Convention Special Issue - 2019

J. Indian Chem. Soc.,
Vol. 96, October 2019, pp. 1265-1277

Peroxo complexes of niobium(V) as potent inhibitors of acid phosphatase and calcineurin enzyme activities†

Sandhya Rani Gogoi, Hiya Talukdar and Nashreen S. Islam*

Department of Chemical Sciences, Tezpur University, Napaam, Tezpur-784 028, Assam, India

E-mail: nsi@tezu.ernet.in, nashreen.islam@rediffmail.com

Manuscript received online 03 October 2019, accepted 10 October 2019

Our recent achievements concerning the synthesis, characterization and exploration of some biochemically significant features of peroxo niobium (PNb) complexes are highlighted here. Water soluble heteroleptic PNb complexes in a variety of ligand environment were screened for their activity with two types of enzymes viz. calcineurin and acid phosphatase. Employing wheat thylakoid ACP as the model enzyme, it has been demonstrated that peroxoniobium derivatives serve as active inhibitors of phosphatase (IC_{50} values 2–9 μM). Enzyme kinetics data revealed that compounds exert mixed type of inhibition on ACP activity ($K_{ii} > K_i$). Calcineurin inhibitors represent a valuable tool for elucidating CN dependent cellular processes. *In vitro* effect of the complexes on CN activity was examined using physiological substrate RII-phosphopeptide. As revealed by enzyme kinetic investigation, hydrogen peroxide as well as the peroxoniobium derivatives, irrespective of the nature of their ligand environment, inactivated calcineurin exclusively via an uncompetitive pathway. .

Keywords: Calcineurin, acid phosphatase, peroxoniobium complex, enzyme kinetics, RII-phosphopeptide.

Introduction

Peroxo compounds of niobium have been attracting great deal of contemporary importance mainly owing to their efficiency as versatile oxidation catalysts[1–4] as well as their utility as valuable water soluble precursors to obtain Nb-based oxide materials[1,5,6]. It is however surprising that although Nb belongs to the same periodic group as vanadium and has been reported to be practically non-toxic to animals[7], very few studies dealing with exploration of biochemical potential of peroxo compounds of niobium[8,9] are available. In contrast, compounds of vanadium, particularly the peroxovanadates have been receiving tremendous importance mainly as antineoplastic and insulin-mimetic agents[10–17], although most of the synthetic PV compounds are unstable under physiological condition and are moderately toxic[10,11,14]. It is notable that vanadate and niobate have been reported to be potent inhibitors of the sarcoplasmic reticulum (SR) Ca^{2+}-ATPase that can influence variety of cellular processes including calcium homeostasis and cell signalling[18].

Enzyme inhibition has already been recognized as one of the important mechanisms of action of inorganic therapeutic agents[19]. For example, insulin-mimetic activity of vanadium compounds has been corelated to the phosphatase inhibitory ability of V-compounds[20]. In fact, owing to the crucial role of phosphatases in signal transduction pathways[21,22], this group of enzymes have become a key target for studying metabolism[21,22], for modifying cell signalling[22,23] and for treatment of diseases. As observed in a recent review on efficacy of vanadium based phosphatase inhibitors by McLauchlan and co-workers[22], not withstanding the existence of numerous studies dealing with relative inhibitory potency such as EC_{50} of V as well as Mo and W derivatives, the reports in which mode of inhibition and K_i values have been defined are still very limited.

Pertinent here is to mention that in recent years, our research group has investigated several bio-relevant aspects of peroxometallates comprising of d^0 metal ions viz. V, Nb, Mo and W[24–37] including their antibacterial[25] and vasomodulatory activities[28]. We have carried out detailed inhibition kine-

†Professor D. P. Chakraborty 60th Birthday Anniversary Lecture (2018).

tics of phosphatases involving monomeric[31–33], dimeric[24,31] as well as macromolecular complexes[24,34–37]. It has also been demonstrated that peroxovanadate immobilized on poly(acrylic acid) becomes a highly potent inhibitor of growth of lung carcinoma cells (A_{549})[35]. Very recently, we have synthesized a series of water soluble PNb complexes in biogenic co-ligand environment[36,38]. Moreover, we could successfully anchor PNb moieties on water soluble polymers to generate a set of well defined and highly stable immobilized PNb macro complexes[37,39]. Several of these complexes served as efficient and highly selective catalysts for organic oxidations[38,39]. These interesting observations reveal that a lot remains yet to be explored regarding other possible chemical and biochemical aspects of such systems.

In this article, we present a summary of our interesting findings on some of the heretofore unexplored biologically important features of PNb derivatives[36,37]. We have investigated their inhibitory effect on phosphatase using wheat thylakoid membrane ACP as model enzyme[36]. Moreover, we have studied the *in vitro* effect of free as well as polymer bound synthetic peroxo compounds of Nb(V), on dephosphorylation activity of calcineurin enzyme[37]. Calcineurin (CN), also known as protein phosphatase 2B, is a serine/threonine phosphatase with the ability to dephosphorylate a broad range of proteins[40–43]. 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[40–43]. It has already been established that CN inhibitors are indispensable for preventing organ transplant rejection and to treat dermatologic and autoimmune disorders[37,43–47]. 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 FK_{506} (tactolimus)[43]. Since these drugs often lead to severe adverse side effects[37,43,44], there has been a continued search for more specific, safer and effective alternative CN inhibitors. Till date, we have come across only one report showing the inhibitory effect of a peroxovanadate complex on CN activity[48], whereas to the best of our knowledge, no data exists about inhibition of CN in cell-free assays or in cell involving other peroxometal systems. These interesting observations inspired us to explore the possibility of developing a new class of inhibitors of activity of calcineurin using free as well as polymer bound synthetic peroxo compounds of Nb(V).

Synthesis and characterization of free monomeric and polymer anchored peroxo complexes of niobium

An appropriate preference of the co-ligand is a significant prerequisite in order to obtain stable and well-defined peroxometallates. Keeping in view our primary goal to explore some bio-relevant properties of PNb compounds, we have endeavoured to gain an access to PNb derivatives with biogenic species as co-ligands.

Although a variety of heteroleptic PNb complexes have been synthesized in recent years[1,49–52], there appears to be a paucity of information regarding well defined synthetic peroxoniobium complexes with ancillary ligands such as amino acids and other related species[5,53]. In nature, amino acids usually promote solubility of metals in aqueous environment and impart stability by complexation and increase their bioavailability. Moreover, carboxylate containing species have been known to be excellent co-ligands for stabilizing PNb species[1,54–56].

We have established viable synthetic routes to obtain stable and water soluble heteroleptic triperoxoniobium complexes in ligand sphere comprising of amino acids viz. L-arginine, L-alanine, L-valine or nicotinic acid (also known as niacin) as potential co-ligands[36,38] of the type, $Na_2[Nb(O_2)_3L]$ [L = alaninato (NbAla) (**1**) or valinato (NbVal) (**2**)], $Na_2[Nb(O_2)_3(arg)]\cdot 2H_2O$ [arg = arginate (NbA) (**3**)], and $Na_2[Nb(O_2)_3(nic)(H_2O)]\cdot H_2O$ [nic = nicotinate (NbN) (**4**)]. Water soluble precursor complex, $Na_3[Nb(O_2)_4]\cdot 13H_2O$, used as the source of niobium, has been prepared by the literature method[4]. The maintenance of pH of *ca.* 6 and limiting the amount of water to that contributed by 30% H_2O_2 appeared to be the key parameters responsible for the successful synthesis of the compounds in an aqueous medium. The near neutral pH apparently favoured the formation of the triperoxo Nb species and co-ordination of the amino acids in their anionic form.

The title compounds have been comprehensively characterized by spectroscopic and other conventional methods including ^{93}Nb NMR and EDX analysis. On the basis of the collective evidences gathered from these investigations structures of the type shown in Fig. 1 has been proposed for the

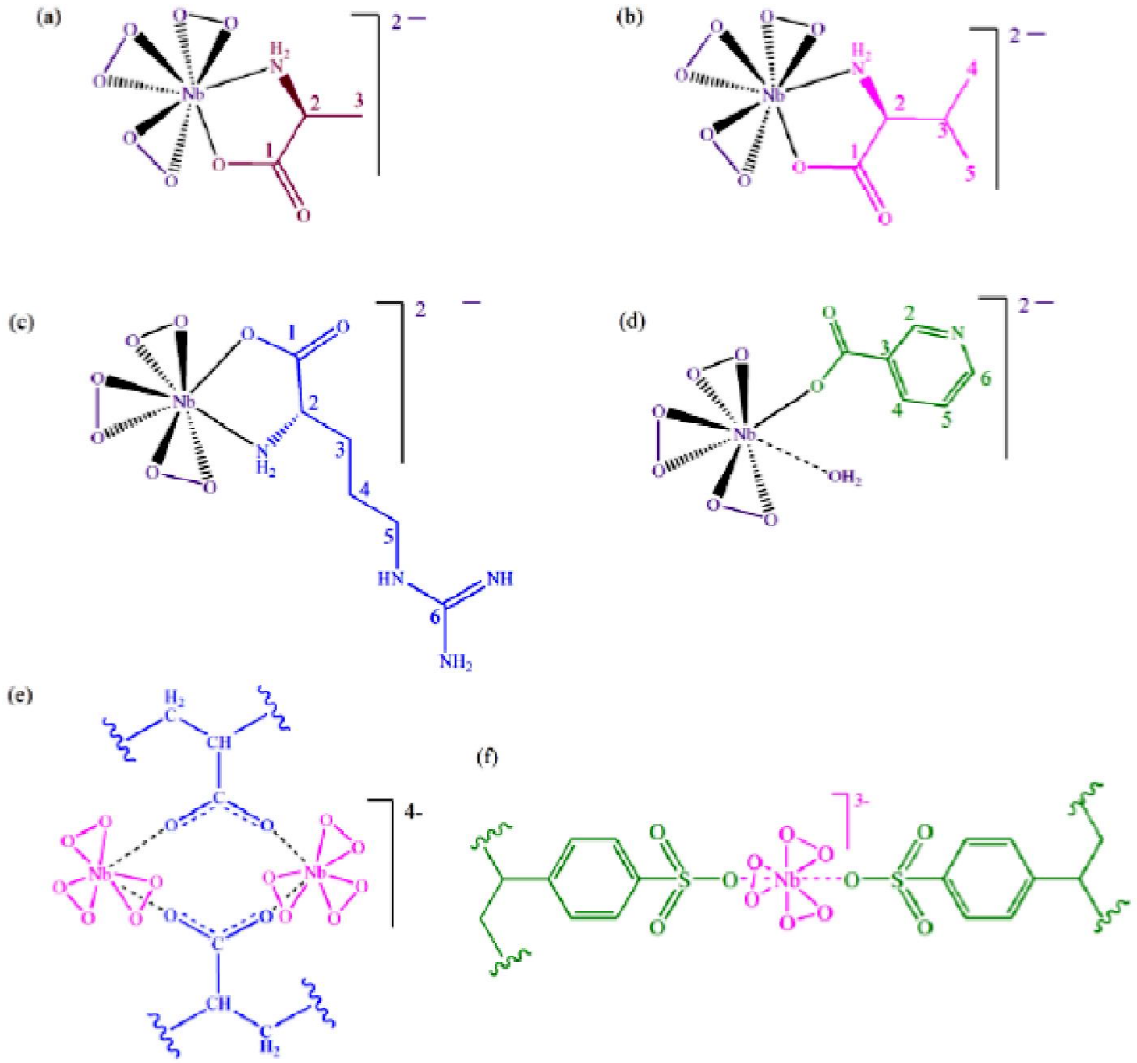


Fig. 1. Proposed structure of (a) NbAla (**1**), (b) NbVal (**2**), (c) NbA (**3**), (d) NbN (**4**) and soluble polymer anchored PNb complexes, (e) PANb (**5**) and (f) PSSNb (**6**). PA = poly(sodium acrylate), PSS = poly(sodium styrene sulfonate) and "〰" represents polymer chain. Reprinted from Ref.[36–39]. Ref. [36], Copyright (2017), with permission from Elsevier, Ref.[37], Copyright (2017), with permission from John Wiley and Sons.

compounds **1** to **4**. In each of the complexes side-on bound peroxo groups and the coordinated ancillary ligands complete the eight-fold coordination around the Nb(V) centre.

The density functional theory (DFT) calculation has been performed to verify the feasibility of the proposed structures of the synthesized PNb complexes (Fig. 2). The results of our theoretical studies substantiated the experimental observations imparting validity to the proposed geometries of the complexes.

In addition to the free monomeric peroxoniobates, we have prepared novel water soluble polymer-anchored PNb complexes, $[Nb_2(O_2)_6(carboxylate)2]$-PA [PA = poly(sodium acrylate)] (PANb) (**5**) and $[Nb(O_2)_3(sulfonate)_2]$-PSS [PSS = poly(sodium styrene sulfonate)] (PSSNb) (**6**) by following a procedure summarized in Scheme 1[39].

It is worthy to mention here that our group has previously synthesized series of stable and well-defined macrocomplexes with peroxo species of vanadium (PV)[24,25,57], molybdenum (PMo)[24,57–59] and tungsten (PW) species[34] anchored to the insoluble cross-linked as well as water soluble polymer (WSP)

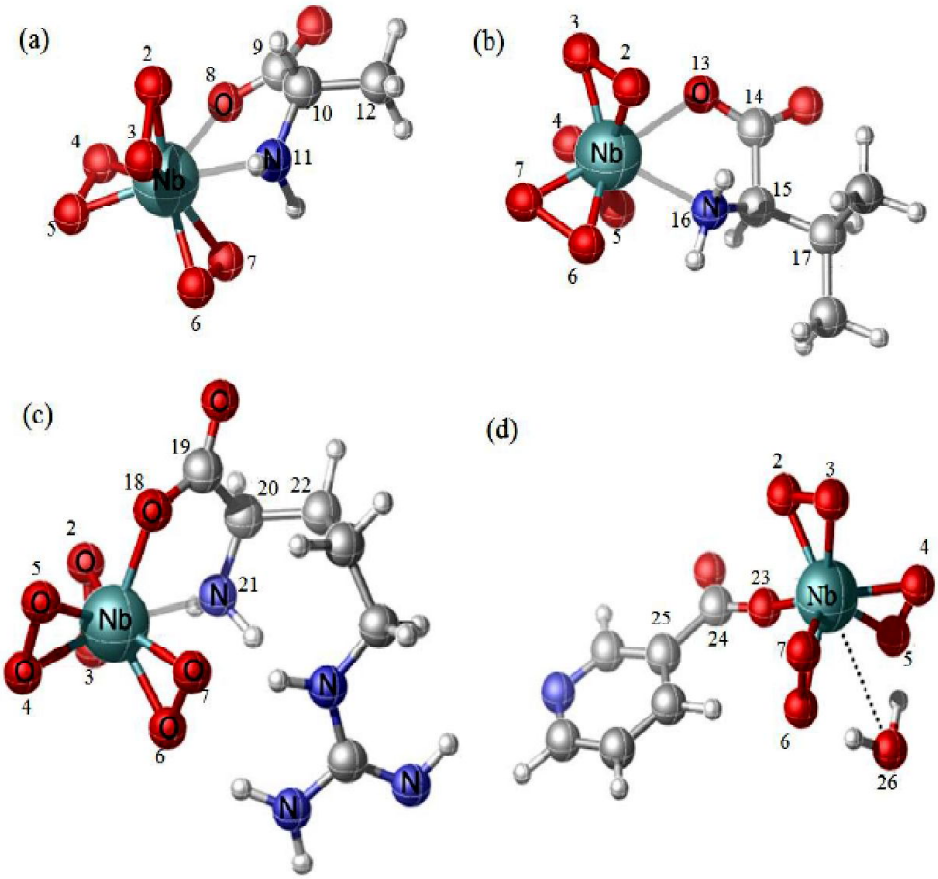
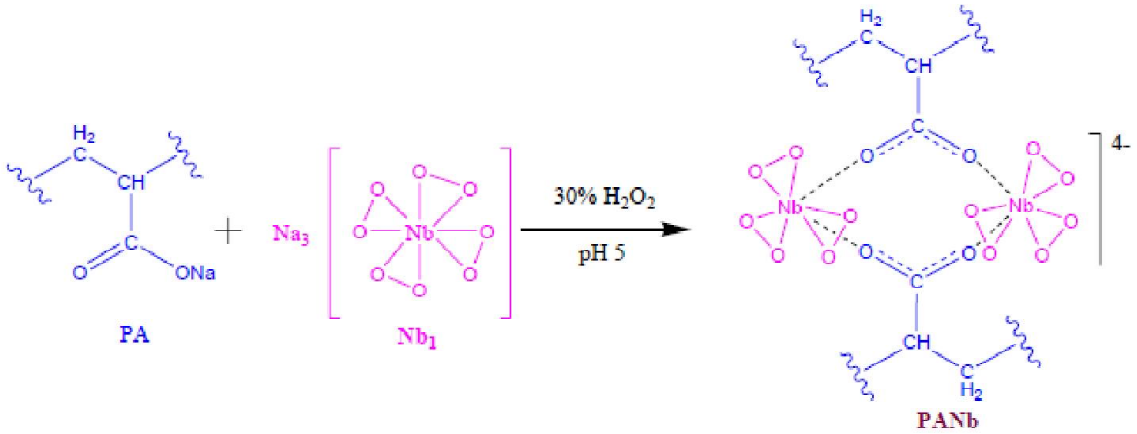


Fig. 2. Optimized geometry of (a) NbAla (**1**), (b) NbVal (**2**), (c) NbA (**3**) and (d) NbN (**4**). Reprinted from Ref. [36,38]. Ref. [36], Copyright (2017), with permission from Elsevier.



Scheme 1. Synthesis of soluble polymer anchored PNb complex, PANb, PA = poly(sodium acrylate), Nb_1 = $Na_3[Nb(O_2)_4]\cdot 13H_2O$. Reprinted from Ref.[37]. Ref.[37], Copyright (2017), with permission from John Wiley and Sons.

matrices. To the best of our knowledge, these complexes have been the first reported examples of peroxometallates attached to water soluble polymers[24,25,34,57]. Several of these macro complexes exhibited unique biochemical as well as

catalytic properties[24–29,34,35,57]. In addition to their application in diverse fields of chemistry, environment and biology[60–62], utility of WSP as supports to stabilize metal complexes is increasingly being realized in recent years. The developed macromolecular PNb complexes were characterized by elemental analysis (CHN, ICP and energy-dispersive X-ray spectroscopy), spectral studies (IR, ^{13}C and ^{93}Nb NMR studies), thermogravimetric analysis (TGA) as well as SEM studies. The structures envisaged for the macro complexes PANb (**5**) and PSSNb (**6**) are depicted in Fig. 1e and 1f. The structure of complex PANb has also been investigated employing density functional theory (DFT) method[37,39]. On the basis of the experimentally derived structural information a model complex has been generated representing a section of the polymer. The results of theoretical calculations were found to be consistent with the observed spectral data for the complex.

Inhibitory effect of PNb compounds on acid phosphatase

Phosphorylation/dephosphorylation of a biomolecule is a key reaction in biology as phosphate ester bond functions as an extremely important linkage within the living cells[63–66]. It participates in storage and transfer of the genetic information, carries chemical energy and regulates the activity of enzymes and signalling molecules in the cell[63]. Enzymes capable of acting on ester bonds and catalyzing the cleavage of phosphate esters constitute the range of enzymes collectively named phosphatases. Acid phosphatases, a group of membrane associated enzymes, catalyze the dephosphorylation of phosphate ester bond at an optimum pH of 4.9–6.0 and are widespread in nature[67]. Thylakoid membrane ACP is essential to photosynthesis, whereas human acid phosphatases, normally found at low concentrations, has been known to be diagnostically useful as serological and histological markers of diseases[68]. These enzymes have been recognised as excellent models to probe the metal induced inhibitory effect in membrane proteins[67,69,70].

In our study, the *in vitro* phosphatase inhibitory effect of the triperoxoniobium compounds **1-4** as well as parent tetraperoxoniobate, NaNb upon activity of the wheat thylakoid membrane ACP was evaluated employing standard enzyme assay system and *p*-nitrophenyl phosphate *p*-NPP as substrate[22,34,69,71]. One of the specific interests was to explore whether binding of low molecular weight PNb species to macromolecular ligands would alter their affinity as enzyme inhibitor. The findings of our investigation on dose response inhibition of the model enzyme activity by the PNb complexes[36] are illustrated in Fig. 3.

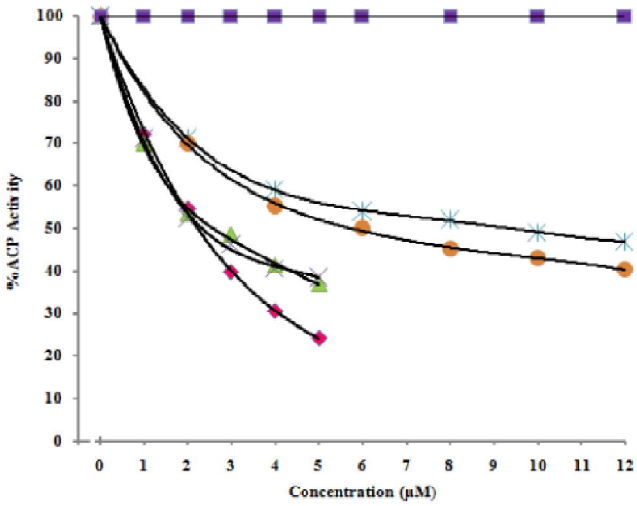


Fig. 3. The effect of ( ) NaNb, ( ) NbA, (X) NbN, (X) NbAla, ( ) NbVal ( ) free ligand on activity of ACP. The ACP catalyzed rates of hydrolysis of *p*-NPP at pH 4.6 were determined at 30°C by measuring A_{405} in a reaction mixture containing ACP (18.38 μg mL^{-1}) and *p*-NPP (2 mM) in acetate buffer (0.1 *M*, pH 4.6) in the absence or presence of stated concentrations of the inhibitors (for NaNb, NbA, NbN and the respective free ligand, compound concentrations: 1, 2, 3, 4 and 5 μM and for NbAla, NbVal and the respective free ligand, compound concentration: 2, 4, 6, 8, 10 and 12 μM). The data are presented as the means ±SE from three separate experiments. Reprinted from Ref.[36]. Ref.[36], Copyright (2017), with permission from Elsevier.

The inhibitor potential of the test compounds was quantified in terms of the half-maximal inhibitory concentration (IC_{50}). The IC_{50} values recorded, being within the range of 1–9 μM, implied that the compounds are highly potent inhibitors of the enzyme (Fig. 3 and Table 1). Thus the data obtained indicated the following sequence of inhibitor potency for ACP inhibition: NaNb > NbN > NbA > NbVal > NbAla. While the compounds NbAla (**1**) and NbVal (**2**) were found to be relatively less potent, IC_{50} value displayed by triperoxoNb complexes with arginine (NbA, **3**) and nicotinic acid (NbN, **4**) as co-ligands as well as tetraperoxo species were observed to be comparable. Since individually the free ligands had no observable effect on the enzyme activity, under the employed assay conditions, the above observations indicate that the inhibitory effect of the PNb compounds indeed originate from

Table 1. Half-maximal inhibitory concentration (IC_{50}) and inhibitor constants (K_i and K_{ii}) values for PNb compounds[a]. Reprinted from Ref.[36]. Ref.[36], Copyright (2017), with permission from Elsevier

Compd.	IC_{50} (μM)	K_i (μM)	K_{ii} (μM)	K_{ii}/K_i	Types of inhibition
NaNb	2.24	2.75	12.85	4.67	Mixed inhibition
NbA	2.59	2.70	7.10	2.63	Mixed inhibition
NbN	2.35	2.45	22.90	9.35	Mixed inhibition
NbAla	8.85	3.15	22.20	7.05	Mixed inhibition
NbVal	5.65	5.60	32.45	5.79	Mixed inhibition

[a]Note: the ACP catalyzed rates of hydrolysis of *p*-NPP at pH 4.6 were determined at 30°C by measuring A_{405} in a reaction mixture containing ACP (18.38 μg mL^{-1}) and *p*-NPP (50–300 μM) in acetate buffer (0.1 *M*, pH 4.6) in the presence of stated concentrations of the inhibitors (as shown in Figs. 3, 4 and 5).

the interaction of the intact metal complexes with the enzyme and the ligand environment influences the inhibitor potency of the tested complexes.

Kinetics of ACP inhibition by the PNb complexes

The mode of enzyme inhibition such as competitive, non-competitive, uncompetitive or mixed type can usually be determined[71,72] by varying the inhibitor concentration and measuring the enzyme activity at various substrate concentrations. To establish the mechanism of inhibition for the acid phosphatase catalyzed hydrolysis of *p*-NPP by the inhibitor species, the steady state kinetics of this process were investigated and kinetic parameters, Michaelis constant (K_m) and maximum velocity (V_{max}) were determined[71–73]. The Lineweaver-Burk (L-B) plots of the reciprocal initial velocity versus the reciprocal substrate concentration in the absence and presence of the inhibitor complexes at various concentrations are shown in Fig. 4 (for NaNb, NbN and NbAla) and Fig. 5 (for NbA and NbVal).

As can be seen from these figures, kinetic measurements yielded straight lines with point of intersection in the second quadrant in each case. Both V_{max} and K_m values are affected by the presence of each of the PNb complexes showing an increase in K_m and decrease in velocity V_{max} values with increasing inhibitor concentration. This behaviour is characteristic of mixed type of inhibition combining competitive and non-competitive modes. The above observations are consistent with the mode of inhibition exhibited by monomeric peroxo compounds of V, Mo and W reported previously from our laboratory[24,34,57].

We have also determined the inhibitor constants K_i and K_{ii} in order to assess the affinity of the enzyme for the inhibitors. The inhibitor constant K_i is a measure of the affinity of the inhibitor for the free enzyme whereas, K_{ii} reflects its affinity for the enzyme-substrate complex. The ratio of K_{ii} and K_i values were used to assess the relative competitiveness of each inhibitor. As shown in the inset to Fig. 4 and Fig. 5, the inhibitor constant K_i for the competitive part of inhibition, was obtained from the secondary plot of the slope of the primary plot (1/*V* versus 1/[S]) against the inhibitor concentration with the intercept on the inhibitor axis being $-K_i$. The value of K_{ii}, inhibitor constant for non-competitive inhibition, was obtained from the replot of slope versus inhibitor concentration, with the inhibitor axis intercept being equivalent to $-K_{ii}$. The kinetic results observed for monomeric PNb compounds (Table 1), show that for each of the inhibitors $K_{ii} > K_i$, confirming that PNb species exert mixed type of inhibition on ACP activity.

A competitive inhibitor usually shares a close structural similarity with the natural substrate of the enzyme. A non-competitive inhibition on the other hand occurs when the inhibitor binds the enzyme reversibly at a site far removed from the active site causing a conformational change in the overall three-dimensional shape of the enzyme[72]. Apart from the nature of the enzyme, one or more of the various factors such as oxidation state of the metal, coordination geometry and stability of the compounds under physiological conditions have been reported to influence the phosphatase inhibitory ability of a metal complex[24,74].

Acid phosphatases, isolated from various plant and animal sources, contain dinuclear iron active sites and highly conserved amino acid sequences with a histidine residue at the active site[68,69,75]. Oxy anions of V, Mo and W having penta or hexa coordinated structures have mostly been known to be competitive inhibitors of phosphatases due to their structural analogy with phosphate[24,75–77]. Oxidative interaction between the highly oxidative compounds with the Fe^{2+} centre resulting in its oxidation to ferric form and consequent inactivation of the enzyme in a noncompetitive manner was suggested as an additional possibility of inhibitory effect of these complexes[74,75]. As the peroxoniobium compounds under investigation have already demonstrated their ability to oxidise organic substrates[38,39] we have surmised that a similar mechanism involving oxidation of Fe^{2+} centre by the

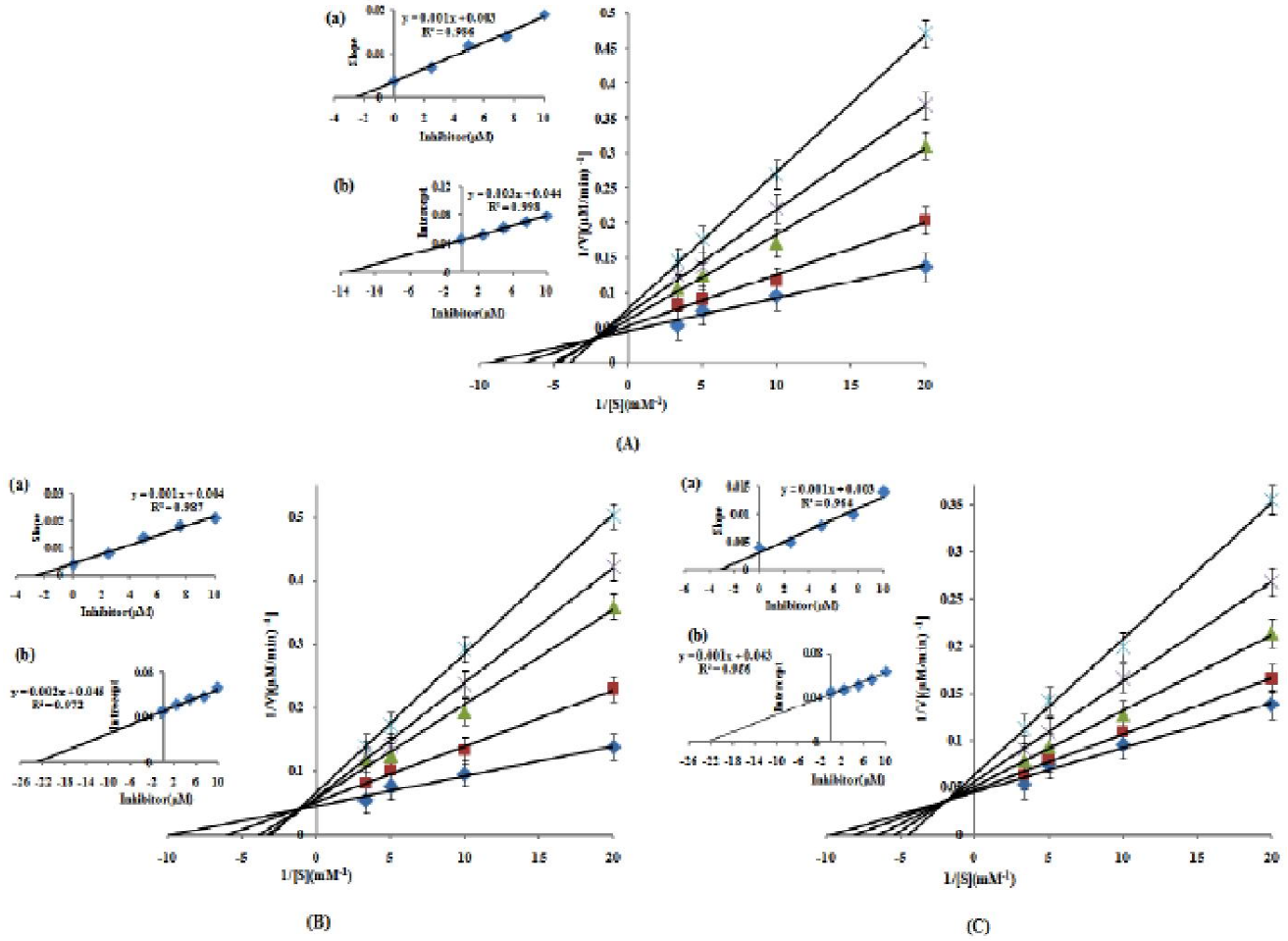


Fig. 4. Lineweaver-Burk plots for the inhibition of ACP activity in the absence and presence of (A) NaNb, (B) NbN and (c) NbAla. The inset represents the secondary plot of the initial kinetic data of the Lineweaver-Burk plot. The reaction mixture contained acetate buffer (0.1 *M*, pH 4.6) and *p*-NPP (50–300 μM). The reaction was started by adding ACP (18.38 μg mL^{-1}) to the reaction solution, which was pre-incubated for 5 min and the rate of hydrolysis in the presence of ( ) 0 μM, ( ) 2.5 μM, ( ) 5 μM, (X) 7.5 μM, (X) 10 μM inhibitors were obtained. The values are expressed as the mean ±SE from three separate experiments. Inset: (a) The slopes were plotted against inhibitor concentrations and K_i values were obtained from the x-intercepts of these re-plots. (b) The vertical intercepts were plotted against the inhibitor concentration and K_{ii} values were obtained from the x-intercepts of these re-plots. Reprinted from Ref.[36]. Ref.[36], Copyright (2017), with permission from Elsevier.

PNb species is perhaps operative, contributing to their mixed type of inhibition of ACP function, combining competitive and non-competitive pathways. There are ample evidences in the literature highlighting the importance of redox properties of peroxovanadium compounds in inhibition of protein phosphatases[20,74] which lend credence to our hypothesis.

Effect of peroxoniobium complexes on the activity of calcineurin

Calcineurin contains iron and zinc as intrinsically bound metal ions and consists of two subunits – the enzymatic subunit and the regulatory subunit[40,78–81]. The enzymatic subunit encloses a calmodulin binding site and an autoinhibitory domain, which blocks the catalytic centre of the enzyme[40]. Binding of Ca^{2+} ions and calmodulin to calcineurin leads to conformational change and a subsequent unmasking of the active centre[40]. The ligand environment of the dinuclear metal centre of CN has been reported to resemble the active site coordination environment of purple acid phosphatase[40,82].

Interestingly, apart from several synthetic and natural CN inhibitors developed recently[40–47,83–87], it has been reported

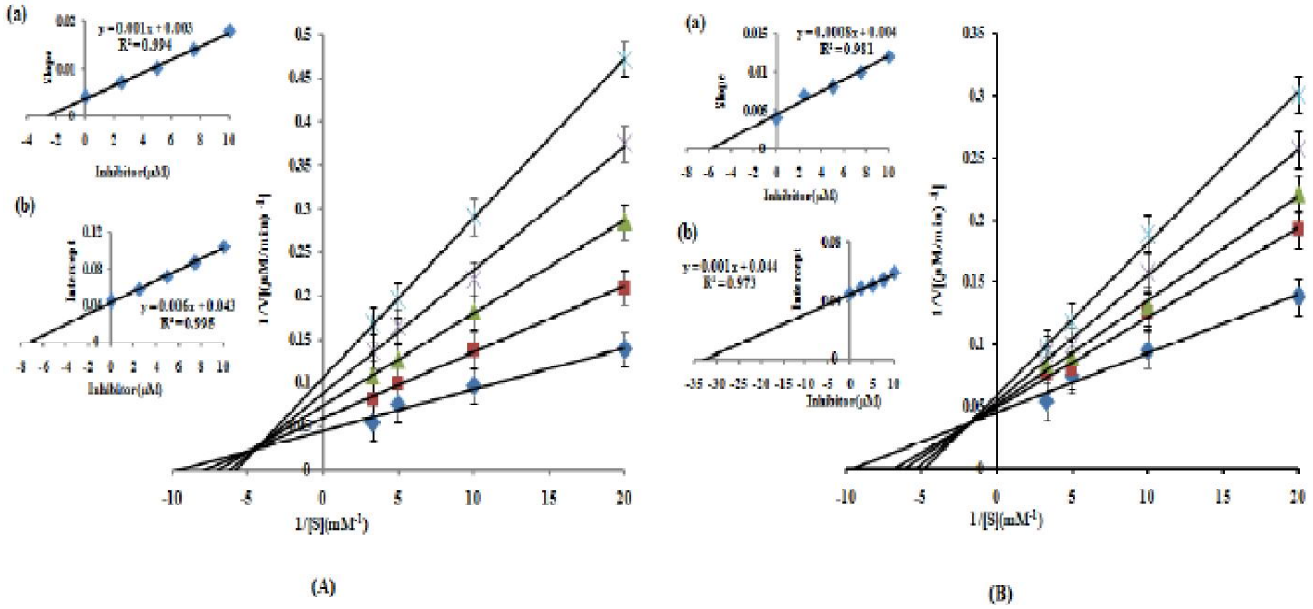


Fig. 5. Lineweaver-Burk plots for the inhibition of ACP activity in the absence and presence of (A) NbA and (B) NbVal. The inset represents the secondary plot of the initial kinetic data of the Lineweaver plot. The reaction mixture contained acetate buffer (0.1 *M*, pH 4.6) and *p*-NPP (50–300 μM). The reaction was started by adding ACP (18.38 μg mL^{-1}) to the reaction solution, which was pre-incubated for 5 min and the rate of hydrolysis in the presence of ( ) 0 μM, ( ) 2.5 μM, ( ) 5 μM, (X) 7.5 μM, ( ) 10 μM inhibitors were obtained. The values are expressed as the mean ±SE from three separate experiments. Reprinted from Ref.[36]. Ref.[36], Copyright (2017), with permission from Elsevier.

that reactive oxygen species (ROS), superoxide and hydrogen peroxide inhibit CN activity[48,88–93]. However, the underlying molecular mechanisms of such inhibition[48,87] by extracellular oxidants, in particular H_2O_2 are yet to be elucidated in detail mainly due to the complex enzymology of CN.

The ability of peroxoniobium compounds to inhibit the calmodulin mediated dephosphorylation activity of calcineurin phosphatase, was assessed using tetraperoxoniobate, $Na_3[Nb(O_2)_4]\cdot 13H_2O$ (NaNb) and the polymer bound $[Nb_2(O_2)_6(carboxylate)_2]$-PA [PA = poly(sodium acrylate)] (PANb), as representatives of monomeric and macromolecular PNb complexes, respectively. The effect of the inhibitor species was examined *in vitro* using the substrates viz. RII-phosphopeptide, a physiological substrate of calcineurin as well as a non-protein substrate *p*-nitrophenyl phosphate (Fig. 6).

As seen in Fig. 6, 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. 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, is seen in Table 2. On the other hand, H_2O_2 with IC_{50} of approximately 32.5 μM (for RII-phosphopeptide) was nearly 6-fold weaker as 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. 6 and Table 2). 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[91], for inhibition of intracellular CN activity by H_2O_2 in T-lymphocytes. The IC_{50} value for the polymer bound PANb (**5**) is reported on the basis of its actual peroxometal loading.

Kinetics of calcineurin inhibition by H_2O_2 and PNb compounds

In order to gain an insight into the mechanism of inhibition of CN catalyzed dephosphorylation by the PNb com-

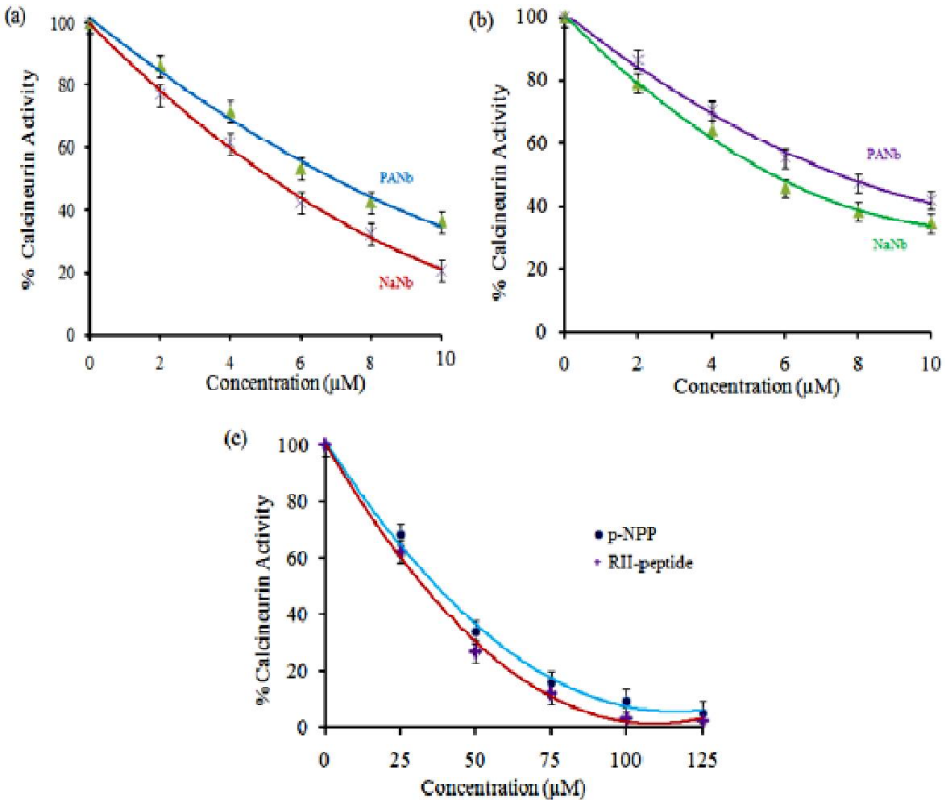


Fig. 6. The effect of PNb compounds and H_2O_2 on calcineurin activity, (a) effect of NaNb and PANb (**5**) on calcineurin activity with RII-phosphopeptide as substrate, (b) effect of NaNb and PANb (5) on calcineurin activity with *p*-NPP as substrate and (c) effect of H_2O_2 on calcineurin activity. 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 (**5**) 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 means ±SE from three separate experiments.

Table 2. Half maximal inhibitory concentration (IC_{50}) and inhibitor constant (K_i) values for PNb compounds and H_2O_2 against calcineurin[a]

Compd.	Substate	IC_{50} (μM)	K_i (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

[a]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.

pounds, we have carried out steady state kinetics of the process using the natural substrate RII-phosphopeptide. From the kinetic measurements at several different substrate concentrations in the presence of the inhibitors, as demonstrated by the Lineweaver-Burk double reciprocal plots (Fig. 7) 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. These results demonstrate that the mode of inhibition of CN by each of the tested peroxo species, including H_2O_2 is of the "uncompetitive" type. The value of inhibitory constant K_i for the inhibitors, was obtained from the secondary plot of the intercepts of the primary plot (1/V versus 1/[S]) against inhibitor concentration. From the K_i values

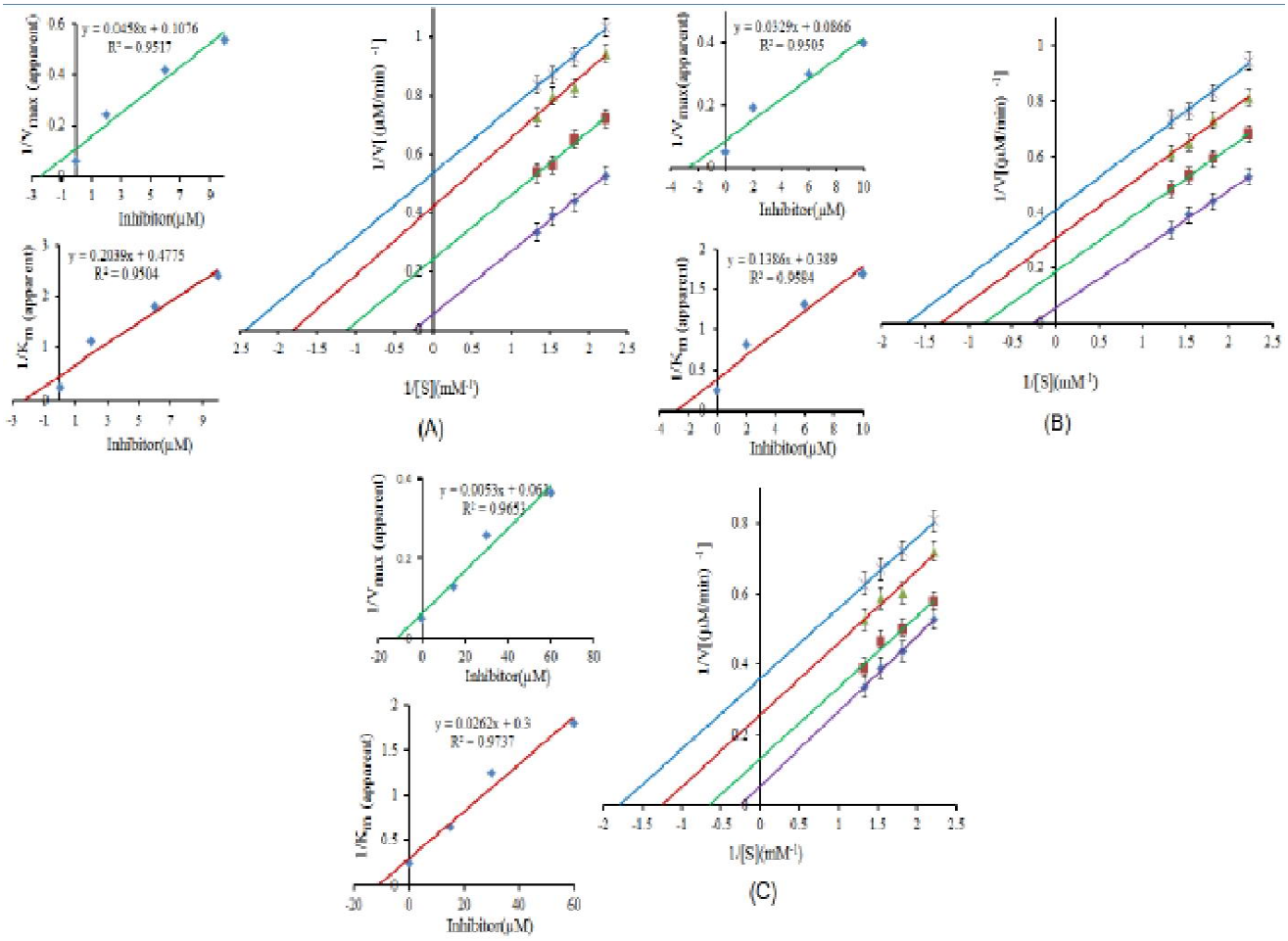


Fig. 7. Lineweaver-Burk plots for inhibition of calcineurin activity in absence and presence of (A) NaNb, (B) PANb and (C) H_2O_2. The inset represents the secondary plot of the initial kinetic data of the Lineweaver-Burk plot. The reaction mixture contained calcineurin (0.2 U/μL), calmodulin (1 mM), RII-phosphopeptide (450–750 μM) in 2X assay buffer of pH 7.5. The rate 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 means ±SE from three separate experiments. Inset: (a) The vertical intercepts ($1/V_{max}$) were plotted against inhibitor concentrations and K_i 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. Reprinted from Ref.[37]. Ref.[37], Copyright (2017), with permission from John Wiley and Sons.

(Table 2), it was further confirmed that the tested peroxoniobate complexes are stronger inhibitors of calcineurin compared to H_2O_2, as has been indicated by their IC_{50} values. 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, as well as previous findings of our group on the inhibition of ACP and ALP by peroxo-metallates, 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[24,30–35].

In uncompetitive mode of inhibition, an inhibitor binds to only the enzyme-substrate complex, but the free enzyme is not a target of inhibition[94]. Although this mode of inhibition is much less common in nature[95], compared to other pathways such as competitive or noncompetitive modes, there have been examples of uncompetitive inhibition occurring in the case of CN[96] as well as of alkaline phosphatases activities in the presence of various inhibitor species[97,98]. In contrast

to this, the CN inhibitor FK_{506}, that leads to immunosuppression has been shown to bind to CN at a region far removed from the enzyme active site[45].

Previous work from other 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[48,92,93]. 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[87,91–93]. 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[87,91].

Keeping in view these information it appears possible that the peroxo compounds examined herein may inactivate calcineurin by several potential mechanisms. It is likely 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[87,91]. We are however constrained in commenting on the exact mechanism of inhibition in absence of direct evidence, as well as due to the complexity of the species involved in these processes.

Conclusion

In this brief account of our work we intended to demonstrate that water soluble peroxoniobium compounds, irrespective of their co-ligand environment, induce strong inhibitory effect on activity of acid phosphatase. Most importantly, we have introduced for the first time neat as well as polymer anchored peroxo derivatives of niobium as a novel class of potent inhibitors of function of calmodulin binding serine/threonine phosphatase, calcineurin. It is remarkable that inhibitory potency of PNb compounds were observed to be comparable to peroxovanadium compounds in similar co-ligand environment, with respect to inhibition of dephosphorylation of the physiological substrate RII-phosphopeptide as well as *p*-NPP by calcineurin[37]. Enzyme kinetics analysis data revealed that the title compounds inhibit the activities of the two types of enzymes via distinct pathways. The monomeric PNb compounds exerted mixed-type of inhibition on ACP function, combining competitive and non-competitive modes of inhibition. On the other hand, the PNb derivatives as well as H_2O_2 displayed mechanistic preference to inactivate CN in an uncompetitive manner. Undoubtedly, many more experiments would be needed to shed more light into these highly complex processes, nevertheless, our findings could provide a basis for further investigation on the role of oxidant species in regulation of CN dependent cellular processes to carry forward to *in vivo* studies.

Acknowledgements

Financial support from Department of Biotechnology, New Delhi, India is gratefully acknowledged. We wish to thank Prof. T. Ramasarma, INSA Hony. Scientist, Indian Institute of Science (IISc), Bangalore, for valuable discussion.

References

1. D. Bayot and M. Devillers, *Coord. Chem. Rev.*, 2006, **250,** 2610.
2. H. Egami, T. Oguma and T. Katsuki, *J. Am. Chem. Soc.*, 2010, **132**, 5886.
3. H. Egami and T. Katsuki, *Angew. Chem. Int.*, 2008, **47**, 5171.
4. L. C. Passoni, M. R. H. Siddiqui, A. Steiner and I. V. Kozhevnikov, *J. Mol. Catal. A: Chem.*, 2000, **153**, 103.
5. G. Stavber, B. Mali and M. Kosec, *Green Chem.*, 2011, **13**, 1303.
6. E. R. Leite, C. Vila, J. Bettini and E. Longo, *J. Phys. Chem. B*, 2006, **110**, 18088.
7. K. Rydzynski and D. Pakulska, "Patty's industrial hygiene and toxicology", John Wiley & Sons Press, 2012.
8. H. Thomadaki, A. Lymberopoulou-Karaliota, A. Maniatakou and A. Scorilas, *J. Inorg. Biochem.*, 2011, **105**, 155.
9. A. Maniatakou, S. Karaliota, M. Mavri, C. Raptopoulou, A. Terzis and A. Karaliota, *J. Inorg. Biochem.*, 2009, **103**, 859.
10. D. C. Crans, in: "Vanadium Compounds Chemistry, Biochemistry and Therapeutic Application", eds. A. S. Tracey and D. C. Crans, Oxford University Press, New York, 1998, p. 60.
11. C. Djordjevic, N. Vuletic, M. L. Renslo, B. C. Puryear and R. Alimard, *Mol. Cell. Biochem.*, 1995, **153**, 25.
12. A. S. Tracey, G. R. Willsky and E. S. Takeuchi, "Vanadium: chemistry, biochemistry, pharmacology and practical applications", CRC Press and Taylor & Francis Group, Boca Raton, 2007.
13. C. E. Heyliger, A. G. Tahiliani and J. H. McNeill, *Science*, 1985, **227**, 1474.
14. K. H. Thompson, J. H. McNeill and C. Orvig, *Chem. Rev.*, 1999, **99**, 2561.
15. Y. Shechter, I. Goldwaser, M. Mironchik, M. Fridkin and D. Gefel, *Coord. Chem. Rev.*, 2003, **237**, 3.
16. T. Ramasarma, *Proc. Indian Natl. Sci. Acad. B: Biol. Sci.*,

2003, **69**, 649.

17. K. H. Thompson, J. Lichter, C. LeBel, M. C. Scaife, J. H. McNeill and C. Orvig, *J. Inorg. Biochem.*, 2009, **103**, 554.
18. G. Fraqueza, C. A. Ohlin, W. H. Casey and M. Aureliano, *J. Inorg. Biochem.*, 2012, **107**, 82.
19. A. Y. Louie and T. J. Meade, *Chem. Rev.*, 1999, **99**, 2711.
20. D. C. Crans, J. J. Smee, E. Gaidamauskas and L. Yang, *Chem. Rev.*, 2004, **104**, 849.
21. A. R. Saltiel, *Cell*, 2001, **104**, 517.
22. C. C. McLauchlan, B. J. Peters, G. R. Willsky and D. C. Crans, *Coord. Chem. Rev.*, 2015, **301**, 163.
23. T. Hunter, *Cell*, 2000, **100**, 113.
24. J. J. Boruah, D. Kalita, S. P. Das, S. Paul and N. S. Islam, *Inorg. Chem.*, 2011, **50**, 8046.
25. D. Kalita, S. Sarmah, S. P. Das, D. Baishya, A. Patowary, S. Baruah and N. S. Islam, *React. Funct. Polym.*, 2008, **68**, 876.
26. A. K. Haldar, S. Banerjee, K. Naskar, D. Kalita, N. S. Islam and S. Roy, *Exp. Parasitol.*, 2009, **122**, 145.
27. N. Chatterjee, S. Kiran, B. M. Ram, N. Islam, T. Ramasarma and G. Ramakrishna, *Mech. Ageing Dev.*, 2011, **132**, 230.
28. A. Misra, S. Srivastava, S. R. Ankireddy, N. S. Islam, T. Chandra, A. Kumar, M. K. Barthwal and M. Dikshit, *Redox Rep.*, 2013, **18**, 174.
29. V. Khanna, M. Jain, M. K. Barthwal, D. Kalita, J. J. Boruah, S. P. Das, N. S. Islam, T. Ramasarma and M. Dikshit, *Pharmacol. Res.*, 2011, **64**, 274.
30. P. Hazarika, D. Kalita and N. S. Islam, *J. Enzyme Inhib. Med. Chem.*, 2008, **23**, 504.
31. P. Hazarika, S. Sarmah, D. Kalita and N. S. Islam, *Trans. Met. Chem.*, 2008, **33**, 69.
32. D. Kalita, S. P. Das and N. S. Islam, *Biol. Trace Elem. Res.*, 2009, **128**, 200.
33. P. Hazarika, D. Kalita and S. Sarmah, *Mol. Cell Biochem.*, 2006, **284**, 39.
34. S. P. Das, S. R. Ankireddy, J. J. Boruah and N. S. Islam, *RSC Adv.*, 2012, **2**, 7248.
35. N. Chatterjee, T. Anwar, N. S. Islam, T. Ramasarma and G. Ramakrishna, *Mol. Cell. Biochem.*, 2017, **424**, 209.
36. S. R. Gogoi, G. Saikia, K. Ahmed, R. Duarah and N. S. Islam, *Polyhedron*, 2017, **121**, 142.
37. G. Saikia, S. R. Gogoi, J. J. Boruah, B. M. Ram, P. Begum, K. Ahmed, M. Sharma, G. Ramakrishna, T. Ramasarma and N. S. Islam, *ChemistrySelect*, 2017, **2**, 5838.
38. S. R. Gogoi, J. J. Boruah, G. Sengupta, G. Saikia, K. Ahmed, K. K. Bania and N. S. Islam, *Catal. Sci. Technol.*, 2015, **5**, 595.
39. S. R. Gogoi, K. Ahmed, G. Saikia and N. S. Islam, *J. Indian Chem. Soc.*, 2018, **95**, 801.
40. F. Rusnak and P. Mertz, *Physiol. Rev.*, 2000, **80**, 1483.
41. C. B. Klee, T. H. Crouch and M. H. Krinks, *Proc. Natl. Acad. Sci.*, 1979, **76**, 6270.
42. F. Shibasaki and F. McKeon, *J. Cell Biol.*, 1995, **131**, 735.
43. M. Sieber and R. Baumgrass, *J. Cell Commun. Signal.*, 2009, **7**, 25.
44. J. R. Azzi, M. H. Sayegh and S. G. Mallat, *J. Immunol.*, 2013, **191**, 5785.
45. J. P. Griffith, J. L. Kim, E. E. Kim, M. D. Sintchak, J. A. Thomson, M. J. Fitzgibbon, M. A. Fleming, P. R. Caron, K. Hsiao and M. A. Navia, *Cell*, 1995, **82**, 507.
46. J. M. Hanifin, M. R. Ling, R. Langley, D. Breneman, E. Rafal and T. O. S. Group, *J. Am. Acad. Dermatol.*, 2001, **44**, S28.
47. F. Erdmann, M. Weiwad, S. Kilka, M. Karanik, M. Pätzel, R. Baumgrass, J. Liebscher and G. Fischer, *J. Biol. Chem.*, 2010, **285**, 1888.
48. M. Carballo, G. Márquez, M. Conde, J. Mart1n-Nieto, J. Monteseir1n, J. Conde, E. Pintado and F. Sobrino, *J. Biol. Chem.*, 1999, **274**, 93.
49. C. Djordjevic and N. Vuletic, *Inorg. Chem.*, 1968, **7**, 1864.
50. D. Bayot, B. Tinant and M. Devillers, *Catal. Today*, 2003, **78**, 439.
51. N. Vuletic, E. Prci and C. Djordjevic, *Z. Anorg. Allg. Chem.,* 1979, **450**, 67.
52. Y. Narendar and G. L. Messing, *Chem. Mater.*, 1997, **9**, 580.
53. T. F. Vin, A. V. Tarakanova, O. V. Kostyuchenko, B. N. Tarasevich, N. S. Kulikov and A. V. Anisimov, *Theor. Found. Chem. Eng.*, 2008, **42**, 636.
54. D. Bayot, B. Tinant, B. Mathieu, J. P. Declercq and M. Devillers, *Eur. J. Inorg. Chem.*, 2003, **2003**, 737.
55. D. Bayot, B. Tinant and M. Devillers, *Inorg. Chem.*, 2005, **44**, 1554.
56. A. Maniatakou, C. Makedonas, C. A. Mitsopoulou, C. Raptopoulou, I. Rizopoulou, A. Terzis and A. Karaliota, *Polyhedron*, 2008, **27**, 3398.
57. N. S. Islam and J. J. Boruah, *J. Chem. Sci.*, 2015, **127**, 777.
58. J. J. Boruah, K. Ahmed, S. Das, S. R. Gogoi, G. Saikia, M. Sharma and N. S. Islam, *J. Mol. Catal. A: Chem.*, 2016, **425**, 21.
59. J. J. Boruah, S. P. Das, S. R. Ankireddy, S. R. Gogoi and N. S. Islam, *Green Chem.*, 2013, **15**, 2944.
60. N. Murthy, J. Campbell, N. Fausto, A. S. Hoffman and P. S. Stayton, *J. Controlled Release*, 2003, **89**, 365.
61. B. R. Twaites, C. de las Heras Alarcón, D. Cunliffe, M. Lavigne, S. Pennadam, J. R. Smith, D. C. Górecki and C. Alexander, *J. Controlled Release*, 2004, **97**, 551.
62. K. Haupt and K. Mosbach, *Chem. Rev.*, 2000, **100**, 2495.
63. In: "Dynamics of bone and cartilage metabolism: principles and clinical applications", eds. M. J. Seibel, S. P.

Robins and J. P. Bilezikian, Academic Press, 2006.

64. T. Hunter, *Philos. T. R. Soc. B,* 2012, **367**, 2513.
65. E. Torres and M. K. Rosen, *Mol. Cell,* 2003, **11**, 1215.
66. N. Tanaka, Z. Hasan, A. F. Hartog, T. van Herk and R. Wever, *Org. Biomol. Chem.*, 2003, **1**, 2833.
67. D. E. Wilcox, *Chem. Rev.*, 1996, **96**, 2435.
68. H. Bull, P. G. Murray, D. Thomas, A. M. Fraser and P. N. Nelson, *J. Clin. Pathol.,* 2002, **55**, 65.
69. M. J. Fei, J. S. Chen and X. Y. Wang, *J. Integr. Plant Biol.*, 2006, **48**, 294.
70. W. N. Lipscomb and N. Sträter, *Chem. Rev.*, 1996, **96**, 2375.
71. H. U. Bergmeyer, J. Bergmeyer and M. Grassl, "Methods of Enzymatic Analysis", Verlag Chemie, Weinheim, Deerfield Beach, FL, 1983.
72. I. H. Segel, "Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady State Enzyme Systems", Wiley, New York, 1975.
73. H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 1934, **56**, 658.
74. D. C. Crans, in: "Vanadium compounds: Chemistry, biochemistry, and therapeutic applications", eds. A. S. Tracey and D. C. Crans, Oxford University Press, UK, 1998, p. 82.
75. J. B. Vincent, M. W. Crowder and B. A. Averill, *Biochemistry*, 1991, **30**, 3025.
76. P. J. Stankiewicz and M. J. Gresser, *Biochemistry*, 1988, **27**, 206.
77. R. L. VanEtten, P. P. Waymack and D. M. Rehkop, *J. Am. Chem. Soc.*, 1974, **96**, 6782.
78. Y. Zhao, H. Yang, K. Meng and S. Yu, *Int. J. Biol. Macromol.,* 2014, **64**, 453.
79. M. M. King and C. Y. Huang, *Biochem. Biophys. Res. Commun.*, 1983, **114**, 955.
80. M. M. King and C. Y. Huang, *J. Biol. Chem.*, 1984, **259**, 8847.
81. R. C. Gupta, R. L. Khandelwal and P. V. Sulakhe, *FEBS Lett.,* 1984, **169**, 251.
82. C. R. Kissinger, H. E. Parge, D. R. Knighton and C. T. Lewis, *Nature*, 1995, **378**, 641.
83. J. Cen, M. Wang, G. Jiang, Y. Yin, Z. Su, L. Tong, Y. Ma, Y. Gao and Q. Wei, *Biochimie*, 2015, **111**, 119.
84. N. Ito, N. Shibuguchi, R. Ishikawa, S. Tanaka, Y. Tokita, J. Nakajima-Shimada and K. Hosaka, *Biosci. Biotechnol. Biochem.*, 2013, **77**, 954.
85. M. Morioka, K. Fukunaga, T. Kawano, S. Hasegawa, K. Korematsu, Y. Kai, J. I. Hamada, E. Miyamoto and Y. Ushio, *Biochem. Biophys. Res. Commun.*, 1998, **253**, 342.
86. J. Huang, D. Zhang, W. Xing, X. Ma, Y. Yin, Q. Wei and G. Li, *Anal. Biochem.*, 2008, **375**, 385.
87. R. Bogumil, D. Namgaladze, D. Schaarschmidt, T. Schmachtel, S. Hellstern, R., Mutzel and V. Ullrich, *FEBS J.*, 2000, **267**, 1407.
88. X. Wang, V. C. Culotta and C. B. Klee, *Nature*, 1996, **383**, 434.
89. K. Furuke, M. Shiraishi, H. S. Mostowski and E. T. Bloom, *J. Immunol.*, 1999, **162**, 1988.
90. D. Sommer, K. L. Fakata, S. A. Swanson and P. M. Stemmer, *FEBS J.*, 2000, **267**, 2312.
91. T. A. Reiter and F. Rusnak, *J. Biol. Inorg. Chem.*, 2002, **7**, 823.
92. D. Namgaladze, H. W. Hofer and V. Ullrich, *J. Biol. Chem.*, 2002, **277**, 5962.
93. T. A. Reiter, R. T. Abraham, M. Choi and F. Rusnak, *J. Biol. Inorg. Chem.*, 1999, **4**, 632.
94. C. G. Whiteley, *Biochem. Educ.*, 2000, **28**, 144.
95. A. Cornish-Bowden, *FEBS Lett.*, 1986, **203**, 3.
96. Y. Tanaka, E. Otsuka, K. Hosaka and S. Tanaka, *Trace Nutrients Res.,* 2009, **26**, 70.
97. N. K. Ghosh and W. H. Fishman, *J. Biol. Chem.*, 1966, **241**, 2516.
98. M. F. Hoylaerts, T. Manes and J. L. Millán, *Biochem. J.*, 1992, **286**, 23.