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ENTPDase inhibitors: therapeutic potential in infectious, inflammatory, and neuroinflammatory diseases

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Abstract

Ectonucleoside triphosphate diphosphohydrolases (ENTPDases), members of the cluster of differentiation 39 (CD39) family, are key regulators of purinergic signaling through the hydrolysis of tri and diphosphate nucleotides. These enzymes are expressed on the cell surface, extracellular environment, or within intracellular organelles such as the Golgi apparatus. ENTPDases play critical roles in modulating immune responses, inflammation, and neuroinflammation by controlling extracellular nucleotide availability in mammals. Moreover, they contribute to adenosine-mediated signaling in cooperation with 5'-nucleotidases (CD73). Pathogenic microorganisms also express ENTPDases, manipulating host purinergic signaling, suppressing adenosine triphosphate (ATP)-driven inflammation, and promoting immune evasion via increased adenosine production. Pathogenic parasites also express ENTPDases, manipulating host purinergic signaling, suppressing ATP-driven inflammation, and promoting immune evasion via increased adenosine production. Given their involvement in infection and inflammatory diseases, ENTPDases have emerged as promising pharmacological targets. This review comprehensively analyzes the ENTPDases from mammals and pathogenic parasites, emphasizing their role in purinergic signaling and their potential as therapeutic targets. While ENTPDase inhibitors hold promise for modulating inflammation and infection, their clinical translation faces challenges, including selectivity, off-target effects, and systemic alterations in purinergic homeostasis. Addressing these concerns through targeted drug delivery, allosteric modulation, and improved inhibitor specificity is crucial for therapeutic advancements.

Keywords

ENTPDase, inhibitors, purinergic signaling, infection, inflammation, neuroinflammation, immune modulation

Introduction

Prof. Burnsstock's group first described purinergic signaling, which regulates various physiological and pathological processes. Among the key regulators of this system are the ectonucleoside triphosphate diphosphohydrolases (ENTPDases), which catalyse the hydrolysis of adenosine triphosphate (ATP) and adenosine diphosphate (ADP) to their diphosphate and monophosphate derivatives, ultimately contributing to the production of adenosine (ADO) through the sequential action of 5'-nucleotidases [cluster of differentiation 73 (CD73)]. By modulating the availability of extracellular nucleotides, ENTPDases influence immune responses, inflammation, and neuroinflammation. Given their central role in purinergic signalling, many investigators have highlighted the applicability of ENTPDase inhibitors to modulate biological processes mediated by purinergic signaling. This review aims to explore the functional significance of ENTPDases in mammalian hosts and pathogenic organisms, addressing their relevance in disease progression and immune regulation. It will also highlight the potential of ENTPDase inhibitors in infectious and non-infectious diseases such as cancer, diabetes, and neuroinflammation. The review will also discuss the challenges associated with developing these inhibitors, considering aspects such as specificity, selectivity of the inhibitors, and purinergic signaling.

Mammalian ENTPDases and their physiological functions

Of the eight NTPDases encoded in the mammalian genome, only four are located on the cell surface, acting as typical ectonucleotidases: NTPDase1 (CD39), NTPDase2 (CD39L1), NTPDase3 (CD39L3), and NTPDase8 (liver canalicular ecto-ATPase) [1]. These enzymes are integral membrane glycoproteins that have two transmembrane domains—one located at the N-terminal end and another at the C-terminal end—and flanking a large extracellular loop that contains the catalytic domain, characterized by five conserved apyrase regions (ACRs). In contrast, NTPDases 4–7 are found intracellular and regulate nucleotide/nucleoside levels within organelles. Secreted forms of NTPDase5 and NTPDase6 have also been reported [2].

The transmembrane domains of ENTPDases are essential for catalytic activity, substrate specificity, and enzyme oligomerization. They undergo conformational changes during nucleotide binding and hydrolysis, influencing the catalytic domain's structure [3]. These domains also mediate the formation of enzyme complexes, ranging from dimers to tetramers, which often display higher catalytic efficiency than monomeric forms [4]. The oligomerization state varies among isoforms, and different forms may coexist within the plasma membrane [2].

Essentially, ENTPDases are widely distributed across tissues, often with overlapping expression patterns. NTPDase1 is found on B cells and various immune cells, including natural killer cells, monocytes, dendritic cells, macrophages (such as Kupffer cells and microglia), and activated T-cell subsets [1]. NTPDase1 is co-expressed with NTPDase2 in blood vessels. Still, each isoform predominates in distinct locations: NTPDase1 is mainly found in endothelial and smooth muscle cells, whereas NTPDase2 is present in adventitial cells, pericytes, and subendocardial space [5]. NTPDase2 is also found in portal fibroblasts, taste buds, muscles, testes, brain, and activated hepatic stellate cells [4]. NTPDase3 is co-expressed with NTPDase2 in salivary glands, gastrointestinal epithelium, and enteric nervous system cells, and with NTPDase1 in human airways [5]. Additionally, NTPDase3 is found in neurons producing the neuropeptide hypocretin-1/orexin-A which regulates feeding and the sleep-wake cycle, as well as in sensory neurons, kidney epithelium, reproductive tissues, and pancreatic Langerhans islets [2, 3]. NTPDase8, the last ectonucleotidase to be characterized, has a more restricted distribution, predominantly expressed in bile canaliculi, intestines, and kidneys [6].

Table 1 summarizes the tissue distribution, biochemical properties, and physiological roles of NTPDases 1, 2, 3, and 8. This review will focus on these NTPDase isoforms that are ecto-localized (ENTPDase) in the membrane of cells or secreted and seem to be associated with infectious diseases, the immune system, and the control of inflammation.

Table 1. Main distribution, biochemical properties, and physiological roles of mammalian E-NTPDases 1, 2, 3, and 8

ENTPDase	Main distribution	Biochemical properties	Main physiological functions	References
NTPDase1/CD39	Immune system cells (monocyte/macrophages, microglia, T and B cells, neutrophils, leukocytes), endothelial and smooth muscle cells	There is no clear preference between Ca^{2+} and Mg^{2+}. Lower activity at acidic pH. Hydrolyzes ATP and ADP about equally well. Km for ATP: 17 μM	Prevention of thrombosis. Modulation of vascular tone. Modulation of vascular inflammation and immune responses. Neuroprotection and cardioprotection.	[2, 5, 7, 11, 12, 28]
NTPDase2/CD39L1	Cells of the vascular adventitia, microvascular pericytes, subendocardial space, portal fibroblasts, astrocytes, taste buds, and neural precursor cells	There is no clear preference between Ca^{2+} and Mg^{2+}. High preference for ATP over ADP (preferential ecto-ATPase). Km for ATP: 70 μM	Regulation of vascular hemostasis. Involvement in controlling neurogenesis and neural differentiation. Role in taste information transmission from taste buds to gustatory nerves. Modulation of portal fibroblast proliferation.	[2, 5, 7, 14–17, 28]
NTPDase3/CD39L3	Hypocretinergic neurons, sensory neurons, islets of Langerhans, and renal and reproductive epithelia	Preference for Ca^{2+} and ATP over ADP. Km for ATP: 75 μM	Modulation of purinergic neurotransmission, regulation of nociceptive circuits, involvement in the development of circadian rhythms in the hypothalamus, and role in energy metabolism.	[2, 5, 7, 19, 20]
NTPDase8/Liver canalicular ecto-ATPase	Bile canaliculi, intestinal, and renal epithelia	Preference for Ca^{2+} and ATP over ADP. Km for ATP: 81 μM	Regulation of the inflammatory response in intestinal diseases.	[2, 5, 8, 22]

ENTPDase: ectonucleoside triphosphate diphosphohydrolase; ADP: adenosine diphosphate; ATP: adenosine triphosphate; CD39: cluster of differentiation 39

Biochemical properties

The biochemical characterization of ENTPDases is crucial in developing inhibitors that can be used in pathophysiological conditions. One of the main challenges in the characterization of ENTPDases is the loss of enzymatic activity in the presence of detergents, destabilizing oligomeric complexes, leading to the formation of monomers with significantly reduced catalytic activity. To address this issue, researchers performed transient transfection of COS-7 cells, allowing the analysis of ENTPDases in their native, membrane-bound forms [2–4, 7, 8]. Here, we will focus on the biochemical characterization data of human ENTPDases.

ENTPDases 1, 2, 3, and 8 differ in substrate specificity, pH sensitivity, and preference for Ca^{2+} or Mg^{2+} as cofactors. They all hydrolyze nucleoside triphosphates such as ATP and UTP, but their hydrolysis rates for nucleoside diphosphates vary significantly among subtypes [3, 7]. NTPDase1 hydrolyzes ATP and ADP equally well, converting ATP directly to adenosine monophosphate (AMP) with minimal ADP accumulation [2]. In contrast, NTPDase2 strongly prefers ATP, leading to sustained ADP accumulation before its conversion to AMP. NTPDases 3 and 8 favor ATP over ADP, allowing transient ADP accumulation. These enzymes follow Michaelis-Menten kinetics, with Km values for ATP of 17, 70, 75, and 81 μM for NTPDases 1, 2, 3, and 8, respectively, indicating that NTPDase1 has the highest ATP affinity [2, 7, 8].

Their cofactor preferences also vary. While NTPDases 1 and 2 show no clear preference between Ca^{2+} and Mg^{2+}, NTPDases 3 and 8 demonstrate a preference for Ca^{2+} [7, 8]. All enzymes exhibit reduced or abolished activity in the presence of ethylenediaminetetraacetic acid (EDTA) and EGTA and hydrolyze uracil nucleotides more efficiently in the presence of Ca^{2+}. They are also highly active at physiological pH, though with some differences: NTPDase1 functions within a more restricted range, predominantly active under neutral to slightly alkaline conditions [7].

Structurally, ENTPDases share about 40% sequence identity among family members and consist of approximately 500 amino acid (aa) residues, with the molecular mass of glycosylated monomers ranging from 70 kDa to 80 kDa [2]. All ENTPDase family members undergo *N*-glycosylation, essential for proper folding, stability, membrane targeting, and enzymatic activity [4]. However, the specific glycan structures of

these enzymes remain unknown, and research in this area is still limited. The predicted numbers of *N*-glycosylation sites are 6, 6, 7, and 8 for NTPDases 1, 2, 3, and 8, respectively [2].

There are some splicing variants for human NTPDases 2 and 3. NTPDase2 has three variants: NTPDase2α (495 aa), NTPDase2β (472 aa), and NTPDase2γ (450 aa) [9]. Only NTPDase2α is active, as the sequence missing in NTPDase2β and NTPDase2γ variants contains a conserved cysteine residue (C399), essential for disulfide bond formation. Although NTPDase2 is still called CD39L1, the original sequence of human CD39L1 corresponds to the inactive NTPDase2β. For NTPDase3, there are two variants: NTPDase3α (529 aa) and NTPDase3β (459 aa) [10]. The shorter variant, NTPDase3β, lacks the C-terminal end containing the ACR5 domain and does not exhibit measurable activity. However, it may still play an important modulatory role in the activity of NTPDase3α.

Understanding the molecular mechanisms involved in nucleotide hydrolysis and the development of inhibitors has advanced significantly following the resolution of the crystal structure of the extracellular domain of *Rattus norvegicus* NTPDase2 in 2008 [11]. The crystal structures with substrate analogs reveal two structural domains, each with an extended RNase H fold, a pattern also found in other members of the actin structural superfamily. The two domains are characterized by a central mixed β-sheet and a peripheral layer of predominantly alpha helices [1]. The binding site for the divalent metal ion and di- and triphosphate nucleosides is located in the cleft between the two domains, allowing precise interactions between the substrates and residues of the ACR regions. However, to date, no high-resolution structures, including the transmembrane domains, are available, and they can play a crucial role in modulating the rotational movements of the catalytic domains.

A general view of ENTPDases in pathological conditions

Ecto-NTPDases play a key role in purinergic signaling due to their ability to hydrolyze extracellular di- and triphosphate nucleotides, which act as ligands for P2 receptors involved in pro-inflammatory responses, promoting the release of cytokines and the activation of immune cells [12]. By tightly regulating the availability of nucleotides, ENTPDases act as key modulators of immune responses, preventing excessive inflammation and tissue damage. The activity of ENTPDases is complemented by the cooperative action of 5'-nucleotidases, which hydrolyze AMP to produce ADO, the primary ligand for P1 receptors responsible for anti-inflammatory effects by limiting excessive immune responses and promoting tissue repair. The precise balance between P2 and P1 receptor activation highlights the essential role of ENTPDases in immune homeostasis and inflammatory and neuroinflammatory diseases.

Dysregulation of ENTPDase activity is associated with various pathologies, particularly those involving vascular and immune dysfunction. In endothelial cells, NTPDase1 plays a critical role in modulating vascular inflammation and thrombosis by degrading ADP, the major recruiter for forming occlusive thrombi by activating $P2Y_1$ and $P2Y_{12}$ receptors on platelets [13]. This function is essential for maintaining vascular integrity, as an imbalance in NTPDase1 activity can lead to excessive thrombus formation and haemorrhagic tendencies. Additionally, this isoform controls vasorelaxation and is the primary enzyme responsible for regulating nucleotide metabolism on the surface of vascular smooth muscle cells, playing an essential role in the local modulation of vascular tone [2].

Like NTPDase1, NTPDase2 is also expressed in blood vessels, predominantly in vascular adventitial cells, microvascular pericytes, and the subendocardial space [14]. However, it acts predominantly as an ATPase, modulating nucleotide-mediated signaling in vascular and neural tissues. By converting ATP to ADP, NTPDase2 facilitates platelet aggregation and thrombus formation, playing an opposite role to NTPDase1 in regulating hemostasis. Thus, NTPDases 1 and 2 coordinate the regulation of pro- and anti-thrombotic responses, with NTPDase1 inhibiting platelet aggregation and recruitment in intact vessels, whereas expression of NTPDase2 promotes microthrombus formation in areas of extravasation after vascular injury.

In addition, studies show that NTPDase1 plays a critical role in creating an immunosuppressive environment in the tumor microenvironment that promotes cancer growth and progression. The

conversion of ATP to ADO through the combined action of CD39 and CD73 favors the suppression of effector T cell activation, the promotion of regulatory T cells (Tregs), and the polarization of macrophages toward the M2 tumor phenotype and angiogenesis. High expression levels of these enzymes have been found in various tumors such as colorectal cancer, pancreatic cancer, and chronic lymphocytic leukemia. CD39 also exists in tumor exosomes and enhances the immunosuppressive effect by activating A2A and A2B receptors [15].

NTPDase2 also regulates ATP levels in the taste buds, preventing desensitization of P2X2 and P2X3 receptors and ensuring proper taste transmission [16]. Its deletion impairs this signaling. In the brain, it is present in germinal zones, suggesting a role in cell proliferation and neuronal differentiation [17]. In liver physiology, its expression by portal fibroblasts can inhibit the activation of P2Y receptor, limiting biliary epithelial proliferation, a process relevant to preventing cholangiopathies [18].

NTPDase3 is prominent in energy metabolism. Its global deletion in mice is associated with resistance to diet-induced obesity and obesity-associated glucose intolerance. This result was not caused by the expression of NTPDase3 in pancreatic beta cells, but probably by metabolic changes in adipocytes [19]. This alteration results from increased expression of uncoupling protein 1 (UCP-1) in brown adipose tissue and increased "browning" in inguinal white adipose tissue with positive regulation of UCP-1 and genes related to thermogenesis. In addition, NTPDase3 may be involved in the regulation of homeostatic functions, since its expression in the hypothalamus coincides with the onset of eating and sleeping rhythms in young rats [20], and there is evidence for its involvement in nociceptive circuits, modulating somatosensory purinergic transmission via P2X and P2Y receptors [21].

Using a model of colitis induced by DSS (dextran sulfate sodium), NTPDase8 was essential for nucleotide hydrolysis in the colon, preventing excessive activation of the pro-inflammatory $P2Y_6$ receptor [22]. Mice deficient in NTPDase8 showed more intense inflammation, more significant histological damage, and higher expression of pro-inflammatory cytokines compared to wild-type mice, while intra-rectal injection of the enzyme conferred complete protection against colitis. Daily administration of a $P2Y_6$ antagonist also reduced inflammation in a dose-dependent manner, and bone marrow transplantation from normal mice to $P2Y_6$-deficient mice also demonstrated a protective effect against inflammation.

ENTPDases in neuroinflammation and neuroinflammatory disorders

Inflammation is a complex signaling cascade in which the innate immune system responds to pathogenic or sterile insults. These processes are mediated by pattern recognition receptors (PRRs), which interact with pathogen-associated molecular patterns (PAMPs) and environment-derived danger-associated molecular patterns (DAMPs) from the environment. In the central nervous system (CNS), microglia and astrocytes are the primary mediators of neuroinflammatory responses, expressing PRRs such as Toll-like receptors (TLRs) that sense extracellular danger signals [23]. During inflammatory responses, inflammasomes are assembled, activating the pro-inflammatory caspase-1, promoting the maturation and release of the cytokines interleukin-1β (IL-1β) and IL-18. In addition, caspase-1 activity contributes to the release of extracellular nucleotides such as ATP and ADP, which amplify inflammatory signaling through purinergic receptors [24].

Neuroinflammation is associated with several CNS disorders, particularly those with a neurodegenerative component, such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), and Huntington's disease (HD) [25]. NTPDases play a critical role in modulating neuroinflammatory pathways by regulating the availability of extracellular nucleotides, thereby influencing microglial activation and neuronal survival [26]. NTPDase1 is highly expressed in microglia, where it regulates extracellular nucleotide concentrations, limits excessive activation of pro-inflammatory P2 receptors, and helps reduce neuroinflammation [18]. In contrast, NTPDase2 is predominantly expressed in astrocytes in rats, which may contribute to the modulation of inflammatory responses within the CNS [27].

In the experimental autoimmune encephalomyelitis (EAE) animal model used to study MS, overexpression of NTPDase1 is associated with microglial polarization toward an anti-inflammatory

phenotype, suggesting a possible role in promoting neuroprotection [28]. Studies also show that MS is involved in the release of ADP, which binds to the $P2Y_{13}$ receptor and activates neuronal survival pathways such as the ERK1/2 pathway [29]. During the progression of EAE, the expression of ENTPDase2 is reduced, limiting the activation of these receptors and favoring the inflammatory response mediated by Th1 and Th17 cells, promoting demyelination and axonal damage [30].

In AD, the accumulation of ATP in the extracellular environment can lead to sustained activation of P2X7 receptors on microglia, promoting a chronic inflammatory state and exacerbating neurodegeneration. NTPDase1 can counteract this process by hydrolyzing ATP, potentially reducing P2X7 receptor overstimulation and subsequent neuronal damage. Data also indicate that in AD, there is increased expression of NTPDase2 and decreased expression of NTPDase3 at different stages of the disease, suggesting an imbalance in purinergic metabolism associated with synaptic impairment and cognitive loss [31]. These enzymes have also been implicated in the modulation of synaptic plasticity and memory, functions that are severely impaired in AD [32].

Although there are few studies on ENTPDases in HD, there is evidence that alterations in ADO metabolism may contribute to pathogenesis. CD73 shows reduced expression, with an imbalance in extracellular levels of hypoxanthine and inosine, possibly associated with hyperactivity of adenosine deaminase (ADA) and a reduction in purine nucleoside phosphorylase [33]. The action of ENTPDases in conjunction with CD73, which provides AMP for the production of ADO, may directly influence the inflammatory and neurodegenerative mechanisms, which are still poorly understood in this disease. Given this scenario, modulating the activity of ENTPDases represents a promising strategy to control inflammation in neurodegenerative and autoimmune diseases.

Several reviews have elaborated on the role of these enzymes in different pathological conditions, including cancer, inflammatory bowel disease, cardiovascular, metabolic, autoimmune, and neurodegenerative diseases, highlighting ENTPDases as therapeutic targets [15, 34, 35]. However, there are still gaps in our understanding of the mechanisms that regulate the expression, localization, and activity of ENTPDases in different tissues and disease stages. Identifying selective modulators—inhibitors or activators—is essential to developing more effective and specific therapies. Further functional and pharmacological studies on ENTPDases are critical to translating their biological potential into concrete clinical applications.

ENTPDases of pathogens and their relationships with infectious diseases

ENTPDases have been identified on the cell surfaces of several pathogenic parasites, including *Trichomonas vaginalis*, *Trypanosoma* spp., *Leishmania* spp., *Schistosoma* spp., and *Toxoplasma gondii*. These enzymes can help purine scavenging for parasite development because they depend on the purine salvage pathway. In addition, they can modulate the host's purinergic signaling, protecting the parasites against immune responses and host platelet activation, contributing to their virulence and infectivity [36–39]. The pathogen ENTPDases exhibit different isoforms and distinct substrate specificities. These enzymes can generally hydrolyze ATP and ADP to AMP, and the parasites have the 5'-NT-generating ADO. This enzymatic system can modulate the host's inflammatory response by preventing P2 receptor activation and stimulating P1 receptors, triggering an anti-inflammatory response [40, 41]. Table 2 summarizes examples of parasite ENTPDases and purinergic signaling that favor pathogen infection.

In the protozoan *T. gondii*, the causative agent of toxoplasmosis, NTPDases, referred to as TgNTPDases 1 and 2, are secreted into the lumen of the parasitophorous vacuole (PV). TgNTPDase 1 exhibits low expression in the bradyzoite form of the parasite, whereas its expression is significantly elevated in the tachyzoite form, which is characterized by active replication [41]. TgNTPDase 1 and 2 isoforms are expressed in tachyzoites and can hydrolyze ATP and ADP. Enzymatic inactivation using sodium azide has been shown to enhance the host's immune response, reducing parasite virulence [42]. During *T. gondii* infection, the P2X7 receptor, a component of the purinergic signaling pathway, exerts protective effects through parasite control. However, modulation of the inflammatory response by *T. gondii* NTPDases enables the parasite to reach the CNS, where ADO generated by the host 5'-ectonucleotidase activity

Table 2. Examples of pathogen E-NTPDases in infectious diseases and the purinergic signaling associated with each disease

Pathogen	ENTPDase	Purinergic signaling	Infectious disease	References
Toxoplasma gondii	TgNTPDase1, TgNTPDase2	Reduces P2X7 activation, promotes P1-A2A activation in the host	Toxoplasmosis	[41–46]
Trichomonas vaginalis	TvNTPDase1, TvNTPDase2	Activates P1-A2A receptor in the host	Trichomoniasis	[44, 45]
Leishmania spp.	LdNTPDase1, LdNTPDase2	Reduces P2X7 activation and promotes P1-A2 receptor activation in the host	Leishmaniasis	[36, 49, 50]
Trypanosoma cruzi	TcNTPDase1	Activates P1-A2 receptors in the host	Chagas disease	[36, 52–55]
Plasmodium falciparum	E-NTPDase (PF3D7_1431800)	Activates P1 receptors in the host, expresses PfENT1 transporter and G protein-coupled receptors (GPCRs)	Malaria	[55–58]
Schistosoma mansoni	SmATPDase1, SmATPDase2	Increased expression of NTPDase2, NTPDase3, and $P2Y_1$ receptors; reduced P2X7 receptor in the host	Schistosomiasis	[37, 60–63]

ENTPDase: ectonucleoside triphosphate diphosphohydrolase

contributes to the dissemination and persistence of the parasite. This leads to the most severe form of the disease, associated with significant neurological impairments [43].

In the protozoan *T. vaginalis*, the pathogen responsible for trichomoniasis, NTPDases (TvNTPDases 1 and 2) and ecto-5'-nucleotidase (E-5'-NT) expressed on the cell surface play roles in parasite-host interactions and extracellular nucleotide metabolism through the purine salvage pathway [44]. Purinergic signaling modulation occurs via the joint action of NTPDase and E-5'-NT, which hydrolyze nucleotides and increase ADO levels. ADO activates the A2A (P1) receptor on neutrophils, reducing pro-inflammatory signals, including nitric oxide (NO) production, thereby affecting neutrophil responses to infections [45]. The pathogenic mechanisms of *T. vaginalis* are multifactorial, with immune response modulation critical for infection maintenance, highlighting ectonucleotidases like NTPDase as essential targets for combating the parasite [46].

In pathogenic trypanosomatids such as *Leishmania* and *T. cruzi*, causative agents of Leishmaniasis and Chagas disease, respectively, ectonucleotidases, including ENTPDases, play essential roles in infection and virulence. They are crucial for nutrition through purine and nucleotide salvage pathways [36, 47, 48]. Genes encoding NTPDases have been identified in all sequenced *Leishmania* species available in NCBI and TriTrypDB protein databases, including *L. infantum*, *L. braziliensis*, *L. donovani*, *L. mexicana*, *L. major*, *L. amazonensis*, and *L. tropica*. Additionally, 5'-nucleotidase activity on the plasma membrane has been reported in trypanosomatids such as *L. tropica*, *L. amazonensis*, *L. braziliensis*, *L. major*, *L. infantum*, *Trypanosoma cruzi*, *T. rangeli*, and *T. brucei* [49].

The infection by *Leishmania* spp. is detected by TLRs present in the membrane of host cells, which induce the release of ATP into the extracellular environment through pannexin-1 channels. Extracellular ATP is then metabolized by ectonucleotidases, such as NTPDases (*Leishmania* NTPDase 1 and/or 2) and E-5'-NT, located on the membrane of *Leishmania* spp., resulting in the accumulation of ADO in the environment. ADO interacts with A2 receptors on host cells, promoting the expression of cyclooxygenase-2 (COX-2) and the consequent production and release of prostaglandin E_2 (PGE_2). This PGE_2, in turn, can activate its receptors through autocrine or paracrine mechanisms, leading to the decreased expression of inducible NO synthase (iNOS) and reduced NO production. This effect favors the establishment and growth of *Leishmania* spp. in the host [50]. Thus, inhibitors of NTPDases and 5'-nucleotidases emerge as a promising therapeutic approach to enhance the immune response against leishmaniasis. In *L. infantum*, the causative agent of the most dangerous form of Leishmaniasis (visceral form), the knockout of NTPDase1 or 2 (LiNTPDase1, LiNTPDase2) led to lower levels of adhesion and infection of the host cell macrophage and decreased NO production [51]. In addition, the double knockout of both isoforms was not viable, highlighting that these enzymes are good pharmacological targets.

The literature indicates an association between the activity of NTPDase (TcNTPDase1) and the virulence of *T. cruzi*, particularly in severe cases of Chagas disease characterized by cardiac and neurological involvement. An increase in NTPDase activity has been observed in the cortex of infected mice, suggesting its participation in ATP and ADP hydrolysis during infection, with implications for neuronal function and modulation of the immune response [52]. Furthermore, the overexpression of the *TcNTPDase1* gene has been correlated with increased virulence and cardiac inflammation. In contrast, the knockout of this gene reduced infection severity and protected the heart from structural and functional damage [53]. Additional findings suggest that increased activities of E-NPP (ecto-nucleotide pyrophosphatase/phosphodiesterase) and E-5'-NT, along with decreased E-ADA (ecto-adenosine deaminase) activity, play a crucial role in modulating platelet aggregation, indicating that the purinergic system is involved in thrombo-regulation in patients with heart failure due to Chagas disease (HFCD). The final product of ATP hydrolysis, ADO, is known for its cardioprotective and vasodilatory effects, which may help prevent the clinical progression of Chagas disease [54]. These findings highlight the relevance of the purinergic signaling system in the pathophysiology of Chagas disease, consolidating ectoenzymes, including NTPDases, as potential therapeutic targets for interventions to mitigate disease progression and the associated cardiac and neurological damage.

In *Plasmodium falciparum*, genome database analyses have identified a gene encoding a single ENTPDase (PF3D7_1431800). It has been reported that an increase in Ca^{2+} levels in *P. falciparum*, induced by extracellular ATP, modulates parasite invasion into red blood cells [55]. The enzymatic activity of this protein was experimentally demonstrated using specific ENTPDase inhibitors, which interfere with the parasite's development in erythrocytes by reducing its capacity to hydrolyze ATP during the asexual cycle stages. This highlights the role of this enzyme in the parasite-host interaction [56]. Furthermore, purinergic signaling, primarily mediated by ATP, plays a significant role in parasite metabolism. ATP is an energy source, while derived nucleosides are used as substrates for synthesizing new nucleotides. These nucleosides pass through nonspecific vacuolar pores in the PV membrane (PVM) and are transported into the parasite via the PfENT1 transporter located in the parasite plasma membrane (PPM) [57].

During *P. falciparum* infection, ATP also functions as a signaling molecule, influencing critical processes in host cell interactions and promoting parasite survival strategies [58]. Recent studies have identified the presence of G protein-coupled receptors (GPCRs) in *P. falciparum* cells, which can detect variations in extracellular ATP levels and trigger intracellular signaling cascades. These mechanisms allow the parasite to respond to environmental stimuli, such as the presence of host immune cells or fluctuations in nutrient concentrations, ensuring its adaptation and survival [59]. Therefore, given their critical roles in metabolism, survival, and host interaction, the ENTPDase enzyme, the PfENT1 transporter, and GPCRs emerge as critical therapeutic targets for parasite elimination.

In *Schistosoma mansoni*, a trematode parasite responsible for schistosomiasis, two NTPDase isoforms (Sm ATPDases 1 and 2) have been identified. Sm ATPDase 1 is located on the parasite's surface, while Sm ATPDase 2 is in the tegument and is secreted by eggs and adult worms. NTPDase activity in *S. mansoni* can be observed throughout the parasite's life cycle, influencing metabolism and host-parasite interactions [37]. The longevity and survival of *S. mansoni* are associated with ectoenzymes on the tegumental surface, which directly interact with the host microenvironment. Biochemical characterization revealed that the nucleotide pyrophosphatase/phosphodiesterase (SmNPP5) can cleave nicotinamide adenine dinucleotide (NAD), forming nicotinamide mononucleotide (NMN) and AMP. Meanwhile, the glycohydrolase (SmNACE) cleaves NAD, generating nicotinamide (NAM) and ADO diphosphate ribose (ADPR). Moreover, these enzymes enhance the processing of the products of each other's catalytic reactions. SmNACE cleaves NMN, producing NAM and ribose phosphate, while SmNPP5 cleaves ADPR, forming AMP and ribose phosphate. Metabolomic analyses conducted on plasma containing adult worms confirmed that these cleavage pathways are active in vivo. These results suggest that the primary function of SmNPP5 is NAD cleavage, contributing to the modulation of the host's immune function. In contrast, SmNACE primarily cleaves NMN, supplying NAM (vitamin B3), an essential nutrient for worm survival and efficient absorption [60].

Mesenteric endothelial cells are modified by *S. mansoni* infection, creating a pro-inflammatory phenotype characterized by increased expression of NTPDases 2 and 3 and $P2Y_1$ receptors. NTPDases 2 and 3 enhance extracellular ATP hydrolysis and ADP formation by endothelial cells, favoring parasite development by modulating purinergic inflammatory responses and increasing mononuclear cell adhesion to endothelial tissue. Meanwhile, ADP's $P2Y_1$ receptor expression and activation promote leukocyte adhesion to the endothelial monolayer [61]. Reduced expression of the P2X7 receptor was also identified in mesenteric endothelial cells, where impaired P2X7 receptor function is correlated with the anti-inflammatory cytokine TGF-β1, which may be induced by $P2Y_1$ receptor activation [62]. These factors contribute to mesenteric inflammation and schistosomiasis morbidity. Based on these findings, the purinergic signaling pathway represents potential pharmacological targets for reducing schistosomiasis-related morbidity, including inhibitors of NTPDases and the CD39-ADP-$P2Y_1$/$P2Y_{12}$ receptor axis linked to hepatic and mesenteric inflammatory exacerbations [63].

Developing new molecules to prevent and control parasitic infections remains a crucial challenge in biomedical research. In this context, various investigations have focused on identifying virulence biomolecules as promising therapeutic targets, particularly enzymes such as E-NTPDases, which are secreted or ecto-localized on the parasites' plasma membrane. Inhibiting these enzymes represents a strategic approach, as it could compromise the parasites' ability to manage purine resources and evade the host's immune defenses, increasing their vulnerability to available treatments.

ENTPDase inhibitors: therapeutic applications, challenges, and perspectives

Given the critical role of ENTPDases in various pathophysiological contexts, searching for and developing selective inhibitors for these enzymes has been extensively explored as a promising therapeutic strategy. In pathological conditions, the overexpression or dysfunction of ENTPDases can contribute to the progression of diseases such as cancer, cardiovascular disorders, type 2 diabetes, and infectious diseases [64–66]. Inhibiting these enzymes can modulate purinergic signaling in a controlled manner, reducing ADO production and, consequently, mitigating the immunosuppressive effects associated with its elevated concentrations, as observed in tumor and infectious microenvironments [67, 68]. Moreover, ENTPDase inhibitors hold utility beyond therapeutic applications, serving as valuable tools for the functional study of these enzymes in complex biological processes [69].

The development of ENTPDase inhibitors requires compounds with selectivity for specific isoforms, avoiding off-target effects such as inhibiting P2X/P2Y purinergic receptors or other ectoenzymes. Potency is also an essential criterion, with ideal IC_{50} and Ki values in the micromolar or nanomolar range guaranteeing clinical efficacy [70]. In addition, metabolic stability and good bioavailability are essential requirements for the inhibitor to act effectively on its target while maintaining a low toxicity profile [67].

Classical inhibitors such as suramin, sodium azide (NaN3), EDTA, EGTA, BG0136 (1-naphthol-3,6-disulfonic acid), reactive blue, and ATP analogs (ARL67156 and 8-BuS-ATP) increase extracellular levels of ATP and ADP, creating a pro-inflammatory environment. However, many show low selectivity for ENTPDases and act as purinergic receptor antagonists, limiting their therapeutic application [71–73]. Although the compounds ARL67156 and 8-BuS-ATP have demonstrated ENTPDase inhibition without significant interference with the receptors, their potency is suboptimal, and they are susceptible to hydrolysis by other enzymes such as alkaline phosphatases [67, 74]. Nevertheless, these compounds are valuable for structural and functional studies [67, 75].

Other compounds have demonstrated improved selectivity based on ATP analogs or derivatives. For instance, 2-hexylthiomethylene-ATP exhibited higher selectivity for isoform 2, with minimal inhibition (5–15%) of 1, 3, and 8 [76]. Similarly, uridine-5'-carboxamide mimetics, such as compound PSB6426, successfully inhibited ENTPDase 2 without affecting other isoforms [75]. Although promising, these compounds still require more comprehensive preclinical and clinical studies to validate their efficacy and safety in treating diseases such as cancer and cardiovascular diseases.

In the search for more potent and selective inhibitors, other classes of compounds have been investigated, including polyoxometalates (POMs) [77–79], tryptamine-derived Schiff bases [69], quinolines [80, 81], and anthraquinone derivatives [73, 82], thiadiazole amide derivatives [83], thiadiazole pyrimidones [66], thienopyrimidine derivatives [64, 84], sulfopolysaccharides [85], sulfamoyl benzamides [86], carboxamides [70] and a protein kinase inhibitor, Certinib [87]. These compounds exhibit varying degrees of selectivity and potency, rendering them valuable for functional studies and potential therapeutic applications. Specific details regarding their inhibitory potential, specificity, and applications are summarized in Table 3. In addition to small molecules, monoclonal antibodies represent another promising avenue for specific ENTPDase inhibition. Monoclonal antibodies targeting human NTPDase2, 3, and 8 have been developed, with those against NTPDase3 showing remarkable inhibitory properties (Table 4) [88–90]. The following sections show more detailed data on the inhibitors targeting human or parasite enzymes.

Inhibitors of human ENTPDases and therapeutic applications

Inhibition of NTPDase1 has been shown in experimental models to prevent thrombus formation by maintaining elevated ADP levels, acting as a protective mechanism against vascular complications [4]. Moreover, blocking the conversion of ATP to ADO restores the pro-inflammatory environment and increases the efficacy of immunotherapies. Compounds such as POM-1 have been evaluated in tumor models and shown to reduce tumor progression, strengthening their therapeutic potential [91]. However, low selectivity for specific isoforms and effects on other ectoenzymes remain significant barriers to clinical development, highlighting the need for optimizations that maximize efficacy and minimize toxicities. Furthermore, in addition to preventing ATP degradation, POM-1 blocks central synaptic transmission, an effect not directly related to ENTPDase inhibition [77, 79]. Other limitations include low bioavailability and a lack of advanced clinical trials, with most studies limited to in vitro and animal models.

Other compounds, such as Schiff bases [69] and thiadiazole pyrimidones (8b and 8f), have shown good inhibitory activity against NTPDase1, with potential in the treatment of cancer and cardiovascular diseases such as thrombosis [66]. These compounds showed high specificity for this isoform, with Ki values in the nanomolar range (Table 3). Quinoline derivatives and thienotetrahydropyrimidines also showed good selectivity, with the latter exhibiting dual inhibitory activity, including CD73 inhibition [64, 80]. However, further studies using cellular and animal models must confirm their therapeutic applicability in NTPDase1-related diseases.

In type 2 diabetes, NTPDase3 regulates insulin secretion in pancreatic β-cells. Preclinical studies using specific antibodies against this isoform have shown increased insulin secretion and improved glycaemia control in experimental models [90]. Recently, Afzal et al. [70] demonstrated the potential of carboxyamides to inhibit NTPDase3 with IC_{50} in the submicromolar range. In vitro tests on pancreatic cells showed that NTPDase3 inhibition modulates insulin secretion [70].

NTPDase2 and NTPDase3 have also been explored in neurodegenerative and neuroinflammatory diseases, aiming to modulate inflammation without disrupting ADO homeostasis [4]. Anthraquinone derivatives have shown moderate selectivity for these isoforms, with IC_{50} in the nanomolar range (see Table 3). These inhibitors may help regulate inflammatory responses in the CNS by promoting extracellular ADP accumulation, indirectly activating $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors in immune and inflammatory modulation. However, additional studies in animal models and cellular systems are needed to validate their therapeutic effects [82].

Monoclonal antibodies are also emerging as promising tools due to their high specificity and ability to target defined molecular epitopes. Anti-NTPDase3 antibodies have shown potential in metabolic diseases such as type 2 diabetes by modulating insulin secretion in pancreatic islets [90]. Antibodies against NTPDase2 may contribute to anti-tumor immunotherapy by inhibiting enzymatic activity and decreasing immunosuppressive ADO levels within the tumor microenvironment. They may also regulate intestinal motility, suggesting therapeutic potential for gastrointestinal disorders [88].

Table 3. ENTPDase inhibitors—non-nucleotide analogs

Derivatives class	Inhibitors	IC_{50}[a] or Ki[b] (μM)	Specificity	Applications	Experimental context
Polyoxometalates (POMs) [77–79]	POM-1	0.8000[b]	N1 > N3 >> N2	Potential anticancer, cardiprotection. ENTPDases functional studies.	Inhibition assays with recombinant protein and in vitro assays using metastatic cells.
	PSB-POM142 (POM-5)	0.0039[b]	N1 >> N2 > N3		
Anthraquinone derivatives [73, 82]	PSB-069	15.70[a]	N1 > N2 > N3	Therapeutic potential for treating neurodegenerative and neuroinflammatory diseases and inflammation.	In vitro inhibition assays using capillary electrophoresis (CE) and the malachite green assay (MG).
	PSB-071	12.80[a]	N2 > N3 >> N1		
	PSB-06126	1.500[a]	N3		
	PSB-16131	0.539[a]	N2		
	PSB-2020	0.551[a]	N2		
	PSB-1011	0.390[a]	N3		
	PSB-2046	0.723[a]	N3		
Tryptamine-derived Schiff bases [69]	C# 1	0.030[b]	N1	Potential for use in the treatment of cancer and thrombosis.	Inhibition assays with recombinant enzymes (MG) and molecular docking.
	C# 4	0.080[b]	N1		
	C# 5	0.071[b]	N3 > N1 = N8		
	C# 13	0.280[b]	N8		
	C# 14	0.170[b]	N3 > N1 > N8		
	C# 22	0.490[b]	N8		
Thiadiazole pyrimidones [66]	8b	0.04[b]	N1	Study of insulin secretion and purinergic modulation.	Inhibition assays with recombinant human enzymes (MG), docking studies, and in vitro tests on pancreatic islets.
	8c	0.03[b]	N1 > N3 >> N8		
	8f	0.03[b]	N1		
	8i	0.17[b]	N2 > N1		
	8j	0.02[b]	N1 > N2		
	8k	0.07[b]	N2		
	8m	0.16[b]	N3 >> N1		
Carboxamides [70]	2a	1.60[a]	N8 > N2 > N3 > N1	Treatment of type 2 diabetes, inflammatory and metabolic disorders, and isoform-specific studies.	Inhibition assays with recombinant human enzymes (MG), in vitro tests on pancreatic islets, and molecular docking studies.
	2b	2.82[a]	N3		
	2d	0.15[a]	N2 > N1		
	2f	0.70[a]	N1		
	2h	0.12[a]	N1		
	2i	1.46[a]	N1 > N3		
Quinoline derivatives [80]	2c	0.86[a]	N3 > N8 > N1	Therapeutic potential to treat cancer and other diseases associated with ENTPDase overexpression.	Inhibition assays with recombinant human enzymes (malachite green).
	2h	0.36[a]	N3 > N1 > N2 > N8		
	3b	0.55[a]	N1 > N2		
	3f	0.20[a]	N1 > N3		
	4	0.51[a]	N3 > N1 > N2		
	5c	0.65[a (b)]	N8		
Sulfopolysac-charides [85]	Compound 5	1.72[b]	N1, but also inhibited NPP1	Therapeutic potential to treat cancer.	Inhibition assays on enzymes (NTPDases and NPPs) and U87 glioblastoma cells using CE and MG.
	Compound 6	0.408[b]	NPP1 > N1		
	Compound 7	12.3[b]			

Table 3. ENTPDase inhibitors—non-nucleotide analogs (*continued*)

Derivatives class	Inhibitors	IC_{50}[a] or Ki[b] (μM)	Specificity	Applications	Experimental context
Tienotetraidropirimidina derivatives [64]	Compound 32	45.2[a]	Dual inhibitor for N1 (CD39) and CD73	Therapeutic potential to treat cancer.	Inhibition assays with recombinant enzymes using CE and MG.
Thiadiazole amide derivatives [83]	5a	0.05[a]	N1 >> N2 > N8	Potential in cancer immunotherapy and the treatment of immunological and thrombotic diseases.	Inhibition assays with recombinant human enzymes (MG) and molecular docking studies.
	5b	0.06[a]	N2 > N1		
	5c	0.08[a]	N2 > N1		
	5e	0.05[a]	N8 >> N1 > N3 >> N2		
	5g	0.04[a]	N2 >> N8		
	5j	0.07[a]	N8 >> N2 > N1 > N3		
Glicinatos de tienopirimidina [84]	3j	0.11[a]	N1	Potential in cancer immunotherapy and the treatment of inflammatory diseases, diabetes, and thrombosis.	Inhibition assays with recombinant human enzymes (MG) and molecular docking studies.
	3k	3.00[a]	N3		
	3l	1.00[a]	N8 >> N1		
	3n	0.40[a]	N2		
	4	0.13[a]	N2		
Protein kinase inhibitors [87]	Certinib	11.3[a]	N1 >> N3 = N8	Potential in cancer immunotherapy and a basis for the development of new inhibitors.	Inhibition assays with recombinant and native enzymes (tumor cell cultures) using CE and MG.
Sulfamoyl benzamides [86]	2d	0.28[a]	N8	Potential in cancer immunotherapy and the treatment of type 2 diabetes and thrombosis.	Inhibition assays with recombinant human enzymes (MG) and molecular docking studies.
	3f	0.27[a]	N2 > N3		
	3i	0.72[a]	N3 > N1		
	3j	0.29[a]	N2 > N3		
	4d	0.13[a]	N2 > N3 > N8		

N1, N2, N3, and N8 refer to NTPDases 1, 2, 3, and 8, respectively. CE: capillary electrophoresis; MG: malachite green method; ENTPDases: ectonucleoside triphosphate diphosphohydrolases; CD39: cluster of differentiation 39; NPP: nucleotide pyrophosphatase/phosphodiesterase

Table 4. ENTPDase inhibitors—monoclonal antibodies

Antibodies	Inhibition potential	Specificity	Application	Experimental context
hN3-$B3_S$ or hN3-$H10_S$ [90]	76–77%	hNTPDase3	Potential for the treatment of type 2 diabetes through insulin secretion modulation and application as a research tool	Inhibition assays in hNTPDase3 and in pancreatic islet cells (Langerhans cells)
hN2-$D5_S$ [89]	50%	hNTPDase2	Tools for studying NTPDase2 (expression and function), therapeutic potential in cancer, and regulation of intestinal motility	Enzymatic assays using malachite green, specificity evaluated by western blot, flow cytometry, and immunocytochemistry
Anti-hNTPDase8 [88]	No inhibition of NTPDase8 activity	hNTPDase8	Tools for the localization and study of NTPDase8 function in human and murine tissues	Enzymatic assays using malachite green, specificity evaluated by western blot, flow cytometry, and immunocytochemistry

ENTPDase: ectonucleoside triphosphate diphosphohydrolase

However, essential gaps must be addressed to expand the possible clinical use of these antibodies. Studies such as those by Munkonda et al. [90] (2009) and Pelletier et al. [88] (2017) have demonstrated the exceptional specificity of antibodies against NTPDases. However, in vivo validations are lacking to assess therapeutic effects in more complex biological systems. In addition, challenges such as low tissue penetration in specific organs and high production costs can limit their clinical use. Advances in molecular engineering and improvements in inhibitory functionality are critical to turning these antibodies into effective therapeutic interventions.

Inhibitors of parasite ENTPDases and their therapeutic potential

The development of specific inhibitors targeting parasitic ENTPDases represents a promising strategy for controlling infections. These enzymes regulate extracellular nucleotide levels, modulating purinergic signaling that influences critical immunological and inflammatory processes. Through this regulation, parasites create an immunosuppressive microenvironment that supports their survival and replication within the host. Inhibiting these enzymes can turn off the immune evasion mechanisms of parasites, restore the host's immune response, and enhance the efficacy of conventional therapies. Additionally, ENTPDase inhibitors can act as adjuvants, reducing the doses and side effects of traditional medications, which is particularly advantageous in chronic infections where the immune system is compromised [36, 37].

Classic inhibitors such as DIDS, sodium azide, suramin, and ARL67156 have effectively inhibited ENTPDases and are widely used in characterization studies [46]. However, these compounds exhibit low specificity and non-selectivity on mammalian and parasitic ENTPDases, limiting their therapeutic application. In vitro studies have shown that suramin significantly reduces *T. cruzi* infectivity [47]; however, in murine models, it induced adverse effects, including exacerbated inflammation, worsened myocarditis, and increased mortality. These findings suggest that suramin's broad interference with host purinergic signaling may lead to undesirable systemic outcomes [92], underscoring the need for more selective inhibitors.

Derivatives of *ent*-isoquercetin, particularly compound 16, demonstrated 94% inhibition of recombinant TcNTPDase1 with a Ki of 8.39 μM. Molecular docking studies confirmed that the compound binds at the catalytic site and interacts with critical residues such as Gly369, Gly370, and Ser371, reinforcing its potential for treating Chagas disease [93]. Furthermore, this inhibitor may support structural studies to deepen the understanding of enzyme conformation and catalytic mechanisms. Given the structural similarity between *Leishmania* ENTPDases and TcNTPDase1 [94], these compounds may also be applicable against *Leishmania* spp. Nonetheless, further studies in cellular and animal models and specificity assessments against human ENTPDases are needed to ensure therapeutic efficacy and safety.

In *S. mansoni*, investigations involving cardamonin and alkylaminoalkanesulfonic acid derivatives (AAATs) have highlighted the importance of NTPDase inhibition. Cardamonin exhibited an IC_{50} of 23.54 μM against SmATPDase1 and significantly reduced parasite burden (46.8%) and egg production (54.5%) in murine models. AAATs demonstrated strong inhibitory activity, with efficacy influenced by their lipophilicity and interactions with cysteine residues. Despite these encouraging results, many studies have used indirect enzymatic models, such as potato apyrase, limiting conclusions regarding SmNTPDase isoform specificity. Furthermore, there is a lack of pharmacokinetic and toxicity data in experimental models—critical factors in drug development [95–97].

In the case of *T. gondii*, resveratrol (RSV) has emerged as a modulator of NTPDases, reducing ATP and ADP hydrolysis by approximately 20% and 10%, respectively, while partially restoring ADA activity. These findings suggest RSV can regulate the inflammatory microenvironment and oxidative stress without significantly decreasing brain cyst load. Thus, RSV's effects appear to relate more to neuroprotection and immunomodulation than direct parasitic clearance, reaffirming the therapeutic potential of NTPDases in neurological infections caused by *T. gondii* [98].

For *T. vaginalis*, the alkaloids lycorine and aldimine significantly inhibited extracellular nucleotide hydrolysis. Lycorine exhibited an IC_{50} of 32 μM, while aldimine inhibited up to 77% of ATP hydrolysis. However, lycorine's cytotoxicity in human cells indicates the need for chemical modifications to improve its selectivity. Moreover, targeting the purinergic system, including ADA inhibition, has reduced reactive oxygen species (ROS) and inflammatory cytokines in neutrophils exposed to the parasite, indicating that combinatorial strategies may enhance infection control [99–101].

Although compounds like cardamonin and flavonoid derivatives have shown promise, the repertoire of specific inhibitors for parasitic ENTPDases remains extremely limited, and the field lacks sufficient chemical diversity. Notably, there are no dedicated studies on selective ENTPDase inhibitors for *Leishmania*

spp. and *P. falciparum*, constituting a significant research gap. Since these enzymes play central roles in the purinergic metabolism of these parasites, they represent attractive targets for therapeutic innovation.

Advances in hNTPDases research offer a valuable framework for developing inhibitors targeting parasite isoforms. Mechanistic, structure-activity relationship (SAR), and molecular dynamics data from human enzymes can be adapted to identify functional and structural divergences in parasitic isoforms. Computational tools such as molecular docking and homology modeling—already widely applied in hNTPDase research—will be instrumental in this process [102]. Such an integrated approach may facilitate the development of novel therapeutic strategies against pathogenic parasites.

Challenges and future perspectives in the development of ENTPDase inhibitors

Despite recent progress, the development of ENTPDase inhibitors—especially for infectious and inflammatory disease contexts—still faces significant challenges that hinder clinical translation. A key issue is the low selectivity of existing compounds, directly resulting from the high structural conservation among human and parasitic ENTPDase isoforms. This homology compromises molecular specificity and increases the likelihood of adverse effects, particularly considering the widespread distribution of these enzymes across vital tissues such as the brain, liver, heart, intestine, and immune system [67].

Cross-reactivity with other elements of the purinergic signaling cascade, including P2 receptors and E-5'-NTs, presents another significant limitation. This is especially concerning in physiological contexts where ENTPDases play protective roles, as indiscriminate inhibition may lead to harmful systemic consequences. As mentioned earlier, compounds like suramin exemplify this issue, showing severe adverse effects in animal models due to widespread interference with host purinergic signaling [92]. Furthermore, many candidate compounds remain restricted to in vitro evaluations and lack in vivo validation for efficacy, toxicity, and pharmacokinetics [36, 37]. The lack of high-resolution structural data, such as crystal structures and accurate 3D models, further limits rational drug design strategies, making it harder to progress toward developing high-affinity and highly selective inhibitors [46, 67].

Targeting the CNS poses additional challenges. Most ENTPDase inhibitors have poor blood-brain barrier (BBB) permeability, limiting their use in neuroinflammatory disorders like AD, MS, and parasitic encephalitis. While monoclonal antibodies offer high specificity, they face practical constraints including high production costs, poor tissue penetration, and limited inhibitory function [35].

For parasitic ENTPDases, the scenario is further complicated by external factors such as limited funding, low commercial interest, and pharmaceutical disinvestment in neglected diseases like leishmaniasis and Chagas disease [36].

Despite these obstacles, recent developments provide encouraging perspectives. Allosteric modulators represent a promising alternative to overcome the selectivity limitations of competitive inhibitors. These compounds fine-tune enzymatic activity instead of entirely blocking it, potentially reducing off-target effects and improving tissue-specific activity [46].

Computational approaches have been central to these advances. Robust biochemical assays, including malachite green-based methods, supply reliable potency data [80]. At the same time, molecular docking and dynamics simulations enable precise analysis of ligand-target interactions, guiding the rational design of next-generation inhibitors [102]. X-ray crystallography has also been vital in characterizing active sites, facilitating the creation of stable and selective compounds [103]. More recently, artificial intelligence and machine learning have accelerated high-throughput screening, enhancing affinity, bioavailability, and toxicity predictions while addressing species-related extrapolation issues [87]. Additionally, novel delivery systems—such as nanostructured carriers, transport vectors, and intranasal administration—are being explored to improve drug penetration into hard-to-reach tissues, including the CNS [104].

Conclusion

This study underscores the therapeutic potential of ENTPDases inhibitors across diverse pathological conditions, including infectious diseases, inflammatory disorders, neurodegenerative syndromes, and

cancer. These enzymes play essential roles in modulating pathogen-host interactions, immune evasion mechanisms, and systemic inflammation by regulating extracellular nucleotide levels. In contexts marked by chronic inflammation or tumor progression, ENTPDase inhibitors have shown potential in enhancing immunotherapeutic responses and reactivating suppressed pro-inflammatory signaling pathways. Nevertheless, substantial challenges still hinder clinical translation. A particularly critical gap is the absence of highly selective inhibitors for parasitic ENTPDases. Structural similarities with human isoforms complicate the development of safe and effective compounds, while many current inhibitors exhibit poor specificity, systemic toxicity, and limited bioavailability. Moreover, the lack of suitable preclinical models and clinical data further restricts therapeutic validation. To advance the field, integrated, multidisciplinary strategies are needed. Structure-based computational modeling should be prioritized to identify and optimize selective inhibitors. Allosteric modulation offers a complementary approach to improve specificity and reduce systemic side effects. Monoclonal antibodies, though underutilized, hold promise for achieving isoform-specific inhibition, especially when directed against parasite-specific epitopes. Overcoming the BBB is another priority, particularly for treating neuroinflammatory and neuroinfectious conditions, and may be addressed through innovative drug delivery systems such as nanocarriers or intranasal routes. A critical evaluation of translational barriers—including regulatory, pharmacological, and economic challenges—is essential. Coordinated efforts between academia, industry, and public health agencies will be required to support innovation and foster the clinical development of ENTPDase-inhibitors targeting therapies.

In conclusion, although ENTPDase inhibitors show remarkable therapeutic promise, their clinical implementation depends on overcoming significant scientific and logistical challenges. Interdisciplinary efforts combining biochemical, pharmacological, and computational expertise will be key to unlocking this promising therapeutic class's potential for treating parasitic, inflammatory, and neuroinflammatory diseases.

Abbreviations

aa: amino acid

AD: Alzheimer's disease

ADA: adenosine deaminase

ADO: adenosine

ADP: adenosine diphosphate

AMP: adenosine monophosphate

ATP: adenosine triphosphate

BBB: blood-brain barrier

CD39: cluster of differentiation 39

CNS: central nervous system

COX-2: cyclooxygenase-2

EAE: experimental autoimmune encephalomyelitis

EDTA: ethylenediaminetetraacetic acid

E-NPP: ecto-nucleotide pyrophosphatase/phosphodiesterase

ENTPDases: ectonucleoside triphosphate diphosphohydrolases

GPCRs: G protein-coupled receptors

IL-1β: interleukin-1β

MS: multiple sclerosis

NAD: nicotinamide adenine dinucleotide

NAM: nicotinamide

NMN: nicotinamide mononucleotide

NO: nitric oxide

NPP: nucleotide pyrophosphatase/phosphodiesterase

POMs: polyoxometalates

ROS: reactive oxygen species

RSV: resveratrol

Declarations

Author contributions

ICR: Conceptualization, Investigation, Writing—original draft, Writing—review & editing. ALdA: Writing—original draft. VdAR: Writing—original draft. MSLL: Writing—original draft. JLRF: Conceptualization, Writing—review & editing, Supervision. All authors read and approved the submitted version.

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The authors declare that they have no conflicts of interest.

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