Therapeutic Potentials of Ecto-Nucleoside Triphosphate Diphosphohydrolase, Ecto-Nucleotide Pyrophosphatase/Phosphodiesterase, Ecto-5′-Nucleotidase, and Alkaline Phosphatase Inhibitors

Mariya al-Rashida[#] and Jamshed Iqbal

Department of Pharmaceutical Sciences, COMSATS Institute of Information Technology, Abbottabad, 22060, Pakistan

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Abstract: The modulatory role of extracellular nucleotides and adenosine in relevance to purinergic cell signaling mechanisms has long been known and is an object of much research worldwide. These extracellular nucleotides are released by a variety of cell types either innately or as a response to patho-physiological stress or injury. A variety of surface-located ecto-nucleotidases (of four major types; nucleoside triphosphate diphosphohydrolases or NTPDases, nucleotide pyrophosphatase/phosphodiesterases or NPPs, alkaline phosphatases APs or ALPs, and ecto-5′-nucleotidase or e5NT) are responsible for meticulously controlling the availability of these important signaling molecules (at their respective receptors) in extracellular environment and are therefore crucial for maintaining the integrity of normal cell functioning. Overexpression of many of these ubiquitous ecto-enzymes has been implicated in a variety of disorders including cell adhesion, activation, proliferation, apoptosis, and degenerative neurological and immunological responses. Selective inhibition of these ecto-enzymes is an area that is currently being explored with great interest and hopes remain high that development of selective ecto-nucleotidase inhibitors will prove to have many beneficial therapeutic implications. The aim of this review is to emphasize and focus on recent developments made in the field of inhibitors of ecto-nucleotidases and to highlight their structure activity relationships wherever possible. Most recent and significant advances in field of NTPDase, NPP, AP, and e5NT inhibitors is being discussed in detail in anticipation of providing prolific leads and

[#]Present address: Department of Chemistry, Forman Christian College (A Chartered University), Ferozepur Road, Lahore 54600, Pakistan.

Correspondence to: Jamshed Iqbal, Department of Pharmaceutical Sciences, COMSATS Institute of Information Technology, Abbottabad 22060, Pakistan. E-mail: drjamshed@ciit.net.pk.

relevant background for research groups interested in synthesis of selective ecto-nucleotidase inhibitors. Med. Res. Rev., 00, No. 00, 1–41, 2013

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1. INTRODUCTION

All cells have the impetus to release extracellular nucleotides especially ATP either innately or as a pathological or physiological response to injury or stress. Extracellular nucleosides and nucleotides derived from the purine (ADP and ATP) or pyrimidine moiety (UDP and UTP) function as ubiquitous extracellular signaling molecules for very sophisticated cell signaling mechanisms designed for effective intercellular communication. These signaling molecules modulate a variety of physiological functions, such as blood clotting, inflammation, immune reactions, pain perception, smooth muscle contraction, cell proliferation, and cancer.[1,2] Cellular responses are induced by activation of P1 (adenosine-activated type 1 receptor having four subtypes A_1, A_{2A}, A_{2B}, and A_3)[3] or P2 (nucleotide-activated type 2 ligand-gated or ionotropic P2X ion channels having seven receptor subtypes P2X1, P2X2, P2X3, P2X4, P2X5, P2X6, P2X7) and G protein-coupled or metabotropic P2Y receptors (having eight subtypes known to date $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$).[2,4–6] This classification for P2X and P2Y receptors has been suggested by NC-IUPHAR (International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification) subcommittee on P2X and P2Y receptors, respectively.[7,8] A short term effect, neurotransmission, is mediated by P2X receptors that bind mainly ATP, whereas long-standing effects, such as cytotoxicity, cell proliferation, differentiation and migration, occur mainly through P2Y receptors that bind both purine and pyrimidine nucleotides.[4,9] Extracellular nucleotides also stimulate immune responses. P2 receptors are expressed by all immunological cells.[10] P2 receptor activation, triggered by extracellular nucleotides is terminated by a cascade of cell surface-localized enzymes collectively known as ecto-nucleotidases, these enzymes hydrolyze nucleoside tri-, di-, and mono-phosphates to their respective nucleosides.[11–14] The four main classes of ecto-nucleotidases are (i) ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), (ii) ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), (iii) alkaline phosphatases (APs) and (iv) ecto-5′-nucleotidase (e5NT; Fig. 1).[5,15]

2. ECTO-NUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASES

This family of ecto-enzymes is also commonly referred to as ecto-apyrase, NTPase, and E-ATPase family. The feature common to all NTPDases is the existence of highly conserved domains of apyrase-conserved regions (ACR1–5) that are involved in catalytic processes and are divalent metal ion dependent (usually Ca^{2+} or Mg^{2+}).[16] All surface-located NTPDases need Ca^{2+} or Mg^{2+} ions for optimal functioning and are deactivated in the absence of these ions. NTPDases hydrolyze nucleoside di- and triphosphates but do not hydrolyze monophosphates. Based on differences in cellular localization and substrate specificity there are eight different NTPDases. These NTPDases are NTPDase1 (CD39), NTPDase2 (CD39L1), NTPDase3 (CD39L3), NTPDase4, NTPDase5 (CD39L4), NTPDase6 (CD39L2), NTPDase7, and NTPDase8. It is important to note here that the use of term CD39L1–4 (CD for cell/cluster differentiation) is misleading for all but NTPDase1 (CD39) and should not be used for other NTPDases.[17] Thus, only NTPDase1 truly belongs to a cluster of differentiating antigens.

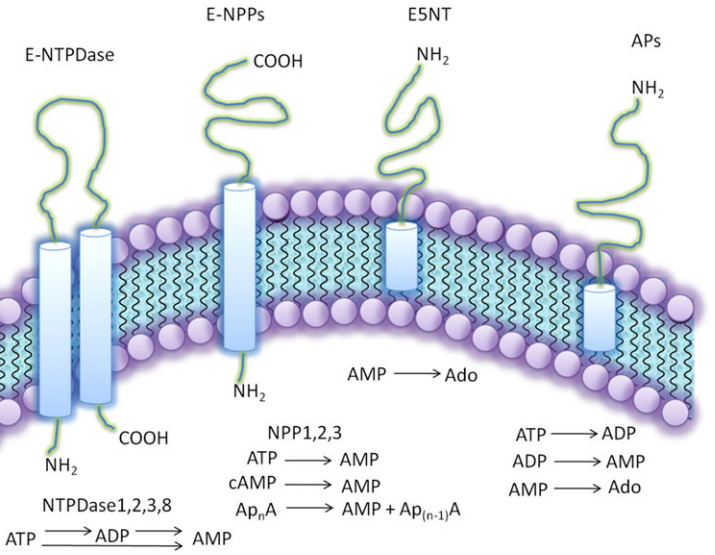


Figure 1. The nucleotide-hydrolysing pathway consisting of four ecto-enzymes, E-NTPDase, E-NPP, e5NT, and AP.

The terminal phosphate group of nucleoside triphosphates (NTPs) is hydrolyzed by NTPDases at different rates to nucleoside di- and monophosphates.[18,19] NTPDase1–3 and NTPDase8 are bound to plasma membrane with an extracellular active site and are the dominant ecto-nucleotidases that terminate nucleotide signaling through the hydrolysis of nucleotide agonists of the P2 receptors. Two major domains (I and II) have been suggested for the transmembrane NTPDases such that interdomain motion/rotation is responsible for substrate binding and product release. Crystal structure of catalytically active extracellular domain of rat (*Rattus norvegicus*) NTPDase2 has been reported in 2008.[20] This allows for most critical assessments to be made regarding substrate binding (ATP and ADP but never AMP), hence shedding light on factors necessary for "specific" substrate binding and specificity of NTPDases and allowing propositions to be made regarding the design of NTPDase subtype specific inhibitors. In 2012, crystal structure of rat NTPDase1 in complex with polyoxometalates (POMs; decavanadate and heptomolybdate) was determined.[21] These POMs were found to bind to a loop that is involved in the binding of nucleobase. Crystal structure of NTPDase from pathogens, such as *Toxoplasma gondii*[22] and *Neospora caninum*[23] has also been determined.

NTPDase1 (a major thromboregulatory molecule) hydrolyzes adenosine tri- and diphosphates to monophosphate. NTPDase1 hydrolyzes ATP almost completely to AMP with minimum accumulation of transitory ADP; however, significant amounts of UDP are known to accumulate when UTP is hydrolyzed. NTPDase2 also has a strong preference for NTPs (ATP), however, in contrast to NTPDase1, ATP to AMP hydrolysis by NTPDase2 proceeds via transitory accumulation of significant quantities of ADP.[4,12] Preferred substrate for NTPDase3 and 8 is also ATP rather than ADP and the rate of accumulation of transitory diphosphates by NTPDase3 and 8 is intermediate between that of NTPDase1 and 2. NTPDase4–7 are mainly associated with intracellular organelles and preferentially hydrolyze nonadenosine-based nucleoside tri- and diphosphates.[9] Secreted forms of NTPDase5 and 6 are known. The putative involvement of NTPDase5 and NTPDase6 in regulation of P2Y receptors in rat cochlea was investigated[24] in 2010 and only NTPDase5 was suggested to be involved in the regulation of P2Y receptor signaling. The contribution of NTPDase4–7 towards hydrolysis of extracellular nucleotides, in relevance to purinergic signaling, cannot expected to be of much significance owing to their high K_m values and low specific activities.[4,11]

Despite the availability of crystal structures of a number of NTPDases from diverse sources, the exact catalytic mechanism and specificity for theses enzymes remain elusive. In NTPDases, the same catalytic site is involved in the hydrolysis of NTPs as well as diphosphates, a rather peculiar feature for enzymes belonging to large superfamily of actin-related phosphotransferases. A comparison (using X-ray crystallographic studies) of various binding modes of nonhydrolysable nucleotide analogues in the active site of NTPDases (extracellular catalytic domain of rat NTPDase2 and NTPDase1 of bacterium *Legionella pneumophilla*) has helped map out a plausible reaction pathway for NTPDases.[25] This dual NTP/NDP (where NDP is nucleoside diphosphate) specificity of NTPDases can be attributed to nonspecificity of the base, which is logical in the sense that if the base were to form any *specific* contacts (hydrogen bonds) with the residues in its vicinity, it would compromise nucleoside binding flexibility. Closely associated with base specificity is the triphosphate/diphosphate specificity. This is typical for intracellular NTPDases4–7 that show preference for either triphosphate or diphosphate.

Recently the entpd5 gene, encoding expression for NTPDase5, was found to be expressed in zebrafish osteoblasts, indicating its involvement in regulation of bone mineralization. Phosphate, a byproduct of NTPDase catalyzed hydrolysis of nucleotides, is a promoter of bone formation and mineralization, whereas pyrophosphate (PP_i), a byproduct of NPP1 function, is a strong inhibitor of mineralization. It was therefore demonstrated that a concerted relationship between entpd5 and enpp1 was necessary for normal bone formation.[26]

3. ECTO-NUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASES

There are seven enzymes (NPP1–NPP7) under the umbrella of NPP family;[27] However, only three nucleotide hydrolyzing NPPs, NPP1 (PC-1 [where PC-1 is plasma cell glycoprotein 1]), NPP2 (autotaxin [ATX], phosphodiesterase1α), and NPP3 (CD203c) are important in purinergic cell-signaling mechanisms.[27–29] NPP1 and NPP3 are structurally related (type II membrane proteins) and are made up of three domains, an intracellular N-terminal domain, a transmembrane, and an extracellular domain. NPP2 is not membrane bound and exists in secreted form. NPP2 and extracellular domains of NPP1 and NPP3 are made up of two somatomedin-B-like domains (SMBDs), a catalytic domain, and a nuclease-like C-terminal domain (Fig. 2)[30] The active site contains two zinc metal ions. NPP1–3 have broad substrate specificity (e.g., ATP, ADP, ADP-ribose, and diadenosine polyphosphates among other substrates) and release AMP and the remaining part of the molecule as main hydrolysis products.[31,32] Both purine and pyrimidine nucleotides are hydrolyzed. Some of the important physiological processes associated with NPP1–3 besides modulation of purinergic signaling are recycling of extracellular nucleotides, regulating levels of PP_i, and cell motility activation. Other suggested roles include involvement in insulin receptor signaling and modulation of ecto-kinase activity.[33]

NPP1 (PC-1) is a membrane bound glycoprotein that is important in regulating bone mineralization by virtue of its ability to generate inorganic PP_i via hydrolysis of nucleoside triphosphates. NPP1 is located on cells of the distal convoluted tubule of the kidney, chondrocytes, osteoblasts, epididymis, and hepatocytes. NPP1 is more expressed in matrix vesicles where it regulates mineralization.[34] It is also implicated in inhibiting insulin signaling.[35,36] Therefore, NPP1 (PC-1) inhibition may represent a possible approach towards treatment of diabetes.[37] However, such inhibition may present a less than ideal situation owing to the fact that NPP1 (PC-1) inhibition or deficiency also leads to abnormal calcification[38] and osteoarthritis.[39] Therefore, much research is warranted before NPP1(PC-1) can be made an established drug target for treating diabetes.

In human aortic valve stenosis (AS), elevated levels of NPP1 have been found. The expression of NPP1 can be correlated to the extent of mineralization/calcification. In a rat model

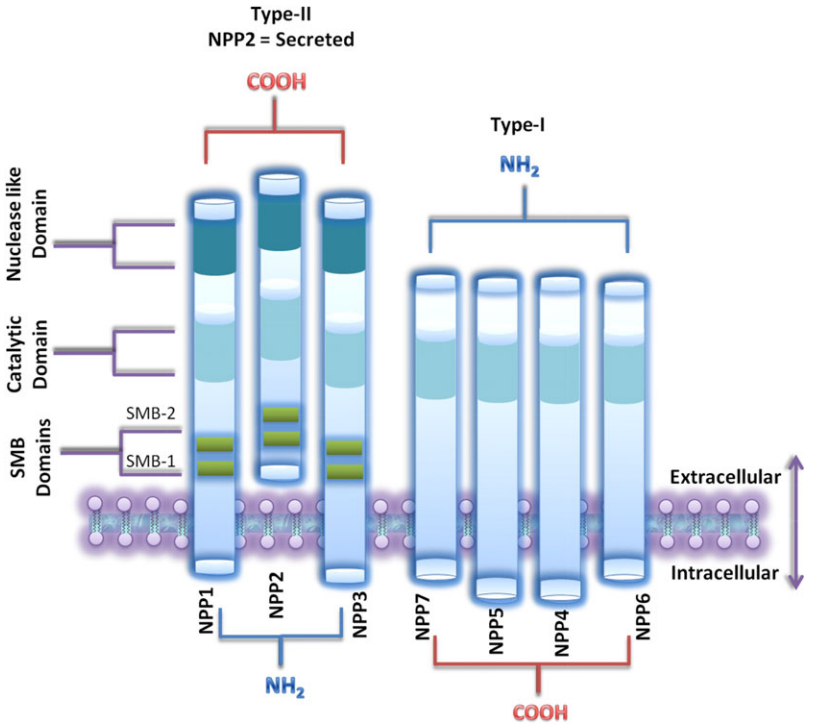


Figure 2. Domain structure and plasma membrane orientation of NPP1–7.

of cardiovascular calcification, ARL 67156 (**1**) significantly reduced calcification of the aortic root and valve cusps. Studies made by Côté et al.[40] parallel these observations. Mineralization of the aorta/aortic valve was documented in warfarin-treated rats and was accompanied by the development of aortic stenosis. These changes were due to an increased expression of NPP1. It was suggested that this ecto-nucleotidase may represent a novel target in the treatment of calcific aortic valve disease (for which there is no cure to date). These suggestions were based on the observation that ecto-nucleotidase inhibition caused by ARL 67156 also prevented the growth of calcific aortic valve in warfarin-treated rat model.[40]

Crystal structure of extracellular domain of mouse NPP1 (PC-1) in complex with different nucleotide monophosphates has been determined.[41,42] Crystal structure of mouse NPP1 that gives most detailed understanding of structural basis for the catalytic activity of NPP1, dimer formation, and secretion has been recently determined.[43] Moreover, critical differences in binding sites of NPP1 and NPP2, that allow NPP2 to hydrolyze lipids but not NPP1, were also revealed.

NPP2 (ATX) also has lysophospholipase D (lysoPLD) activity and is involved in the production of an important bioactive lipid molecule lysophosphatidic acid (LPA, a lipid mediator that induces various cellular responses) via hydrolysis of lysophosphatidylcholine (LPC).[44] There are three firmly established G-protein-coupled receptors (GPCR) of LPA (LPA_1, LPA_2, and LPA_3). Most interestingly, a new and fourth LPA receptor (LPA_4) has been identified that although belongs to GPCR family, but does not share much identity with other LPA receptors; it is instead very closely related to the purinergic GPCR family (GPR23/P2Y9).[45] Overexpression of ATX–LPA receptors is implicated in progression of many cancerous tumors.[46–48] This ATX–LPA is a newly emerging and attractive drug target. In this regard an excellent review has recently appeared[49] that focuses on potential strategies of controlling cancer by means

of ATX–LPA axis. For NPP2, the substrate selectivity (nucleotide vs. phospholipid) has been rather elusive. However, Hausmann et al.,[50] in 2011 provided structural basis for substrate discrimination between nucleotide and phospholipid by NPP2. Indications about the location of nucleotide-binding site can be obtained from crystal structure of *Xa*NPP in complex with nucleotide substrate AMP.[51] A hydrophobic lipid-binding pocket was identified from the crystal structure of rat NPP2 (ATX) in complex with inhibitor HA15533. This allowed for significant assumptions to be made regarding substrate selectivity between nucleotides and phospholipids.[50,52] Crystal structure of mouse NPP2 alone and in complex with different LPAs has been reported.[53,54] To better understand and resolve the issues still left unclear by crystal structure analysis (regarding role of nuclease-like domains and *N*-glycosylation in catalytic activity) Koyama et al.[55] have carried out molecular dynamics simulation studies of NPP2.

NPP3 is a plasma-membrane associated nucleotide hydrolyzing glycoprotein that is recognized by the monoclonal antibody RB13–6 (hence also known as $gp130^{RB13-6}$). NPP3 is more closely related in structure and function to NPP1 than NPP2.[56] NPP3 has been associated with carcinogenesis of human colon cancer and was even suggested to be a tumor marker of colon carcinoma.[57] It also acts as an activation marker of human basophils and mast cells.[58,59] Soluble forms of NPP1, NPP3, and NPP6 have also been known, whereas NPP2 exists as secreted form. Physiological and pathophysiological roles of NPPs have been summarized in a review.[33]

NPP6–7 are known to hydrolyze phosphodiester bonds in phospholipids, nucleotides are not known to be hydrolyzed by NPP6–7. More specifically, NPP6 has a choline-specific glycerophosphodiester phosphodiesterase activity and NPP7 has alkaline sphingomyelinase activity. Exact substrates and physiological roles for NPP4 and NPP5 are unknown.[60,61] However, this much is known with certainty that neither NPP4 nor NPP5 posses any lysoPLD or lysophospholipase C (lysoPLC) activity toward LPC. They also do not have any phosphodiesterase activity toward glycerophosphorylcholine (GPC).[61] NPP4 located at the surface of vascular endothelium was found to hydrolyze Ap_3A and Ap_4A into ADP and ATP, respectively, along with the formation of AMP.[62]

4. ALKALINE PHOSPHATASES

APs are ubiquitous metalloenzymes and represent a protein family of nonspecific phosphomonoesterases with a broad substrate specificity.[5] They can be divided into two groups, the tissue nonspecific alkaline phosphatase (TNAP) and tissue-specific APs that include placental AP (PLAP), intestinal AP (IAP), and germ cell AP (GCAP).[63] The tissue specific APs (PLAP, GCAP and IAP) share 90–98% sequence identity, whereas TNAP shares only 50% sequence identity with these tissue-specific APs.[64] Catalytic roles of AP involve breakdown of various nucleotides to liberate inorganic phosphate (P_i).[17] Mammalian APs have optimum activities at alkaline pH and exhibit a wide range of substrate specificity ranging from phosphomonoesters to an assortment of phosphate containing compounds, such as inorganic polyphosphates, glucose-phosphates, phosphatidates (containing fatty acid side chains), and bis(*p*-nitrophenyl) phosphate. APs also produce P1 receptor agonist adenosine by sequential dephosphorylation.[65]

Unequivocal evidence in favor of involvement of AP in purinergic signaling is lacking. An important role for TNAP in embryonic neurogenesis and regulation of purinergic signaling was suggested[66] based on its selective expression in certain parts of murine brain. But a major role for mammalian APs in purinergic signaling and hydrolysis of extracellular nucleotides may not be plausible as mammalian enzymes function best at alkaline pH and also have relatively greater K_M and specific activity values.[17] Differences in structure and function of NPP and AP have been compared in detail by Zalatan et al.[51]

5. COMPARISON OF METALLIC CONTENT IN THE ACTIVE SITES OF NUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASE, NUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASE, ECTO-5′-NUCLEOTIDASE, AND ALKALINE PHOSPHATASE

NPPs belong to AP superfamily and both have a bimetallic Zn^{2+} ion catalytic center, APs additionally have a third Mg^{2+} ion, which is absent in NPPs. e5NT belongs to a large superfamily of distantly related metallophosphoesterases with a dinuclear metal (Zn^{2+}) center. There are two domains in e5NT, a dimetallic Zn^{2+} ion containing N-terminal domain and a nucleotide-binding C-terminal domain. The active site of e5NT is located in between the two domains. These two domains are linked together by an α-helix chain.[67] The crystal structure of *Escherichia coli* e5NT in complex with AMPCP (α,β-methylene-ADP) has provided structural insights into the possible mechanism of nucleotide binding inside enzyme's active site.[68] The AMPCP is bound to the active site (which is located at the interface of two domains) such that the nucleoside is bound to the C-terminal domain and the phosphate chain is bound to the metal ions in the N-terminal domain. Figure 3 shows comparison of dimetallic Zn^{2+} ion containing catalytic site of AP, NPP, and e5NT.

However, unlike e5NT, APs and NPPs, the catalytic active site of NTPDase, neither contains a zinc bimetallic center nor belongs to metallophosphoesterase superfamily. Instead, the NTPDases belong to the ASKHA (acetate and sugar kinases, heat shock proteins hsp70 and actin) structural superfamily. Members of this family have a common ATPase domain and are known to undergo large interdomain movement for catalysis purpose.[15,69,70]

A characteristic feature of all NTPDases is the presence of ACRs that are thought to be important for the catalytic process. The major feature common among NTPDases and actin/hsp70/sugar kinase superfamily is the conservation of two nucleoside phosphate-binding motifs (DXG) in the catalytic domain.[4] These motifs are found in ACR1 and ACR4 for all NTPDases.[71,72] Moreover, there are also many close similarities in the secondary structure topology (of domain I and II) of NTPDases with the actin/hsp70/sugar kinase superfamily. Vorhoff et al.[73] have carried out detailed studies on comparison of secondary structure of NTPDase3 with other members of NTPDase family as well as with actin. The activity of all NTPDases depends upon the presence of divalent metal cations (as cofactors) usually Ca^{2+} or Mg^{2+} ions. The substrate and metal ion-binding site is located in between the cleft of two domains. In the crystal structure of NTPDase2, the Ca^{2+} cofactor was found to be in octahedral coordination. The four coordination sites were occupied with water molecules and remaining coordination sites were occupied by oxygen atoms of phosphate group. This binding of phosphate oxygen to the Ca^{2+} ion is thought to polarize the phosphorus oxygen bond, thus, aiding in the catalysis process.[20] The metal ion-binding geometries of *Lp*NTPDase1, in complex with the inhibitor molecule AMPPNP and Ca^{2+} ion (metal co-factor), and *Rn*NTPDase2, in complex with AMPPNP-Ca^{2+} and AMPPNP-Zn^{2+}, are very closely related to one other. The active site residues and six water molecules (four coordinating to the metal ion cofactor, one nucleophilic water molecule, and one water molecule in vicinity of phosphate group of the substrate) are also conserved between the two enzymes (Fig. 4).[25]

6. ECTO-5′-NUCLEOTIDASE

e5NT is wide spread in various bacteria, vertebrates, and plants where it displays much diversity in the nature of substrate hydrolyzed.[18,67,74] The evidence for the existence of transmembrane e5NT came from studies on cell lines that showed aberrant insertion of 5′-nucleotidase. On this basis, existence of a domain capable of interacting with intracellular actin (cytoskeleton)

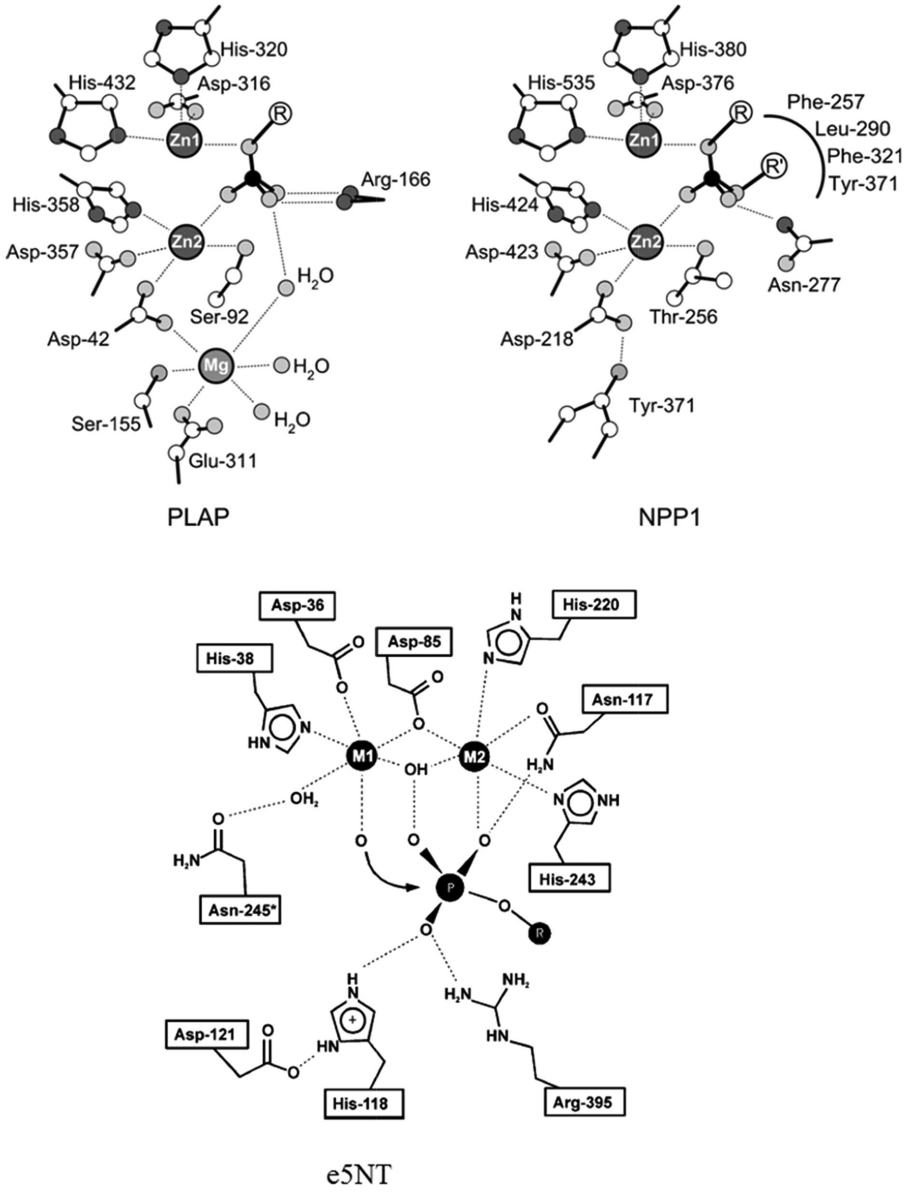


Figure 3. Comparison of the active site structures of human placental AP (PLAP), human NPP1,[60] and e5NT.[67] The active site structure of human NPP1 differs from PLAP mainly by the absence of the magnesium ion-binding site, the replacement of Arg-166 by Asn-277, and the presence of a substrate specific site formed by Tyr 371, Phe-321, Phe-257, and Leu-290. This figure was reproduced with permission from the publisher.

was proposed.[75] But later on, Baron et al.[76] carried out detailed studies on transmembrane topography of rat hepatocyte e5NT using glycoprotein labeling and limited proteolysis technique, and found no evidence for direct interaction with actin. It was suggested that previous effects on actin were due to contamination from other ATPases. Most studies agree on at least three different types of e5NT, a membrane-anchored form, a soluble form derived from the membrane-anchored form, and a cytosolic form (c5NT which is structurally different from surface-located e5NT).[67] This enzyme has been found intracellularly in hepatocytes, adipocytes,

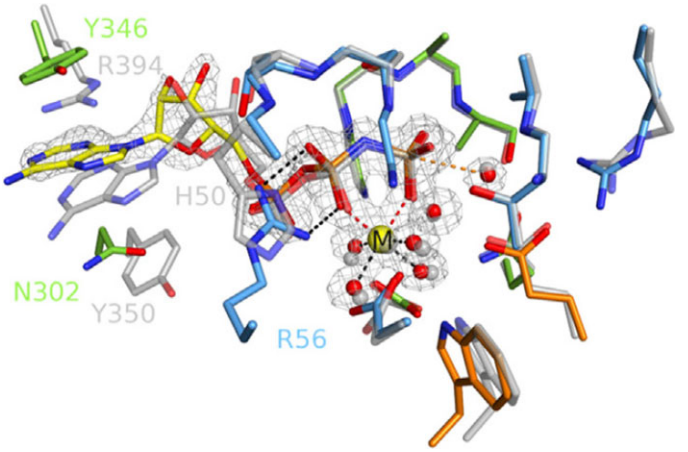


Figure 4. Superposition of *Lp*NTPDase1 in complex with AMPPNP-Mg^{2+} and *Rn*NTPDase2 in complex with AMPPNP-Zn^{2+}.[25]

and lymphocyte endocytotic recycling pools;[77] Golgi apparatus;[78,79] and in lysosomes.[80] Inosine monophosphate (IMP) and guanosine mpnophosphate (GMP) specific c5NT isolated from calf thyums was also found to have a phosphotransferase activity.[81] Crystal structures of human c5NT II[82] and human mitochrondrial deoxyribonucleotidase (dNT-2)[83] have been determined. Studies made by Pesi et al.[84] highlight the regulatory role of cytosolic 5NT. Surface-located e5NT is anchored to the plasma membrane via glycosyl-phosphatidylinositol (GPI) linkages[85,86] and is distributed in a wide assortment of cells, such as hepatocytes, fibroblasts, endothelial cells, lymphocytes, and glial cells.[87] Based on the amounts of inositol found in soluble e5NT (which is comparable to the amounts of inositol quantified in GPI-anchored e5NT), it can be suggested that the soluble form of e5NT was previously membrane anchored via GPI linkage.[88] Phosphatidylinositol-specific phospholipase C (PI-PLC) seems to be responsible for the generation of soluble form of e5NT via hydrolysis of the GPI anchor.[89] It was also found that anchoring of e5NT via GPI linkage to the lipid bilayer strongly affects its catalytic efficiency.[90] It has been confirmed that the soluble e5NT isolated from electric organ of electric ray and bovine cortex has been derived from GPI anchored form via phospholipase C cleavage.[91]

X-ray crystal structure of e5NT isolated from *E. coli* has revealed the presence of an N-terminal domain, housing catalytic Zn metal ions in divalent state, and a C terminal domain containing nucleotide-binding pocket. The adenosine group of nucleotide substrate binds to this pocket such that the substrate occupies interdomain region.[67] This arrangement is very closely related to the e5NT from human source.[92]

The majority of extracellular adenosine comes from e5NT via hydrolysis of AMP.[67,68] The hydrolysis of AMP by e5NT is stereoselective hydrolyzing only nucleoside 5′ (and not 2′ and 3′) monophosphates.[93] It is therefore one of the enzymes (among other ecto-ATPases) that act to conclude the action of extracellular nucleotide signaling molecules acting on P2X and P2Y receptors.[18,67] e5NT generates adenosine that acts via adenosine receptors (P1)[94] in a variety of physiological signaling processes.[48] Accordingly, synchronized activation and suppression of e5NT and the A_{2A} adenosine receptor has been observed in human B-cell lines.[95]

Adenosine plays an important role in therapeutically relevant functions, such as antinociceptive,[94] anti-inflammatory,[96–98] immunosuppressive,[99] vasodilatatory, and antidiuretic effects.[100] e5NT has been linked to other pathological conditions, such as cancer[101] and myocardial ischemia.[100] It has been reported that e5NT is involved in drug resistance[102]

and tumor-promoting functions.[103] It is proposed that e5NT inhibitors may be suitable drug candidates for the treatment of hepatic fibrosis,[104] bacterial diarrhea, and gastrointestinal infections.[105] The potential applications of e5NT inhibitors include therapies for cancer, including melanomas, gliomas, and breast cancers.[106]

7. INHIBITORS OF ECTO-NUCLEOSIDE TRIPHOSPHATE DIPHOSPHOHYDROLASE AND ECTO-5′-NUCLEOTIDASE

One of the ways pathogens interfere with human immune response is by disruption of extracellular ATP levels in host thereby interrupting the purinergic signaling pathways. These extracellular ATP levels are strictly controlled by NTPDases (which are almost exclusively present in eukaryotes). However, some pathogenic prokaryotes also contain these enzymes. The pathogenic NTPDases are therefore attractive drug targets. In this regard, the crystal structure of pathogenic NTPDase from *Legionella pneumophila* (Lpg1905/Lp1NTPDase) has been determined.[107] The study highlights the host–pathogen relation and provides insights into the basis of NTPDase specific inhibition. Lp1NTPDase has been shown to be inhibited by the ATP analogue ARL 67156 (**1**). An excellent review highlights the effects of microbial NTPDases on host–pathogen interactions.[108] X-ray crystal structure of periplasmic-5NT form *E. coli* has also been determined.[109] In 2007, evidence was provided for incorporation of host NTPDase1 (CD39) into HIV type 1 particles where they preserve their activity.[110] It was proposed that modulation of host extracellular ATP/ADP levels by HIV type 1 virus could suggest one of the many ways by which immunological deficiencies are observed in infected individuals.

There is a dire requirement for the availability of ecto-nucleotidase inhibitors in order to be able to map out exact mechanisms and consequences associated with the release of nucleotides.[18,111–113] Similarly, NTPDase subtype specific inhibitors are also required to investigate patho-physiological roles of NTPDase and to explore the potential of such compounds as therapeutic immunomodulatory agents for the possible treatment of cancer, cardiovascular, and central nervous system related disorders.[114]

NTPDase inhibitors can modulate P1 and P2 receptor activation.[112] Many P2 receptor agonist/antagonists also act as inhibitors of ecto-nucleotidase but ideally such inhibitors should have little or no effect on P2 receptor activation or deactivation. However, that is usually not the case and one often finds in literature non-subtype specific inhibitors of NTPDase that are frequently also inhibitory toward e5NT. A nonhydrolysable ATP analogue ARL-67156 (**1**) is the only commercially available compound that does not significantly affect purinoreceptors.[4] The significant lack of specific NTPDase inhibitors is a major roadblock toward developments in modulators of purinergic cell signaling mechanisms for therapeutic purposes.

Some of the commonly known compounds that inhibit extracellular nucleotide hydrolysis via inhibiting NTPDases include suramin (**2**) and its derivatives,[115] pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) (**3**),[116] 1-naphthol-3,6-disulfonic acid (BG0136) (**4**),[114] and reactive blue 2 (RB2; **5**).[18,113,117] Cibacron blue (3GA) (**6**), uniblue A (**7**), and reactive blue 19 (**8**) inhibit ecto-nucleotidases with IC_{25}% values in the range 17 and 62 μM.[117] These compounds were nonselective inhibitors of NTPDases and were also found to act as nonselective antagonists of nucleotide receptors.[113,116,118] Some aromatic isothiocyanato-sulfonates were found to inhibit of NTPDases with IC_{25}% values between 10 and 464μM.[119]

In another study, P2 receptor antagonists such as RB2 (**5**), suramin (**2**), NF279 (**9**), NF449 (**10**) and MRS2179 (**11**) were also found to be non-subtype-selective inhibitors against recombinant human and mouse NTPDase.[120] However, human NTPDases were more susceptible to inhibition than mouse NTPDases. RB2 (**5**) and NF279 (**9**) were found to be most potent

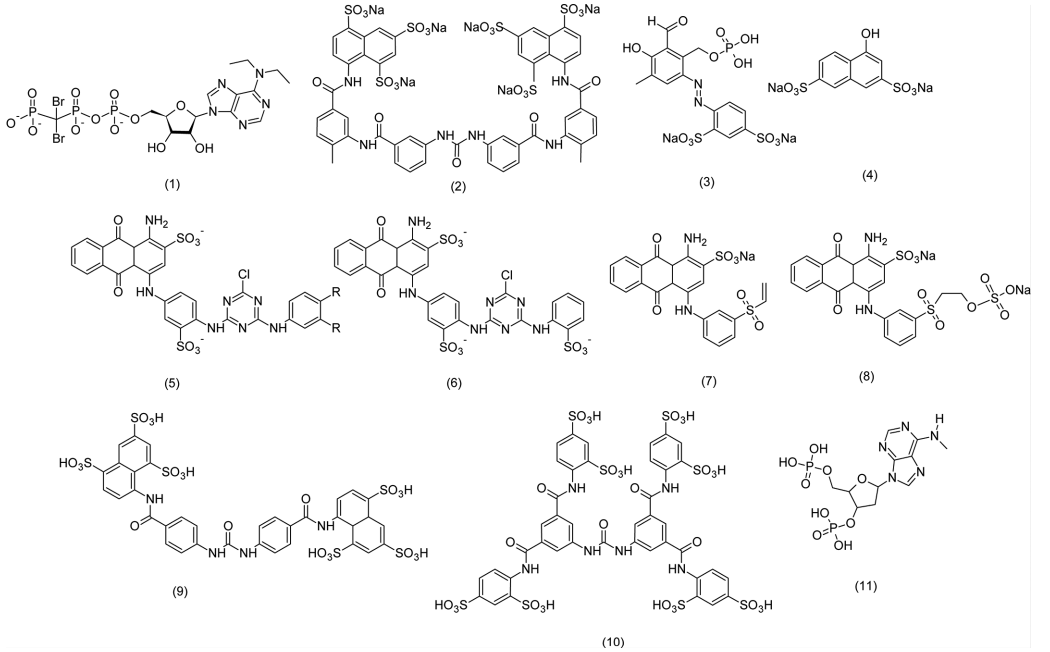


Figure 5. Structures of inhibitors **1**–**11**.

inhibitors that completely inhibited the enzymatic activity at the concentration of 100 μM (Fig. 5).

Some potent cytosolic 5′-nucleotidase inhibitors were identified[121] via initial in silico screening of available chemical databases followed by in vitro enzymatic assays and crystallographic studies of resulting enzyme–inhibitor adduct. The most potent compounds identified include anthraquinone-2,6-disulfonic acid sodium salt (AdiS) (**12**; K_i = 2.0 mM), Na salt of 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid (PDTdiS) (**13**) and 7-amino-1,3-naphthalene disulfonic acid (ANdiS) (**14**). AdiS (**12**) also exhibited different levels of cytotoxicity on several cancer cell lines in vitro. Two more sulfonic acid containing compounds reactive red 2 (**15**) and acid red 33 (**16**) were also found to inhibit ecto-nucleotidase and reduce degradation of ATP in rat vas deferens and guinea-pig taenia coli.[122]

Among naturally occurring compounds, two alkaloids, lycorine (**17**) and candimine (**18**), were shown to be strong inhibitors of NTPDase and e5NT from human pathogenic parasite *Trichomonas vaginalis*.[123] It was suggested that the vulnerability of *T. vaginalis* to host immune response could be amplified in the presence of these alkaloids (**17**) and (**18**; Fig. 6).

Most NTPDase inhibitors are also inhibitors of e5NT, for example, RB2 (**5**) inhibits rat e5NT with a K_i value of 3.07 μM and acid blue 25 (**19**) with K_i value of 15.2 μM.[106] Among anthraquinone derivatives two of the most potent and competitive rat e5NT inhibitors identified were 1-amino-4-[4-fluoro-2-carboxyphenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (PSB-0952) (**20**) and 1-amino-4-[2-anthracenylamino]-9,10-dioxo-9,10-dihydroanthracene-2-sulfonate (PSB-0963) (**21**).[106]

Human NTPDase2 was found to be inhibited by *p*-chloromercuriphenylsulfonate, a sulfhydryl reagent. The extracellular domain of NTPDase contains ten conserved cystein residues and only one free cystein (C26) that resides in N-terminal domain. Inhibitory potential due to *p*-chloromercuriphenylsulfonate can be attributed to membrane perturbation that inhibits NTPDase activity by interfering with this free cystein, therefore, rendering it

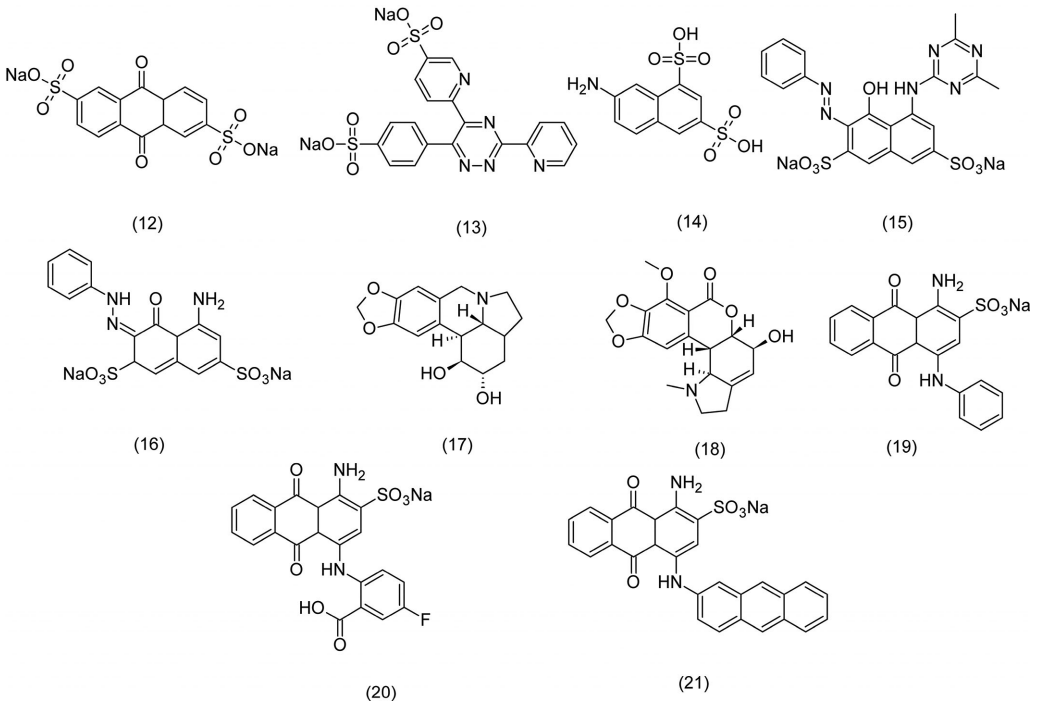


Figure 6. Structure of inhibitors **12**–**21**.

nonfunctional. This point is further proven by the fact *p*-chloromercuriphenylsulfonate has no inhibitory potential against mutated NTPDase2 in which cystein C26 is replaced by serine.[124]

Some naturally occurring polyphenols were found to act as weak inhibitors of e5NT.[125] It was also reported that myricetin (**22**) and quercetin (**23**) inhibited e5NT with IC_{50} values of 1.1 and 1.4 μM, respectively.[126] In another study, quercetin was reported to be an inhibitor of e5NT with a K_i value of 45.3 μM, it also exhibited antiproliferative action in the U138MG glioma cell line.[127]

Recently with the help of computational screening approach 13 sulfonamide derivatives were identified as potent and competitive inhibitors of e5NT. The most potent inhibitor was 6-chloro-2-oxo-N-(4-sulfamoylphenyl)-2H-chromene-3-carboxylic acid amide (**24**) that exhibited an IC_{50} value of 1.90 μM.[128] Raza et al., identified 11 sulfonic acid derivatives as potent inhibitors of rat and human e5NT.[129] The most active compounds in the series were sodium 2,4-dinitrobenzenesulfonate (**25**) with K_i value of 0.66 μM and *N*-(4-sulfamoylphenylcarbamothioyl) pivalamide (**26**) with K_i value of 0.78. Two clinically used antiplatelet thienopyridine-based drugs clopidogrel (**27**) and ticlopidine (**28**) were also found to have vascular ecto-nucleotidase (NTPDase1 and e5NT) inhibitory activity.[130] These drugs also inhibit $P2Y_{12}$ receptor but only after metabolic activation. It was suggested that ticlopidine (**28**) could even be used as a selective NTPDase1 inhibitor since at 100 μM concentration it did not inhibit other NTPDases.

Substituted naphthalene sulfonates were investigated for their NTPDase inhibitory potential.[114,131] According to these studies, a *m*-sulfonate group relative to a hydroxyl group on naphthalene core were deemed essential for enhanced inhibitory potential. Consequently, naphthalene sulfonate derivatives BG0124 (**29**) and BG0136 (**30**) were found to be the best

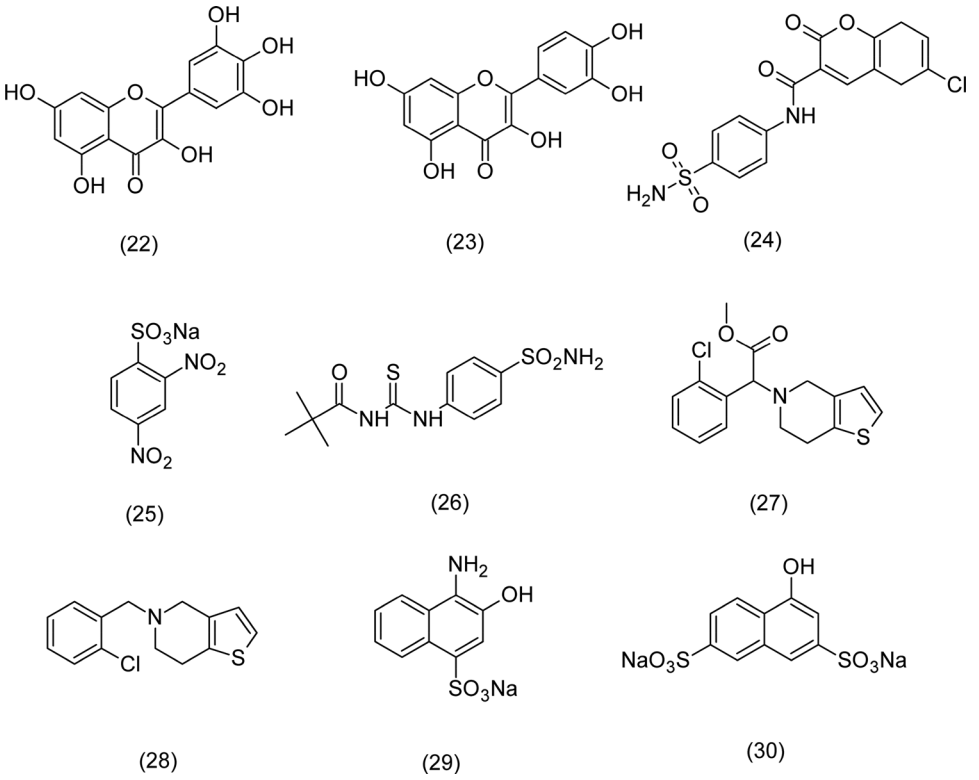


Figure 7. Structures of inhibitors **22**–**30**.

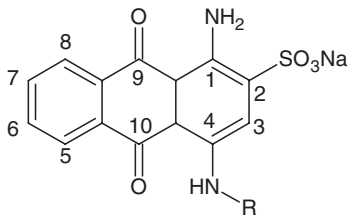
inhibitors (Fig. 7). Both studies confirmed that the presence of a sulfonate group on naphthalene core as well as the presence of a hydroxyl group is essential for NTPDase inhibition.

8. STRUCTURE ACTIVITY RELATIONSHIP FOR RB2 DERIVATIVES: LEADS FOR DEVELOPMENT OF MORE POTENT AND POSSIBLY SPECIFIC INHIBITORS

RB2 (**5**) meets the criteria for small molecule with drug like properties, and is therefore a good candidate for lead molecule optimization. It was found that RB2 (**5**) had about 20-fold more inhibitory potential toward NTPDase3 ($K_i = 1.10\ \mu M$) as compared to NTPDase1 ($K_i = 20.0\ \mu M$) and NTPDase2 ($K_i = 24.2\ \mu M$).[7] Consequently, Baqi et al.,[113] in 2009, while building on similar concepts, carried out detailed structure–activity relationship (SAR) studies based on systematic modulation of anthraquinone scaffold (the very heart of RB2 (**5**) molecule). It was observed that sulfonate group on 2-position of anthraquinone ring was indispensable for increased inhibitory potential, since compounds for which 2-sulfonate group was replaced with other groups had little or no activity. The 4-chlorophenylamino derivative **(38)** was a nonselective inhibitor of NTPDase1–3, whereas 4-(1-napthylamino) derivative **(40)** was a very potent and selective inhibitor of NTPDase3 (Table I, Fig. 8).

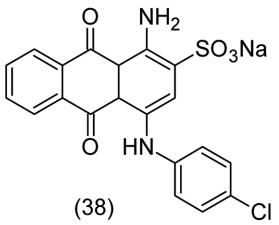
Similar observations regarding SAR of anthraquinone derivatives from above-mentioned studies have been summarized in Figure 9.

Table I. Inhibitory Potencies of Selected Anthraquinone Derivatives against Rat NTPDase1, 2, and 3[113] and e5NT[106] Determined by Capillary Electrophoresis



31-42

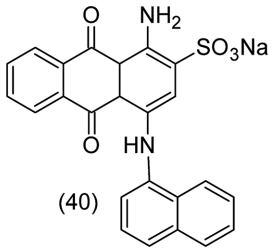
		K_i [μM] ± SEM			
Inhibitor	*R*	e5NT	NTPDase1	NTPDase2	NTPDase3
31	Phenyl	15.2 ± 0.95	49.1 ± 5.1	35.8 ± 6.1	14.3 ± 1.5
32	2-Methylphenyl			25.7 ± 5.1	23.0 ± 2.5
33	3-Methylphenyl	12.3 ± 3.7	51.5 ± 0.4	12.8 ± 0.9	19.1 ± 6.0
34	2,3-Dimethylphenyl	—	—	22.7 ± 3.4	38.5 ± 5.0
35	2,4-Dimethylphenyl	—	—	15.6 ± 2.5	41.8 ± 5.5
36	2-Methoxyphenyl	—	—	53.8 ± 5.7	17.6 ± 6.6
37	2-Ethoxyphenyl	—	—	40.8 ± 11.1	58.0 ± 12.7
38	4-Chlorophenyl	4.81 ± 0.34	15.7 ± 3.4	18.0 ± 2.0	16.4 ± 1.6
39	4-Acetylaminophenyl	—	—	486 ± 18	343 ± 63
40	1-Naphthyl	0.53 ± 0.03	—	—	1.5 ± 0.1
41	2-Naphthyl	1.47 ± 0.33	—	—	—
42	3,4-Dimethoxy-phenethyl	—	173 ± 6	54.1 ± 6.8	23.4 ± 0.4
RB-2 (**5**)		3.07 ± 0.24	20.0 ± 0.0	24.20 ± 0.06	1.10 ± 0.03



(38)

NTPDase 1: 15.7 ± 3.4
NTPDase 2: 18.0 ± 2.0
NTPDase 3: 16.4 ± 1.6

K_i [μM] ± SEM



(40)

NTPDase 1: Inactive
NTPDase 2: Inactive
NTPDase 3: 1.5 ± 0.1

Figure 8. Structures of inhibitors **38** and **40**.

9. NUCLEOTIDE-BASED INHIBITORS

A few nucleotide and nucleoside analogues have been identified as inhibitors of e5NT,[132] but usually such compounds are better inhibitors of NTPDase. ADP itself appears as a potent inhibitor of e5NT with K_i value of 0.91 μM, whereas ATP is a less potent inhibitor with K_i value of 8.90 μM.[133] The ADP analogue 5′-[α,β-methylene]diphosphate(β-methylene)-ADP, also known as AOPCP (**43**) in which the β-phosphate ester bond has been replaced with a methylene group has been identified as a potent and competitive inhibitor of e5NT (K_i value is

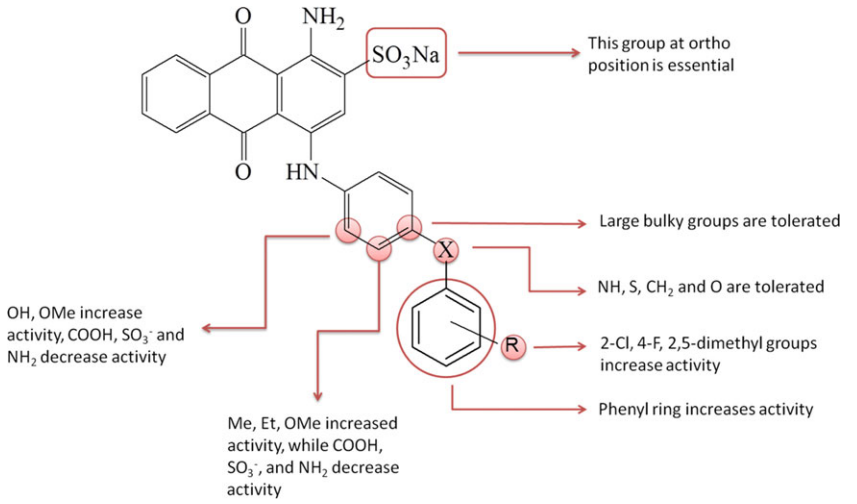


Figure 9. Important structure–activity relationships for NTPDase inhibitory activities of anthraquinone derivatives.

0.87 μM).[74,134,135] It has negative charges at physiological pH and can therefore not be perorally absorbed; it may also be hydrolyzed to yield the adenosine receptor agonist adenosine.[133]

Nucleotide derivatives and analogues, derived from ATP are metabolically unstable as they quickly suffer dephosphorylation.[136,137] However, this problem has been partially solved by employing ATP analogues that are nonhydrolysable, for example, ATP analogue ARL67156 (6-N,N-diethyl-β-γ-dibromomethylene-D-adenosine-5′-triphosphate) (**1**)[138,139] and 8-thiobutyladenosine-5′-triphosphate (8-BuS-ATP) (**44**).[136] These compounds show inhibitory potential without significantly affecting nucleotide receptors. In later studies ARL 67156 (**1**) was found to be a weak competitive inhibitor of human NTPDase1 (K_i = 11 μM) and NTPDase3 (K_i = 18 μM), but had almost no activity against NTPDase2.[136,139] More importantly, ARL 67156 (**1**) was also found to be stable and not susceptible to hydrolysis by human NTPDase1–3, NTPDase8, NPP1, or NPP3. The other compound, 8-thiobutyladenosine-5′-triphosphate (**44**) is a competitive inhibitor (K_i value of 10 μM) against NTPDase from bovine spleen[136] and is stable against other NTPDases; however, it can be hydrolyzed by ecto-APs.[113] A metabolically stable, uncharged nucleotide mimetic derived from uridine-5′-carboxylate PSB-6426 (**45**; Fig. 10) was a selective and potent inhibitor of human NTPDase2 (K_i = 8.2μM) as it showed little or no activity against NTPDase1, NTPDase3, and NTPDase8.[112]

Recently, Lecka et al.[140] synthesized a series of 8-BuS adenine nucleotide derivatives with shorter phosphate chains, that is, 8-BuS-ADP (**46**) and 8-BuS-AMP (**47**) and 8-BuS-ATP derivatives with an imido (**48**) or methylene group (**49**) in between the phosphate chain (Fig. 11). The compounds (**46**), (**47**), and (**49**) were found to be stable toward hydrolysis by human NTPDase1,3, and 8. Although compound (**48**) could not be hydrolyzed by NTPDase1, 2, and 3, it was only slightly hydrolyzed by NTPDase8. Compounds (**46**) and (**47**) were potent inhibitors of NTPDase1.

10. POLYOXOMETALATE-BASED INHIBITORS

POMs are anionic metal complexes that are relatively stable in aqueous solutions at physiological pH. POMs typically contain transition metals, such as vanadium, molybdenum, tungsten,

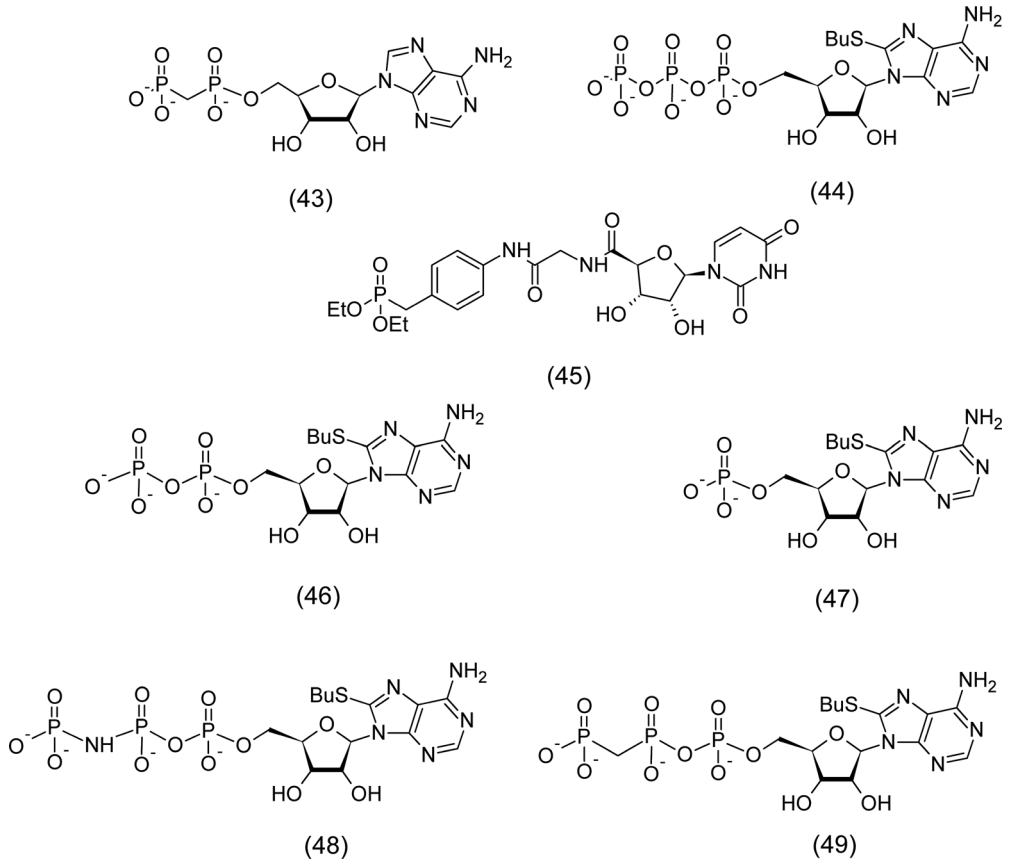


Figure 10. Structures of inhibitors **43**–**49**.

etc., which are bridged by negatively charged oxygen atoms.[141] POMs were identified as a novel class of subtype selective NTPDase inhibitors.[111] It was suggested that since the negative charge of POMs is a quality that these compounds have in common with nucleotides, therefore, this negative charge may actually have a contribution in increasing the susceptibility of POMs toward nucleotide-binding enzymes.[142–145] A series of polyoxotungstates bearing different charge, size, and shape were investigated for their inhibitory activities against NTPDase1–3. The compound with most activity was K_6H_2 [$TiW_{11}CoO_{40}$], exhibiting K_i values of 0.140 μM against NTPDase1, 0.910 μM against NTPDase2, and 0.563 μM against NTPDase3. Compound $(NH_4)_{18}$[$NaSb_9W_{21}O_{86}$] was more selective toward NTPDases2 and 3 as compared to NTPDase1.[111] Moreover, the investigated POMs did not significantly affect P2 receptors and were instead much more inhibitory toward NTPDases. While these POMs successfully inhibit ATP break down, in an independent study,[142] some of these compounds were also found to have the potential to block central synaptic transmission. It is important to note here that this synaptic transmission is not related to NTPDase inhibition. This consideration severely limits the usefulness of POMs for therapeutic purposes (Table II).

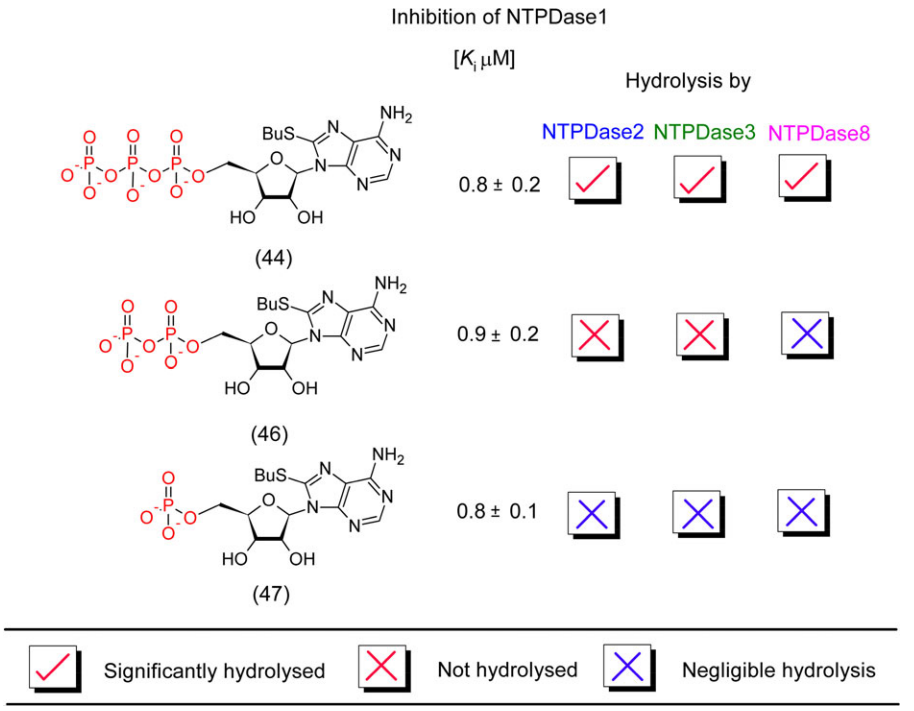


Figure 11. Comparison of NTPDase1 inhibition activity and hydrolysis by other NTPDases (NTPDase2,3,8) of 8-BuS-ATP (**44**), 8-BuS-ADP (**46**), and 8-BuS-AMP (**47**).

Table II. NTPDase Activity of Selected Polyoxometalates[111]

	K_i [μM] ± SEM		
Inhibitor	NTPDase1	NTPDase2	NTPDase3
$Na_6[H_2W_{12}O_{40}]$	2.58 ± 0.30	28.8 ± 0.2	3.26 ± 0.18
$H_3[PW_{12}O_{40}]\cdot H_2O$	3.49 ± 0.23	6.17 ± 0.15	8.72 ± 1.81
$K_7[Ti_2W_{10}PO_{40}]$	2.00 ± 0.34	37.4 ± 1.3	4.0 ± 0.26
$K_6H_2[TiW_{11}CoO_{40}]\cdot 13\ H_2O$	0.14 ± 0.021	0.91 ± 0.041	0.563 ± 0.113
$K_{10}[Co_4(H_2O)_2(PW_9O_{34})_2]\cdot 22\ H_2O$	0.48 ± 0.010	1.53 ± 0.20	2.61 ± 0.97
$(NH_4)_{18}[NaSb_9W_{21}O_{86}]$	(15% inhibition at 1 mM)	3.94 ± 0.78	3.77 ± 0.52

11. MODE OF INHIBITION OF POLYOXOMETALATES

The mechanism suggested for inhibitory effect of POMs comes from studies of NTPDase1 crystallized in complex with inhibitors, such as sodium vanadate (Na_3VO_4) and ammonium heptamolybdate $(NH_4)_6[Mo_7O_{24}]$).[21] It was found that these POM inhibitors bind electrostatically to a loop (originally engaged in the binding of nucleobase substrate) located at the positively charged patch at the active site cleft. This positively charged patch is probably what helps to attract the negatively charged nucleotides. The metallic clusters are in contact with two lysine residues K406 and K408. It has been known since long that a domain movement mechanism is also involved in proper functioning of these enzymes.[146] So, the overall effect of binding of POMs to NTPDase may be a restricted or impaired domain movement or flexibility, which is critical for normal substrate binding and product release.

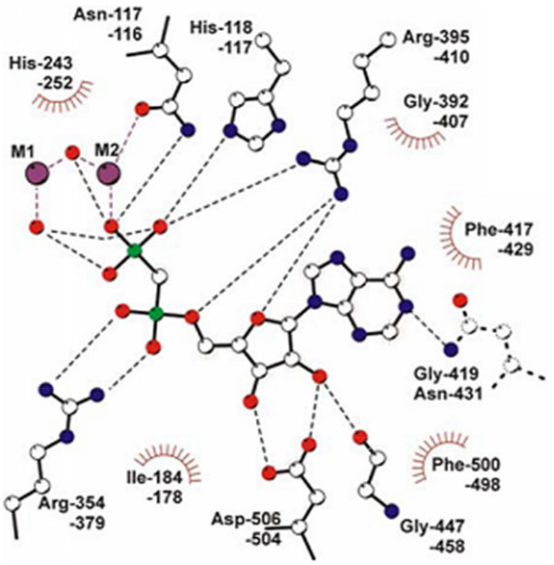


Figure 12. Interactions of inhibitor molecule with the active site of *E. coli* e5NT.[67] Figure is reproduced with permission from the publisher.

To aid in the design of structure-based inhibitors and to better understand the catalytic mechanism of cell signaling, single crystal X-ray structure of the extracellular domain of rat NTPDase2 in complex with bound substrate analogue, products, and cofactor was determined.[147] This approach may be very useful for the designing of subtype-specific NTPDase inhibitors. Similarly, structural and catalytic mechanisms of NTPDase have also been determined by crystallization of the catalytic domain of *R. norvegicus* NTPDase2 after refolding from bacterial inclusion bodies.[147]

12. STRUCTURAL INSIGHTS INTO E. COLI ECTO-5′-NUCLEOTIDASE INHIBITION

The catalytic site of *E. coli* e5NT contains a dimetallic center. e5NT isolated from three different sources showed the two metal atoms (per dimer) to be of zinc.[148] Earlier studies[149] reported slightly less amount of Zn than required to suggest the existence of two Zn atoms per dimer, this is possibly due to the loss of one of the Zn atoms owing to its low binding affinity. In monomeric e5NT isolated from *E. coli*, there is a clear evidence of two Zn atoms occupying the catalytic site.[150] An aspartate side chain and a water molecule serve to bridge together the two metal ions of the active site. Metal ion 1 is additionally coordinated by three amino acid residues aspartate (Asp-36), histidine (His-38), and a glutamine (Gln-254 is not conserved and corresponds to Asn-245 instead). Metal ion 2 is coordinated by a water molecule and three amino acid residues, two of which are histidines (His-220 and His-243) and one is an asparagine (Asn-117). During the formation of Michaelis complex, the phosphate group of the substrate replaces the water molecule.[67]

Crystal structure of *E. coli* e5NT crystallized with the nucleotide analogue inhibitor molecule (α,β-methylene-ADP) has served to shed light on the interactions that take place inside the enzyme's substrate binding C-terminal domain. As can be seen from the Figure 12, the adenine moiety is hydrophobically stacked among two phenylalanines. The carboxamide

group of Asn-431 interacts with the nitrogen atom (N1) of adenine ring, while the phosphate groups (α- and β-) of the nucleotide analogue are held in place by two arginines.

Important clues about substrate specificity of monophosphates and inhibitory potential of di- and triphosphates can be derived from comparison of crystal structures of e5NT of both human and *E. coli* in complex with adenosine analogue inhibitor. Assuming that ADP adopts a similar binding mode as AMPCP, that is, it binds to human e5NT by replacing a water molecule such that hydrolysis can no longer occur, then this can not only explain the substrate specificity, but also the inhibitory role of di- and triphosphates toward human e5NT. Another idea is that substrate specificity of human e5NT is dictated (at least in part) by domain movements. It was therefore suggested that computational approaches toward structure-based e5NT inhibitors must also make provisions to allow for domain rotations.[92] Accordingly there may even be a possibility for the existence of separate inhibitors of open and closed forms. Inhibitors that bind to both domains may need to "hold" the enzyme in its closed form thus compromising the domain flexibility.

13. STRUCTURAL INSIGHTS INTO HUMAN ECTO-5′-NUCLEOTIDASE INHIBITION: IDENTIFICATION OF A NEW BINDING POCKET FOR STRUCTURE-BASED DESIGN OF e5NT INHIBITORS

Crystal structures of human e5NT in both open and closed conformations have been determined at 1.55–2.00 Å resolution.[92] The crystal structures indicate a dimeric form of e5NT. Each subunit of the e5NT dimer is made up of an N-terminal domain and a C-terminal domain. The larger N-terminal domain contains the metal ion-binding site, whereas the C-terminal domain contains substrate-binding site and dimerization interface. A single α helix, comprising a small hinge region, serves to connect the two domains enabling the enzyme to undergo large domain movements (upto 114°) and thus switch between the open and closed conformations. The active site (containing both metal and substrate-binding sites) is located at the interface of N- and C-terminal domains. The active site in its closed state is largely solvent inaccessible. This extensive active site closure movement is thought to be necessary for catalysis, permitting the binding of substrate and the release of product. It may also explain the substrate (AMP) specificity of e5NT. The dimerization interface involves only the C-terminal domains and is oriented in such a way that the GPI-anchored C-termini are facing the cell membrane.

An interesting binding pocket that may be explored further to design potent inhibitors has been identified. This large pocket (about 210 Å) with bound water molecules is formed entirely from the C-terminal domain. This pocket has a predominantly hydrophobic base and polar or charged sides. To aid in the structure-based development of selective human e5NT inhibitors, the binding mode of the nucleotide-derived inhibitor PSB11552 (**50**) and flavonoid-based inhibitor baicalin (**51**) in complex with human e5NT were determined crystallographically (Fig. 13–14). It was suggested that larger substituents at the C2 carbon atom (next to the phenyl ring of baicalin) may be introduced such that it extends into the hydrophobic base of the pocket. Alternatively, this substituent may contain polar groups that interact with the charged residues lining the side walls of the binding pocket.

14. INHIBITION OF ECTO-NUCLEOTIDASES BY ANTIBODIES

One of the earliest reports of e5NT inhibition by a monoclonal antibody specific for human e5NT was in 1984.[151] This IFH-5N1 antibody belongs to IgG 1 subclass. For purified e5NT, the optimal inhibition was found to be 90%, whereas for the enzyme on lymphoblast, 80%

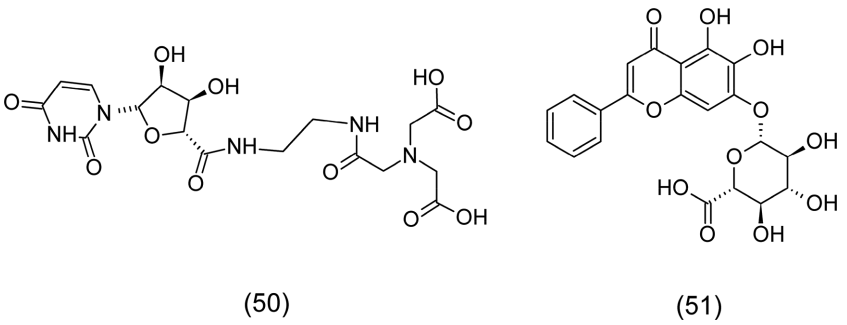


Figure 13. Structures of inhibitors **50**–**51**.

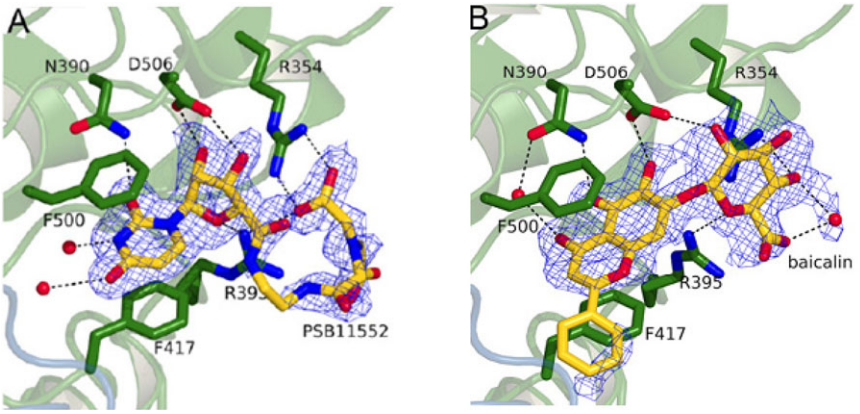


Figure 14. Views of PSB11552 (**50**) (A) and baicalin (**51**) (B) bound within the substrate-binding site of human e5NT.[92] The figure is reproduced with permission from the publisher.

inhibition was observed. Polyclonal antibodies specific for human, rat, and mouse NTPDase3 were developed,[73,152–156] but they were not inhibitory in nature. In 2009, Munkonda et al.[157] reported for the first time IgG2b monoclonal antibodies (hN3-B3$_s$ and hN3-H10$_s$) having selective inhibition against human NTPDase3. Such antibodies not only have applications in immunological techniques, but after proper epitope recognition, these inhibitory antibodies may also help in mapping out structural requirements for NTPDase inhibition. These antibodies may even be developed further to produce other inhibitory antibodies specific against different NTPDase isozymes. In order to characterize the structural elements responsible for NTPDase3 inhibition, the binding site of this monoclonal antibody on the surface of NTPDase3 was identified via epitope mapping using site-directed masking.[158] The epitope was found to be located adjacent to the active site and a noncompetitive mechanism of inhibition that involved steric hindrance was suggested.

An antigenic conserved domain (r82–121) was identified on NTPDase1 from pathogenic parasite *Leishmania (Viannia) braziliensis* promastigote.[159] Polyclonal antibodies anti-LbB1LJ and anti-LbB2LJ were used for this purpose. The ATPase and ADPase activity of the parasite was found to be reduced by 43–79% by the antibody anti-LbB1LJ, whereas the other anti-LbB2LJ antibody exhibited relatively less inhibition of only 18–47%. Moreover, the immune sera were also found to be cytotoxic (upto 67% by anti-LbB1LJ and 33% by anti-LbB2LJ) and reduced the growth of promastigotes in vitro. In 2013, Maia et al.[160] identified an antigenic

domain on NTPDase1 from *Leishmania infantum* as a target for inhibition by antibodies. These studies identify important target sites on NTPDases for design of specific inhibitors.

15. ECTO-NUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASE INHIBITORS

NPPs have attracted interest as potential druggable targets.[27] The use of NPP1 inhibitors has been suggested to alleviate effects of hypophosphatasia,[161,162] chondrocalcinosis,[163] and insulin resistance.[164–166] An over expression of NPP1 has been observed in brain tissues of aged rats.[167] Based on this observation, the inhibition of NPP1 can be proposed for the possible treatment of neurodegenerative diseases.

Expression of NPP2[168] and NPP3[50] has been linked with metastasis and carcinogenesis. Accordingly, NPP2 inhibitors may prove to be helpful in preventing cancer metastasis.[169–171] NPP3 has been suggested to be a potential target for treating allergies.[27] The expression of NPP2, in the brain cortex from human Alzheimer patients, was found to be amplified.[172] In this regard, some inhibitors of NPP2 have been designed to target neurodegenerative diseases.[173] At present only a few NPP isozyme selective inhibitors are known. There is a need to design NPP specific inhibitors to test the hypothesis that these inhibitors can be used as therapeutic agents against cancer and other neurodegenerative diseases.

16. NON-NUCLEOTIDE NPP INHIBITORS NPP INHIBITORS

One of the first NPP2/ATX inhibitor to be identified in vitro was the amino acid L-histidine,[174] a metal chelator that probably acts by scavenging the metal ions necessary for catalysis. Accordingly, other common metal chelating agents, such as EDTA and *o*-phenanthroline, have also exhibited in vitro inhibitory effects on NPP2/ATX.[44] Another major class of NPP2/ATX inhibitors is lipid-based phosphonates,[175] however, the usefulness of this class of compounds as drugs is severely limited because, due to their lipid-like nature, they can also act on LPA receptors,[176,177] therefore, presenting a less than ideal situation.

Iqbal et al.[29] have identified RB2 (**5**) and suramin (**2**) as potent and selective inhibitors of NPP1 and 3. RB2 (**5**) was stronger inhibitor of NPP1 (K_i = 0.52 μM) than NPP3 (K_i = 0.71μM). However, suramin (**2**) exhibited K_i value of 0.26 and 0.04 μM against NPP1 and 3, respectively. In another study, suramin was reported to reduce the level of hydrolysis of p-Nph-5′-TMP by about 46% at 250 μM.[178] RB2 and derivatives of suramin cannot be considered as strictly specific inhibitors of NPPs as they are antagonist of most P2 receptors as well as strong inhibitors of NTPDases.[19,120] Biscoumarin derivatives of type (**52**), where R represents an assortment of variously substituted aromatic and heterocylic rings, were identified as noncompetitive inhibitors of human recombinant NPP1 with K_i and IC_{50} values in the range 50–1000 and 164–1000 μM, respectively.[179]

Phenolic glycosides, such as benzoyl salireposide (**53**) and salireposide (**54**), were shown to have inhibitory potential against human NPP1 with K_i values of 360 and 1000 μM and IC_{50} values of 90 $\pm$ 0.04 μM and 383 $\pm$ 0.03 μM, respectively. Moreover, both compounds were also shown to be nontoxic up to concentrations of 500 μM/mL.[180] A series of 1,3,4-oxa(thia)diazole-2(3*H*)-thiones were identified to be noncompetitive inhibitors of NPP1. Compounds [4-(*t*-butyldimethylsilyloxy)-phenyl]-1,3,4-oxadiazole-2 (3*H*)-thione (**55**) and 5-[4-(*t*-butyldimethylsilyloxy)-phenyl]-1,3,4-thiadiazole-2(3*H*)-thione (**56**) were most potent among the series with IC_{50} values of 66.47 and 368 μM and K_i values of 100 and 360 μM, respectively (Fig. 15, Table III).[181]

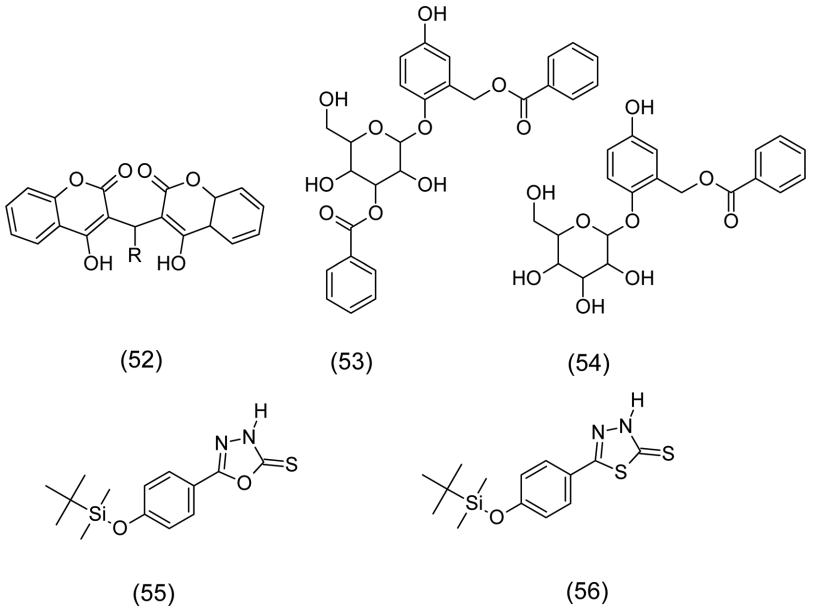


Figure 15. Structures of NPP inhibitors (**52–56**).

17. NUCLEOTIDE ANALOGUES AS NUCLEOTIDE PYROPHOSPHATASE/PHOSPHODIESTERASE INHIBITORS

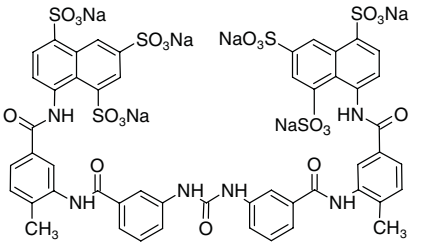
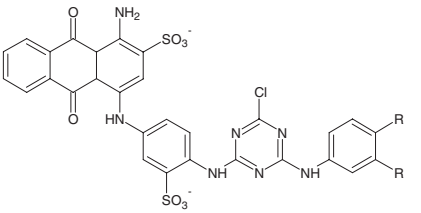
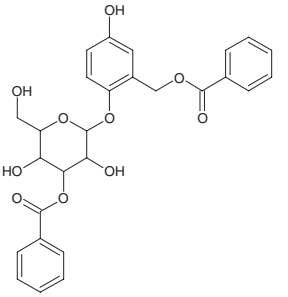
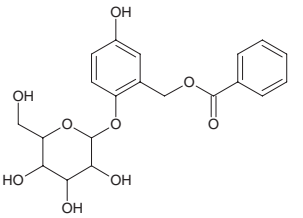
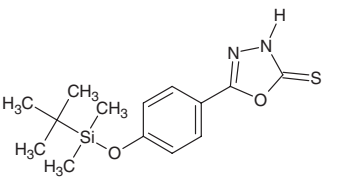
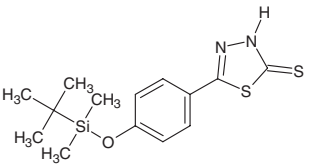
Nucleotide derivatives can be specific and effective inhibitors of NPPs. Some dinucleotide analogues were synthesized and tested as potential NPP inhibitors. It was observed that diadenosine 5′,5″-(boranated)polyphosphonate analogues (**57–61**, Fig. 16) were potent and selective inhibitors of NPPs.[182] Some of these derivatives reduced the hydrolysis of thymidine-5′-monophosphate *p*-nitrophenyl ester (pNP-TMP) by intact osteocarcinoma and colon cancer cells that express some of these enzymes.

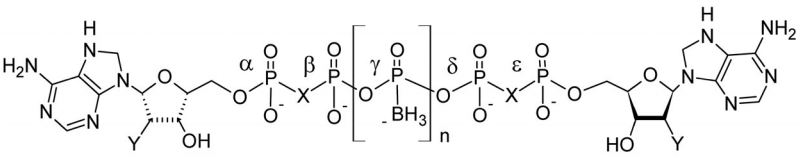
Importantly, these diadenosine 5′,5″-(boranated)polyphosphonate analogues had little effect on major NTPDases (i.e., NTPDase1–3 and NTPDase8), ecto-5′-nucleotidase, and P2Y1 and P2Y2 receptors. These compounds showed insignificant hydrolysis (less than 5%) by human NTPDase1, NTPDase2, NTPDase3, and NTPDase8. Similarly, NPP1, NPP3, and e5NT activities were also significantly unaffected by these compounds, instead all compounds inhibited about 80% of the NPP2-dependent hydrolysis.

18. ALKALINE PHOSPHATASE INHIBITORS

Inhibitors of APs can help map out the exact mechanisms and origins of pathological calcification, thus, defining footsteps that can lead to novel therapies based on inhibition of AP.[183–185] The most well known and commonly used inhibitors of AP are levamisole (**62**, K_i = 16 μM) and theophilline (K_i = 82 μM).[186] Some pyrazole derivatives (**63–66**) were found to be potent and selective inhibitors of TNAP. The most active compound had IC_{50} value of 5 nM.[63] In silico docking studies were carried out to gain insights into the binding mechanisms by docking 3-(2,4-dichlorophenyl)-N-(2-hydroxyethyl)-1H-pyrazole-5-carboxamide with TNAP active site. In the absence of TNAP crystal structure, a homology model was built using PLAP crystal structure as a template (Fig. 17). Among other small molecule inhibitors of AP,

Table III. NPP1 Inhibitory Activity of Different Molecules

Compound code	Structure	NPP1 inhibition (K_i, μM)
2		0.26
5		0.52
53		360
54		1000
55		100
56		360



57; Y=OH X=CH_2 n=1

58; Y=H X=CH_2 n=1

59; Y=OH X=CH_2 n=0

60; Y=H X=CH_2 n=0

61; Y=OH X=0 n=1

Figure 16. Structures of nucleotide based NPP inhibitors (**57**–**61**).

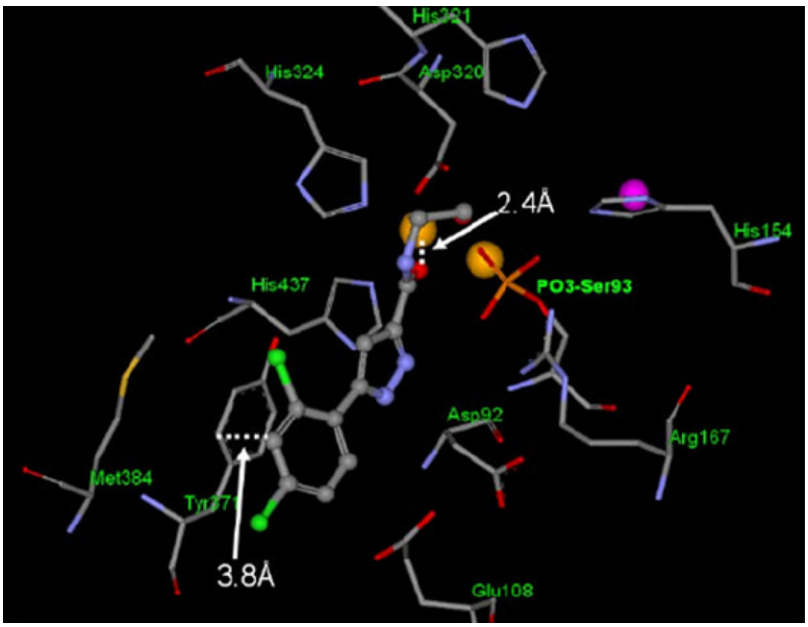


Figure 17. Proposed binding mode of 3-(2,4-dichlorophenyl)-N-(2-hydroxyethyl)-1H-pyrazole-5-carboxamide in the catalytic site of the enzyme.[63] Figure reproduced with permission from the publisher.

thiopheno-imidazo[2,1-b]thiazole derivatives were found to be potent against TNAP with IC_{50} in the range 42 ± 13 μM.[187] Similarly related benzo[*b*]thiophene derivatives were evaluated as TNAP inhibitors, benzothiopheno-tetramisole and -2,3-dehydrotetramisole with K_i values of 85 and 135 μM, respectively.[185]

Sergienko et al.,[188] have identified a number of active compounds via high-throughput screening (HTS) and studied their SAR in detail. Of all compounds screened three major active scaffolds, a biaryl sulfonamide (**67**,**68**), substituted pyrazoles (**69**,**70**), and triazoles (**71**) were established (Fig. 18). Compounds were assayed using a novel luminescent TNAP assay that is superior to the well-established colorimetric assay as it allows a much greater number

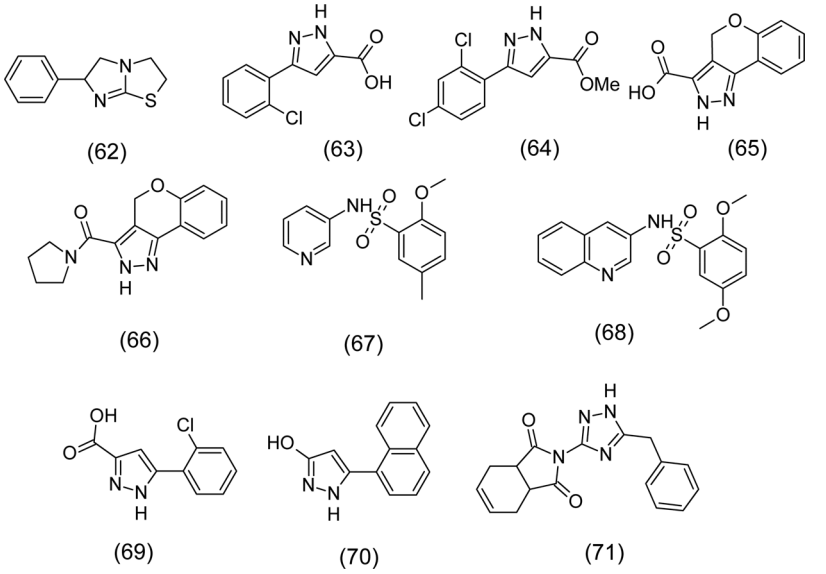


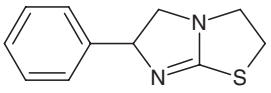
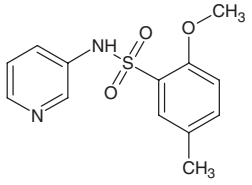
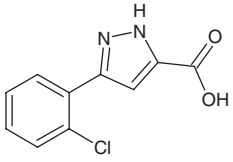
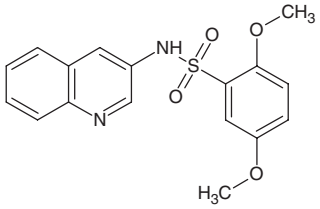
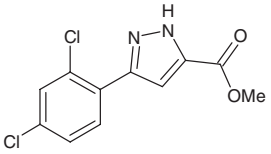
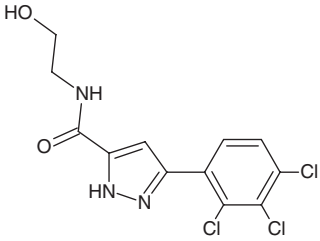
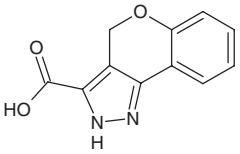
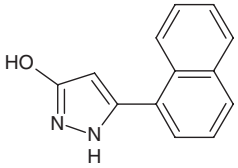
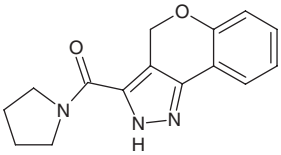
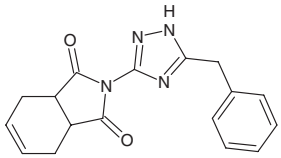
Figure 18. Structures of inhibitors (**62**–**71**).

of compounds to be screened in a time-effective manner. For compounds based on biaryl sulfonamide scaffold, an alkoxy side chain on benzene ring and a pyridine/quinoline ring were found to be mandatory for activity. For pyrazole-based scaffold position four was best left unsubstituted. Among triazoles, thio-phenyl-triazole-based compounds were found to be active (Table IV). Lanier et al.,[64] identified some potent and selective PLAP inhibitors (**72–75**, Fig. 19).

Quite a few metal containing inhibitors of AP have been identified. Sodium vanadate is a well known reversible inhibitor of AP with K_i of 4 μM against rat IAP[189] and K_i of 12 μM against *E. coli* AP.[190] AP is known to be inhibited by insulin in a noncompetitive manner. It was suggested that this AP inhibition may be a consequence of direct interaction between the AP and insulin, either through disulfide cross linkages or by metal chelating potential of insulin.[191] Many vanadium compounds can mimic the action of insulin and are important in glucose regulation mechanisms. The effect seems to be probably due to the inhibition of enzymes responsible for catalyzing phosphate ester displacement in the insulin signaling pathway.[192] Vanadyl (IV) carboxylate complexes $Na_2[VO(Fer)_2(CH_3OH)_2]$ and $Na_2[VO(Cin)_2(CH_3O)_2]$ with ferulic and cinnamic acid, respectively, were prepared and had inhibitory potencies against bovine IAP.[193] Some peroxo–tungsten complexes of type $A_2[W_2O_3(O_2)_4(\text{cystine})]\cdot 4H_2O$ (A = Na, K) with cystine as a bridging coligand were recognized as potent inhibitors of rabbit IAP.[194] Same research group also reported peroxotungstate (VI) complexes of type $[WO(O_2)_2(\text{dipeptide})(H_2O)]\cdot 3H_2O$, where the dipeptide used was glycyl–glycine or glycyl–leucine.[195] Similarly diperoxovanadate (V) complexes of type $A[VO(O_2)_2(\text{peptide})(H_2O)]\cdot H_2O$ (A = Na, K) with the dipeptides auxiliary ligands (glycyl–glycine or glycyl–leucine) were also found to have AP inhibition activity.[196] Such compounds were fairly stable at physiological pH. Recently some polyoxotungstates were assayed as inhibitors of tissue-specific and nonspecific AP and showed promising results with inhibitory potencies in nanomolar range.[197] For tissue specific AP, $Na_{10}[H_2W_{12}O_{42}]\cdot 27H_2O$ was found to be the most potent inhibitor with K_i value of 313 ± 7 nM. Although for TNAP Na_{33} $[H_7P_8W_{48}O_{184}]\cdot 92H_2O$ was most active (K_i = 135 ± 10 nM).

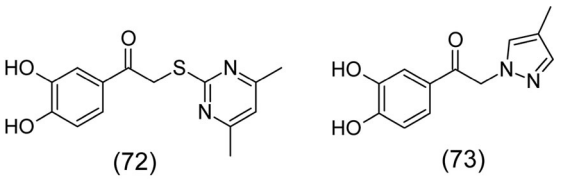
It was observed that several sulfonamides (both antimicrobials and diuretics) also inhibit AP.[198] Also, it was suggested that at least some of the action of these sulfonamides may actually

Table IV. TNAP Inhibitory Activities of Some Molecules

	Structure	TNAP inhibition (IC_{50}, μM)		Structure	TNAP inhibition (IC_{50}, μM)
(62)		16	**(67)**		1.8
(63)		0.98	**(68)**		0.16
(64)		0.5	**(69)**		0.005
(65)		1.3	**(70)**		3.2
(66)		0.5	**(71)**		0.24

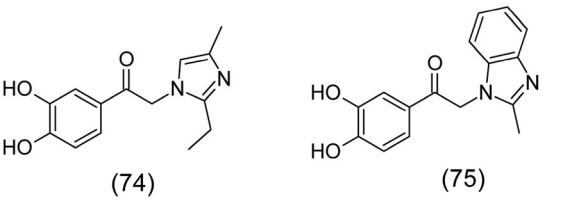
be due to the inhibition of AP rather than CA (carbonic anhydrase that is notably inhibited by sulfonamides). Similarly, acetazolamide (**76**; $K_i = 8.4$ mmol), furosemide (**77**; $K_i = 7.0$ mmol), ethacrynic acid (**78**; $K_i = 2.8$ mmol), and chlorothiazide (**79**) diuretics ($K_i = 0.1$ mmol) were also found to reversibly inhibit AP.[199] Dahl et al.,[200] have identified a series of arylsulfonamides as potent inhibitors of TNAP via high-throughput screening (Fig. 20).

In literature relatively few inhibitors of IAP (including L-amino acids) are known. The first IAP selective inhibitors were reported by al-Rashida et al.[201] These compounds were based on chromone containing sulfonamides (**80**–**83**, Fig. 21). All such compounds had a marked preference for IAP as compared to TNAP. Substitution by halogens at 6-position of



(72) (73)

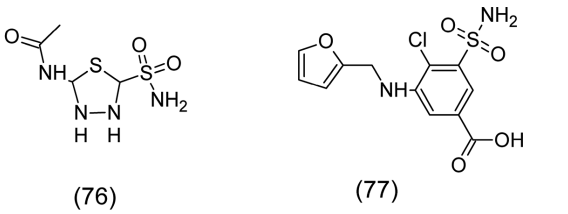
PLAP IC_{50}	2.5 μM	2.1 μM
TNAP IC_{50}	5.6 μM	>100 μM
IAP IC_{50}	49 μM	53.3 μM



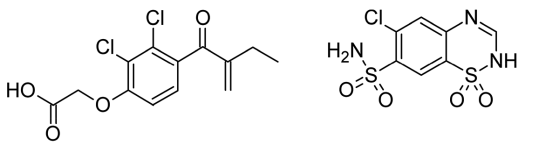
(74) (75)

PLAP IC_{50}	3.7 μM	2.5 μM
TNAP IC_{50}	>100 μM	20 μM
IAP IC_{50}	>100 μM	>100 μM

Figure 19. Structures of AP inhibitors (**72**–**75**).



(76) (77)



(78) (79)

Figure 20. Structures of AP inhibitors (**76**–**79**).

the chromone ring was found to significantly enhance the IAP inhibition activity. A comparison of IAP and TNAP inhibition activities of some of the representative compounds from the series is given in Table V. Interestingly, all these compounds were also potent inhibitors of CA.[202]

Cimetidine (**84**), a histamine H2-receptor antagonist (clinically used for the treatment of gastrointestinal diseases) was found to have inhibitory effect on mouse renal AP activity with K_i and IC_{50} values of 0.5 and 0.52 mM, respectively.[203] It was found that methylenebisphosphonic acid moieties integrated on to calix4arene scaffold were able to inhibit bovine IAP. Inhibition constant K_i of calix4arene bis(methylenebisphosphonic) acid (**85**) at pH 9.0 against bovine IAP was found to be 0.38 μM.[204] Later on, a series of thioureido derivatives of methylenebisphosphonic acid was prepared and found to inhibit bovine IAP and human PLAP. Molecular docking studies of (3-phenylthioureido)methylenebisphosphonate, a potent compound among the series, revealed that the increased inhibitory potential was due to the fixation of phenyl

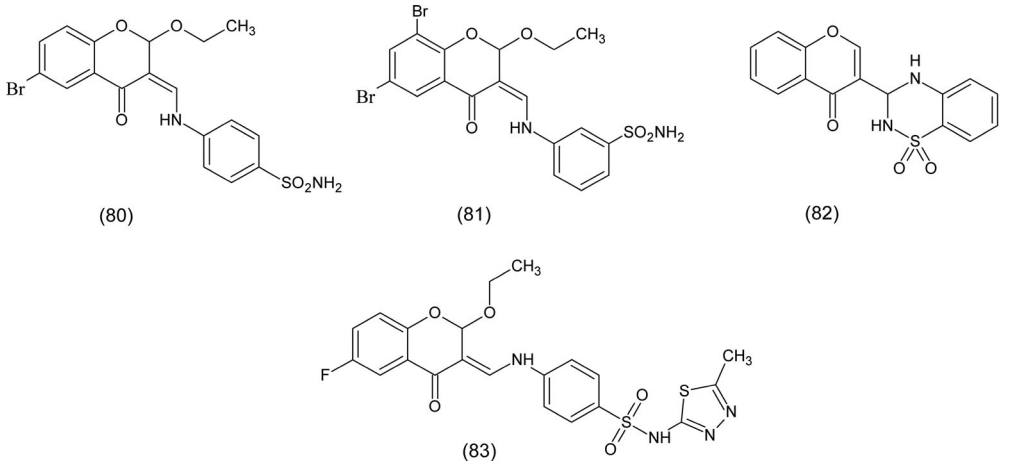


Figure 21. Structures of selective IALP inhibitors (**80**–**83**).

Table V. Chromone-Based Sulfonamides as IALP Selective Inhibitors

	$K_i \pm$ SEM (μM)	
Compound	IAP (bovine intestine)	TNAP (bovine kidney)
80	0.41 ± 0.002	48 ± 13
81	0.8 ± 0.001	26 ± 9.9
82	0.049 ± 0.007	27 ± 5.8
83	0.01 ± 0.001	21 ± 7.8

substituent in the active site of enzyme.[205] Okadaic acid (**86**) is a metabolite responsible for shellfish poisoning in humans and is also known to be a protein phosphatase inhibitor. In keeping with its protein phosphatase activity, its AP inhibition potency was also checked against APs from diverse sources and was found to be a noncompetitive inhibitor with K_i value of 360 nM against *E. coli* AP, K_i of 2.05 μM against human PLAP, and K_i of 3.15 μM against bovine IAP. AP inhibition by okadaic acid is suggestive of its role in regulation of phosphorylation that is relevant to maintenance of homeostasis.[206]

Imipenem (**87**; Fig. 22) is an intravenous β-lactam antibiotic (the only β-lactam antibiotic among other antibiotics tested, such as ertapenem, meropenem, ampicillin, or penicillin G), which was found to have inhibitory potential against AP isolated from cold-adapted Vibrio strain (VAP) with IC_{50} of 44 ± 4 μM. Interestingly the same compound had no inhibitory potential against AP from *E. coli* and shrimp.[207] A series of 4,5-disubstituted-2,4-dihydro-3H-1,2,4-triazole-3-thiones were also found to be active against AP. The IC_{50} value of most active compound was found to be 44 ± 1 nM.[208]

19. SUMMARY

Surface located ecto-nucleotidases, that is, nucleoside triphosphate diphosphohydrolases (NTPDases), nucleotide pyrophosphatase/phosphodiesterases (NPPs), alkaline phosphatases (APs) and ecto-5′-nucleotidase (e5NT) are important class of enzymes responsible for

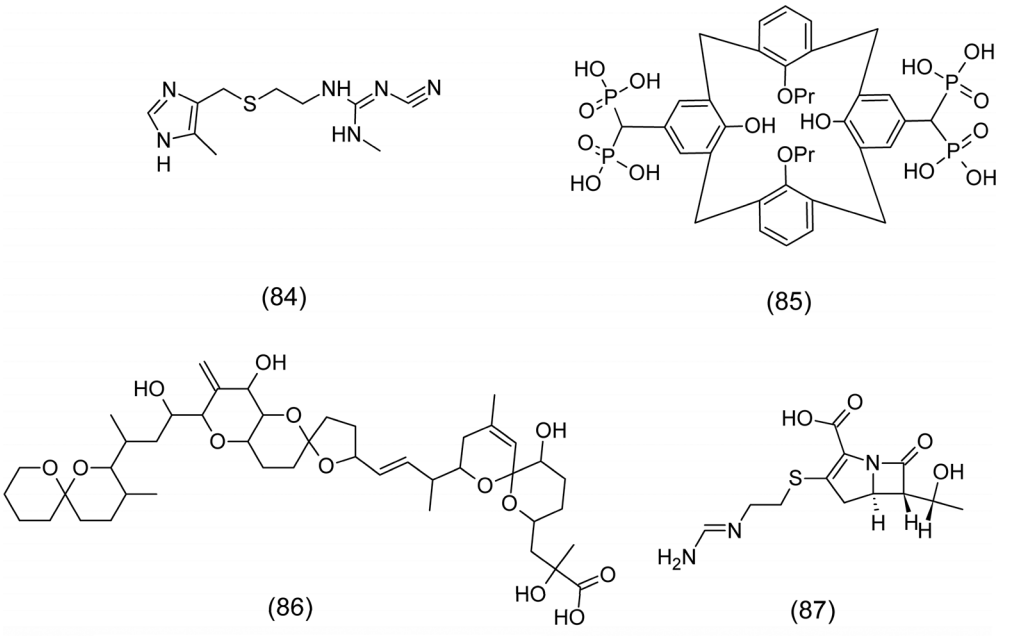


Figure 22. Structures of AP inhibitors (**84**–**87**).

controlling and regulating levels of extracellular nucleotides. These extracellular nucleotides are important signaling molecule between different cell types and are responsible for normal cell functioning. Many physiological disorders including patho-physiological responses and cell proliferation have been linked with overexpression of these ecto-enzymes. Inhibition of these ecto-nucleotidases presents an attractive drug target for therapeutic purposes. Accordingly, inhibitors of ecto-nucleotidases have many applications as anticancer agents, immunomodulatory agent for the treatment of cardiovascular and central nervous system disorders.

One of the greatest challenges encountered in the development of these inhibitors is their nonselectivity and there is a dire need to come up with novel, potent, and selective ecto-nucleotidase inhibitors. Ideally such inhibitors should have little or no effect on P1 and P2 receptors, but one frequently finds in literature non-subtype selective inhibitors. Recently elaborated crystal structures of ecto-nucleotidases in complex with their natural substrate or inhibitor molecule can provide important leads about selectivity of these enzymes and have been discussed here in detail. Most recent and significant advances in the field of NTPDase, NPP, AP, and e5NT inhibitors has been discussed in detail in anticipation of providing prolific leads and relevant background for research groups interested in synthesis of selective ecto-nucleotidase inhibitors.

20. ABBREVIATIONS

ACR	apyrase-conserved region
AOPCP	5′-[α,β-methylene]diphosphate(β-methylene)-ADP
AP/s	alkaline phosphatase/s
ATX	autotaxin
CA	carbonic anhydrase
e5NT	ecto-5′-nucleotidase

GCAP	germ cell AP
GPC	glycerophosphorylcholine
GPCR	G-protein-coupled receptor
GPI	glycosyl-phosphatidylinositol
IAP	intestinal AP
LPA	lysophosphatidic acid
LPC	lysophosphatidylcholine
LysoPLD	lysophospholipase D
NDP	nucleoside diphosphate
NPP/s	nucleotide pyrophosphatase/phosphodiesterase/s
NTP	nucleoside triphosphate
NTPDase/s	nucleoside triphosphate diphosphohydrolase/s
PC-1	plasma cell glycoprotein 1
P_i	inorganic phosphate
PI-PLC	phosphatidylinositol-specific phospholipase C
PLAP	placental AP
POM/s	polyoxometalate/s
PPADS	pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid
PP_i	pyrophosphate
RB2	reactive blue 2
SAR	structure–activity relationship
SMBD	somatomedin-B-like domain
TNAP	tissue nonspecific alkaline phosphatase

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Mariya al-Rashida *completed Ph.D. in chemistry from the Institute of Chemistry, University of the Punjab, Lahore, Pakistan in 2011. Research interests include synthesis of enzyme inhibitors (carbonic anhydrase, alkaline phosphatases, and ecto-nucleotidases). Currently working as an Assistant Professor in the Department of Chemistry, Forman Christian College (A Chartered University), Lahore, Pakistan.*

Jamshed Iqbal *received a Ph. D. degree in pharmaceutical chemistry at the University of Bonn in 2005. Dr. Iqbal then continued as a postdoc at the Pharmaceutical Chemistry Department at the University of Bonn till 2008. From 2009 to 2010, he did postdoc at Dalian Institute of Chemical Physics, Chinese Academy of Sciences, China. Since 2010, he has been an Associate Professor of Pharmaceutical/Medicinal Chemistry at the COMSATS Institute of Information Technology, Abbottabad, Pakistan. His current research focuses on the medicinal chemistry and drug design.*