensitivity to MCLR which inhibited CaN activity with an $IC_{50}$

of 2 nM. This mutant was nearly 600-fold more sensitive to toxin binding than WT enzyme. The simplest explanation for this large increase in sensitivity may involve the formation of a hydrogen bond between the carboxylic acid of the Masp residue of MCLR and the Tyr-160 residue of the mutant; also there appeared to be reduced steric hindrance of the mutated β12–13 loop. The β12–13 loop participates in formation of the active site of the PPP family of phosphatases.[59, 94, 180, 305, 308] This loop is highly variable among Ser/Thr protein phosphatases, and has been shown to play an important role in natural toxin binding.[74, 76, 169, 320] Further truncation of the CaN quadruple mutant (residues 26–344) had a negligible effect, resulting in an $IC_{50}$ of 1.8 nM; this suggests that additional regulatory domains of CaN have no impact in its interaction with microcystins.

Sensitivity of the mutant CaN to OA was also dramatically increased, resulting in an $IC_{50}$ of 330 nM, about 40-fold more sensitive than WT CaN. Mutation of only two residues provided this sensitivity to OA, L312C:Y315L of the β12–13 loop. Mutagenesis of other active site residues (Y159I and F160Y) impedes interaction with OA. Interestingly, truncation of the quadruple mutant CaN resulted in sensitivity similar to the L312C:Y315L double mutant form of CaN. This suggests that, unlike MCLR, the additional regulatory domains may have a negative impact on OA interaction with the enzyme. One possibility is that this could be due to competition with the autoinhibitory domain of CaN. This result is not seen with MCLR as it binds more tightly than OA to CaN.

Alternatively, truncation of the terminal segments of CaN could have affected the architecture of the active site. This result is consistent with the fact that a hyperactive form of CaN is produced when the CaM and CaN-B regulatory domains are deleted, suggesting that they affect the structure of the catalytic core. Such deletions could have a direct bearing on OA binding as well.[300, 302, 309] N-terminal end of CaN has also been shown to bear impact in substrate dephosphorylation and OA sensitivity.[304]

The work here explains why CaN is less sensitive than PP-1c and PP-2Ac to OA and MCLR. This understanding provides the basis for chemical modification of OA and MCLR toxins to generate congeners that would inhibit CaN but not PP-1c and PP-2Ac. Alternatively such congeners could be synthesized *de novo*. Such an approach has been attempted by others to generate a form of microcystin more sensitive to PP-1c than PP-2Ac.[3] Considering the negative impact of the CaN active site architecture on sensitivity to OA and MCLR, altered forms of these toxins that would inhibit CaN would very likely not inhibit PP-1c and PP-2Ac. In view of the numerous problems encountered with the current clinical regimen of CaN inhibitors, generation of CaN specific OA or MCLR analogs would be an important step forward in the search for novel CaN suppressants.

How currently existing natural toxins should be chemically modified merits further discussion. The first question is which toxin would be a more

suitable candidate, OA or microcystin, for chemical manipulation. The choice of OA is problematic because it is difficult to obtain. Dinoflagellates which produce OA are not easily cultured, and isolation and quantification of the OA is time and labor intensive. Microcystins are available in the ubiquitous blue-green algae and the most common microcystin, MCLR, is easy to isolate. However, MCLR is too hydrophilic to pass into cells, and in mammals it is targeted specifically at hepatocytes. Liver cells can be successfully targeted through delivery of MCLR by the bile ducts.[78, 86]

The cellular permeability of more hydrophobic forms of microcystin, for instance, microcystin-LL (MCLL) has not been studied but it is possible that they could cross the cell membrane. A dihydroform of MCLL would exclude the possibility of the toxin's covalent bonding to Ser/Thr protein phosphatases (see chapter one). Microcystin is a more potent inhibitor of CaN ($IC_{50}$ ~1 μM) than OA ($IC_{50}$ ~12 μM) (see chapter two), and thus would be a better candidate for chemical manipulation.

A covalent attachment of microcystin to CaN, although not likely for therapeutic use, could be used to study toxin delivery to cells, and therefore establish if microcystin could be used as an immunosuppressant. Studies described in chapter two suggest that Tyr-315, present in CaN but not in other Ser/Thr protein phosphatases, might lend itself to covalent bond linkage. For instance, an alkene group attached to MCLL in a position where it would be

proximal to Tyr-315 in the CaN-MCLL complex could promote an attack on the Tyr-315 hydroxyl group, producing a covalently bound MCLL-CaN complex. Effects on all protein phosphatases would have to be tested to see if the modified toxin reacts specifically with CaN.

Our model for predicting how CaN binds to dihydro-MCLA (see Figure 2.6, p. 102) suggests that Tyr-315 is in close proximity to many structural elements of microcystin. Nearly the entire backbone of microcystin from D-Glu residue 4 to D-Ala residue 6 is within 4 Å of Tyr-315. Thus, addition of an alkene group to any carbon atom along this portion of the toxin, with subsequent generation of an alkyl free radical, could potentially lead to a covalent bond between the toxin and the hydroxyl group on Tyr-315 of CaN. The L-Leu residue 7 of microcystin would be a good target for chemical modification as two of the side chain carbon atoms are 3.4 and 4.3 Å away from Tyr-315 of CaN.

Polar groups introduced to microcystin in the areas described above could participate in hydrogen bonding with Tyr-315, creating noncovalent associations between microcystin and CaN. In addition, Tyr-159 of CaN, while too distant from bound microcystin to expect covalent binding, is 5.5–6.5Å away from two carbon atoms at the base of the Adda residue 3 side chain of microcystin; this area of microcystin could be modified to allow polar interactions with Tyr-159.

The introduction of polar groups to microcystin could compromise the compound's hydrophobicity and prevent cellular delivery of the molecule. One way to alleviate this problem would be to remove the carboxyl groups from the first and fourth amino acids of microcystin (see Figure 1.13B, p. 34). This would have an added bonus of reducing the potency of the toxin against other Ser/Thr protein phosphatases, as binding of phosphatase depends on hydrogen bonding with the carboxyl group of the D-Masp residue of microcystin.

In another approach, novel forms of microcystin could be generated through genetic manipulation of the operon that codes for the enzymes involved in microcystin synthesis. Catalogues of gene clusters that encode enzymes involved in the production of natural products can help to determine the function of these enzymes. This provides an opportunity to engineer new compounds. In addition, by swapping genes from gene clusters responsible for the synthesis of different molecules, new natural product variants could be synthesized.[63] The microcystin biosynthesis gene cluster has been sequenced for different strains of the *Microcystis* genera and *Planktothrix agardhii*, all of which can produce several different forms of microcystin.[61] Christiansen et al. (2003) successfully isolated a novel form of microcystin generated through genetic manipulation of the cyanobacterial genome. This provides a way to obtain analogs of microcystin not found in nature which could be tailored to be CaN specific.[61]

A further benefit of this research is described below. Microcystin can be covalently linked to Sepharose resin; the modified resin can then be used to trap proteins that bind microcystin. Microcystin sensitive CaN mutant could be separated from a mixture of components by applying the mixture to microcystin resin. Microcystin-Sepharose would trap CaN at its active site, leaving other areas of CaN, including the regulatory tail, exposed for potential interactions with other proteins from cell extracts of choice. Microcystin-Sepharose has been used successfully to trap PP-1c, and the resulting microcystin-sepharose-PP-1c entity has been used to probe cell extracts for binding partners of PP-1c.[283]

Binding of the CaN quadruple mutant to the microcystin-Sepharose resin (with addition of CaN-B/CaM/$Ca^{2+}$) was recently demonstrated. Finding novel cellular binding partners of CaN by trapping CaN on microcystin-Sepharose resin is a reasonable goal given the increase in microcystin sensitivity achieved in CaN mutants in this research. It is possible that a CaN mutant even more sensitive to MCLR could be generated with further mutagenesis of the β12–13 loop as outlined in chapter three.

## 5.2 Interaction of inhibitor-1 with calcineurin is mediated via the PXIXIT motif

NFAT proteins, recognized as principal regulators of the activation of the mammalian immune system, are the most studied substrates of CaN.[12, 109, 141, 206, 262] The mode of interaction between NFAT and CaN was shown to require a short

stretch of amino acids in the NFAT protein, termed the PXIXIT motif.[155, 193, 277] Multiple other proteins have since been shown to interact with CaN via this motif. Comparison of these PXIXIT sites shows that the PXIXIT motif allows a certain level of degeneracy, including residue insertions. Currently only a handful of CaN binding partners are known to contain this motif, but it is expected that more will be discovered as several PXIXIT containing proteins have been uncovered in the last few years (see Table 3.1, p. 121). I-1 contains a PXIXIT-like sequence, PSRKIQFT.[5] I-1 appears to be of vital importance in the regulation of a phosphatase cascade in heart that can lead to hypertrophy and heart failure.[98, 99] Therefore knowledge how CaN and I-1 interact with one another has important implications with respect to how this medical condition could be treated. Proving that I-1 contains a PXIXIT-like motif was approached in three ways.

A 16 residue peptide containing a VIVIT sequence was shown to be an inhibitor of NFAT interactions with CaN. The peptide was based on the NFAT PXIXIT motif sequence but the VIVIT sequence was substituted to obtain greater affinity for CaN.[11, 13] The VIVIT peptide inhibits interactions of CaN with proteins containing the PXIXIT motif. Our research showed that the VIVIT peptide inhibited I-1 dephosphorylation with an $IC_{50}$ of 8.79 μM. Since it is known that VIVIT targets CaN at the PXIXIT motif binding groove, this strongly suggested that the VIVIT peptide disrupted the binding of I-1 by binding at CaN's PXIXIT motif binding groove as well.[155, 193, 277]

To further show that I-1 uses a PXIXIT motif to interact with CaN, dephosphorylation of I-1 peptides that had either full or partial deletion of the PXIXIT motif was compared with dephosphorylation of peptides that contained the entire motif. The peptide encompassing the entire PXIXIT motif was shown to be dephosphorylated more efficiently than the ones that contained truncations, including complete removal of the motif. Removal of the motif from the peptide diminished its dephosphorylation rate approximately 2-fold.

The I-1 peptide assay developed to study the impact of the PXIXIT motif deletion on peptide dephosphorylation by CaN could be an important future research tool. Currently there is no clinically validated non-radioactive procedure for pharmacodynamic monitoring of CaN activity. There is a mounting call for use of CaN activity as a biomarker of immunosuppression.[122, 270, 285, 288] The I-1 peptide assay could be adapted to fulfill such a role.

The final approach to prove the interaction of I-1 with CaN via its PXIXIT motif involved mutagenesis of residues in the PXIXIT motif of the I-1 protein, or mutagenesis of selected residues in the PXIXIT motif binding groove of CaN-A. The effects of CaN mutagenesis could not be measured directly as the mutants showed decreased enzymatic activity against various substrates. The effects of the mutagenesis could be assessed when CaN's ability to dephosphorylate WT I-1 substrate was compared with the dephosphorylation of mutant PXIXIT motif I-1 substrate. I-1 mutant had all but one residue of the PXIXIT motif converted to

alanine residues.

The largest difference in dephosphorylation rates between WT I-1 and mutant I-1 substrates was observed with a sextuple mutant of the CaN PXIXIT motif binding groove (Y288A:M290A:F299A:330-NIR-332→330-AAA-332), with ~2–3-fold reduction in dephosphorylation of the PXIXIT motif mutant I-1 as compared to WT substrate. Together, the studies presented in this thesis indicate that I-1 indeed contains a PXIXIT motif that is related to that seen in NFAT proteins and other CaN binding partners.

The major difference between the I-1 PXIXIT motif and those previously described is presence of additional amino acid (see Table 3.1, p. 121). Like I-1, the PXIXIT motif of AKAP79 also contains an extra residue from the canonical motif length (PIAIIIT).[64, 109] I-1 and AKAP79 are the only proteins known to contain an additional amino acid in their PXIXIT motifs. Recently released structure of the AKAP79 PXIXIT motif peptide bound to CaN not only provides an understanding of how such insertion can be accommodated, but also provides important clues of how the I-1 PXIXIT motif could be binding to CaN.[192]

The structure revealed that the PIAIIIT motif of AKAP79 interacts with the PXIXIT motif binding groove of CaN in a similar fashion as was observed for the VIVIT peptide. The major difference observed between the structures is that the hydrophobic pocket that was occupied by a proline residue in the VIVIT

structure, is occupied by the first isoleucine residue of the PIAIIIT motif in the AKAP79 peptide structure (see Figure 5.1, p. 213).

This raises two important considerations. First, this structural arrangement demonstrates flexibility of the PXIXIT motif binding groove to accommodate variety of different sequences. Second, this questions the need of the proline residue in the AKAP79 PXIXIT motif. While the proline no longer interacts with the binding groove, this is the most conserved residue in the PXIXIT motif. This could suggest that the proline residue in the PIAIIIT motif was retained as an evolutionary byproduct. Alternatively, this proline could act as an initial docking ligand for the motif. It could perhaps first interact with the binding groove of CaN, but be subsequently displaced by the adjacent isoleucine as remainder of the motif structurally aligns itself into the groove. In support of the proline docking hypothesis, the only described PXIXIT motif that is missing the proline residue (GAITID of Rcn1) exhibits lowest binding affinity of all such currently known regulatory partners.[194] In another study, substitution of proline residues in the PVIVIT peptide for alanines, has resulted in ~100-fold increase in $K_d$.[195]

What can be learned about I-1's PXIXIT motif binding to CaN from the AKAP79 structure? AKAP79 structure suggests that the proline residue of the PRKIQFT motif of I-1 would not be involved in binding to the hydrophobic groove. However, the adjacent amino acid, arginine, being charged, might not be an appropriate fit in the hydrophobic environment of the PXIXIT motif binding

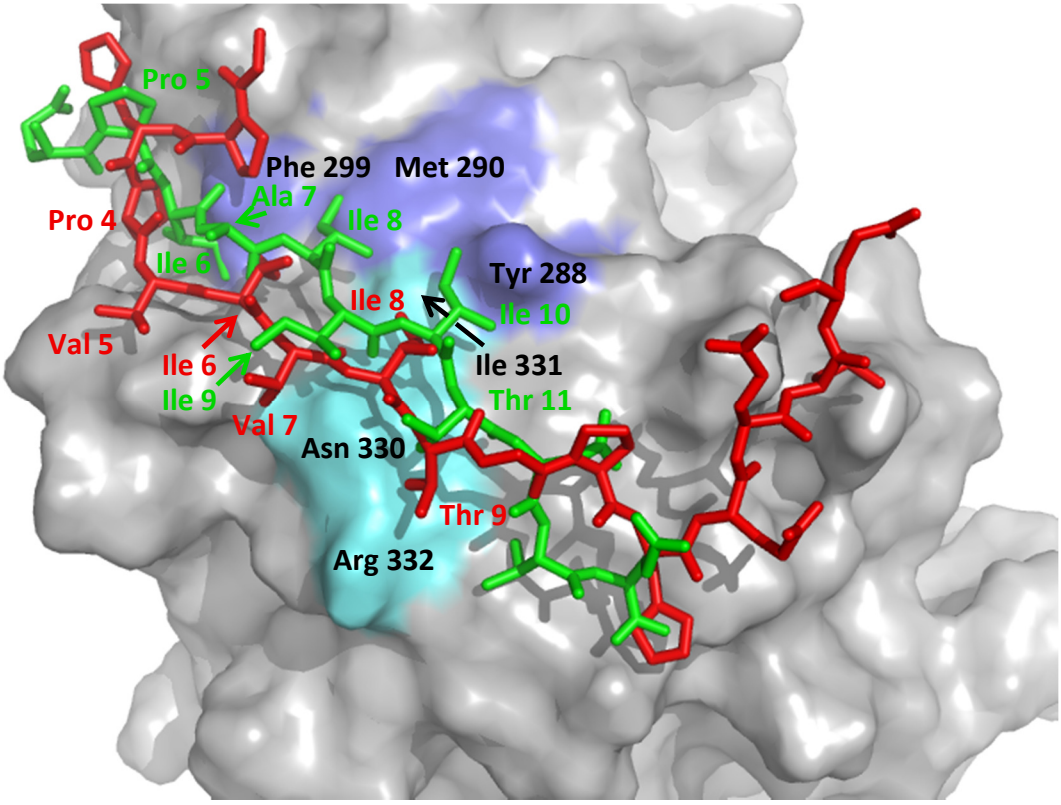



**Figure 5.1: Structural comparison of the PXIXIT motif binding to calcineurin demonstrates calcineurin's flexibility to accommodate diverse motif sequences.** The VIVIT peptide (red stick model) is shown interacting with CaN's PXIXIT motif binding groove in the same orientation as in Figure 1.19. The VIVIT peptide residues are labeled in red. AKAP79 peptide spanning the PIAIIIT motif is shown in green stick model with its amino acids labeled in green. CaN residues (labeled in black) of the sextuple mutant Y288A:M290A:F299A:330-NIR-332→330-AAA-332 are colored as follows: Y288A:M290A:F299A in slate, and 330-NIR-332→330-AAA-332 in pale cyan. The hydrophobic pocket that accommodates Pro-4 of the VIVIT peptide is occupied by the first isoleucine of the PIAIIIT motif in the AKAP79 structure.[192, 277]

groove. One possibility is that I-1's motif PRKIQFT binds via the proline, with adjacent arginine and lysine polar residues placed into cellular space by forming half a helix, allowing IQFT sequence to participate in a conventional β-strand binding as observed for the VIVIT and AKAP79 peptides. Alternatively, I-1 could be binding to the PXIXIT motif binding groove via the IQFT sequence only, and the proline residue could be used as an anchoring point only, similarly to what is suggested for the AKAP79 PXIXIT motif.

The sequence of the PXIXIT motif could also be a hint of whether such motif could interact with one or more CaN at a time. Li et al. (2007) reported a crystal structure of two CaN-A subunits binding to a single VIVIT peptide. The hydrophobic nature of the peptide allowed for its interaction with two separate PXIXIT motif binding grooves.[195] Authors attributed this to be a crystal artifact. However, the possibility of the PXIXIT motif binding two CaNs is supported by recent research on an AKAP79 scaffolding protein involvement in multi-protein complex formation. Gold et al. (2011) reported that a single AKAP79 protein binds two CaN molecules, and a required segment of AKAP79 is one that contains the PXIXIT motif.[123] Authors observed that the hydrophobic sequence composition of the PXIXIT motif should determine if one or two CaNs can be involved in the interaction. The PXIXIT motif of I-1 is less hydrophobic (PRKIQFT) than that of the VIVIT peptide or AKAP79 protein (PIAIIIT); this could suggest that I-1 associates with only a single molecule of the phosphatase.

It is of interest that I-1 is a substrate for CaN but an inhibitor of PP-1c. This is despite the fact that I-1 appears to use similar modes of interaction with CaN and PP-1, that is, PXIXIT motif and RVXF motif binding pockets, respectively. Structural data for I-1 interactions with CaN and PP-1c phosphatases does not exist. However, some information can be gained from the study of a model depicting a myosin phosphatase bound to a specific phosphorylated inhibitor CPI-17.[107] The myosin phosphatase is composed of a PP-1 catalytic subunit, a myosin targeting subunit MYPT1, and an accessory subunit M21. Phosphorylated CPI-17 (CPI-17-P) is a potent inhibitor of the myosin phosphatase. In the model of CPI-17-P bound to the myosin phosphatase, CPI-17-P occludes the active site of PP-1c with a phosphorylated Thr-38 residue. In the best fit configuration the Thr-38 group is 0.5Å away from the active site where a phosphate group would be located when a substrate is bound. The authors argued that binding of MYPT1 alters the configuration of CPI-17-P to shift the distance of Thr-38 from the catalytic center so the phosphate on CPI-17-P can no longer be removed by the phosphatase. If the model is correct, then only a small distance determines whether a phosphate group is removed from a substrate or acts as an inhibitor blocking the active site. This explanation of inhibition of the myosin phosphatase is especially compelling as other PP-1 holoenzymes are known to dephosphorylate CPI-17-P.[106, 107]

A similar scenario could account for the fact that phosphorylated I-1 inhibits PP-1c but is a substrate for CaN. How far the phosphorylated Thr-35

residue of I-1-P is inserted into the active sites of CaN and PP-1c could determine whether it is a substrate or an inhibitor. This would suggest that the phosphorylated residue of I-1-P is placed in the active site of CaN deep enough to be dephosphorylated by the catalytic center of the enzyme. In PP-1c the same phosphorylated residue must occlude the active site but be too far from the catalytic center for dephosphorylation to occur.

The mutagenesis of CaN described in chapter two provides certain clues to understanding what affects the distance of I-1-P Thr-35 insertion into the active site of CaN. In an effort to make CaN more sensitive to inhibition by OA and MCLR toxins, key CaN residues were mutated to the corresponding PP-1c residues. In all instances, substitution of PP-1c amino acids in CaN made CaN more efficient in dephosphorylating I-1 substrate (see Table 2.3, p. 86). In fact, the mutagenesis work implies that, with respect to I-1, PP-1c should be a more efficient phosphatase than CaN. This suggests the architecture of the active site of CaN is not responsible for the more advantageous placement of Thr-35 for dephosphorylation. Therefore other structural elements of CaN and PP-1c phosphatases must account for the difference in their interactions with I-1.

It is possible that the binding of I-1 to CaN and PP-1c via their structurally related binding grooves could determine how far the phosphorylated Thr-35 residue is inserted into the active site of the enzyme. Comparison of previous structural data encompassing binding of substrates and inhibitors to the related

clefts in CaN and PP-1c suggests that the PXIXIT motif binding groove of CaN can interact with more residues than the RVXF motif binding groove of PP-1c.[95, 155, 277, 280] But other binding sites may be involved. Specifically, in CaN, a pocket formed by Tyr-288, Ile-331, Asn-330, and Arg-332 can accommodate Thr-13 of I-1, steering the remaining part of the protein in the direction toward the active site. In PP-1c, Thr-13 appears to take a divergent pathway, inserting itself in the pocket formed by Met-290, Phe-257, and Phe-293 (see Figure 1.20, p. 54). It is possible that the additional interactions of CaN with I-1 could contribute to the PXIXIT motif binding resulting in insertion of the I-1 Thr-35 residue deep enough for dephosphorylation.

Establishing that I-1 contains a PXIXIT motif introduces important avenues into further research. Paramount is whether this knowledge will be an advantage in finding ways to inhibit the deregulation of a β-adrenergic pathway that is characteristic in failing hearts. PP-1c does not lend itself to drug inhibition due to its ubiquitous functions in the cell. However, a search for drugs that would target the PXIXIT motif binding groove of CaN in an analogous manner to the VIVIT peptide would also target the interaction of CaN with I-1 directly. Modulation of I-1-P levels inhibiting PP-1c as a therapeutic tool for the treatment of heart failure has been receiving more critical attention.[52, 99, 229, 301] However, the latest research suggests that transgenic manipulation of I-1 is not without limitations, as such approach in mice resulted in aberrant cardiac function and maladaptive changes.[301] This further underscores the need for compounds that

could target I-1 interaction with CaN as an alternative therapeutic method. Such compounds would have the added advantage of being immunosuppressants.

## 5.3 Marine organisms as a source of inhibitory compounds of calcineurin

CsA and FK506 are currently the most widely used medical inhibitors of CaN, especially in the field of organ transplantation.[129] However, these drugs cause severe toxic side effects, and can even compromise the survival times of both graft and patient.[23, 145, 175] Furthermore, the efficacy of these drugs varies widely among patients, with chronic rejection a continuing problem that plagues the success rate of transplantation.[22, 23, 128, 167] CsA and FK506 have had a tremendous impact in medicine, greatly extending the life-span of patients, but the need for new drugs that would act less ubiquitously and/or produce fewer associated problems is immense.[129]

The search for novel immunosuppressants has been intense in the last two decades with over 60 compounds identified. These compounds include inhibitors of CaN, inhibitors of NFAT function, compounds acting outside the CaN signaling transduction pathway, and several antibodies that act on the T-cell surface receptors. The majority of these promising drugs were isolated from existing chemical libraries, and several others are chemically modified versions of previously known compounds (see Appendix B, p. 281).[50, 104, 213, 220, 225, 264]

A unique way of looking for potential regulators of CaN is to seek candidate drugs in the extracts of marine organisms. The marine environment is vastly complex; life is extremely concentrated in the ocean fringes, requiring many inhabitants to develop a chemical means of defense, as competition for food and living space is fierce.[8, 266] The sea is an abundant and quite unexplored ecosystem where a large number of specimens can be collected at one time.[63]

The laboratory where I did this research has established over the years a fruitful collaboration with Dr. Raymond Andersen of the University of British Columbia, one of the leading chemists in the study of unique compounds derived from marine organisms. Working with Dr. Andersen gave us access to hundreds of marine samples. This was a unique opportunity to test the hypothesis that the marine environment could be a source of novel inhibitors of CaN.

A pilot study was first undertaken, a sampling of 205 marine extracts collected by Dr. Andersen's research team in Indonesia. Analysis showed that many samples potently inhibited CaN. Selected extracts were purified further by HPLC and eluted fractions were tested for inhibition of both CaN and PP-1c; many of the extracts inhibited both phosphatases. One HPLC fraction inhibited CaN specifically, with little activity against PP-1c. Further analysis of this sample could not be carried out due to limited source material.

Additional study of 281 marine samples from Papua New Guinea was undertaken. Many were shown to inhibit CaN, and to a lesser extent PP-1c. One sample, RJA-03-193, was pursued further as it was available in the largest amount. Bioassay-guided fractionation lead to identification of 3 separate compounds in this single extract, all of which modestly inhibited both CaN and PP-1c, but not PP-2Ac. These compounds were identified by the Andersen laboratory. They belong to a unique and not well known class of chemicals termed sesterterpene sulfates and include halisuflate-7, hipposulfate C, and irregularsulfate; none have previously been shown to inhibit Ser/Thr phosphatases. Sesterterpene sulfates were chemically modified in an attempt to increase the potency and specificity of these compounds. These modifications resulted in increasing potency and specificity against PP-1c only. The research described in this thesis indicates that the marine environment can yield novel phosphatase inhibitors, including inhibitors of CaN. The method of bioassay-guided isolation of novel compounds is a viable technique in the search for drugs that regulate CaN.

Based on our successful research with marine organisms, attempts were made to establish collaboration with a pharmaceutical company, Isotechnika (Edmonton, AB). Isotechnika is a world leader in the search for novel inhibitors of CaN. Interest was expressed by Isotechnika, however, difficulties over intellectual property rights prevented this collaboration from coming to fruition. As means of novel CaN drug discovery has been clearly established in this thesis,

this new area of research has a potential to yield new CaN specific inhibitors with judicious investment of capital and human resources. Furthermore, the bioassay could be modified to a kit for on-site analysis of collected samples. This would provide an opportunity to collect samples specific to CaN inhibition, and to collect organisms of interest in large quantities.

The location of sesterterpene sulfate binding on CaN has not been determined. The likely candidate area could be the active site of the Ser/Thr protein phosphatases or a nearby site such as hydrophobic groove. The first argument in support of this theory is the presence of a sulfate group on each member of the sesterterpene family of compounds, a group that could mimic the phosphate group of a substrate and be inserted in the catalytic center of the enzyme. The equipotency of inhibition of CaN and PP-1c supports this notion. Alternatively, it has been previously suggested that the γ-hydroxybutenolide ring of manoalide and luffariellolide sesterterpenes could act as a phosphate surrogate in inhibition of Cdc25. The equivalent moiety in halisulfate-7 and hipposulfate C is the furan ring.[29]

Another argument is that CaN, PP-1c, and PP-2Ac, the Ser/Thr protein phosphatases tested for inhibition by the sesterterpene sulfates, are not known to share structurally related binding sites. The PXIXIT motif binding pocket of CaN, although related to the RVXF motif binding pocket of PP-1c, is structurally different enough to exclude common binding partners between these two

phosphatases. One notable exception to this rule is I-1 as established in chapter three of this thesis. The same binding pocket in PP-2Ac more closely resembles the architecture of the PP-1c binding cleft, although several residues are also different between PP-1c and PP-2Ac in this region. Therefore it is unlikely that this region could interact with the sesterterpene compounds. The active site and the hydrophobic groove appear to be the most evolutionarily preserved areas among these three protein phosphatases.

One possible way to determine if sesterterpene inhibitors bind to the active site of CaN would be to determine if sesterterpene compounds inhibit dephosphorylation of *p*NPP substrate. Binding of inhibitors to other areas of CaN should not affect dephosphorylation of this small compound unless a global conformational change of the enzyme is induced by inhibitor binding.

Sesterterpene sulfates provide and exciting addition to a catalogue of phosphatase inhibitors. They appear to be biologically active immunosuppressive compounds that can inhibit IL-2 production. Although their precise mode of inhibition is yet to be established, it is likely that inhibition of T-cell response is mediated via inhibition of CaN.

Furthermore, the possibility of sesterterpene compounds binding in a location different from the active site would be a very valuable finding. While many active site-targeting inhibitors of phosphatases have been described,

compounds shown to bind at any other area of phosphatases are rare. However, peripherally binding compounds that do not target the active site could hold great potential in regulation of phosphatase function. Such compounds, for instance, could disrupt binding of regulatory proteins important for modulation of the enzyme activity. Furthermore, the emerging image of phosphatase function in eukaryotic cells is that these enzymes participate in numerous complex multi-protein interactions. CaN interaction with NFAT proteins serves as an example of such complexity. NFAT proteins are known to use at least two different docking sites to bind to CaN, the PXIXIT and the LXVP binding motifs.[11, 201, 237] Recent evidence suggests that the LXVP binding motif of NFAT proteins interacts with a hydrophobic pocket that is formed by residues contributed from both CaN-A and CaN-B subunits.[201, 245] This means that assembly of NFAT protein on the surface of CaN leads to a unique interface of three different proteins (see Figure 5.2A, p. 224). Ability of a compound to compete for such a specific site of interaction would be very valuable for a precise modulation of signal transduction pathways.

FK506 and CsA are examples of compounds that actually do not target the active site. In fact, inhibition of CaN by FK506 and CsA also appears to generate interface contributed from all three proteins that are required for the immunosuppression to occur: CaN-A and CaN-B subunits, and an immunophilin.[166, 180] Interestingly, this interface is spatially adjacent to where the NFAT substrate would be expected to form a three protein interface of its own. However, in the case of the immunosuppressive compounds, their role is to

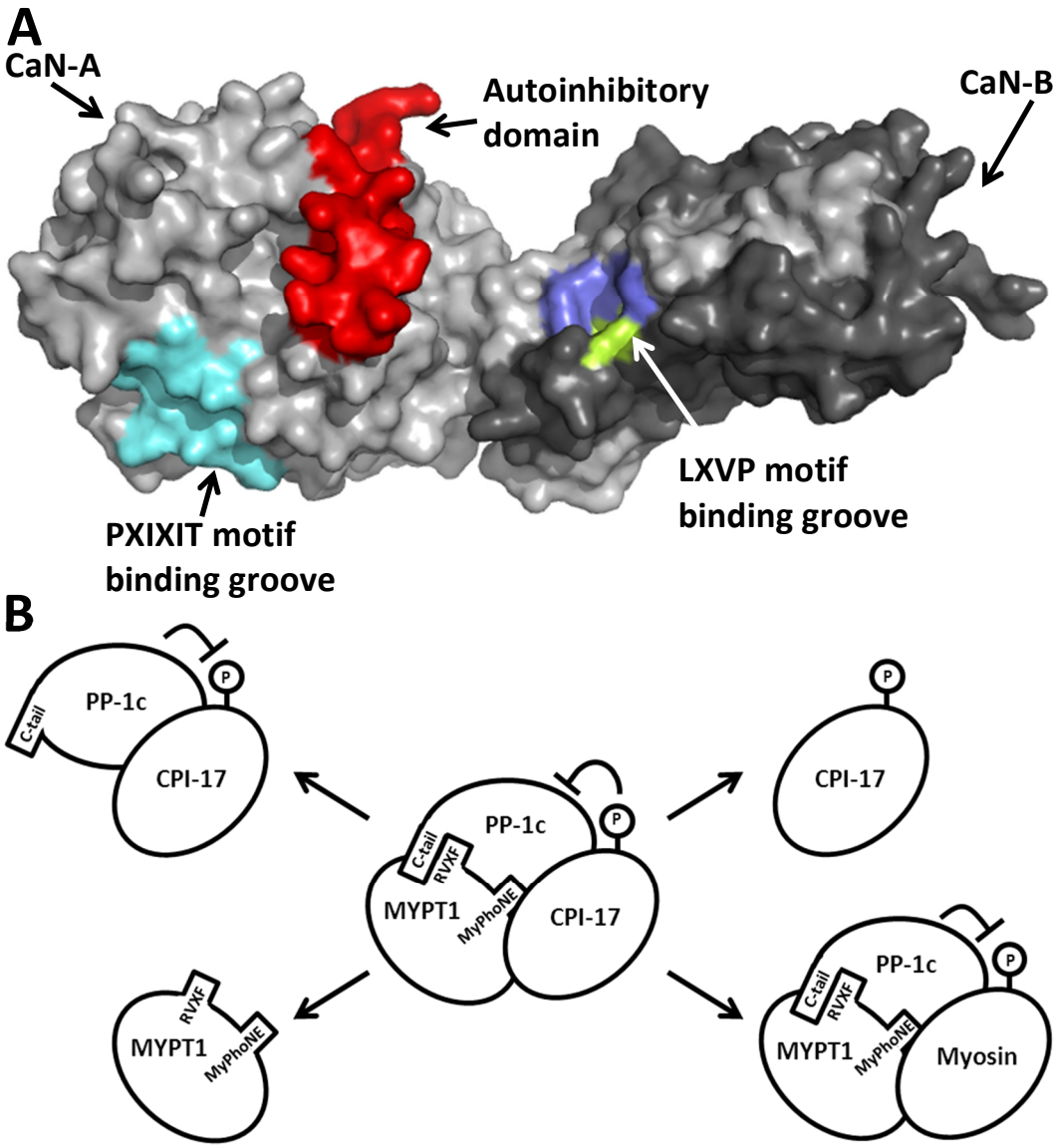



**Figure 5.2: Ser/Thr protein phosphatases participate in complex multi-protein interactions.** (A) Interaction of NFAT substrate with CaN requires at least two binding sites, including interface between three distinct proteins. These binding sites include the PXIXIT motif binding groove (highlighted in aquamarine color) and the LXVP binding groove formed by residues from both the CaN-A and -B subunits (shown in slate and lime color, respectively). The autoinhibitory domain occluding the active site is represented in red. The CaN-B regulatory subunit is shown in darker shade of gray than the catalytic subunit. Surface area appears contiguous between independent components of the enzyme complex. (B) This panel depicts the complex regulation of a myosin phosphatase. Depending on PP-1c binding partner, CPI-17 regulatory protein, when phosphorylated, can be either a substrate or an inhibitor. The known motifs involved in protein interaction are listed. CPI-17 = C-kinase-activated PP-1 inhibitor of 17 kDa, MyPhoNE = Myosin phosphatase N-terminal element, MYPT1 = myosin phosphatase target subunit 1.[106, 107, 180]

promote the formation of a multi-protein complex, stabilizing the binding of immunophilins to CaN, and leading to CaN inhibition by restricting access to the active site.

Inhibition of myosin phosphatase by phosphorylated CPI-17 provides another example of a fine-tuned structural composition of multiple different proteins. CPI-17-P can only inhibit PP-1 that is bound to MYPT1 regulatory protein; otherwise CPI-17-P is a substrate (see Figure 5.2B, p. 224).

Although binding of MYPT1 does not have a noticeable effect on the shape of PP-1c itself, it does alter the architectural landscape around the active site of PP-1c by its own proximity. MYPT1 associates with PP-1c using at least three distinct locations: the RVXF motif, the myosin phosphatase N-terminal element (MyPhoNE) motif found in several binding proteins of PP-1c, as well as interaction with the flexible C-terminal tail of PP-1c.[280] How CPI-17-P interacts with such a heterodimer still awaits structural confirmation, however, this interaction requires the MyPhoNE motif.[107, 248] Binding of MYPT1 also appears to have an impact on the overall charge distribution, altering the electrostatic surface composition of both proteins.[280] This is also expected to facilitate the binding of CPI-17-P, as the electrostatic surface potential of CPI-17-P complements the acidic surface generated by MYPT1 and PP-1c participation.[106]

Coincidently, as the number of known PP-1c binding partners increase, currently standing at ~200, the knowledge of PP-1c's involvement in multi-protein complexes is also growing.[133] Many of these interactions use more than one PP-1c participant at a time. Various such multifaceted interactions have been described recently.[118, 238, 287] One such complex deserving further attention is an interaction of myosin phosphatase with NUAK1 kinase, as it utilizes the three most common PP-1c binding motifs used by the regulatory proteins. These include the RVXF motif, the most frequent used motif for PP-1c contact, MyPhoNE motif, as well as the GILK motif. NUAK1 kinase binds PP-1c via the GILK motif (see Figure 5.3A, p. 227).[133, 248, 314]

As is the case with CPI-17-P, NUAK1-P kinase is a substrate of PP-1c, but upon binding of MYPT1, NUAK1-P leads to myosin phosphatase inhibition. However, unlike CPI-17-P, NUAK1 does not block active site of PP-1c itself directly. Instead, NUAK1-P phosphorylates MYPT1 which promotes binding of 14-3-3 protein to MYPT1 which leads to inhibition of PP-1c. NUAK1 kinase regulation of myosin phosphatase thus promotes a formation of even more elaborate multi-protein complex (see Figure 5.3B, p. 227).[314]

The size scale of such multi-protein complexes can be illustrated by a recently described example of AKAP79 scaffolding protein. AKAP79 was shown to bind two CaNs, one CaM and two PKA protein segments, in a collaborative manner. In addition, AKAP79 could dimerize to generate a structure of 466 kDa

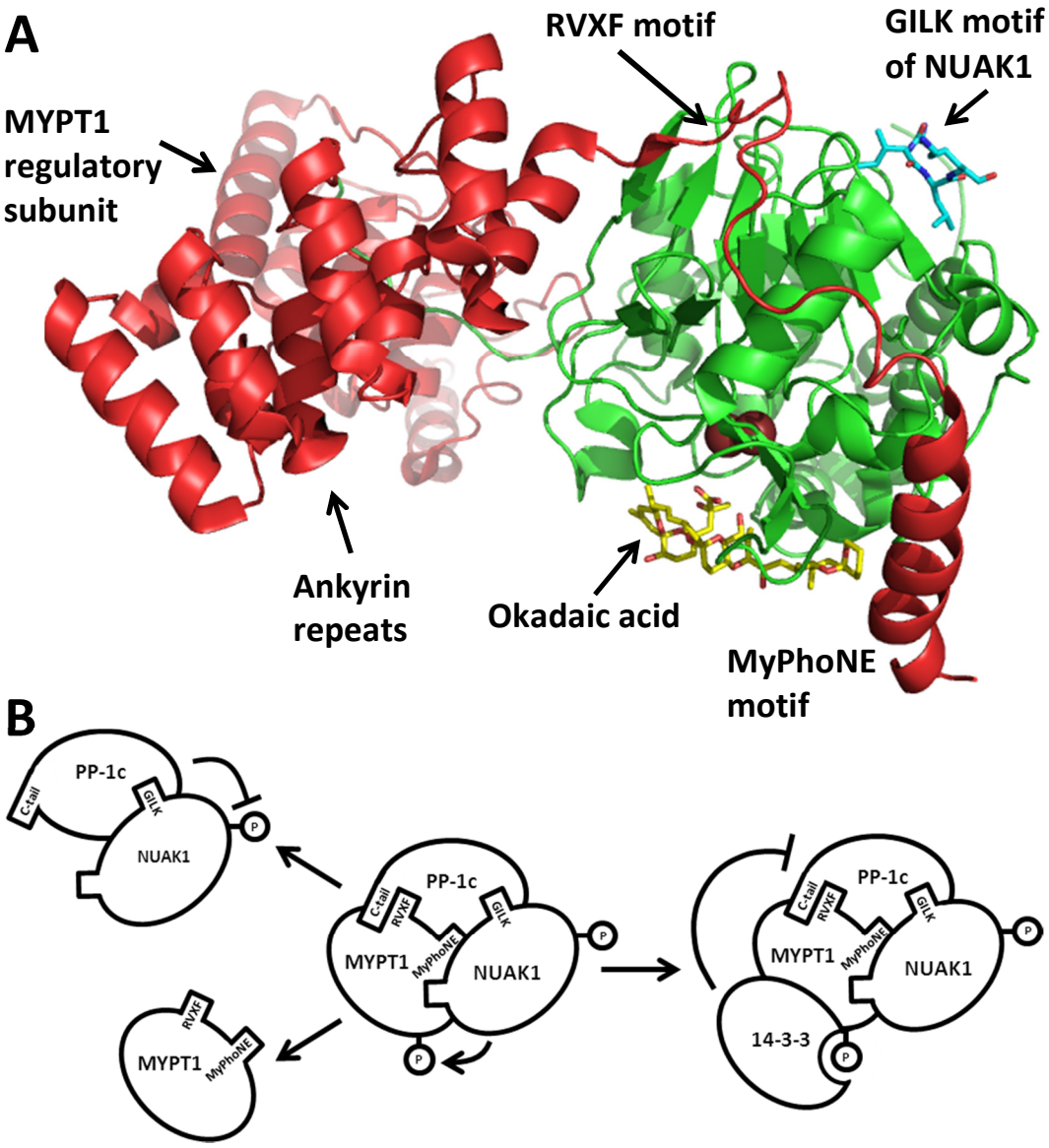



**Figure 5.3: PP-1c is a frequent central scaffolding partner of multi-protein complexes.** (A) This panel demonstrates PP-1c's ability to interact with multiple proteins. PP-1cβ (lime color) is shown interacting with MYPT1 regulatory subunit (red color) which makes at least three contacts with PP-1c. The GILK binding motif via which NUAK1 kinase interacts with PP-1c is shown in cyan stick model. Active site, marked by a presence of two $Mn^{2+}$ ions (ruby spheres) is seen occluded by okadaic acid (yellow stick model). (B) Model of myosin phosphatase regulation by phosphorylated NUAK1 kinase (NUAK1-P). NUAK1-P binding to PP-1c via the GILK motif allows it to be dephosphorylated. Upon binding of MYPT1 regulatory protein NUAK1-P kinase can instead lead to myosin phosphatase inhibition. The known motifs or structural elements involved in protein interaction are listed. C-tail = PP-1c C-terminal tail, MyPhoNE = Myosin phosphatase N-terminal element, MYPT1 = myosin phosphatase target subunit 1, NUAK1 = AMP-activated protein kinase-related kinase 5.[314]

molecular mass. These examples highlight the intricacy of multi-protein interactions that phosphatases participate in.[123]

The increasing complexity of protein contact and regulation suggests that in order to manipulate such multi-protein complexes for a therapeutic purpose, it will require use of compounds of more refined targeting capabilities than just blocking the active site of the enzyme of interest. Inhibition of CaN dephosphorylation of NFAT by specific compounds is an example of medicinal need for molecules that influence specific multi-protein interactions. Precise drug targeting of NFAT dephosphorylation could reduce side effects experienced by patients undergoing immunosuppression. Such compounds could target any site of interaction between the two proteins, including the PXIXIT motif. Compounds that target the PXIXIT motif would be potential immunosuppressants as they would disrupt interaction between NFAT and CaN.

Determination that I-1 contains the PXIXIT motif is thus of great significance. This allows the use of I-1 as an *in vitro* substrate in search of target compounds that inhibit CaN function, either through blocking the active site or by blocking the protein-protein interaction between CaN and I-1 by obstructing the PXIXIT motif binding groove of CaN. This facilitates the search for inhibitors of CaN:NFAT interactions without compromising the enzymatic activity of CaN.

The crystal structure of I-1 bound to CaN would be an important aid in the development of chemical compounds that block access to the PXIXIT motif binding groove of CaN. Currently there is no structural information that demonstrates how a substrate of CaN binds this enzyme. Structural data would allow refinement of the inhibitor via chemical modification to improve the potency of the potential drug. In addition, such data could provide information on how the PXIXIT motif in a full length protein directs the dephosphorylation site to the catalytic cleft. Such a structure could reveal as yet unknown binding sites on CaN.

Information presented in chapter two and three of this thesis suggests that mutagenesis of both CaN and I-1 could improve interaction between the two proteins. The Y159I:F160Y:L312C:Y315L quadruple CaN mutant was shown to be nearly 5-fold more effective at dephosphorylating I-1 than WT CaN. This suggests that the residue changes implemented allowed a closer fit of I-1 substrate in the catalytic center of CaN. Use of thiophosphorylated I-1 which cannot be dephosphorylated by CaN should result in better interaction between the two proteins, possibly stabilizing the complex enough to crystallize it. A crystal structure of thiophosphorylated I-1-CaN would show how the PXIXIT motif interacts with CaN.

Further mutagenesis could be undertaken with I-1 where part or all of the PXIXIT motif could be converted to a VIVIT sequence which is known to interact

with CaN more strongly than the PXIXIT motif.[13]

All three chapters of this work contribute roadways towards the development of CaN specific inhibitors. This goal was approached in two different ways: using mutagenesis of CaN to learn if existing PPP phosphatase inhibitors such as microcystins could be chemically modified to become CaN specific, and using marine environment as a source of novel regulatory compounds. We also establish that I-1 interacts with CaN in analogous manner to NFAT proteins. Use of I-1 as substrate could aid in the discovery of CaN immunosuppressants with fewer toxic side effects than CsA/FK506.

Over 20 years ago, the discovery of CaN specific immunosuppressants changed the face of transplantation medicine. New inhibitors of CaN could once again alter the survival time of grafts and the length and quality of life of patients. It is the highest aspiration of every researcher that his or her work will eventually contribute to the benefit of society. It is hoped that the information presented here will reach that goal.

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# Appendix A

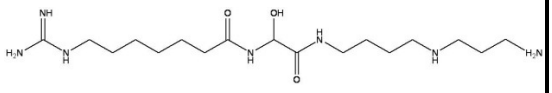
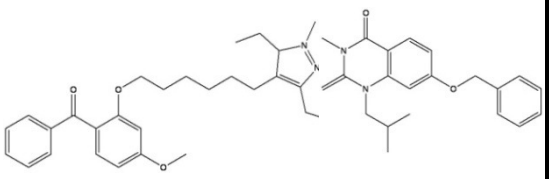
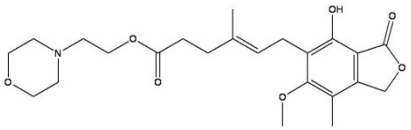
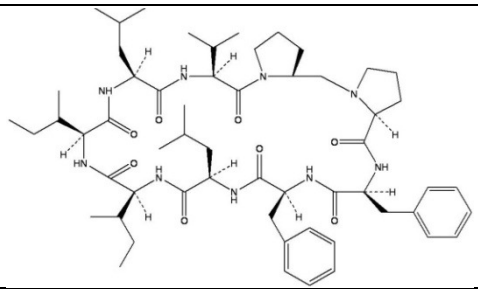
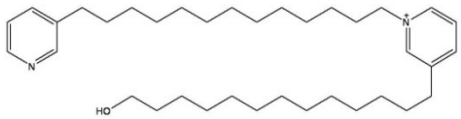
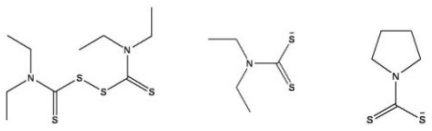
Toxic side effects experienced by patients undertaking immunosuppressive treatment.

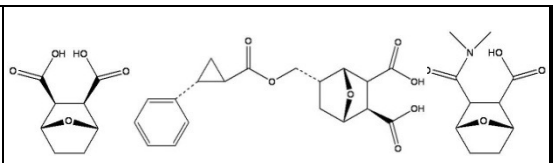
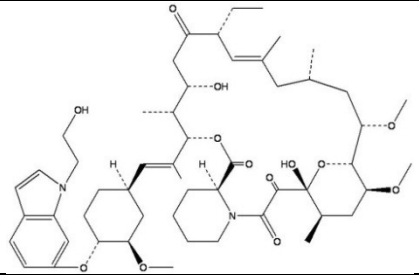
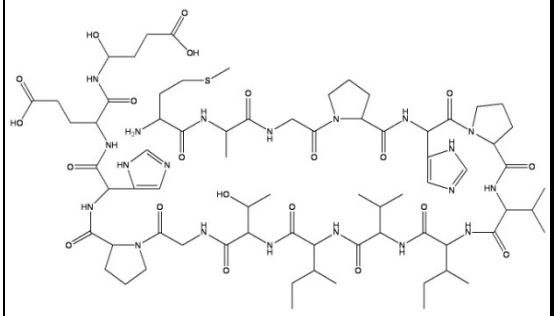
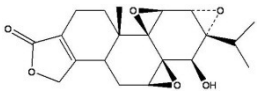
| Immunosuppressant | | | | | |
|---|---|---|---|---|---|
| Both | | CsA | | FK506 | |
| Side effect | Refs | Side effect | Refs | Side effect | Refs |
| Nephrotoxicity[A] | 49, 145, 167, 175 | Hirsutism[B] | 49, 145 | Increased risk of infections | 167, 175 |
| Tremor[C] | 23, 49, 145, 175, 254 | Gingival hyperplasia[D] | 49, 145 | Gastrointestinal disturbances[E] | 145, 175 |
| Mild hepatotoxicity | 49, 145 | Severe muscle weakness[F] | 23, 204 | Hyperkalemia[G] | 145 |
| Bone marrow depletion | 49 | Weight loss[H] | 204 | Hypomagnesemia[I] | 145 |
| Burkitt's lymphoma[J,K] | 81, 145, 167, 236, 244 | Stroke[C] | 254 | Hyperuricemia[L] | 145 |
| Nasopharyngeal carcinoma[J,M] | 81, 167, 175 | Brain edema[C] | 253 | Nightmares[C] | 23 |
| Insomnia | 23, 175 | Somnolence[C,N] | 23 | Vertigo[C] | 23 |
| Severe headaches[C] | 23, 145, 175, 254 | Peripheral neuropathy[C,O] | 23 | Dysesthesia[C,P] | 23 |
| Hyperglycoma[Q] | 175 | Cerebellar syndrome[C,R] | 23 | Photophobia[C] | 23 |
| Hypertension | 145, 175 | Visual disturbances[C] | 23 | Mood disturbances[C] | 23 |
| Altered brain morphology[S] | 23, 253, 254, 267 | Neuralgia[C,T] | 23 | Akinetic mutism[C,U] | 23 |
| Brain ischemia | 204, 219 | Altered level of consciousness[C] | 23 | Hyperflexia[C,V] | 23 |
| Hyperlipidemia, high cholesterol | 145 | Hallucinations [C,W] | 23 | Myalgia[C,X] | 23 |
| Epileptic seizures[C] | 23, 145, 254 | | | | |
| Cardiac complications[Y] | 145, 204, 267 | | | | |
| Aberrant cardiac $Ca^{2+}$homeostasis | 204, 267 | | | | |
| Cortical blindness[C] | 23, 254 | | | | |
| Skin carcinoma[Z] | 297 | | | | |
| Encelopathy[C,a] | 23 | | | | |
| Psychosis[C,b] | 23 | | | | |
| Cerebellar hemorrhage[C] | 23 | | | | |
| Hemiparesis[C,c] | 23 | | | | |
| Dysphasia[C,d] | 23 | | | | |
| Paresthesia[C, e] | 23, 254 | | | | |

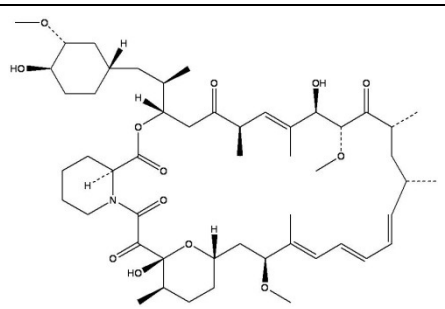
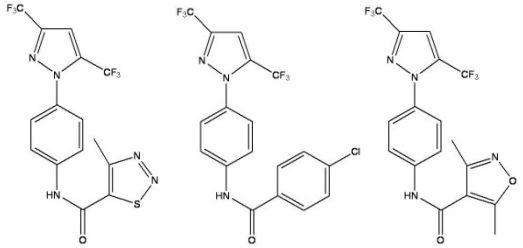
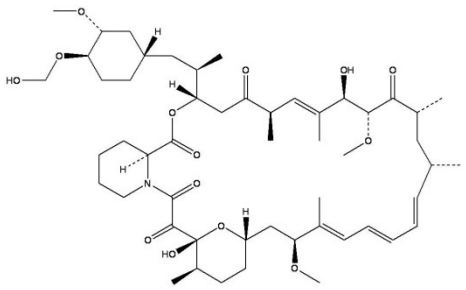
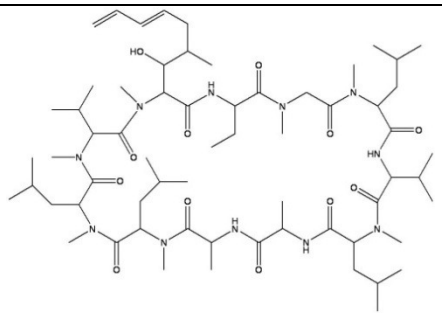
[A]Most common and serious complication, caused primarily due to vasoconstriction; [B]Growth of soft facial and body hair; [C]Ascribed to neurotoxicity. [D]Gum hypertrophy; [E]Leads to nausea, diarrhea, vomiting; [F]CsA prevents muscle regeneration resulting in the muscle weakness; [G]Higher than normal levels of $K^+$ in blood due to nephrotoxicity; [H]Caused by metabolic toxicities imparted by CsA; [I]Low $Mg^{2+}$ blood levels usually due to diarrhea and vomiting; [J]Believed to be caused by Epstein-Barr virus typically suppressed by the immune system; [K]Lymphoma of B-cells; [L]Build up of uric acid in blood; [M]Cancer of the respiratory tract; [N]Sleepiness and feeling drowsy; [O]Affects the peripheral nervous system resulting in tremor of hands and feet; [P]Distortion of senses, especially touch, so that ordinary stimulus becomes unpleasant or painful; [Q]Caused by impaired glucose metabolism, resulting in diabetogenic effects that need to be treated with insulin; [R]Incoordination of voluntary movements; [S]Characterized by white matter disorders, grey matter hypoxic damage, and metabolic encephalopathy seen with administration of CsA; [T]Pain along the course of the nerves; [U]State of apparent alertness but no speech or voluntary motor responses; [V]Exageration of reflexes; [W]Both visual and auditory; [X]Muscle pain; [Y]Complications due to hyperglycoma, hyperlipidemia, high cholesterol and impaired $Ca^{2+}$ homeostasis which alters contractile properties of cardiomycytes; [Z]Most common cancer found in patients; [a]Mental status changes ranging from confusion, delirium, to coma; [b]Illness that prevents people from distinguishing between the real world and imaginary world; [c]Muscular weakness of one side of the body; [d]Difficulty of coordinating and recognizing speech; [e]Abnormal sensation of tingling, prickling or burning on the skin.

# Appendix B

Summary of search for novel immunosuppressive drugs.

| Compound name | Description and Refs | Chemical Formula |
|---|---|---|
| 15-deoxyspergualin (Gusperimus) | Used clinically in both acute and chronic rejection graft remission with 79% success rate. Side effects observed.[10] |  |
| WIN-53071 (left) and WIN-61058 (right) | Found in chemical library screen. Inhibited IL-2 production in T-cells as well as NFAT binding to DNA.[17] |  |
| Mycophenolate mofetil | Shown to be an effective substituent for CsA once onset of nephrotoxicity was observed. Shown to increase the graft survival rate and lead to improvement of renal function. Major side effects include gastrointestinal problems, and increased risk of cancer[23, 96, 129, 269] |  |
| Cyclolinopeptide and MC-172 | MC-172 is a sulfonated analog. Compounds showed inhibition of lymphocyte proliferation. Could inhibit rotamase activity of CyP but not FKBP12. Bound CaN and inhibited CaN function.[24, 121] |  |
| YM-53792 | Found in chemical library screen. Shown to inhibit IL-2 expression which was directly correlated to NFAT DNA binding inhibition.[184] |  |
| Disulfiram (left), diethyl (center) and pyrrolidine (right) dithiocarbamates | Inhibit expression of IL-2 and CD69 as well as IL-2Rα in T-cells, by inhibition of NFAT and NF-κB function. Have been used in HIV treatment to delay AIDS onset but neurotoxic side effects seen. Can cause neuronal cell death.[188, 190, 214] |  |

| | | |
|---|---|---|
| Endothal (left) and derivatives (center and right) | Shown to inhibit CaN at 11.5 μM but is not specific. Center derivative showed $K_i$ of 0.5 μM for CaN, 8-fold lower than for PP-1c. Right compound inhibited IL-2 secretion at 5 μM.[219, 279] |  |
| L-732,531 (indolyl-ascomycin) | Equipotent to FK506. When bound to CaN has increased stability and decreased affinity for immunophilin interaction.[92, 190, 241] |  |
| V7 antibody | Inhibits T-cell proliferation and IL-2 expression by reducing $Ca^{2+}$ influx. Also abrogates NFAT transcriptional activity.[268] | |
| VIVIT peptide | Selectively mutated peptide based on NFAT PXIXIT motif, but is 25-fold more effective in binding CaN than PXIXIT motif. Inhibits NFAT dephosphorylation and cytokine expression in T-cells. Peptide inhibited T-cell proliferation, prolongs graft survival in mouse models with reduced nephrotoxicity phenotype.[13, 190, 222, 232] |  |
| PG-490 (Triptolide) | Inhibits IL-2 secretion in T-cells in a CaN independent manner. Shown to greatly compromise transcriptional activity of NF-κB.[243] |  |
| Campath-1H (Alemtu-zumab) | Anti-CD25 monoclonal antibody. Clinical trials allowed low levels of CsA use which together resulted in low incidence of organ rejection with no major side effects observed.[46, 96] | |

| | | |
|---|---|---|
| Sirolimus (Rapamycin) | Inhibits mTOR in CaN independent manner. Approved for clinical use along with CsA. Reduced acute rejection levels, but known to increase CsA brain and kidney storage, exacerbating its effects. Shows different side effects than CsA/FK506, and decreases rate of malignancy development.[15, 18, 183, 253] |  |
| 3,5-bistrifluoro-methyl pyrazole derivatives: BTP1(left), BTP2 (center, also termed YM-58483), BTP3 (right) | Found in chemical library screen. Inhibited IL-2 production in T-cells. Prevented NFAT function without affecting CaN activity. Compounds were found to inhibit $Ca^{2+}$ capacitative influx. Compounds were altered to generate more potent inhibitors.[90, 161, 190, 284] |  |
| SDZ-RAD (Everolimus) | Rapamycin derivative that inhibits mTOR which leads to inhibition of cell cycle progression. Particularly effective in conjunction with CsA and has been approved for clinical use in Europe but not in United States. Seriously compromises renal function in patients.[15, 96, 254] |  |
| ISA247 (voclosporin, formerly $ISA_{TX}247$) | CaN inhibitor developed from CsA but is 2.5-fold more potent than CsA, showing significant improvement in graft survival in animal models with much reduced toxicity. Anemia is the major side effect. Clinical trials have begun.[14, 27, 28, 125, 162, 271] |  |

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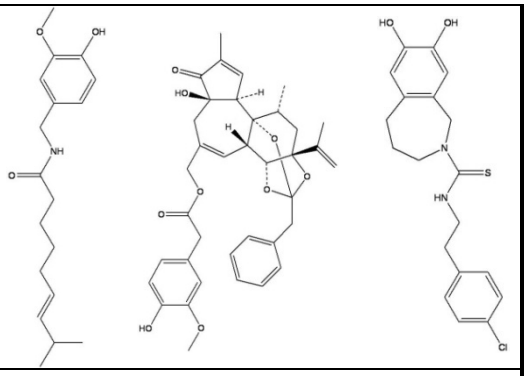
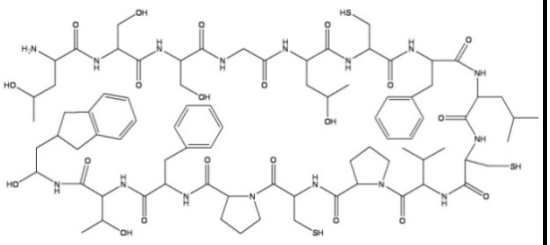
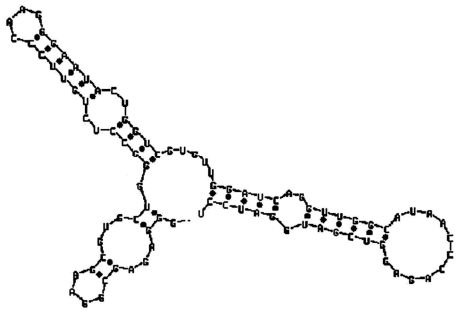
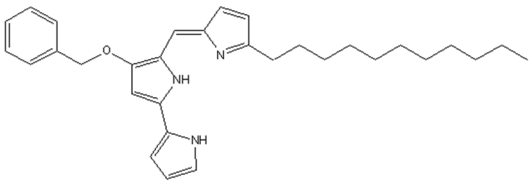
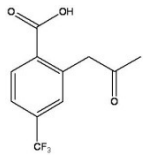
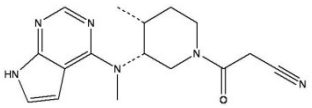
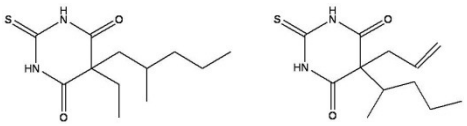
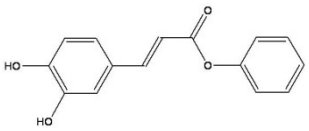
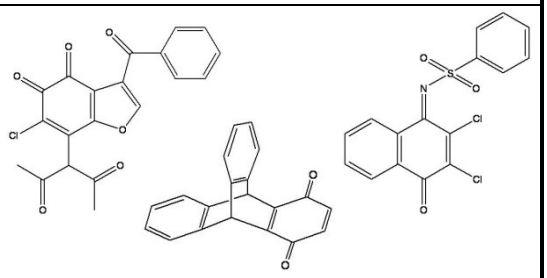
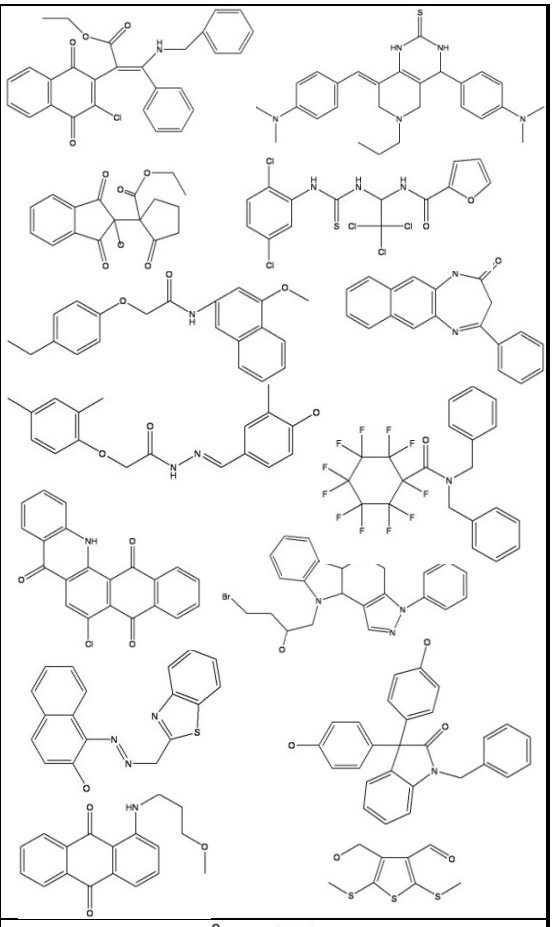
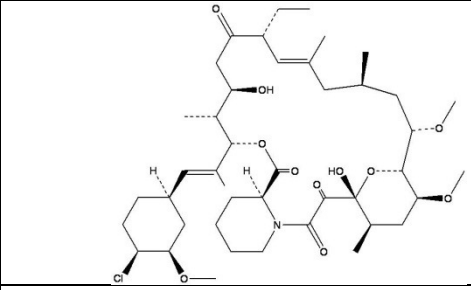
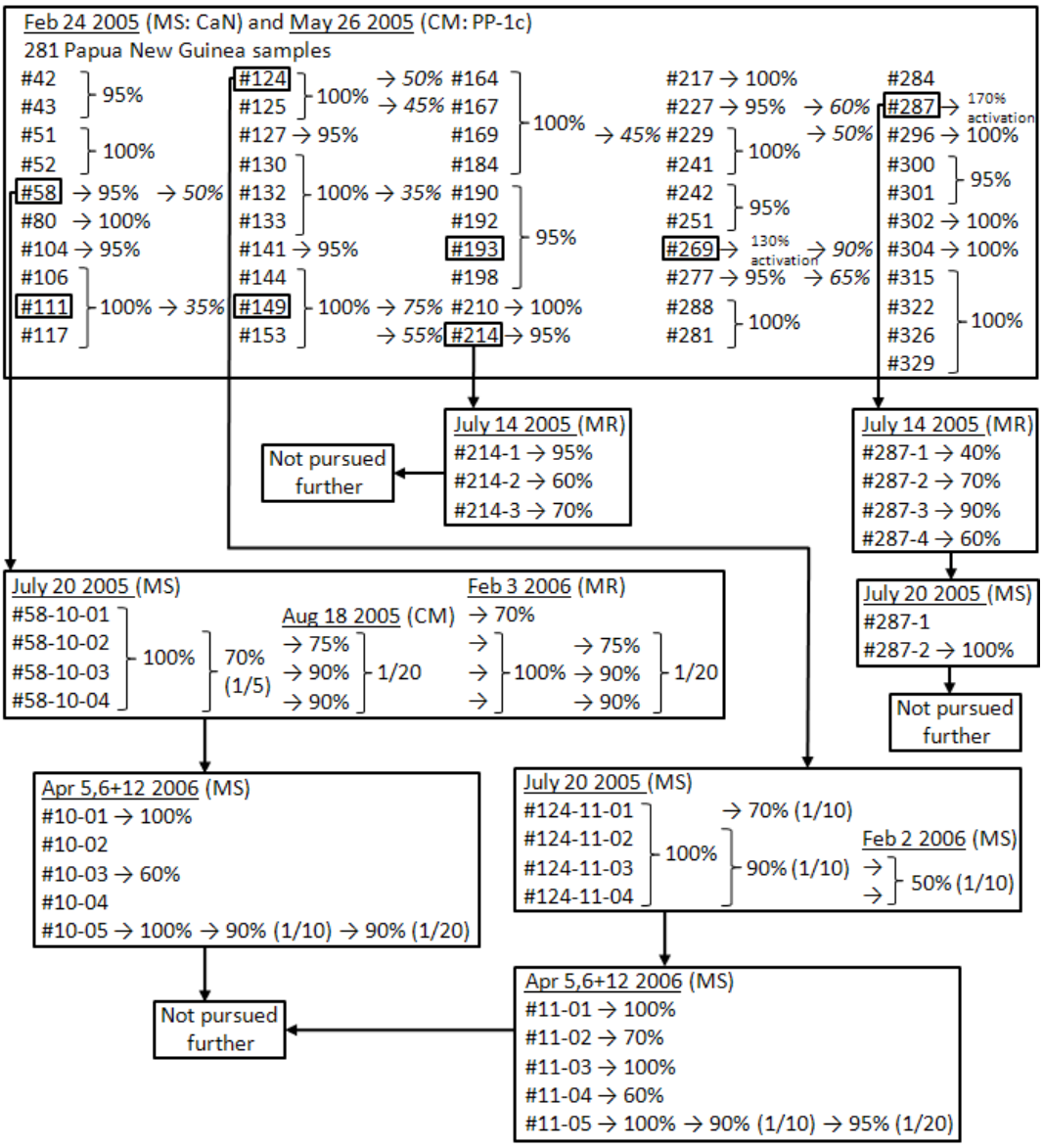
|---|---|---|
| Capsaicin (left), resiniferatoxin (center) and capsazepine (right) | Known to act on sensory nerve cells and has been used to relieve pain. Shown to inhibit capacitative $Ca^{2+}$ entry in T-cells and thus can prevent IL-2 expression.[112] |  |
| Pep3 peptide | 16-mer peptide based on NFAT LXVP motif, a CaN binding site different from PXIXIT motif. Shown to inhibit various cytokines in $T_H2$ helper T-cells as well as to prevent NFAT nuclear translocation and DNA binding.[198] |  |
| RNA aptamers | One aptamer (shown) specifically inhibited NFATc function by preventing DNA binding and activating a reporter gene expression. Other aptamers (not shown) isolated from the RNA library were shown to bind NFATc and NFATp DNA binding domains with even higher affinity.[16, 58, 190] |  |
| Daclizumab (humanized anti-Tac) | Antibody that targets IL-2R and was shown to inhibit T-cell proliferation. Works independently of CaN pathway, instead inhibits the Jak1 and 3 kinase pathways.[282] | |
| PNU156804 | Shown to inhibit T-cell proliferation at 7.5 μM $IC_{50}$ by specifically targeting JAK3 kinase activity. Rats with heterotropic transplants showed improved graft survival when treated with PNU156804 as compared to controls.[273] |  |

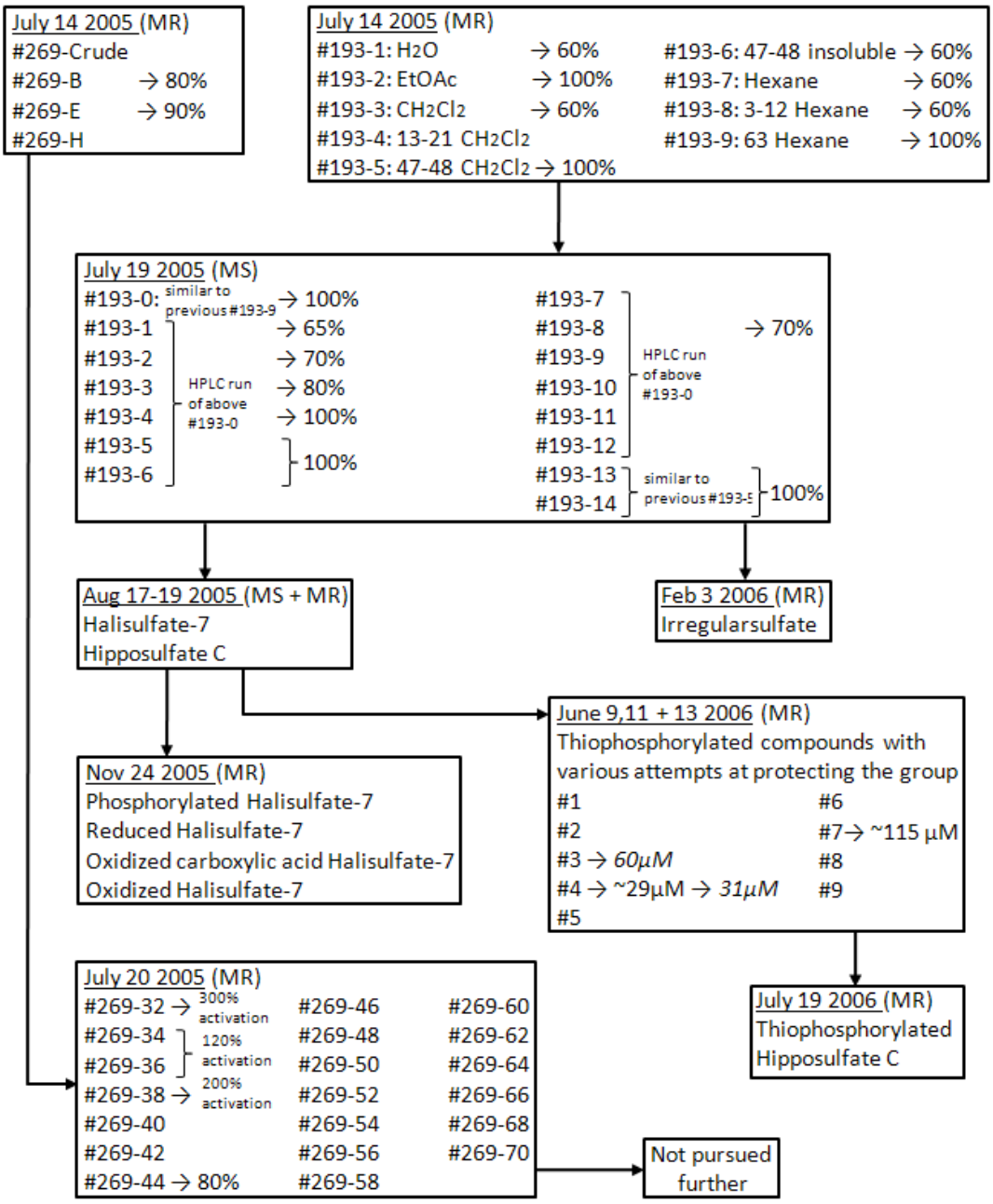
| | | |
|---|---|---|
| LF 15-0195 | Analog of Gusperimus but more stable, more potent, and less toxic. Attenuates humoral rejection which occurs most often in renal transplant patients. High doses still show side effects in animal models, but promotes graft tolerance.[307, 322] | Structure not available |
| 2-acetyloxy-4-trifluoro-methyl benzoic acid (Trifusal) | Anti-inflammatory agent shown to inhibit NFAT-dependent cytokine production in T-cells at 1 mM by interfering with its DNA binding. Other salicylate derivatives were less potent.[2, 190] |  |
| CP-690,550 | JAK3 kinase inhibitor shown to prolong the allograft survival in animal models, and cause reduction in natural killer cells, and lymphocytes. Major side effect is anemia. Clinical trials have begun.[38, 73, 96, 240] |  |
| Thiopental (left) and thiamylal (right) barbiturates | Known to inhibit T-cell proliferation, cytokine production, and NFAT transcriptional activity. Shown to indirectly inhibit CaN at $IC_{50}$ of 1-5 mM by interfering with CaM function.[154] |  |
| Caffeic acid phenyl ester | Shown to inhibit T-cell proliferation and IL-2 production, and this was correlated to inhibition of NF-κB and NFAT function. Pharmacologically safe to use.[212] |  |
| Inhibitors of NFAT-CaN association-1 (left), -2 (center) and -6 (right) | Found in chemical library screen. Three compounds found to displace VIVIT from CaN with 100% effectiveness, termed INCA-1, INCA-2, and INCA-6. INCAs prevented NFAT localization to nucleus and cytokine expression, but were severely toxic to cells.[246, 247] |  |

| | | |
|---|---|---|
| NFAT inhibitors | Found in chemical library screen. 14 compounds found based on their ability to inhibit NFATc3 translocation in BHK cells. 9 prevented dephosphorylation of NFAT independently of CaN. All compounds were shown to inhibit IL-2 production in murine T-cells.[289] |  |
| Pimecrolimus | FK506 derivative used to treat eczema, psoriasis, lupus erythematosus and seborrhoeic dermatitis. Similar to other CaN inhibitory compounds, it shows predisposition to promote skin cancer.[297] |  |
| TAFA93 | "Novel prodrug of the mTOR inhibitor rapamycin which has successfully completed Phase I clinical development." The drug was well tolerated with no adverse effects.[163] | Structure not available |

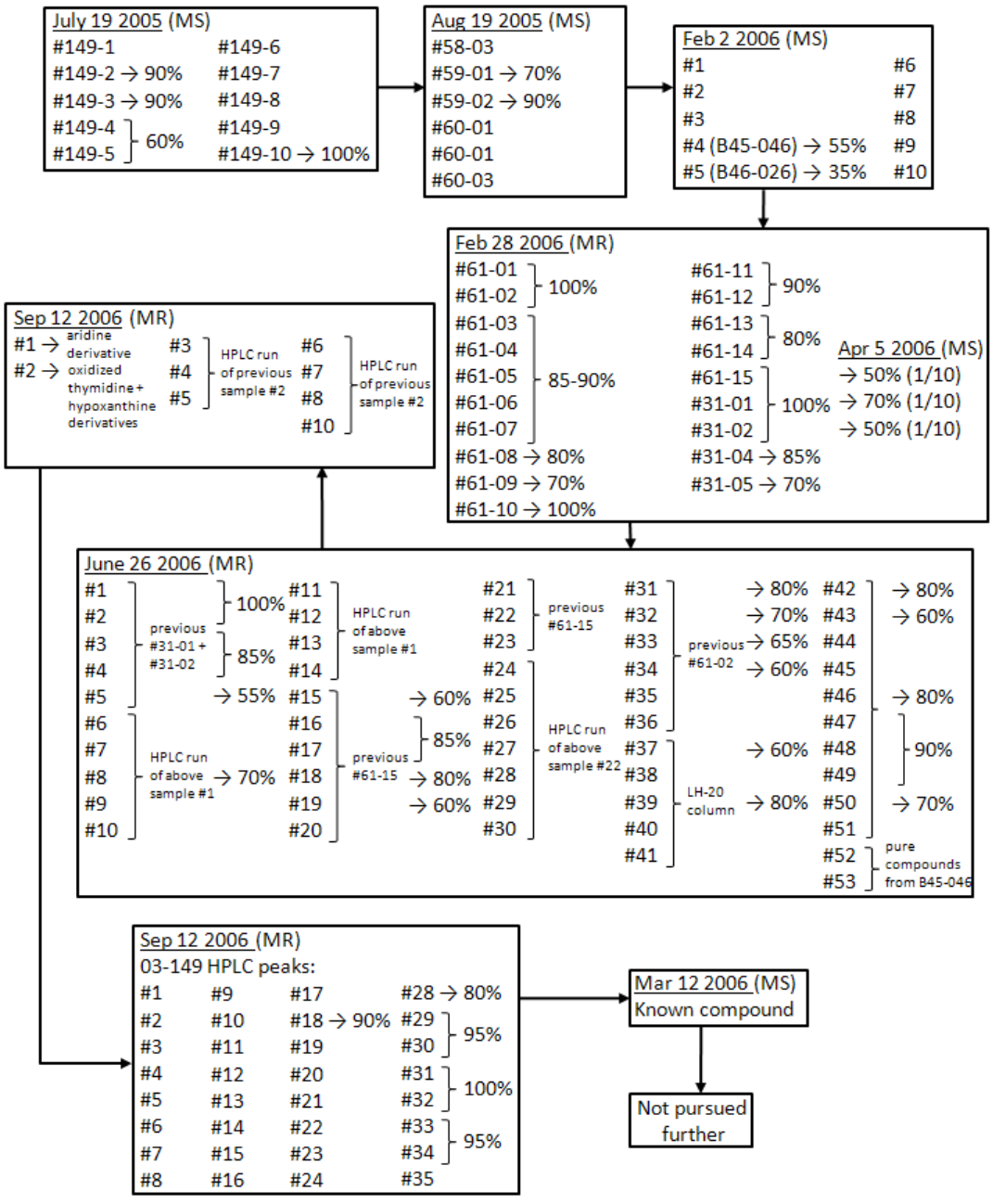
# Appendix C



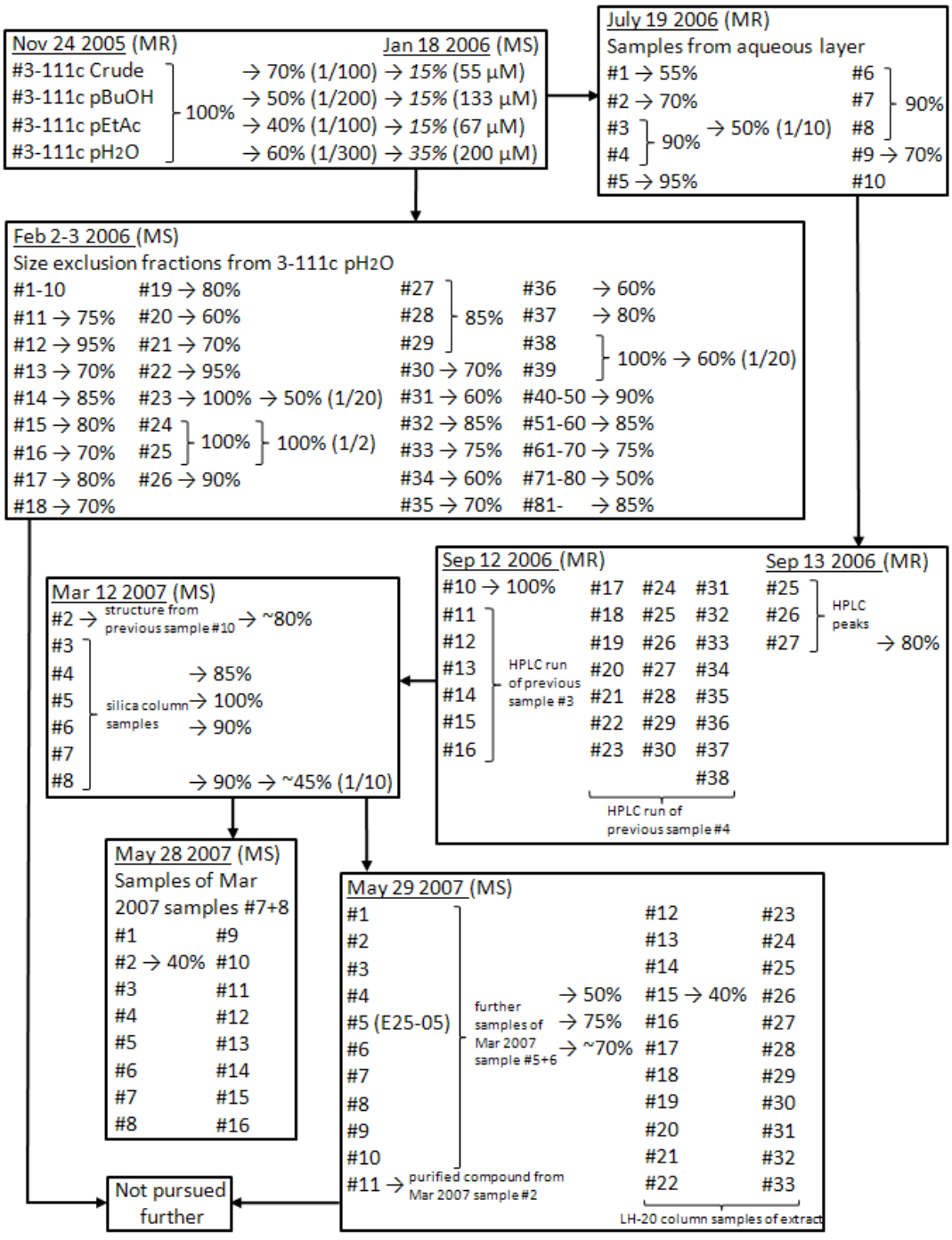

**Schematic representation of pursuit of calcineurin regulatory compounds from RJA-03-58, -124, -214, and -287 marine extracts.** Only marine extracts that were potently inhibiting CaN are listed in the first assay, unless otherwise stated. Open arrows (→) or braces (}) indicate inhibition values unless otherwise stated. Inhibition values of PP-1c are shown in italics. All samples were 1-3 mg/mL. Boxed items with closed arrows ( →►) indicate origin of further assays. Fractions in brackets designate dilution facor used in resulting inhibition numbers. If no value is provided, no inhibition or insignificant inhibition was observed. Additional extracts of interest that are boxed in the very first assay are represented in following pages. Assays were performed by following people: CM = Cheryl McCormik; MR = Mikolaj Raszek; MS = Micheal Shopik.




**Schematic chronological representation of attempted isolation of calcineurin regulatory compounds from RJA-03-193 and -269 marine extracts.** See p. 287 for details.




**Schematic chronological representation of attempted isolation of calcineurin inhibitory compounds from RJA-03-149 marine extract.** See p. 287 for details.




**Schematic chronological representation of attempted isolation of calcineurin inhibitory compounds from RJA-03-111 marine extract.** See p. 287 for details.