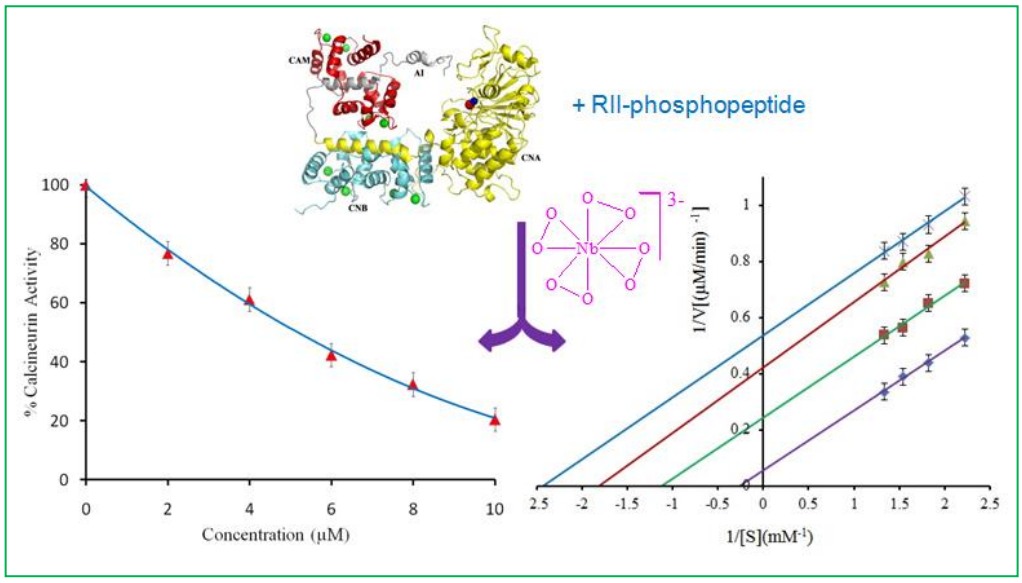
CHAPTER 8

Chapter 8

Peroxoniobium(V) Compounds as Uncompetitive Inhibitors of Calcineurin Activity

Contents

Calcineurin (CN) is a major calmodulin binding serine/threonine phosphatase which plays a crucial role in numerous mammalian signal transduction pathways. Inhibitors of CN represent a valuable tool for elucidating CN dependant cellular processes. This work deals with the identification of a set of peroxoniobium compounds, tetraperoxoniobate, $Na_3[Nb(O_2)_4]\cdot 13H_2O$ (**NaNb**), as well as the polymer-anchored macro complex, $[Nb_2(O_2)_6(carboxylate)_2]$-PA (**PANb**) (**3.1**), as potent inhibitors of calcineurin activity.



8.1 Introduction

Calcineurin (CN), also known as protein phosphatase 2B, is a serine/threonine phosphatase with the ability to dephosphorylate a broad range of proteins [1,2]. Physiologically, CN has been established as a key calmodulin dependent enzyme which plays a critical role in mammalian signal transduction pathways necessary for T-cell activation, nervous system development and function, cardiac growth and cell death mechanism [1-4].

Calcineurin is comprised of two subunits - the enzymatic subunit A and the regulatory subunit B [1,5] (**Fig. 8.1**). The subunit A encloses a calmodulin binding site and an autoinhibitory domain, which blocks the catalytic centre of the enzyme [1]. Binding of Ca^{2+} ions and calmodulin to calcineurin leads to conformation change and a subsequent unmasking of the active centre [1]. It has been established that calcineurin contains iron and zinc as intrinsically bound metal ions, but it requires exogenous metal for effective substrate turnover [6-12]. The ligand environment of the dinuclear metal centre of CN (**Fig. 8.2**) has been reported to resemble the active site co-ordination environment of purple acid phosphatase [1,13].

A number of natural products have been isolated that are potent inhibitors of calcineurin and other serine/threonine protein phosphatases. The most potent, specific, and well-known inhibitors of calcineurin are the immunosuppressant drugs cyclosporine A (CsA) and FK506 (tactolimus) [2] (**Fig. 8.3**). With the ground breaking discovery that CN is the target of these drugs, it has been firmly established that CN inhibitors are indispensable for preventing organ transplant rejection and to treat dermatologic and autoimmune disorders [2,14-17]. On the other hand, since these drugs often lead to severe adverse side effects [2,17], there has been a continued search for more specific, safer and effective alternative CN inhibitors. CN inhibitors are also receiving increasing importance as valuable tool for basic research [1,2,14], as such compounds would help to identify and characterize different targets of CN thereby contributing to the elucidation of CN dependent signalling processes.

Several new synthetic as well as natural CN inhibitors have been developed in recent years although the underlying molecular mechanisms of most of these compounds are yet to be elucidated in detail [2,14,17-27]. Interestingly, a number of groups provided

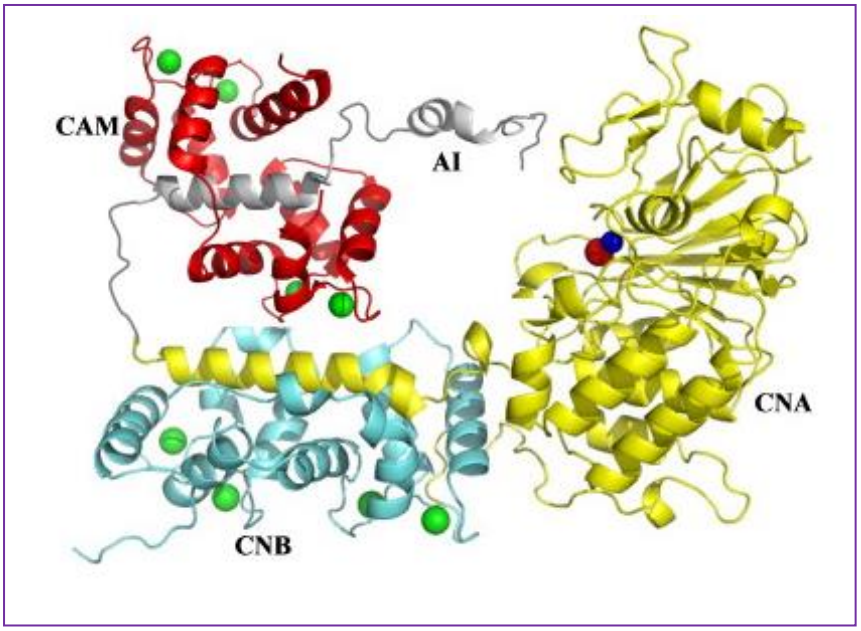


Fig. 8.1 The structure of Ca^{2+}/CaM-CN complex [5]. Calcineurin A (CNA) is shown in yellow and calcineurin B (CNB) in blue. Iron and zinc in the active site are shown as red and blue spheres, respectively. The four Ca^{2+} in the CNB subunit and calmodulin (CaM) sites are shown as green spheres. A region of CNA whose structure has not been determined is indicated schematically (grey).

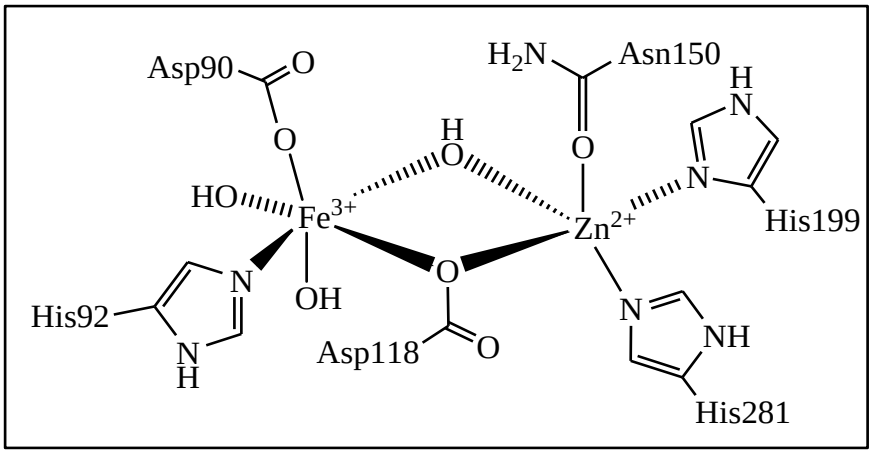


Fig. 8.2 Schematic of the active site of human calcineurin based on the 2.1-Å resolution structure described by Kissinger *et al*. [13].

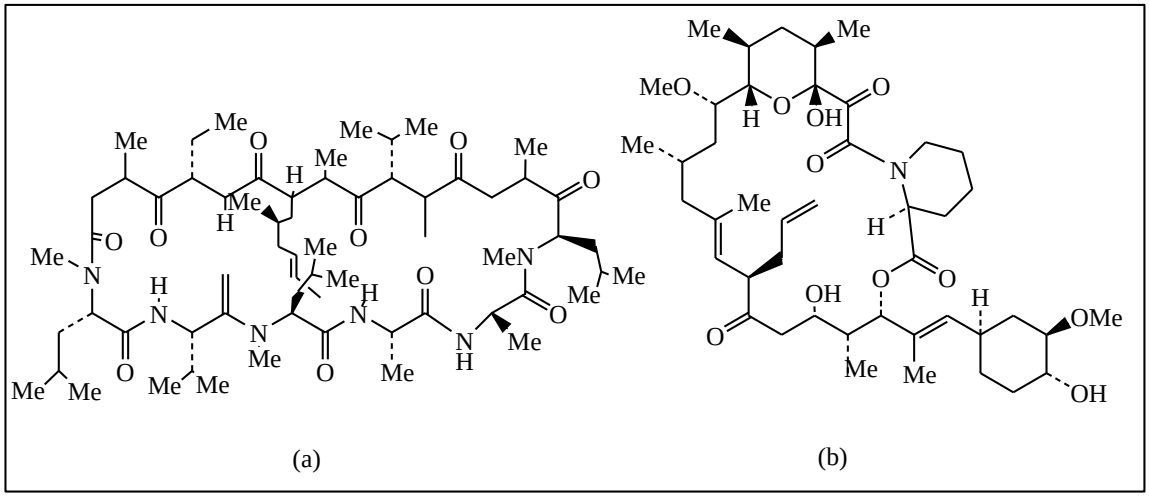


Fig. 8.3 Inhibitors of calcineurin (a) cyclosporine A (CsA) and (b) FK506 (tactolimus) [1,2].

evidence that reactive oxygen species (ROS), superoxide and hydrogen peroxide inhibit CN activity [28-31]. However, there have been conflicting conclusions about mechanism of such inhibition [27,29,32-35] by extracellular oxidants, in particular H_2O_2 mainly due to the complex enzymology of CN. Orthovanadate was reported to inhibit protein serine/threonine phosphatase activity of calcineurin [22]. Carballo and co-workers [29] used a synthetic peroxovanadate compound to study the effect of oxidative stress on calcineurin activity. It is worthy to note that despite the existence of scores of reports dealing with phosphatase inhibition by peroxo compounds of V [36], till date, we have come across only one report showing the inhibitory effect of a pV complex on CN activity [29], whereas no data appears to exist about inhibition of CN in cell-free assays or in cell involving other peroxometal systems.

Inspired by the above interesting findings, in the present work, we deemed it worthwhile to explore the possibility of developing a new class of inhibitors of phosphoester hydrolysis activity of calcineurin using peroxo compounds of niobium *viz*., $Na_3[Nb(O_2)_4]\cdot 13H_2O$ **(NaNb)** and $[Nb_2(O_2)_6(carboxylate)_2]$-PA [PA = poly(sodium acrylate)] **(PANb) (3.1)**, as representative systems. We describe here findings from our comparative study on *in vitro* effect of free as well as polymer bound synthetic peroxo compounds of Nb(V) *vis-a-vis* H_2O_2, on dephosphorylation activity of CN. For our investigation, we have used two different types of CN substrates *viz*.,

RII-phosphopeptide, a physiological substrate of calcineurin and a non-protein substrate, *p*-nitrophenylphosphate *(p*-NPP). Significantly, *in vitro* calcineurin inhibition by the drug complexes CsA and FK506 was reported to occur only when a physiological substrate is used to assay the enzyme such as phosphocasein or phospho-RII peptide, a peptide whose sequence represents the phosphorylation site of the regulatory subunit of cAMP-dependent protein kinase [37]. To the best of our knowledge, the present work is the first report concerning the steady state kinetics of inhibition of CN activity by any synthetic peroxometal compound.

8.2 Experimental section

8.2.1 Calcineurin phosphatase assay

8.2.1.1 Measurement of calcineurin activity

The calcineurin activity was assayed spectrophotometrically using either *p*-NPP or RII-phosphopeptide as substrate, employing established enzyme assay system [38-40]. Calcineurin assay reagents were prepared exactly according to the manufacturer's (Calbiochem) instructions. The indicator, malachite green reagent was prepared by reported procedure [38], adding 3 volumes of 0.045% malachite green in water with 1 volume of 4.2% ammonium molybdate in 4 N HCl. The mixture was stirred and kept for 20 mins in dark followed by addition of tween-20.

In the standard assay, the reaction mixture (50 μL) contained calcineurin (0.2 U/μL), calmodulin (1 mM), *p*-NPP or RII-phosphopeptide (750 μM) in incubation buffer (200 mM NaCl, 100 mM Tris, 12 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 0.05% NP-40, pH 7.5) and different concentrations of peroxoniobium species (concentration ranges between 2 and 10 μM) or H_2O_2 (concentration ranges between 25-125 μM). The inhibitor concentrations are indicated in figure legend (**Fig. 8.4**). The assay without inhibitor was used as control. A phosphate standard curve was run with each assay to calculate the specific activity of the enzyme. Prior to the start of the phosphate assay, CN was incubated in assay buffer along with the inhibitor for 5 min at 30 °C. Calmodulin was then added to the test solution which was further incubated for 10 min. The reaction was initiated by addition of the substrate to the pre-incubated test solution. After 1 h of

incubation of the reaction mixture at 30 °C, the reaction was terminated by addition of malachite green reagent to the test solution and the absorbance at 655 nm (ε_{655}=6700 M^{-1} cm^{-1}) [41] was recorded in order to determine the phosphate released from the substrate. The IC_{50} values were graphically determined as the half-maximal inhibitory concentration of the inhibitor species giving 50% inhibition. All assays were performed in triplicate. The data in the figures are presented as the mean ±SE from three separate experiments.

8.2.1.2 Kinetic analysis

The enzyme kinetic studies were carried out using Cary 100 Bio Enzyme Kinetics software. For the kinetic analysis, the concentration of RII-phosphopeptide was varied from 450-750 μM for each concentration of inhibitor. The inhibitor concentrations used are indicated in figure legend (**Fig. 8.5**). The kinetic constants, maximum velocity (V_{max}) and Michaelis constant (K_m) were determined from Lineweaver-Burk plots by rearranging Michaelis-Menten equation [42-44].

$$\frac{1}{V} = \frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}} \qquad \text{.....1}$$

In the current study, the expression (2) was used to characterize the mode of inhibition, which was found to be of uncompetitive type.

$$V = \left\{ \frac{V_{max} \times [S]}{K_m + [S]\left(1 + \frac{[I]}{K_{iu}}\right)} \right\} \qquad \text{.....2}$$

where V is the velocity, [S] is the RII-phosphopeptide concentration and [I] refers to the inhibitor concentration. K_{iu} is the inhibitory constant for the uncompetitive inhibition. The enzyme inhibitor constant K_{iu} was calculated from the secondary plots of initial rate data by linear regression analysis. The intercepts obtained from Lineweaver-Burk plots were replotted against inhibitor concentration to measure K_{iu} values from x-intercepts of these re-plots.

8.3 Results and discussion

8.3.1 Effect of peroxoniobium complexes on the activity of calcineurin

The ability of peroxoniobium compounds to inhibit the calmodulin mediated dephosphorylation activity of calcineurin phosphatase *vis-a-vis* H_2O_2, was assessed using tetraperoxoniobate (**NaNb**) and the polymer bound **PANb (3.1)**, as representatives of monomeric and macromolecular pNb complexes, respectively. The effect of the inhibitor species was examined *in vitro* using the substrates, RII-phosphopeptide as well as *p*-nitrophenyl phosphate (**Fig. 8.4**). Calcineurin is capable of hydrolyzing *p*-NPP to generate *p*-NP, which can be readily monitored spectrophotometrically.

As seen in **Fig. 8.4**, apart from H_2O_2, each of the pNb compounds, irrespective of being free monomeric or macromolecular species, inhibited CN activity in the enzymatic assay in a dose-dependent manner. The inhibitory potential of the molecules was quantified by determination of the half-maximal inhibitory concentration (IC_{50}) for each inhibitor. Our results demonstrate that the peroxo-Nb complexes are highly potent inhibitors of the enzyme with their IC_{50} values varying within a close range of 5-7.5 μM, as seen in **Table 8.1**. On the other hand, H_2O_2 with IC_{50} of approximately 32.5 μM (for RII-phosphopeptide) was nearly 6 fold less potent inhibitor of the enzyme relative to the tested pNb compounds. It is worth noting that each of the pNb inhibitor species including H_2O_2 inhibited the CN activity towards RII-phosphopeptide to nearly the same extent as its *p*-NPP phosphatase activity with comparable IC_{50} values [**Fig 8.4** and **Table 8.1**]. The IC_{50} value of 32.5 μM obtained for H_2O_2 in the present study is in good agreement with the IC_{50} value of 30-40 μM previously reported by Reiter and Rusnak [32,33], for inhibition of intracellular CN activity by H_2O_2 in T-lymphocytes. The IC_{50} value for the polymer bound **PANb (3.1)** is reported on the basis of its actual peroxometal loading. It is notable that the ligand environment of the complexes did not appear to have significant effect on the inhibitor efficiency of the compounds. This is in contrast to our observations with respect to ACP inhibition by pNb complexes reported in the preceding Chapter, as well as previous findings of our group on the inhibition of ACP and ALP by peroxometallates, where the nature of the ligand sphere of the inhibitors showed a marked influence on the extent and mode of inhibition of these enzyme functions [45-51].

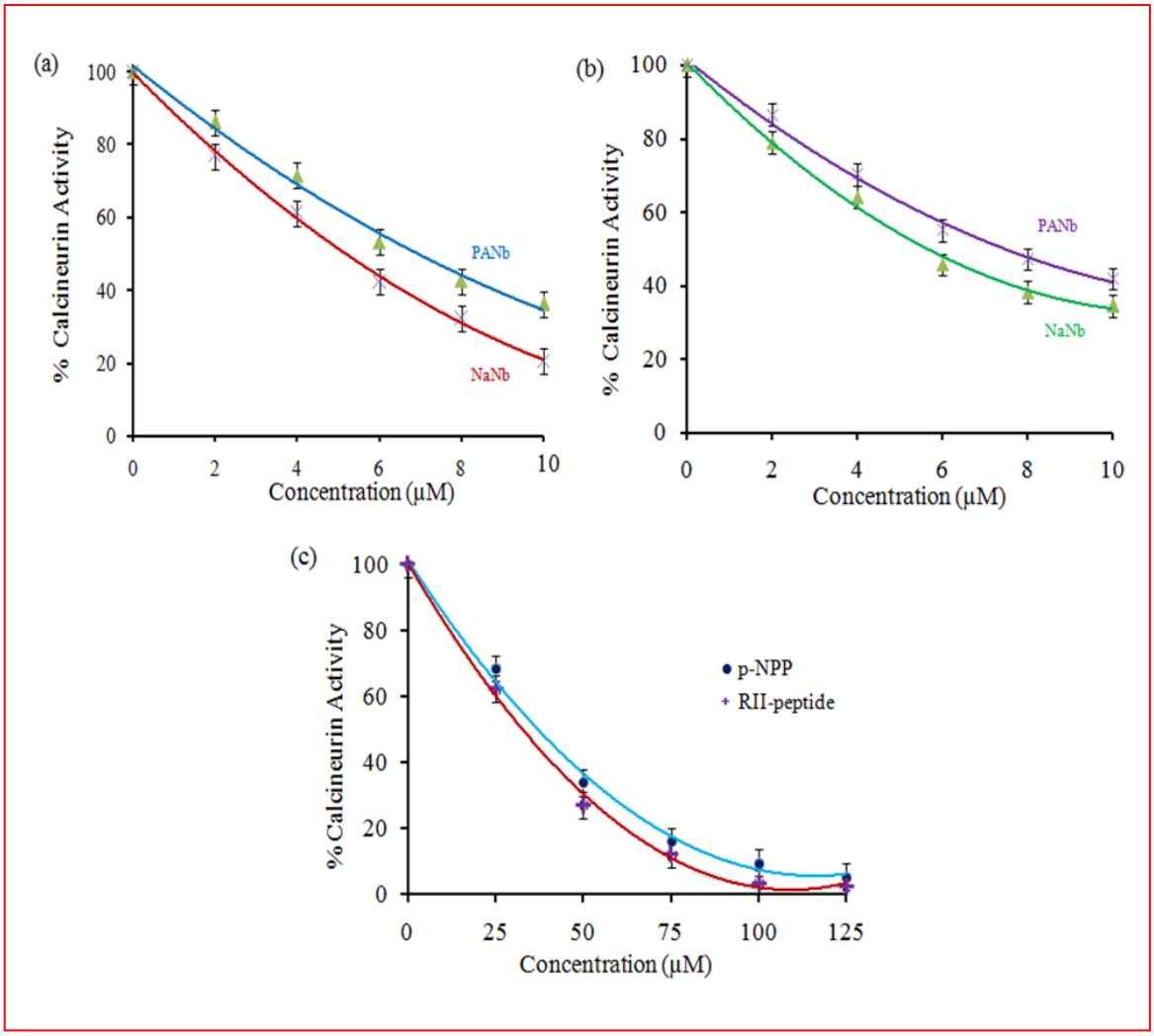


Fig. 8.4 The effect of pNb compounds and H_2O_2 on calcineurin activity, (a) effect of **NaNb** and **PANb (3.1)** on calcineurin activity with RII-phosphopeptide as substrate, (b) effect of **NaNb** and **PANb (3.1)** on calcineurin activity with *p*-NPP as substrate and (c) effect of H_2O_2 on calcineurin activity. Calcineurin catalyzed rates of hydrolysis of substrate were determined at pH 7.5 by measuring A_{655} in a reaction mixture containing calcineurin (0.2 U/μL), calmodulin (1 mM), *p*-NPP or RII-phosphopeptide (750 μM) in incubation buffer (200 mM NaCl, 100 mM Tris, 12 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 0.05% NP-40, pH 7.5) with or without stated concentrations of inhibitors (compound concentrations: 2, 4, 6, 8 and 10 μM for **NaNb** and **PANb (3.1)** and for H_2O_2, concentrations = 25, 50, 75, 100 and 125 μM). Effects of the additions are represented as the percent values (rounded to integers) of control. The data are presented as mean ± SE from three separate experiments.

8.3.2 Kinetics of calcineurin inhibition by H_2O_2 and pNb compounds

There are various ways through which an inhibitor can interact with an enzyme and to explicate the distinction between the inhibition mechanisms of enzyme catalyzed reactions, enzyme kinetic investigation is a major implement. In order to gain an insight into the mechanism of inhibition of CN catalyzed dephosphorylation by the pNb compounds, we have investigated the steady state kinetics of the process. RII-phosphopeptide being a well-characterized reliable physiological substrate for CN [1,37], we have carried out kinetic investigations using RII-phosphopeptide as substrate. In **Fig. 8.5**, the Lineweaver-Burk double reciprocal plots of the reciprocal initial velocity *versus* the reciprocal substrate concentration, with and without the inhibitor, at various concentrations are shown. From the kinetic measurements at several different substrate concentrations in the presence of the inhibitors, straight parallel lines were obtained with constant slope. An increase in concentration of each of the compounds led to decrease in V_{max} with concomitant lowering of Michaelis constant, K_m values. Thus both $1/K_m$ and $1/V_{max}$ changed to the same extent in each case, affording plots consisting of parallel lines. Our findings demonstrate that the mode of inhibition of CN by each of the tested peroxo species, including H_2O_2 is of the "uncompetitive" type. We have further determined the inhibitory constant, K_{iu}, for the inhibitors. The value of K_{iu}, was obtained from the secondary plot of the intercepts of the primary plot (1/V *versus* 1/[S]) against inhibitor concentration. From the data obtained for K_{iu} values **(Table 8.1**), it was further confirmed that the tested niobate complexes are stronger inhibitors of calcineurin compared to H_2O_2, as has been indicated by their IC_{50} values.

The present study thus offers several points of interest. In our previous work, our research group has provided kinetic evidence to demonstrate that free monomeric peroxo compounds of V [45,47-49], Mo [45,47] and W [46,49-51] exerted mixed type of inhibition on activities of phosphohydrolases such as ALP as well as ACP. On the other hand, the polymer immobilized compounds of V and its Mo and W containing analogues inhibited the activity of both of these enzymes non-competitively [45-47]. Consistent with these findings, as demonstrated by the results presented in the previous Chapter, free and polymer bound peroxoniobium complexes too showed a similar trend in the mode of inhibition of ACP. Moreover, H_2O_2 had no observable effect on ACP and ALP

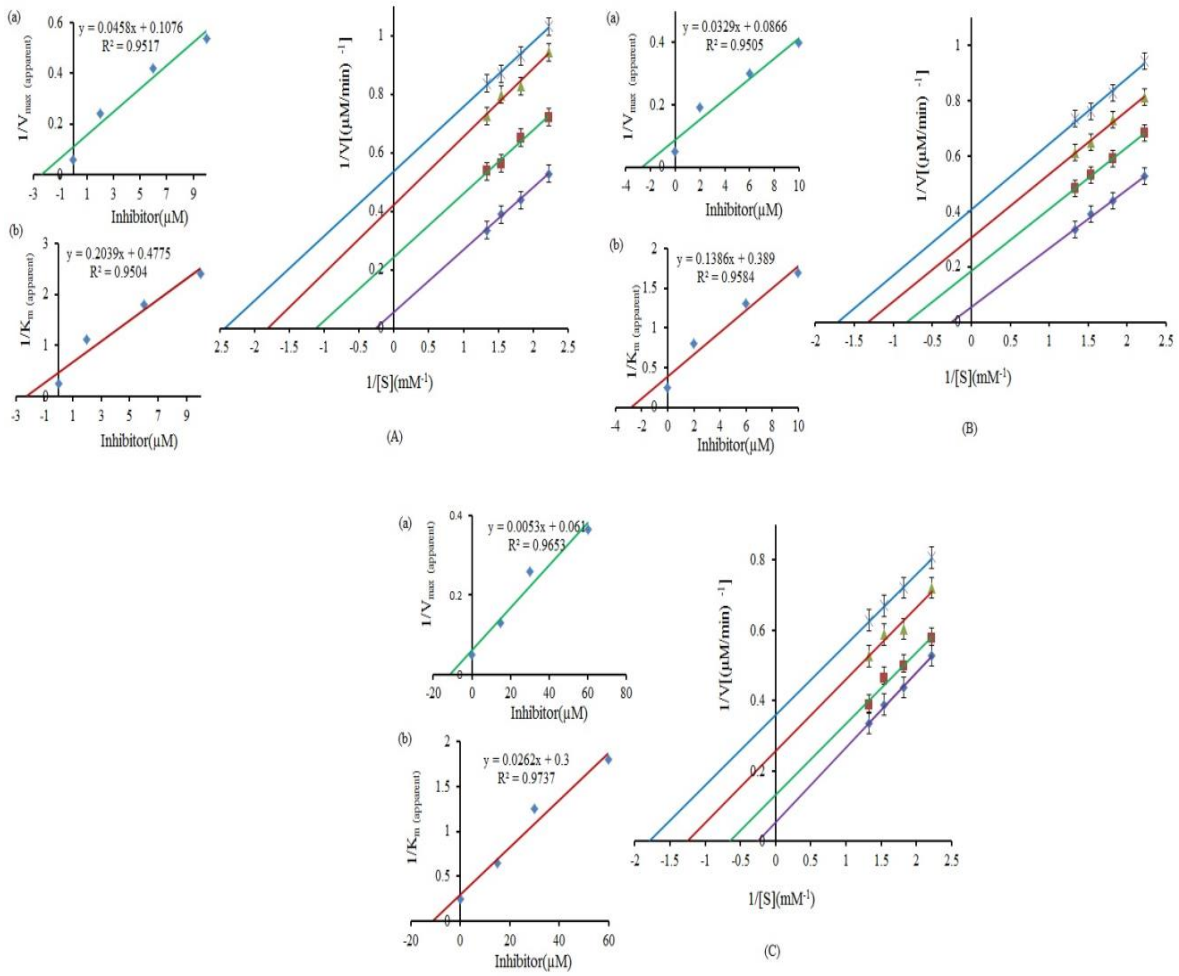


Fig. 8.5 Lineweaver-Burk plots for inhibition of calcineurin activity in absence and presence of (A) **NaNb**, (B) **PANb (3.1)** and (C) H_2O_2. The inset represents the secondary plot of the initial kinetic data of the Lineweaver-Burk plot. The reaction mixture contained calcineurin (0.2 U/μL), calmodulin (1 mM), RII-phosphopeptide (450-750 μM) in 2X assay buffer of pH 7.5. The rates of hydrolysis was obtained in the presence of (♦) 0, (■) 2, (▲) 6, (x) 10 μM concentrations for **NaNb** and **PANb** compounds and (♦) 0, (■) 15, (▲) 30, (x) 60 μM concentrations of H_2O_2**.** The values are expressed as mean ± SE from three separate experiments. (Inset) (a) The vertical intercepts ($1/V_{max}$) were plotted against inhibitor concentrations and K_{iu} values were obtained from x-intercepts of these plots, (b) The $1/K_m$ values were plotted against inhibitor concentrations. For **PANb**, compound concentrations are on the basis of peroxometal loading.

function under our experimental conditions [45,46]. It is important to note that, in the present case as revealed by our kinetic investigation, hydrogen peroxide as well as the peroxo metal derivatives, irrespective of the nature of their ligand environment, inactivated calcineurin exclusively *via* an uncompetitive pathway. The observation also indicates that CN may be specifically sensitive to inactivation by H_2O_2.

In uncompetitive mode of inhibition, an inhibitor binds to only the enzyme-substrate complex, but the free enzyme is not a target of inhibition [52]. Although this mode of inhibition is much less common in nature [53], compared to other pathways such as competitive or non-competitive modes, there have been examples of uncompetitive inhibition occurring in the case of CN [25] as well as of alkaline phosphatases activities in the presence of various inhibitor species [54,55]. Recently, Tanaka *et al*. [25] have reported that Mn^{2+} inhibited the Ni^{2+} stimulated CN activity by uncompetitive inhibition. In contrast to this, the CN inhibitor FK506, that leads to immunosuppression has been shown to bind to CN at a region far removed from the enzyme active site [15].

Table 8.1: Half-maximal inhibitory concentration (IC_{50}) and inhibitor constant (K_{iu}) values for pNb compounds and H_2O_2 against calcineurin

Compound	Substrate	IC_{50} (μM)	K_{iu} (μM)
NaNb	*p*-NPP	5.6	
	RII-phosphopeptide	5.2	2.4
PANb	*p*-NPP	7.4	
	RII-phosphopeptide	6.9	2.7
H_2O_2	*p*-NPP	36.5	
	RII-phosphopeptide	32.5	11.3

Calcineurin catalyzed rates of hydrolysis of *p*-NPP or RII-phosphopeptide at pH 7.5 were determined at 30 °C by measuring A_{655} in a reaction mixture containing calcineurin (0.2 U/µL), calmodulin (1 mM), substrate (750 μM) in incubation buffer (200 mM NaCl, 100 mM Tris, 12 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 0.05% NP-40, pH 7.5) in the presence of stated concentrations of inhibitors. The V_{max} and K_m in the absence of inhibitor were found to be 20 μM/min and 4 mM, respectively.

Previous studies from several laboratories demonstrated that a Fe^{2+}-Zn^{2+} binuclear metal centre is the key component of the native, catalytically active site of CN which is susceptible to redox regulation [29,34,35]. There has been evidence showing that inhibition of CN by reactive oxygen species (ROS) such as superoxide and H_2O_2 originates from oxidation of the Fe^{2+}-Zn^{2+} centre to the inactive Fe^{3+}-Zn^{2+} form [29,32,34,35]. Apart from these physiological oxidants, other oxidant systems such as NaOCl, inhibited CN when added to Jurkat cell lysate with an IC_{50} value of 150-200 μM whereas in a similar system, the IC_{50} value for H_2O_2 inhibition was reported to be 60-80 μM [32]. Carballo and co-workers [29] reported that treatment of human neutrophiles with a synthetic peroxovanadium compound, oxodiperoxo(1,10-phenanthroline)vanadate(V) [pV(phen)] caused a dose- and time-dependant inhibition of CN activity with an IC_{50} of about 30 μM. However, kinetic data or K_i values were not reported in these studies. On the other hand, it has also been proposed that inactivation by H_2O_2 occurs due to the formation of bridging disulfide bond between closely spaced Cys residues in the catalytic subunit of CN-A [27,32].

Thus taken together, the aforementioned observations raise the possibility that the peroxo compounds examined herein may inactivate calcineurin by several potential mechanisms. We surmise that these inhibitor species, possessing the ability to oxidise organic substrates, would modify the redox state of Fe-Zn centre by oxidizing an active site Fe^{2+} to Fe^{3+}, causing inactivation of the enzyme. Calcineurin may also undergo inactivation by the peroxometal species *via* formation of a disulfide bond between the cysteine residues in the catalytic site of the enzyme as proposed previously [27,32]. Although, many more experiments would definitely be needed to shed more light into these highly complicated processes, nevertheless, it may be hoped that the information generated from the present study will provide a basis for further investigation on the role of oxidant species in regulation of CN dependent cellular processes.

8.4 Conclusions

We describe in this study the identification of neat as well as polymer anchored peroxo derivatives of niobium compounds as novel class of potent inhibitors of calcineurin. Our results demonstrated that the pNb compounds examined are highly

efficient, at nearly 6 fold lesser dose than hydrogen peroxide, in inhibition of dephosphorylation of the physiological substrate RII-phosphopeptide as well as *p*-NPP by calcineurin. The most significant finding of the present investigation is that H_2O_2 as well as peroxoniobium compounds tested irrespective of the nature of their ligand environment, display a distinct mechanistic preference to inhibit the function of CN in an uncompetitive manner. In summary, our findings could provide a paradigm to design potent inhibitors of CN, effective at far lower doses compared to H_2O_2, that are easily formed, stable to degradation and applicable to *in vivo* studies.

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