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Highly Potent and Selective Ectonucleotide Pyrophosphatase/Phosphodiesterase I Inhibitors Based on an Adenosine 5'-(α or γ)-Thio-(α,β or β,γ)-methylenetriphosphate Scaffold

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Abstract

Aberrant nucleotide pyrophosphatase/phosphodiesterase-1 (NPP1) activity is associated with chondrocalcinosis, osteoarthritis, and type 2 diabetes. The potential of NPP1 inhibitors as therapeutic agents, and the scarceness of their structure–activity relationship, encouraged us to develop new NPP1 inhibitors. Specifically, we synthesized ATP- α -thio- β,γ -CH₂ (**1**), ATP- α -thio- β,γ -CCl₂ (**2**), ATP- α -CH₂- γ -thio (**3**), and 8-SH-ATP (**4**) and established their resistance to hydrolysis by NPP1,3 and NTPDase1,2,3,8 (<5% hydrolysis) (NTPDase = ectonucleoside

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#Author Contributions

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The authors declare no competing financial interest.

Supporting Information

Table of interactions between analogues **1a**, **1b**, **2a**, **2b**, and **3** in their representative poses with binding site residues within the catalytic site of the human NPP1 model. This material is available free of charge via the Internet at <http://pubs.acs.org>

triphosphate diphosphohydrolase). Analogues **1–3** at 100 μM inhibited thymidine 5'-monophosphate *p*-nitrophenyl ester hydrolysis by NPP1 and NPP3 by >90% and 23–43%, respectively, and only slightly affected (0–40%) hydrolysis of ATP by NTPDase1,2,3,8. Analogue **3** is the most potent NPP1 inhibitor currently known, $K_i = 20 \text{ nM}$ and $\text{IC}_{50} = 0.39 \mu\text{M}$. Analogue **2a** is a selective NPP1 inhibitor with $K_i = 685 \text{ nM}$ and $\text{IC}_{50} = 0.57 \mu\text{M}$. Analogues **1–3** were found mostly to be nonagonists of P2Y₁/P2Y₂/P2Y₁₁ receptors. Docking analogues **1–3** into the NPP1 model suggested that activity correlates with the number of H-bonds with binding site residues. In conclusion, we propose analogues **2a** and **3** as highly promising NPP1 inhibitors.

INTRODUCTION

Two major families of ectonucleotidases, namely, the ectonucleoside triphosphate diphosphohydrolase (NTPDase) family and the ectonucleotide pyrophosphatase/phosphodiesterase (NPP) family, terminate nucleotide signaling through the hydrolysis of nucleotide agonists of the P2X and P2Y receptors.^{1,2} The NTPDase family consists of eight $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent ectonucleotidases (NTPDase1–8) which hydrolyze extracellular nucleoside 5'-triphosphate and nucleoside 5'-diphosphate to nucleoside 5'-monophosphate.¹ The NPP family consists of seven ectoenzymes^{3,4} which exist as membrane glycoproteins with an extracellular divalent-ion containing active site,⁵ yet are also found extracellularly as secreted enzymes.⁶ NPP1–3, the only nucleotide-hydrolyzing members of this group, have a wide tissue distribution⁶ and are capable of hydrolyzing phosphodiester and pyrophosphate bonds of nucleotides. NPPs are more specific for phosphodiester vs phosphomonoester substrates. NPP1 and NPP3 both hydrolyze extracellular nucleoside di/triphosphate derivatives.⁷ NPP1 can catalyze the hydrolysis of ATP to either ADP and P_i or AMP and PP_i .³ NPP2 has a much lower ATPase activity than NPP1/3.

NPP1–3 can control P2 and P1 receptor signaling because their enzymatic action results in the hydrolysis of signaling nucleotides and the generation of new messengers such as AMP, adenosine, or pyrophosphate.⁶

NPP1 is expressed in different tissues. It was reported to exist in bone (osteoblasts) and cartilage (chondrocytes) and has a role in regulating mineralization processes.⁸ Extracellular pyrophosphate (ePP_i), the product of ATP hydrolysis by NPP1, is a likely source of inorganic phosphate to sustain hydroxyapatite (HA) formation when hydrolyzed by phosphatases and is also a potent inhibitor preventing apatite mineral deposition and growth.⁹ The main hydrolyzing agent of PP_i within chondrocytes is tissue-nonspecific alkaline phosphatase (TNAP).³ TNAP is an ectoenzyme highly similar to NPP1 containing a similar catalytic site and hydrolyzing its substrates in a similar mechanism.⁵ Although TNAP's main substrate within chondrocytes is PP_i , TNAP can also act as a phosphodiesterase and hydrolyze nucleoside 5'-triphosphates (NTPs).¹⁰ Although PP_i is required to prevent ectopic mineralization, its overproduction leads to deposition of the pathological mineral calcium pyrophosphate dihydrate (CPPD), most often in the articular cartilage.^{11,12} This condition, known as chondrocalcinosis, frequently accompanies age-related osteoarthritis.³

A role for NPP1 in insulin receptor signaling has also been proposed.¹³ Defective insulin-stimulated insulin receptor autophosphorylation in type 2 diabetes patients was found to result from an overexpression of NPP1.^{13–16} In addition, NPP1 was found in human astrocytic brain tumors and was correlated with tumor gradation.^{17,18}

The activity of NPP1 is endogenously regulated by AMP (i.e., product inhibition),⁸ yet nonendogenous NPP inhibitors have been scarcely reported. Recent reports suggested that quinazoline-4-piperidine-4-methylsulfamide is an NPP1 inhibitor lacking binding affinity for hERG¹⁹ and that 1,3,4-oxadiazole(thiadiazole)-2(3*H*)-thiones are noncompetitive human NPP1 inhibitors with K_i values of 360 μM .²⁰ Previously, we reported that bis(2'-deoxyadenosine) $\alpha,\beta,\delta,\epsilon$ -dimethylenepentaphosphonate (Figure 1A) is an NPP1 inhibitor with an IC_{50} of 13 μM and a K_i of 9 μM .²¹ In addition, we recently reported that adenosine 5'- α -borano- β,γ -methylenetriphosphate (isomer A) (Figure 1B) is a selective inhibitor of NPP1 with a K_i of 0.5 μM .²²

Here, we continue our search for highly potent and selective NPP1 inhibitors. Specifically, we describe the synthesis of a series of new ATP analogues bearing both phosphonate and thiophosphate modifications (Figure 2), the evaluation of their activities as NPP1 inhibitors, and their selectivity for NPP1 vs other ectonucleotidases and representative adenine nucleotide binding receptors, P2Y_{1,2,11} receptors. Furthermore, we performed docking simulations of the new analogues to the catalytic site of a model of human NPP1 and analyzed the resulting poses to deduce the interaction pattern with binding site residues and the origin of NPP1 inhibitory activities.

RESULTS

Design and Synthesis of Potential NPP1 Inhibitors

NPPs hydrolyze $\text{P}_\alpha\text{-P}_\beta$ phosphorodiester as well as $\text{P}_\beta\text{-P}_\gamma$ phosphoroester bonds. In an attempt to confer hydrolytic stability and possibly inhibitory activity of nucleotide ligands at NPP1, we have designed compounds **1** and **2** in which the P_α phosphate moiety was modified to a thiophosphate group and a methylene or dichloromethylene group, respectively, was introduced between P_β and P_γ .

In compound **3** we replaced the nucleotide bridging $\text{P}_\alpha\text{-P}_\beta$ oxygen atom with a CH_2 group and modified the P_γ phosphate moiety to thiophosphate. The catalytic binding site of NPP1 was suggested to contain two Zn^{2+} ions,²³ although other reports also suggested activation by Mg^{2+} and Ca^{2+} ions.^{24,25} Hence, thiophosphate groups were designed to chelate the tentative Zn^{2+} ions.²⁶ For this reason, we further replaced H8 of the adenine base in ATP by a thiol group, analogue **4**.

Synthesis of ATP- α -thio- β,γ -CX₂ Analogues **1** and **2**

Adenosine 5'- α -thio- β,γ -CX₂-triphosphate analogues **1** and **2** were prepared from 2',3'-(methoxymethylidene)adenosine, compound **5** (Scheme 1), to ensure a selective reaction of adenosine at 5'-OH. The 5'-OH of nucleoside **5** was activated with 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one to give compound **6**, which was condensed with bis-(tributylammonium) methylenediphosphonate or dichloromethylenediphosphonate to form

the cyclic triphosphate intermediate, compound **7** (Scheme 1). Treatment of compound **7** with powdered sulfur yielded, after hydrolysis with triethylammonium bicarbonate (TEAB) buffer, analogue **1** or **2** in moderate yield (9–17%).

The identity and purity of the products were established by ^1H and ^{31}P NMR, ESI HR mass spectrometry, and HPLC in two solvent systems. The ^{31}P NMR spectra of analogues **1** and **2** showed a typical P_α signal as a doublet at about 43 ppm ($J = 34$ Hz). The ^1H NMR spectrum of analogue **1** showed methylene hydrogen atoms as a triplet at about 2.3 ppm ($J = 20$ Hz). Due to the chiral center at P_α , analogues **1** and **2** were each obtained as a pair of diastereoisomers in a 1:1 ratio. In both ^1H and ^{31}P NMR spectra, there was a slight difference between the chemical shifts of the diastereoisomers of analogues **1** and **2**. For instance, diastereoisomers **1a,b** (Figure 3) yielded two sets of signals for H8, at 8.67 and 8.62 ppm. These isomers were well separated by reversed-phase HPLC with an about 6–10 min difference in their retention times. The first and second eluting isomers were designated as isomers A and B, respectively.

Assignment of the Absolute Configurations of the **1a,b** and **2a,b** Diastereoisomers

We employed ^1H NMR spectroscopy to elucidate the absolute configuration at P_α of the **1a,b** and **2a,b** diastereoisomers. A difference in the chemical shift of H8 was observed between the two diastereoisomers of ATP- β,γ -methylene- α -S. The signal of H8 of isomer A in the ^1H NMR spectrum was more shielded than that of H8 of isomer B possibly due to the effect of the proximal negatively charged P_α group of thiophosphate (8.62 vs 8.67 ppm). P_α is much further away from H8 in isomer B than in isomer A (Figure 3). Thus, the S_P configuration may be attributed to isomer A, analogue **1a**, and the R_P configuration to isomer B, analogue **1b**.²⁷

Synthesis of P_γ -thio- α,β -CH₂-ATP, Analogue **3**, and 8-SH-ATP, Analogue **4**

Adenosine 5'- α,β -methylene- γ -thiotriphosphate, analogue **3**, was prepared in several steps from protected adenosine, compound **5** (Scheme 2). The 5'-OH of **5** was activated with tosyl chloride and a catalytic amount of (dimethylamino)pyridine. 2',3'- (Methoxymethylidene)-5'-tosyladenosine, compound **8**, was phosphorylated with methylenediphosphonate bis(tributylammonium) salt to form 2',3'- (methoxymethylidene)adenosine 5'- α,β -methylenediphosphonate, compound **9**.²⁸ Next the protecting group was removed with 10% HCl and then with 10% NH_4OH . Activation of compound **10** with carbonyl diimidazole (CDI) for 4–5 h resulted in a phosphoroimidazolide intermediate, the yield of which was ca. 50% by NMR. Subsequently, MeOH was added to decompose the excess CDI, followed by addition of thiophosphate tributylammonium/tri-*n*-octylammonium salt and ZnCl_2 . The latter was added to protect the thiophosphate sulfur atom from reaction with phosphorimidazolide. Finally, compound **3** was obtained in 29% yield after LC separation.

The ^{31}P NMR spectrum of analogue **3** showed a typical P_γ signal as a doublet at about 39 ppm ($J = 32$ Hz). The ^1H NMR spectrum of analogue **3** showed methylene hydrogen atoms as a triplet at about 2.5 ppm ($J = 20$ Hz).

Analogue **4** was obtained in two steps from 8-bromoadenosine (Scheme 3).²⁹ 8-Mercptoadenosine, obtained in a quantitative yield from 8-bromoadenosine upon treatment with 10 equiv of NaSH in wet DMF at 100 °C overnight, was 5'-triphosphorylated first by addition of POCl₃ in the presence of proton sponge in TMP for 3 h and then by the addition of pyrophosphate in DMF for 2 h at –15 °C to give analogue nucleotide **4** in 60% yield.

Analogues 1–3 Are Not Substrates of NTPDase1,2,3,8, NPP1,3, or TNAP

Experiments were conducted with protein extracts from COS-7 cells transfected separately with an expression vector encoding each ectonucleotidase, i.e., NPP1,3 and NTPDase1,2,3,8. The protein extracts of nontransfected COS-7 cells exhibited less than 5% NTPDase or NPP activity compared with those of COS-7 cells transfected with NTPDases or NPPs, thus allowing the analysis of each ectonucleotidase in its native membrane-bound form.³⁰

Analogues **1–3** (100 μM, *n* = 3) were stable to hydrolysis by NTPDase1,2,3,8 when compared to ATP (4.4–5.5% hydrolysis over 1 h, Table 1). Analogues **2** and **3** (100 μM) also were not catabolized by NPP1 or NPP3. Analogue **1a** (100 μM) was fully stable to NPP1 hydrolysis and was hardly hydrolyzed by NPP3 over 1 h (~1%) compared to the physiological substrate ATP. Analogue **1b** was weakly hydrolyzed by both NPP1 and NPP3 (~4%). Analogue **4** was hydrolyzed by NPP1 and NPP3 at 32–54% over 1 h and therefore was not studied further as an inhibitor of these enzymes.

In addition, the metabolic stability of the promising candidates **2a** and **3** was further proven by their resistance to enzymatic hydrolysis by TNAP. Compound **2a** was fully stable to TNAP hydrolysis for 1 h vs 100% hydrolysis of ATP, and **3** was negligibly hydrolyzed (2.5%) (data not shown).

Analogues 1–3 Are Selective Inhibitors of NPP1

The effect of analogues **1–3** on NPP and NTPDase activities and their selectivity were tested using a synthetic substrate (pNPTMP) as well as a natural substrate (ATP), respectively. Analogues **1–3** at 100 μM (*n* = 3) effectively inhibited pNPTMP (100 μM) hydrolysis by NPP1 by over 90% (Figure 4A). The hydrolysis of the physiological substrate ATP by NPP1 was inhibited more potently by analogues **2a** and **3** vs **1a/b** and **2b** (Figure 4B). Similar inhibition was observed when osteocarcinoma cells (HTB85 cells, also known as SaOS 2) were used as a native source of NPP1 (Figure 4C). Analogues **1–3**, at 100 μM, were NPP1-selective inhibitors, since they inhibited NPP3 activity by only 23–43% (Figure 4A,B).

Analogues **1a**, **1b**, **2a**, and **2b** did not interfere with the hydrolysis of ATP by human NTPDase1,2,3,8 (Table 2). Analogue **3** at 100 μM inhibited human NTPDase1,3 by 60% and 40%, respectively (Table 2). Both compounds **2a** and **3** at 100 μM showed low inhibition of TNAP, 17% and 8%, respectively.

We have estimated IC₅₀ (a parameter that shows the ability of a molecule to inhibit an enzyme under specific conditions and substrate concentration) and inhibition constant *K_i* (a kinetic parameter that represents an absolute value for each tested inhibitor) toward NPP1. The kinetic parameters indicate that analogue **3** is the most potent inhibitor of NPP1 with a

K_i value of 20 nM (Table 3, 2aFigure 5). Analogue was also a good NPP1 inhibitor exhibiting a K_i value of 685 nM. Under these experimental conditions the IC_{50} values of both analogues were also the lowest, being 390 and 600 nM for **3** and **2a**, respectively (Table 3). Using the methods of Dixon (Figure 5A and Table 3) and Cornish–Bowden (Figure 5B and Table 3), which estimate the dissociation constant for the enzyme–inhibitor–substrate (EIS) complex (K_i'), we determined that the inhibitors **1b**, **2a**, **2b**, and **3** presented in Table 3 showed mixed-type inhibition, predominantly competitive.^{31,32}

Evaluation of Analogues 1–3 as Agonists of P2Y₁, P2Y₂, and P2Y₁₁ Receptors

Adenine nucleotides are not only substrates of ectonucleotidases but also potent agonists at P2Y₁, P2Y₂, and P2Y₁₁ G-protein-coupled receptors.³³ These receptors are expressed in many tissues and are involved in many diseases. Of relevance to the current study is the expression of P2Y receptors in bone tissues. P2Y₁ receptors are expressed in osteoclasts and osteoblasts,³⁰ P2Y₂ receptors are expressed in osteoblasts, where they block bone formation,³⁴ and P2Y₁₁ receptors are expressed in human osteoclast cultures.³⁵ This expression pattern may lead to selectivity problems for inhibitors if they act as P2YR agonists.

With this in mind, we evaluated the activity of NPP1 inhibitors, analogues **1–3**, at P2Y_{1,2,11} receptors. GFP (green fluorescent protein) constructs of human P2Y₁, P2Y₂, and P2Y₁₁ receptors were expressed in 1321N1 astrocytoma cells, which lack endogenous expression of P2X and P2Y receptors.³⁶ These cells were then incubated with various concentrations of analogues **1–3**. The Ca²⁺ responses to analogues **1–3** were compared with that to ATP. Analogues **1–3** were weak agonists ($EC_{50} > 10 \mu M$) of the P2Y₁ receptor (Table 4). Analogues **1a** and **2a** were not agonists of the P2Y₁₁ receptor. In contrast, analogues **1b** and **3** were 7-fold more potent than the endogenous P2Y₁₁ receptor ligand, ATP ($EC_{50} = 6.7 \mu M$) (Figure 6). In addition, analogue **2b** was a P2Y₁₁ receptor agonist (2-fold more active than ATP). Analogues **2a** and **3** (100 μM) had no activity as agonists at P2Y₂R. These analogues did not inhibit the typical response to UTP observed for the P2Y₂R in 1321N1 cells (data not shown).

Docking simulations at human NPP1

Docking simulations were used to provide insight into the inhibitory activities of the analogues studied in this work. We have previously reported a model of human NPP1 based on the recently solved structure of mouse NPP1²² and demonstrated that docking simulations using Glide were able to successfully reproduce the crystallographic pose of AMP in this structure with an RMSD of 0.73 Å and to provide a plausible binding mode for ATP in which it is stabilized within the binding site through an array of aromatic, hydrophobic, and H-bond interactions with binding site residues. These previous docking simulations suggest that Glide is a suitable docking tool for this system.

Analogues **1–3** were docked into the NPP1 site, and their representative poses were analyzed. These were obtained by first clustering all poses obtained by the docking simulations within the binding site and then by selecting the pose which is closest to the center of the largest cluster. The representative poses of all five analogues (**1a**, **1b**, **2a**, **2b**, **3**)

were found to adopt ATP-like conformations, suggesting that they could compete with ATP for binding site interactions (Figure 7; Table S1, Supporting Information). Furthermore, similar to ATP, all five analogues coordinate Zn1 through their P_{α} -oxygen atom (although other chelation patterns were also observed). An excellent correlation ($R^2 = 0.95$; Figure 8A) was found between Boltzmann-averaged Glide scores of the largest cluster and between experimental G values (calculated from K_i values at the experimental temperature of 37 °C using analogue **3** as a reference) for all five analogues considered in this work. Furthermore, this correlation was largely maintained when only the representative poses from each cluster were considered ($R^2 = 0.8$; Figure 8B). In addition, a very good correlation ($R^2 = 0.95$; Figure 8C) was found between the analogues' activities and the hydrogen bond term, which forms part of Glide's scoring function. A detailed analysis of the interaction patterns of the representative poses of all analogues with binding site residues is provided in Table S1.

DISCUSSION

A series of ATP analogues modified at the $P_{\alpha}/P_{\beta}/P_{\gamma}$ positions by bridging methylene and thiophosphate moieties (analogues **1–3**) or by including an 8-SH group (analogue **4**) were designed and synthesized to identify potent and selective NPP1 inhibitors. Analogue **4** was hydrolyzed by NPP1 and NPP3 at about 50% the rate of ATP (Table 1), and therefore, it could not serve as a good NPP inhibitor. Of the remaining compounds, ATP- α,β -CH₂- γ -S, **3**, was found to be an extremely potent NPP1 inhibitor ($K_i = 20$ nM, Figure 5, Table 3) followed by ATP- α -S- β,γ -CCl₂, **2a** ($K_i = 685$ nM, Figure 5, Table 3), while the other three analogues were less active (**1b**, $K_i = 1.3$ μ M; **1a**, $K_i = 4.5$ μ M; **2b**, $K_i = 15$ μ M; Table 3).

To provide structural insight into these results as well as tools for the design of yet more potent NPP1 inhibitors, analogues **1–3** were docked into the binding site of our previously reported NPP1 model.²² Similar to other non-hydrolyzable ATP derivatives studied by us in the past,²² the representative poses of all five analogues (**1a**, **1b**, **2a**, **2b**, and **3**) adopt ATP-like conformations, suggesting that they could effectively compete with ATP for binding site interactions (Figure 7; Table S1, Supporting Information). Furthermore, similar to ATP, all simulated analogues coordinate Zn1 through their P_{α} -oxygen atom (although other chelation patterns were also observed). While zinc is known to be a “thiophilic” ion, our docking results demonstrate preference for zinc chelation through the nonbridging oxygen atom of the phosphate chain rather than through the sulfur atom, yet we argue that this nontypical chelation pattern may be the consequence of binding site constraints. With this in mind we have searched the PDB for protein complexes where a ligand containing both oxygen and sulfur atoms chelates a zinc ion. Twenty-four such complexes were found out of which seven demonstrated O–Zn chelation. Interestingly, out of this list of ligands, the two ligands most similar to analogues **1–3** (ATP- γ -S, PDB code 3ZEU, and a phosphorothioate derivative, PDB code 1KRP) show zinc chelation via oxygen. In the present study, the potential role of the binding site in controlling the Zn²⁺ chelation pattern is nicely demonstrated for the most active compound, analogue **3**. As the data in Table S1 clearly indicate, zinc chelation via the P_{γ} -sulfur atom, rather than via the P_{α} -oxygen atom, results in loss of important interactions with binding site residues including hydrogen bonds with

Thr256, Lys295, and Tyr340 and an important salt bridge with Lys255. These findings suggest that the role of the sulfur atom may not be to enhance binding by providing better chelation to the zinc ion but rather by affecting the degree of ionization of the terminal phosphate. Indeed, the pK_a of thiophosphate is 5.5, whereas that of phosphate is 6.5.³⁷

The docking results were analyzed using a Boltzmann averaging of glide scores over the largest cluster of each analogue and resulted in an excellent correlation ($R^2 = 0.95$; Figure 8A) with experimental G values calculated from the kinetic parameters (K_i values). This correlation was largely maintained when considering only a single (representative) structure pose for each cluster ($R^2 = 0.8$; Figure 8B). The representative poses of all ligands considered in this work are given in Figure 7, and an analysis of their interaction patterns with binding site residues and key Glide scores (total score, H-bond score, and electrostatic score) are given in Table S1 (Supporting Information). In particular, the hydrogen bond component of the scoring function shows an excellent correlation ($R^2 = 0.95$) with the experimental data (Figure 8C). This is reflected in the hydrogen-bonding and salt bridge patterns of the representative poses of the different ligands (Figure 7 and Table S1). Thus, while the most active compound **3** is hydrogen bonded to five binding site residues (Thr256 $\times$ 2, Asn277, Lys295, and Tyr340), and forms a salt bridge with Lys255, the less active analogues have fewer H-bonds (**2a**, two H-bonds with Asn277 and Tyr340; **1b**, two H-bonds with Asn277 and Lys295; **1a**, one H-bond with Asn277) while maintaining the salt bridge with Lys255, and the least active analogue has only two H-bonds and no salt bridge (**2b**, two H-bonds with Asn277 and Lys295). In addition, analogue **3** features the most favorable electrostatic energy component of the Glide score (-65.7 kcal/mol), although no clear correlation is observed between this score and the binding affinities (Tables 4 and S1). Presumably, for **1b** vs **2b**, the introduction of the bulkier chlorine atoms shifts the position of the **2b** analogue within the binding site, leading to the loss of the important electrostatic interaction with Lys255.

Analogues **2a** (the second most active compound) and **2b** (the least active compound) differ only in the chirality at P_α (*S* and *R*, respectively). While this reversed chirality is reflected in the more favorable H-bond and salt bridge pattern of the more active compound (see above), it also leads to different exposures of polar surface areas to the solvent. As the data in Figure 9 indicate, analogue **2a** exposes a more polar “face” to the solvent than analogue **2b**. This in turn may lead to more favorable ligand–solvent interactions for this analogue and consequently to a stronger protein binding.

A detailed analysis of the representative poses of the five analogues does not provide a clear interpretation of the role of the two chlorine atoms since they are not involved in specific interactions with binding site residues. These atoms were originally introduced into the ATP skeleton to reduce the pK_a of the terminal oxygen of the phosphonate moiety, bringing it closer to that of phosphate (the pK_a values of phosphonate, dichlorophosphonate, and phosphate are 8.4, 6.7–7.0, and 6.5, respectively)^{38,39} and making the compound a more potent inhibitor. This was indeed the case for analogues **1a** and **2a** (4.5 and 0.685 μ M, respectively, Table 3) but not for analogues **1b** and **2b** (1.3 and 15.2 μ M, respectively, Table 3).

Analogues **1–3** at 100 μM selectively inhibited NPP1 vs NPP3 (Figure 4A), inhibiting the former by 90–100% and the latter by only 23–43%, using pNP-TMP as the substrate. Analogues **2a** and **3** at 100 μM inhibited about 90% of ATP hydrolysis by NPP1 (Figure 4B). The results were confirmed by assays with osteocarcinoma cells (Figure 4C).

Analogues **2a** and **3** exhibited IC_{50} values of 685 and 600 nM and K_i values of 20 and 390 nM, respectively. IC_{50} values that do not parallel K_i values may perhaps result from differences in the hydrophobic character of compounds **2a** and **3** under the experimental conditions that may influence the type of observed inhibition (see Figure 7D,F and Table 3).

As previously reported,²² the preferred binding of all analogues to NPP1 rather than to NPP3 could be readily explained by comparing the binding sites of the two proteins. In NPP1, a unique arrangement of Lys residues (seven in total) which is absent in NPP3 (a single binding site Lys residue) results in a highly positive electrostatic potential which renders this site more suitable for binding negatively charged nucleotides.

Analogues **1** and **2** at 100 μM had a minor inhibitory effect on NTPDase1,2,3,8 activity (0–25% inhibition) (Table 2). In contrast, analogue **3** inhibited NTPDase1 by 60% and NTPDase3 by 40%. In addition, the two most promising candidates, **2a** and **3**, had a limited effect on TNAP activity, showing minor inhibition (8–17%).

As adenine nucleotides are also the endogenous agonists of $\text{P2Y}_{1,2,11}$ receptors, we tested analogues **1–3** at these receptors involved in numerous physiological activities.^{40,41} Compounds **1a**, **b** did not activate the P2Y_1 receptor, consistent with earlier reports that a methylene modification tends to reduce agonist potency at P2Y receptors as compared to ATP.^{42–44} Both ATP ($\text{pEC}_{50} = 4.77$) and ATP- γ -S ($\text{pEC}_{50} 5.52$) activate the P2Y_{11} receptor,⁴⁵ with ATP- γ -S being the most potent agonist of the two.³⁰ Consistent with this, ATP- $\alpha,\beta\text{-CH}_2\text{-}\gamma$ -thio, **3**, was found here to be ~7-fold more potent than ATP at this receptor. This finding is also in agreement with our previously reported model of $\text{hP2Y}_{11}\text{R}$ and its preference for ATP- γ -S over ATP binding as a result of the tighter fit of the larger $\text{P}_{\gamma}\text{-S}$ moiety.⁴⁶ Adenosine 5'- α -thio- β,γ -methylenetriphosphate isomer B, **1b**, and adenosine 5'- α -thio- β,γ -(dichloromethylene)triphosphate isomer B, **2b**, showed ~7- and ~2-fold, respectively, higher potency than ATP at the P2Y_{11} receptor. The respective isomers A, **1a** and **2a**, were inactive at the P2Y_{11} receptor.

This is in accordance with our previous findings⁴⁷ reporting that the P2Y_{11} receptor prefers isomer B of P_{α} -substituted ATP derivatives over isomers A. Recently we found that 2-(propylthio)adenosine 5'- α -thio- β,γ -(dichloromethylene)-triphosphate, isomers A and B,⁴⁸ showed ~3- and ~4 fold, respectively, higher potency than ATP at the P2Y_{11} receptor. In accordance with our findings presented here, the α -thio- β,γ - CCl_2 -substituted ATP derivatives were very weak agonists at the P2Y_1 receptor or not active at the P2Y_2 receptor. The nucleotides used in the present study lack the 2-propylthio modification of potent $\text{P2Y}_{11}\text{R}$ agonists,⁴⁸ but have the α -thio- β,γ - CCl_2 substitution. Taken together, these results suggest that the α -thio- β,γ - CCl_2 substitution generally increases the affinity and selectivity of ATP derivatives at the P2Y_{11} receptor.

Site-directed mutagenesis and computational analysis identified some amino acid residues responsible for the ligand recognition of the P2Y₁ and P2Y₁₁ receptor, as reported before.^{27,46,49,50} However, only little is known about the molecular structure of the binding pocket of all the P2Y receptors. Even though they belong to the same phylogenetic family, P2Y receptor subtypes show significant differences in amino acid sequences.^{51,52} P2Y₁, P2Y₂, and P2Y₁₁ receptors differ in their preference for the natural agonists.⁵³ Therefore, different EC₅₀ values are expected for the nucleotide derivatives tested here (Table 4).

The newly identified NPP1 inhibitors were found to be highly potent, as compared to currently available inhibitors. Specifically, analogues **2a** and **3** were 525-fold and 18000-fold, respectively, more potent human NPP1 inhibitors than the recently reported [4-((*tert*-butyldimethylsilyl)oxy)phenyl]-1,3,4-oxadiazole-2(3*H*)-thione.²⁰ Likewise, analogue **3** was found to be 405-fold and 25-fold more potent than our previously reported NPP1 inhibitors —bis(2'-deoxyadenosine) $\alpha,\beta,\delta,\epsilon$ -dimethylenepentaphosphonate (Figure 1A)²¹ and adenosine 5'-*O*- α -borano- β,γ -methylenetriphosphate ($K_i = 0.5 \mu\text{M}$; Figure 1B),⁴⁴ respectively. The greater activity of analogue **3** relative to adenosine 5'-*O*- α -borano- β,γ -methylenetriphosphate could possibly be attributed to its ability to coordinate the M²⁺ (Zn1) ion through its nonbridging P α -oxygen atom. On the basis of docking results, such coordination is not possible for the borano compound since in this compound, the nonchelating borano group at P α points toward the Zn1 ion.²²

The kinetic data presented in this work coupled with the structural insight into the origin of the analogues' activities available from the docking simulations suggest that analogues **3** and **2a**, together with the NPP1 and NPP3 models, are good starting points for the design of efficacious and selective NPP1 inhibitors. Nevertheless, being ATP-based, these analogues are not classical “druglike” compounds, yet related compounds such as thiazole-4-carboxamide adenine dinucleotide and denufosol have found their way into clinical trials.^{54,55} Developing these compounds into drugs may require prodrug approaches,⁵⁵ appropriate formulations, and/or administration modes other than oral. However, even if these compounds are not eventually developed into drugs, they are still likely to serve as important mechanistic tools for the study of the complex process of mineralization.

EXPERIMENTAL SECTION

General Procedures

All commercial reagents were used without further purification, unless otherwise noted. All air- and moisture-sensitive reactions were conducted in flame-dried, nitrogen-flushed, two-neck flasks sealed with rubber septa, and the reagents were introduced with a syringe. Progress of the reactions was monitored by TLC using precoated Merck silica gel plates (60F-253). Reactants and products were visualized using UV light. Compounds were characterized by NMR using a Bruker AC-200, DPX-300, or DMX-600 spectrometer. ¹H NMR spectra were recorded at 200, 300, or 600 MHz. Nucleotides were also characterized by ³¹P NMR in D₂O using 85% H₃PO₄ as an external reference on Bruker AC-200 and DMX-600 spectrometers. High-resolution mass spectra were recorded on an AutoSpec-E FISION VG mass spectrometer. Nucleotides were analyzed using electron spray ionization (ESI) on a Q-TOF microinstrument (Waters). Primary purification of the nucleotides was

achieved on an LC (Isco UA-6) system using a column of Sephadex DEAE-A25, swollen in 1 M NaHCO₃ at 8 °C for 24 h. The resin was washed with deionized water before use. LC separation was monitored by UV detection at 280 nm. Final purification of the nucleotides was achieved on an HPLC (Merck-Hitachi) system using a semipreparative reversed-phase column [Gemini 5u C-18 110A, 250 mm × 10 mm, 5 μm (Phenomenex, Torrance, CA)]. The purity of the nucleotides was evaluated on an analytical reversed-phase HPLC column system [Gemini 5u C-18 110A, 150 mm × 3.60 mm, 5 μm (Phenomenex)] in two solvent systems, I and II. Solvent system I consisted of (A) 100 mM triethylammonium acetate (TEAA) (pH 7) and (B) CH₃CN. Solvent system II consisted of (A) 46 mM PBS (pH 7.4) and (B) CH₃CN. The details of the solvent system gradients used for the separation of each product are provided below. The products, obtained as triethylammonium salts, were generally 95% pure. All reactants for moisture-sensitive reactions were dried overnight in a vacuum oven. 2',3'-O-(Methoxymethylidene)adenosine, compound **5**,⁵⁶ 2',3'-(methoxymethylidene)-5'-tosyladenosine, compound **8**,⁵⁶ 2',3'-(methoxymethylidene)-α,β-methylene-ADP, compound **9**,⁵⁶ and 8-SH-ATP, compound **4**,⁵⁶ were prepared as previously described.

Adenosine 5'-P_α-Thio-β,γ-methylenetriphosphate (Analogues **1a,b**)

A solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (98 mg, 0.48 mmol) in anhydrous DMF (0.75 mL) was added via syringe to a solution of 2',3'-orthoformate-protected adenosine (compound **5**) (100 mg, 0.32 mmol) and anhydrous pyridine (250 μL) in 0.5 mL of anhydrous DMF at 0 °C under nitrogen. After the resulting solution was stirred at rt for 1 h, tributylamine (500 μL) was added, followed by a solution of bis(tributylammonium) methylenediphosphonate (68 mg, 0.38 mmol) in anhydrous DMF (0.5 mL). The reaction mixture was stirred at rt for 2 h, and then sulfur (21 mg, 0.64 mmol) was added at 0 °C. The solution color changed to orange and afterward to brown. After being stirred at room temperature for 1.5 h, the mixture was dripped into a cold 1 M TEAB solution (10 mL) until pH ≈ 7 was attained. The resulting mixture was stirred at room temperature for 30 min. During that time the color of the solution changed to yellow. The solution was extracted (2 × 10 mL) with ether. The aqueous phase was freeze-dried twice. The product was then deprotected by addition of 10% HCl until pH 2.3 was attained, and the mixture was stirred for 3 h. Afterward 24% NH₄OH was added to give pH ≈ 9, and the mixture was stirred for another 45 min and freeze-dried overnight.

The crude residue was separated on a DEAE-Sephadex A25 column with a linear gradient of ammonium bicarbonate (from 0.1 to 0.4 M ammonium bicarbonate, total gradient volume 600 mL). The relevant fraction was freeze-dried four times to afford 20 mg (9% yield) of adenosine 5'-P_α-thio-β,γ-methylenetriphosphate ammonium salt. Final separation of analogues **1a,b** (two diastereoisomers) was carried out by HPLC on a semipreparative reversed-phase column with a TEAA/ CH₃CN gradient from 96.6:3.4 to 95:5 over 22 min at a flow rate of 4.5 mL/min: retention time *t*_R(isomer **1a**) = 15.8 min, *t*_R(isomer **1b**) = 21.4 min. Data for isomer **1a**: ¹H NMR (D₂O, 200 MHz) δ 8.62 (s, H-8, 1H), 8.27 (s, H-2, 1H), 6.15 (d, *J* = 6.0 Hz, H-1', 1H), 5.00 (m, H2', 1H), 4.60 (m, H-3', 1H), 4.42 (m, H-4', 1H), 4.28 (m, H-5', 2H), 2.28 (t, *J* = 20.0 Hz, CH₂, 2H) ppm; ³¹P NMR (D₂O, 81 MHz) δ 42.9 (d, *J* = 33.5 Hz, P_α-S, 1P), 13.0 (br m, P_γ and P_β, 2P) ppm; TLC (2:7:11 NH₄OH/H₂O/2-

propanol) $R_f = 0.22$. The following purity data were obtained on an analytical column: $t_R = 6.48$ min (96% purity) using solvent system I with a TEAA/CH₃CN isocratic elution at 95:5 over 10 min at a flow rate of 1 mL/min; $t_R = 2.94$ min (95% purity) using solvent system II with a PBS/CH₃CN isocratic elution at 98:2 over 8 min at a flow rate of 1 mL/min. Data for isomer **1b**: ¹H NMR (D₂O, 200 MHz) δ 8.67 (s, H-8, 1H), 8.25 (s, H-2, 1H), 6.15 (d, $J = 6.0$ Hz, H-1', 1H), 5.00 (m, H-2', 1H), 4.59 (m, H-3', 1H), 4.41 (m, H-4', 1H), 4.28 (m, H-5', 2H), 2.31 (t, $J = 21.0$ Hz, CH₂, 2H) ppm; ³¹P NMR (D₂O, 81 MHz) δ 43.2 (d, $J = 32.4$ Hz, P_{α} -S, 1P), 13.2 (br s, P_{γ} , 1P), 11.9 (br d, P_{β} , $J = 32.4$ Hz, 1P) ppm; HRMS ESI (negative) m/z calcd for C₁₁H₁₇N₅O₁₁P₃S⁻ 519.9864, found 519.9853; low-resolution mass spectra were measured for both isomers, and the HRMS spectrum was measured for one of the isomers; TLC (2:7:11 NH₄OH/H₂O/2-propanol) $R_f = 0.22$. The following purity data were obtained on an analytical column: $t_R = 9.12$ min (97% purity) using solvent system I with a TEAA/CH₃CN isocratic elution at 95:5 over 15 min at a flow rate of 1 mL/min; $t_R = 4.17$ min (97% purity) using solvent system II with a PBS/CH₃CN isocratic elution at 98:2 over 10 min at a flow rate of 1 mL/min.

Adenosine 5'- P_{α} -Thio- β,γ -(dichloromethylene)triphosphate (Analogues **2a,b**)

A solution of 2-chloro-4*H*-1,3,2-benzodioxaphosphorin-4-one (117 mg, 0.58 mmol) in anhydrous DMF (1 mL) was added via syringe to a solution of 2',3'-orthoformate-protected adenosine (compound **5**) (100 mg, 0.32 mmol) and anhydrous pyridine (260 μ L) in 1.5 mL of anhydrous DMF at 0 °C under nitrogen. After the resulting solution was stirred at rt for 1 h, tributylamine (626 μ L) was added, followed by a solution of bis(tributylammonium) dichloromethylenediphosphonate (127 mg, 0.42 mmol) in anhydrous DMF (1 mL). The reaction mixture was stirred at rt for 2 h, and then sulfur (25 mg, 0.77 mmol) was added at 0 °C. The solution color changed to orange and then to brown with stirring at rt for 1.5 h. The mixture was dripped into a cold 1 M TEAB solution (10 mL) until pH $\approx$ 7 was attained. The resulting mixture was stirred at rt for 30 min. During that time the color of the solution changed to yellow. The solution was extracted with ether (2 $\times$ 10 mL). The aqueous phase was freeze-dried twice. The product was then deprotected by addition of 10% HCl until pH 2.3 was attained, and the mixture was stirred for 3 h. Afterward 24% NH₄OH was added to give pH $\approx$ 9, and the mixture was stirred for another 45 min and freeze-dried overnight.

The crude residue was separated on a DEAE-Sephadex A25 column with a linear gradient of ammonium bicarbonate (from 0.1 to 0.4 M ammonium bicarbonate, total gradient volume 600 mL). The relevant fraction was freeze-dried four times to afford 43 mg (17% yield) of adenosine 5'- P_{α} -thio- β,γ -(dichloromethylene)triphosphate ammonium salt. Final separation of analogues **2a,b** (two diastereoisomers) was carried out by HPLC on a semipreparative reversed-phase column with a TEAA/CH₃CN gradient from 96.5:3.5 to 95.5:4.5 over 31 min at a flow rate of 4.5 mL/min: t_R (isomer **2a**) = 20.3 min, t_R (isomer **2b**) = 30.6 min. Data for isomer **2a**: ¹H NMR (D₂O, 200 MHz) δ 8.72 (s, H-8, 1H), 8.29 (s, H-2, 1H), 6.16 (d, $J = 6.0$ Hz, H-1', 1H) (H-2' and H-3' signals are hidden by the water signal at 4.78), 4.63 (m, H-4', 1H), 4.35 (m, H-5', 2H), 3.35 (t, Et₃N, 24H), 2.05 (s, AcOH, H), 1.42 (d, Et₃N, 36H) ppm; ³¹P NMR (D₂O, 81 MHz) δ 44.0 (d, $J = 35.4$ Hz, P_{α} -S, 1P), 8.0 (d, $J = 19.3$ Hz, P_{γ} , 1P), -1.0 (dd, $J = 19.3$ Hz, $J = 35.4$ Hz, P_{β} , 1P) ppm; HRMS ESI (negative) m/z calcd for C₁₁H₁₅Cl₂N₅O₁₁P₃S⁻ 587.9084, found 587.9073; low-resolution mass spectra were

measured for both isomers, and the HRMS spectrum was measured for one of the isomers; TLC (2:7:11 NH₄OH/H₂O/2-propanol) R_f = 0.35. The following purity data were obtained on an analytical column: t_R = 9.5 min (99% purity) using solvent system I with a TEAA/CH₃CN isocratic elution at 96:4 over 15 min at a flow rate of 1 mL/min; t_R = 3.35 min (99% purity) using solvent system II with a PBS/CH₃CN isocratic elution at 98:2 over 8 min at a flow rate of 1 mL/min. Data for isomer **2b**: ¹H NMR (D₂O, 200 MHz) δ 8.65 (s, H-8, 1H), 8.29 (s, H-2, 1H), 6.16 (d, J = 6.2 Hz, H-1', 1H) (H-2' signal is hidden by the water signal at 4.78), 4.64 (m, H-3', 1H), 4.45 (m, H-4', 1H), 4.38 (m, H-5', 2H), 3.15 (t, Et₃N, 24H), 2.05 (s, CH₃CO₂H, 3H), 1.38 (d, Et₃N, 36H) ppm; ³¹P NMR (D₂O, 81 MHz) δ 43.9 (d, J = 35.5 Hz, P_{α} -S, 1P), 8.00 (d, J = 19.1 Hz, P_{γ} , 1P), -0.9 (dd, J = 19.1 Hz, J = 35.5 Hz, P_{β} , 1P) ppm; TLC (2:7:11 NH₄OH/H₂O/2-propanol) R_f = 0.36. The following purity data were obtained on an analytical column: t_R = 10.0 min (98% purity) using solvent system I with a TEAA/CH₃CN isocratic elution at 95:5 over 15 min at a flow rate of 1 mL/min; t_R = 4.54 min (98% purity) using solvent system II with a PBS/CH₃CN isocratic elution at 98:2 over 10 min at a flow rate of 1 mL/min.

Adenosine 5'- α,β -Methylene- γ -thiotriphosphate (Analogue 3)

Adenosine 5'- α,β -methylenediphosphate tributylammonium/tri-*n*-octylammonium and thiophosphate tributylammonium/tri-*n*-octylammonium salts were prepared by applying the corresponding phosphate derivatives through a column of activated Dowex 50WX-8 (200 mesh, H⁺ form). The eluate was collected in an ice-cooled flask containing tributylamine, tri-*n*-octylamine (1 equiv), and EtOH. The resulting solution was freeze-dried to yield adenosine 5'- α,β -methylenediphosphate tributylammonium/tri-*n*-octylammonium and thiophosphate tributylammonium/tri-*n*-octylammonium salts, each as a viscous oil.

Adenosine 5'- α,β -methylenediphosphate tributylammonium and tri-*n*-octylammonium salts (75 mg, 0.17 mmol) were suspended in anhydrous DMF (2 mL), 1,1'-carbonyldiimidazole (260 mg, 1.64 mmol) was added at rt, and the mixture was stirred for 5 h. Next dry MeOH (66 μ L) was added, and the reaction was stirred for 8 min, followed by addition of anhydrous ZnCl₂ (220 mg, 1.60 mmol) and thiophosphate tributylammonium and tri-*n*-octylammonium salts (170 mg, 0.98 mmol) in dry DMF (1 mL). After 3 h, the reaction was added to an EDTA solution (580 mg, 1.5 mmol in 20 mL of water) and brought to pH $\approx$ 7 with triethylammonium bicarbonate, and the resulting solution was freeze-dried overnight. The residue was separated on a DEAE-Sephadex A25 column with an isocratic elution of ammonium bicarbonate (from 0 to 0.4 M ammonium bicarbonate, total gradient volume 600 mL). The solution was freeze-dried four times to afford adenosine 5'- α,β -methylene- γ -thiotriphosphate ammonium salt (30 mg, 29% yield). Final purification of analogue **3** was carried out by HPLC on a semipreparative reversed-phase column using an isocratic elution of 4% CH₃CN, 96% TEAA over 15 min at a flow rate of 4.5 mL/min: t_R = 10.25 min; ¹H NMR (D₂O, 200 MHz) δ 8.58 (s, H-8, 1H), 8.26 (s, H-2, 1H), 6.09 (d, J = 5.8 Hz, H-1', 1H), 4.90 (m, H-2', 1H), 4.56 (m, H-3', 1H), 4.36 (m, H-4', 1H), 4.20 (m, H-5', 2H), 3.20 (t, Et₃N, 24H), 2.48 (t, J = 20.00 Hz, CH₂, 2H), 1.30 (d, Et₃N, 36H) ppm; ³¹P NMR (D₂O, 81 MHz) δ 39.0 (d, J = 32 Hz, P_{γ} -S, 1P), 18.2 (d, J = 9 Hz, P_{α} , 1P), 6.9 (dd, J = 9 Hz, J = 32 Hz, P_{β} , 1P) ppm; HRMS ESI (negative) m/z calcd for C₁₁H₁₇N₅O₁₁P₃S₂⁻ 519.9853, found 519.9820; TLC (2:7:11 NH₄OH/H₂O/2-propanol) R_f = 0.30. The following purity data were

obtained on an analytical column: $t_R = 5.23$ min (90% purity) using solvent system I with a TEAA/CH₃CN isocratic elution at 96:4 over 10 min at a flow rate of 1 mL/min; $t_R = 6.72$ min (93% purity) using solvent system II with a PBS/CH₃CN isocratic elution at 97:3 over 10 min at a flow rate of 1 mL/min.

Plasmids

The plasmids used in this study have all been described in published reports: human NTPDase1 (GenBank accession no. U87967),⁵⁷ human NTPDase2 (RefSeq accession no. NM_203368),⁵⁸ human NTPDase3 (GenBank accession no. AF033830),⁵⁹ human NTPDase8 (GenBank accession no. AY330313),⁶⁰ human NPP1 (RefSeq accession no. NM_006208),⁶¹ and human NPP3 (RefSeq accession no. NM_005021).⁶²

Cell Culture and Transfection

Ectonucleotidases were expressed by transiently transfecting COS-7 cells in 10 cm plates by use of Lipofectamine (Invitrogen, Burlington, ON, Canada), as previously described.⁶³ Briefly, 80–90% confluent cells were incubated for 5 h at 37 °C in Dulbecco's modified Eagle's medium nutrient mix F-12 (DMEM/F-12) in the absence of fetal bovine serum (FBS) with 6 µg of plasmid DNA and 23 µL of Lipofectamine reagent. The reaction was stopped by the addition of an equal volume of DMEM/F-12 containing 20% FBS, and the cells were harvested 33–72 h later.

Preparation of the Membrane Fraction

For the preparation of protein extracts, transfected cells were washed three times with Tris–saline buffer at 3 °C, collected by scraping in the harvesting buffer (95 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 35 mM Tris at pH 7.5), and washed twice by 300g centrifugation for 10 min at 3 °C. The cells were resuspended in the harvesting buffer containing 10 mg·mL^{−1} aprotinin and sonicated. Nuclei and cellular debris were discarded by centrifugation at 300g for 10 min at 3 °C, and the supernatant (crude protein extract) was aliquoted and stored at −80 °C until it was used for activity assays. The protein concentration was estimated by the Bradford microplate assay using bovine serum albumin (BSA) as a standard of reference.⁶⁴

NPP and TNAP Stability Assay

Evaluation of the hydrolysis percentage (compared to ATP) of analogues **1–4** by human NPP1,3 and of **2a** and **3** by TNAP was conducted at 37 °C in incubation buffer (0.573 mL) containing 1 mM CaCl₂, 130 mM NaCl, 5 mM KCl, and 50 mM Tris (pH 8.5 for NPPs and pH 9 for TNAP) with or without lysates of cells expressing NPP1,3 or TNAP. Analogues **1–4** were used at a final concentration of 100 µM. Recombinant human NPP1,3 or TNAP cell lysates were added to the above mixture and preincubated at 37 °C for 3 min. The reaction was initiated by addition of the analogues or ATP (control). The reaction was stopped after 1 h by addition of 0.375 mL of ice-cold 1 M perchloric acid. The samples were centrifuged for 1 min at 13000g. The supernatants were neutralized with 2 M KOH (0.136 mL) at 4 °C and centrifuged for 1 min at 13000g. Each mixture sample was filtered and freeze-dried once for storage overnight. The samples were dissolved in HPLC water (0.2 mL) and filtered again.

An aliquot of 20 μ L was separated by reversed-phase HPLC to evaluate the nucleotide content of each reaction sample.

Separation and Quantification of Nucleotides by HPLC

An aliquot of 20 μ L of the reaction products (described above) was used for nucleotide analysis on an analytical reversed-phase HPLC column [Gemini 5u C-18 110A, 150 mm $\times$ 3.60 mm, 5 μ m (Phenomenex)]. Analogues **1–4** and their hydrolysis products were separated with a mobile phase consisting of 100 mM TEAA (99–90%, pH 7.0) and 1–10% CH₃CN at a flow rate of 1 mL/min for 20 min. Separated nucleotides were detected by UV absorption at 260 nm, identified, and quantified by their peak integration and by comparison of the retention times with those of appropriate standards.

Inhibition of NTPDase (EC 3.6.1.5): Activity Assays

Activity was measured as described previously¹ in 0.2 mL of Tris–Ringer buffer (120 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 25 mM NaHCO₃, 5 mM glucose, 80 mM Tris, pH 7.3) at 37 °C with or without analogues **1–3** (final concentration 100 μ M) and with or without 100 μ M ATP as a substrate. The analogues were added alone when tested as a potential substrate and with ATP when tested for their effect on nucleotide hydrolysis. NTPDase protein extracts were added to the incubation mixture and preincubated at 37 °C for 3 min. The reaction was initiated by the addition of substrate (ATP and/or analogues **1–3**) and stopped after 15 min with 50 μ L of malachite green reagent. The released inorganic phosphate (P_i) was measured at 630 nm according to Baykov et al.⁶⁵

Inhibition of NPP and TNAP (EC 3.1.4.1; EC 3.6.1.9): Activity Assays

Evaluation of the effect of analogues **1–3** on human NPP1,3 activity was carried out with either pNP-TMP or ATP as the substrate.⁶¹ pNP-TMP hydrolysis was carried out at 37 °C in 0.2 mL of the following incubation mixture with or without analogues **1–3** and/or substrates: 1 mM CaCl₂, 130 mM NaCl, 5 mM KCl, and 50 mM Tris, pH 8.5. Substrates and analogues **1–3** were all used at a final concentration of 100 μ M. Recombinant human NPP1 or NPP3 cell lysates were added to the incubation mixture and preincubated at 37 °C for 3 min. The reaction was initiated by the addition of the substrate. For pNP-TMP hydrolysis, the production of *p*-nitrophenol was measured at 310 nm, 15 min after the initiation of the reaction. The type of inhibition was determined and IC₅₀, *K_i*, and *K_i'* were calculated by plotting the data of three independent experiments using pNP-TMP as the substrate according to the Dixon and Cornish–Bowden methods.^{31,32}

Evaluation of the activities of human NPP1, NPP3, and TNAP with ATP and analogues **1–3** was carried out at 37 °C in 0.2 mL of the following mixture: 1 mM CaCl₂, 140 mM NaCl, 5 mM KCl, and 50 mM Tris, pH 8.5 for NPPs and pH 9 for TNAP. Human NPP1, NPP3, or TNAP extract was added to the reaction mixture and preincubated at 37 °C for 3 min. The reaction was initiated by addition of ATP or analogues **1–3** at a final concentration of 100 μ M. The reaction was stopped after 20 min by transferring a 0.1 mL aliquot of the reaction mixture to 0.125 mL of ice-cold 1 M perchloric acid. The samples were centrifuged for 5 min at 13000g. The supernatants were neutralized with 1 M KOH at 4 °C and centrifuged

for 5 min at 13000g. An aliquot of 20 mL was separated by reversed-phase HPLC to evaluate the degradation of ATP and analogues **1–3** using the conditions described above.

Activity assays at the surface of intact HTB-85 cells were carried out in 0.5 mL of the incubation mixture in 24-well plates. Reaction was initiated by the addition of pNP-TMP to obtain a final concentration of 100 μ M. After 20 min, 0.2 mL of the reaction mixture was transferred to a 96-well plate, and the production of *p*-nitrophenol was measured at 410 nm as described above.

Calcium Measurements

Human 1321N1 astrocytoma cells transfected with the respective plasmid for GFP-P2Y-R expression were plated on glass-bottom dishes, grown to approximately 80% density, and incubated with 2 μ M fura-2 acetoxymethyl ester (fura-2 AM) and 0.02% pluronic acid in HBSS (Hank's balanced salt solution, 5.44 mM KCl, 0.44 mM KH₂PO₄, 0.34 mM NaH₂PO₄, 0.49 mM MgCl₂, 0.41 mM MgSO₄, 132 mM NaCl, 5.56 mM glucose, 10 mM Hepes/Tris, pH 7.4) for 30 min at 37 °C. The cells were superfused (1 mL/min, 37 °C) with different concentrations of nucleotide in HBSS. The nucleotide-induced change of [Ca²⁺]_i was monitored by detecting the respective emission intensity of fura-2 AM at 510 nm with 340 and 380 nm excitations.⁶⁶ The average maximal amplitude of the responses and the respective standard errors were calculated from the ratio of the fura-2 AM fluorescence intensities with excitations at 340 and 380 nm. Microsoft Excel (Microsoft Corp., Redmond, WA) and SigmaPlot (SPSS Inc., Chicago, IL) were used to derive the concentration–response curves and EC₅₀ values from the average response amplitudes obtained in at least three independent experiments.^{47,49} Only cells with a clear GFP signal that yielded typical calcium response kinetics upon agonist pulse application were included in the data analysis. The GFP-tagged P2Y receptors are suitable for pharmacological and physiological studies, as previously reported.^{46,67,68}

Molecular Modeling

A 3-dimensional (3D) model of human NPP1 was previously reported.²² This model was used for the docking of analogues **1–3**. Prior to docking, the protein structure was prepared using the Protein Preparation Wizard in Discovery Studio version 3.5.⁶⁹ Docking simulations were performed using Glide^{70,71} as implemented in Maestro 9.0.⁷² Glide's grid box was centered on the binding site as deduced from the mouse NPP1 crystal structure. A docking grid was generated within the docking box, and ligands were docked into the binding site using Glide's Extra Precision (XP) option with default parameter values.⁷³ Following docking, the resulting poses were clustered in Maestro using all heavy atoms along the analogues' "backbone", and the poses closest to the center of the largest clusters were taken as representatives. Boltzmann-averaged energies were calculated over poses comprising the largest cluster at *T* = 37 °C.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ABBREVIATIONS USED

[Ca²⁺]_i	intracellular Ca ²⁺ concentration
CDI	carbonyldiimidazole
NPP	ectonucleotide pyrophosphatase/phosphodiesterase
NTPDase	ectonucleoside triphosphate diphosphohydrolase
ESI	electron spray ionization
HRMS-MALDI	highresolution mass spectrometry matrix-assisted laser desorption ionization
P2R	P2 receptor
NTP	nucleoside 5'-triphosphate
pNP-TMP	thymidine 5'-monophosphate <i>p</i> -nitrophenyl ester
rt	room temperature
SD	standard deviation
TEAA	triethylammonium acetate
TEAB	triethylammonium bicarbonate
CPPD	calcium pyrophosphate dihydrate
GFP	green fluorescent protein
hERG	human ether-à-go-go-related gene
PDB	Protein Data Bank

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Figure 1.

Dinucleotide and nucleotide analogues previously studied as potential NPP1 inhibitors: (A) bis(2'-deoxyadenosine) $\alpha,\beta,\delta,\epsilon$ -dimethylenepentaphosphonate, (B) ATP-5'-O- α -borano- β,γ -CH₂.

Figure 2.

Adenosine 5'-triphosphate analogues studied here as potential NPP1 inhibitors.

Figure 3.
 S_P and R_P configurations are attributed to ATP-5'- α -S- β , γ -CH₂ diastereoisomers **1a** and **1b**, respectively.

Figure 4.

Analogues **1–3** (100 μ M) inhibit NPP activities. The activity of human NPP1 (hNPP1) and NPP3 (hNPP3) was tested with pNPTMP (A, C) or ATP (B) as the substrate at 100 μ M. Activity of 100% with pNP-TMP alone was 48 ± 4 and 32 ± 2 nmol of *p*-nitrophenol·min⁻¹·mg of protein⁻¹ for NPP1 and NPP3, respectively (A). Activity of 100% with ATP alone was 153 ± 6 and 110 ± 5 nmol of nucleotide·min⁻¹·mg of protein⁻¹ for NPP1 and NPP3, respectively (B). Data presented are the mean $\pm$ SD of three experiments carried out in triplicate. (C) Analogues **1–3** inhibit NPP activity at the surface of HTB85 cells. NPP activity of 100% was set with the substrate alone and was 1.3 ± 0.04 nmol of *p*-

nitrophenol·min⁻¹·well. Data presented are the mean ± SD of results from three experiments carried out in triplicate.

Figure 5.

$K_{i,app}$ determination using Dixon (A) and Cornish–Bowden (B) plots of human NPP1 by analogues **2a** and **3**. pNP-TMP concentrations were 25, 50, and 100 μ M, and the inhibitor concentrations were 0, 25, 50, and 100 μ M. The data of one representative experiment out of three are shown.

Figure 6.

Activity of analogues **1b**, **2b**, and **3** at the P2Y₁₁R. Data were obtained by determining the ligand-induced change in $[Ca^{2+}]_i$ in 1321N1 cells stably expressing the human GFP-P2Y₁₁R. Cells were preincubated with 2 μ M fura-2 AM for 30 min, and the change in fluorescence ($F_{340\text{ nm}}/F_{380\text{ nm}}$) was detected.

Figure 7.

Homology model of human NPP1 and representative poses for analogues **1–3**. (A) 3D model of human NPP1 with ATP docked into its catalytic site. The protein is shown as a ribbon diagram color coded according to its secondary structure. The two tentative Zn ions are shown as orange spheres, and the ATP molecule is depicted in blue. (B–F) Representative poses of analogues **1a**, **1b**, **2a**, **2b**, and **3**, respectively, in the binding site of the human NPP1 model. The two Zn ions are shown as orange spheres, and the ligands are colored according to atom type (nitrogen atoms are colored in blue, oxygen atoms are colored in red, carbon atoms are colored in gray, and phosphorus atoms are colored in purple). H-bonds and π -interactions are shown in green and orange, respectively.

Figure 8.

Correlation between binding free energies (kcal/mol; derived from the experimentally determined K_i values at 37 °C) and (A) Boltzmann averaged Glide scores (kcal/mol) over the largest clusters, (B) Glide scores (kcal/mol) for the representative poses of analogues **1**–**3**, and (C) H-bond component of the Glide score (kcal/mol). G values were calculated using analogue **3** as a reference.

Figure 9. Electrostatic potential of analogues **2a** and **2b**. Electrostatic potentials of analogues **2a** (A) and **2b** (B) within the human NPP1 model were calculated on their respective molecular surfaces. The enhanced negative potential (red) of the solvent-exposed surface area corresponding to analogue **2a** in comparison with analogue **2b** (circled in green) is clearly visible.

Scheme 1. Synthesis of ATP- α -S- β γ -methylene Analogues 1a,b and 2a,b^a

^aReaction conditions: (a) 2-chloro-4H-1,3,2-benzodioxaphosphorin-4-one, DMF, pyridine, rt, 1 h; (b) bis(tributylammonium) methyl-enediphosphonate or bis(tributylammonium) dichloromethylenedi-phosphonate, DMF, tributylamine, rt, 2 h; (c) (1) S₈, 0 °C, 1.5 h; (2) 1 M TEAB, 0.5 h; (3) 10% HCl, pH 2.5, 3 h; (4) 10% NH₄OH, pH 7, rt, 45 min. Analogues **1** and **2** were obtained in 9% and 17% yields, respectively. Compounds **6** and **7** are reaction intermediates and were not isolated.

Scheme 2. Synthesis of ATP- α,β -methylene- γ -thio (3)^a

^aReaction conditions: (a) TsCl, DMAP, DCM, rt, 3 h; (b) bis(tributylammonium) methylenediphosphonate, DMF, rt, 24 h (50% yield); (c) (1) 10% HCl, pH 2.5, rt, 3 h; (2) 10% NH₄OH, pH 7, rt, 45 min; (d) (1) CDI, DMF, rt, 5 h; (2) ZnCl₂, MeOH, rt, 8 min; (3) tri-*n*-octylammonium and tributylammonium thiophosphate, rt, 3 h; (4) EDTA, H₂O, rt, 10 min (29% yield).

Scheme 3. Synthesis of 8-SH-ATP (4)^a

^aReaction conditions: (a) NaSH, DMF 12 h; (b) (1) POCl₃, proton sponge, trimethyl phosphate, -15 °C, 3 h; (2) pyrophosphate, DMF, 2 h (60% yield).

Table 1

Hydrolysis of Analogues 1–4 by Human Ectonucleotidases^a

human ectonucleotidase	relative activity (%) $\pm$ SD of Δ ATP hydrolysis					
	1a	1b	2a	2b	3	4
NTPDase1	4.4 $\pm$ 0.2	5.4 $\pm$ 0.2	5.3 $\pm$ 0.2	5.4 $\pm$ 0.2	5.3 $\pm$ 0.2	43 $\pm$ 2
NTPDase2	4.7 $\pm$ 1.7	5.5 $\pm$ 0.3	5.2 $\pm$ 0.1	5.5 $\pm$ 0.2	4.7 $\pm$ 0.2	73 $\pm$ 2
NTPDase3	4.2 $\pm$ 0.2	4.8 $\pm$ 0.2	5.2 $\pm$ 0.2	5.4 $\pm$ 0.2	4.8 $\pm$ 0.2	51 $\pm$ 3
NTPDase8	4.4 $\pm$ 0.2	5.3 $\pm$ 0.2	4.3 $\pm$ 0.1	5.3 $\pm$ 0.2	5.2 $\pm$ 0.2	73 $\pm$ 1
NPP1	ND	4 $\pm$ 0.8	ND	ND	ND	54 $\pm$ 2
NPP3	1.2 $\pm$ 0.9	4 $\pm$ 0.8	ND	ND	ND	32 $\pm$ 2

^a Adenosine triphosphate analogues **1–4** were incubated in the presence of the indicated ectonucleotidases at a concentration of 100 μ M. The activity with 100 μ M ATP was set as 100%, which was 807 $\pm$ 35, 1051 $\pm$ 45, 240 $\pm$ 17, and 122 $\pm$ 7 nmol of P_i·min⁻¹·mg of protein⁻¹ for NTPDase1,2,3,8, respectively. For NPP1 and NPP3, 100% activity with 100 μ M ATP as the substrate was 67 $\pm$ 5 and 54 $\pm$ 2 nmol of nucleotide·min⁻¹·mg of protein⁻¹, respectively. Data presented are the mean $\pm$ SD of results from three experiments carried out in triplicate. ND = no hydrolysis detected.

Table 2Effect of Analogues 1–3 on Human NTPDase1,2,3,8 Activity^a

enzyme	inhibition (%) ± SD			
	1a	1b	2a	2b
human NTPDase1	18.7 ± 0.8	21.6 ± 1.0	0.5 ± 0.01	0.5 ± 0.02
human NTPDase2	15.4 ± 0.7	12.7 ± 0.5	11.2 ± 0.5	9.9 ± 0.4
human NTPDase3	26.8 ± 1.1	24.0 ± 1.2	21.8 ± 1.0	18.7 ± 0.8
human NTPDase8	0.50 ± 0.02	1.5 ± 0.06	4.9 ± 0.2	0.5 ± 0.01

^a ATP was used as the substrate in the presence of analogue **1a**, **1b**, **2a**, **2b**, or **3**. Both the substrate and analogues **1–3** were studied at 100 μ M. Activity of 100% was set with the substrate ATP alone, which was 807 ± 35 , 1051 ± 45 , 240 ± 17 , and 122 ± 7 nmol of $P_i \cdot \text{min}^{-1} \cdot \text{mg of protein}^{-1}$ for NTPDase1,2,3,8, respectively. Data presented are the mean $\pm$ SD of three experiments carried out in triplicate.

Table 3Kinetic Parameters and IC₅₀ of NPP1 Inhibition^a

inhibitor	K_i (μ M)	K_i' (μ M)	IC ₅₀ (μ M)
1a	4.5 $\pm$ 0.03	4.5 $\pm$ 0.003	16.3 $\pm$ 0.04
1b	1.3 $\pm$ 0.01	-71.5 $\pm$ 0.5	18.7 $\pm$ 0.03
2a	0.685 $\pm$ 0.005	-12.5 $\pm$ 0.1	0.6 $\pm$ 0.01
2b	15.2 $\pm$ 0.1	-192.0 $\pm$ 1	31.2 $\pm$ 0.1
3	0.02 $\pm$ 0.0001	-9.0 $\pm$ 0.05	0.39 $\pm$ 0.001

^aFor K_i and K_i' determinations, pNP-TMP (substrate) and analogues **1–3** were used in the concentration range of 2.5×10^{-5} to 1×10^{-3} M. For IC₅₀ determinations, the pNP-TMP concentration was 5×10^{-5} M and the inhibitor concentration ranged from 5×10^{-7} to 1×10^{-3} M. All experiments were performed three times in triplicate.

Table 4EC₅₀ Values for [Ca²⁺]_i Elevation by Analogues 1–3 Mediated by P2Y_{1,11} Receptors

receptor subtype	nucleotide analogue	EC ₅₀ ± SEM (μM), [Ca ²⁺] _i elevation	increase in efficacy ^a vs ATP
P2Y ₁	1a	not active, nr ^c	
	1b	not active, slight response ^b	
	2a	not active, nr ^c	
	2b	not active, nr ^c	
	3	not active, slight response ^b	
	ATP ^a	0.85 ± 0.047	1
P2Y ₁₁	1a	not active, nr ^c	
	1b	0.9 ± 0.075	7.4
	2a	not active, nr ^c	
	2b	3.0 ± 1.46	2.2
	3	1 ± 0.49	6.7
	ATP ^a	6.7 ± 0.87	1

^a Efficacy is given as the EC₅₀ value of the tested nucleotide in relation to the EC₅₀ value of the endogenous agonist ATP. ATP was selected as the common reference agonist at both P2Y₁ and P2Y₁₁ receptors, although ADP is the preferred endogenous P2Y₁ receptor agonist.

^b No response up to 1 μM; only 10 μM agonist concentration evoked a response corresponding to 40% of the maximal response of endogenous ligand ATP. For technical reasons, higher concentrations were not tested.

^c The agonist showed no response up to 10 μM concentration.