Journal of Medicinal Chemistry Article

J. Med. Chem. **2010,** *53,* 8485–8497 **8485**
DOI: 10.1021/jm100597c

Diadenosine 5′,5″-(Boranated)polyphosphonate Analogues as Selective Nucleotide Pyrophosphatase/Phosphodiesterase Inhibitors[⊥]

Shay Eliahu,[†,∥] Joanna Lecka,[∥,‡] Georg Reiser,[§] Michael Haas,[§] François Bigonnesse,[‡] Sébastien A. Lévesque,[‡] Julie Pelletier,[‡] Jean Sévigny,*[,‡] and Bilha Fischer*[,†]

[†]*Department of Chemistry, Bar-Ilan University, Ramat-Gan 52900, Israel,* [‡]*Centre de recherche en Rhumatologie et Immunologie, Centre Hospitalier Universitaire de Québec (pavillon CHUL), Québec, QC, Canada, and Département de microbiologie-infectiologie et d'immunologie, Faculté de Médecine, Université Laval, Québec, QC, Canada, and* [§]*Institute for Neurobiochemistry, Faculty of Medicine, Otto von Guericke University, Leipziger Strasse 44, D-39120 Magdeburg, Germany.* [∥]*These authors contributed equally to this work.*

Received May 17, 2010

Nucleotide pyrophosphatase/phosphodiesterases (NPPs) hydrolyze extracellular nucleotides and dinucleotides and thus control purinergic signaling. Enhanced NPP activity is implicated in health disorders such as osteoarthritis and cancer. We designed novel diadenosine polyphosphonate derivatives as potential NPP inhibitors. Analogues **1**−**4** bear a phosphonate and/or boranophosphate group and/or a 2′-H atom instead of a 2′-OH group. In comparison to ATP, analogues **1**−**4** were barely hydrolyzed by human NTPDase1, -2, -3, and -8 (< 5% hydrolysis) and NPP1 and -3 (≤13%) and were not hydrolyzed by ecto-5′-nucleotidase, unlike AMP. These derivatives did not affect NTPDase activity, and analogues **1** and **2** did not inhibit ecto-5′-nucleotidase. All analogues blocked ∼80% of the NPP2-dependent hydrolysis of pnp-TMP, a specific NPP substrate, and inhibited the catabolism of pnp-TMP (K_i and IC_{50} both found to be between 10 and 60 μM), Ap_5A, and ATP by NPP1. The activity of NPP3 was inhibited to a lesser extent by the new analogues, with compounds **1** and **4** being the most effective in that respect. The analogues dramatically reduced the level of hydrolysis of pnp-TMP at the cell surface of both osteocarcinoma and colon cancer cells. Importantly, analogues **1**−**4** exhibited significantly reduced agonistic activity toward human $P2Y_{1,11}$ receptors (except for analogue **1**) and no activity with human $P2Y_2$ receptor. Our data provide strong evidence that analogue **2** is the first specific NPP inhibitor to be described.

Introduction

Nucleoside triphosphate diphosphohydrolase-1, -2, -3, and -8 [NTPDase1, -2, -3, and -8, respectively (EC 3.6.1.5)] and nucleotide pyrophosphatase/phosphodiesterase-1 and -3 [NPP1 and -3, respectively (EC 3.1.3.1 and EC 3.6.1.9, respectively)] are the dominant ectonucleotidases that terminate nucleotide signaling through the hydrolysis of nucleotide agonists of the P2X and P2Y receptors.[1−3]

NTPDases catalyze the hydrolysis of the terminal phosphate of nucleoside triphosphates (e.g., ATP and UTP) and diphosphates (ADP and UDP) at different rates. NTPDase1, -2, -3, and -8 are plasma membrane-bound with an extracellular active site. NTPDase1 (CD39/ATPDase/ectoapyrase/ecto-ADPase) hydrolyzes ATP and ADP equally well,[4] while NTPDase2 (ecto-ATPase/CD39L1) is a preferential triphosphonucleosidase.[5] Both NTPDase3 (CD39L3/HB6) and NTPDase8 are functional intermediates between NTPDase1 and NTPDase2.[3] NTPDase4−7 are mainly associated with intracellular organelles and are therefore not expected to significantly affect P2 receptor activation. The product of NTPDase activity, AMP, is further hydrolyzed by ecto-5′-nucleotidase (CD73), giving adenosine, which is the natural ligand of P1 receptors.[6,7]

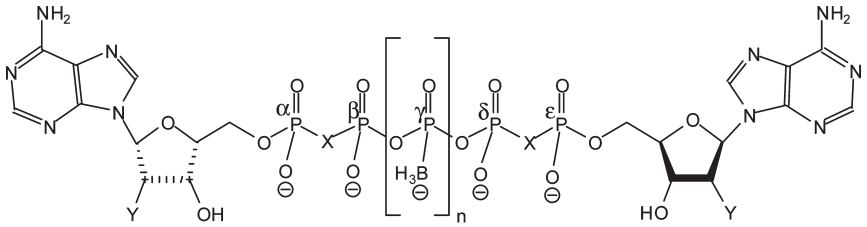
E-NPP[a] family members are conserved eukaryotic enzymes that, as for NTPDases, exist as membrane glycoproteins, with an extracellular active site. Three members of the E-NPP family hydrolyze nucleotides, namely, NPP1 (PC-1), NPP2 (PD-Iα autotaxin), and NPP3 (gp130RB13-6/B10/PD-Iβ). NPP1 and NPP3 are closely related, being ∼50% identical, and are 39 and 31% identical to NPP2, respectively.[8] NPP1−3 have broad substrate specificity. They hydrolyze nucleotides, dinucleotides, and nucleotide sugars, e.g., ATP, ADP, NAD^+, ADP-ribose, and diadenosine polyphosphates, and normally release AMP and the remaining part of the molecule as the main hydrolysis products. Both purine and pyrimidine nucleotides may serve as substrates.[9,10] Thymidine 5′-monophosphate *p*-nitrophenyl ester (pnp-TMP) is often used as a synthetic substrate for NPP activity assays.[11]

[⊥] Patent pending.

*To whom correspondence should be addressed. B.F.: Department of Chemistry, Bar-Ilan University, Ramat-Gan 52900, Israel; fax, 972-3-6353907; telephone, 972-3-5318303; e-mail, bfischer@mail.biu.ac.il. J.S.: Centre de Recherche en Rhumatologie et Immunologie, 2705 Laurier Blvd., Room T1-39, Québec, QC, Canada G1V 4G2; fax, (418) 654-2765; telephone, (418) 654-2772; e-mail, Jean.Sevigny@crchul.ulaval.ca.

[a] Abbreviations: E-NPP, ecto-nucleotide pyrophosphatase/phosphodiesterase; E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; pnp-TMP, thymidine 5′-monophosphate *p*-nitrophenyl ester; P2R, P2 receptor; BP_i, boranophosphate; CDI, carbonyldimidazole; ESI, electron spray ionization; MPLC, medium-pressure liquid chromatography; RT, room temperature; TEAA, triethylammonium acetate; HRMS-MALDI, high-resolution mass spectrometry matrix-assisted laser desorption ionization; FBS, fetal bovine serum; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; SD, standard deviation.

Published on Web 11/22/2010



1 Y = OH X = CH_2 n = 1
2 Y = H X = CH_2 n = 1
3 Y = OH X = CH_2 n = 0
4 Y = H X = CH_2 n = 0
5 Y = OH X = O n = 1

Figure 1. Dinucleotide analogues synthesized and investigated in this study.

NPP2 has a much lower ATPase activity than NPP1 and NPP3 and therefore may not play an important role in the regulation of P2 receptor activation. Nevertheless, NPP1, NPP2, and NPP3 are the major if not only enzymes that have the ability to hydrolyze extracellular diadenosine polyphosphates in vertebrate tissues[12] and may therefore play a crucial role in the regulation of the functions played by these molecules. Other cloned NPPs (NPP4−7) do not hydrolyze nucleotides.[9,13]

NPP1−3 have been implicated in various biological processes. NPP1 plays an important role in the regulation of bone mineralization.[14] NPP1-deficient humans exhibit pathological calcification.[15] Although osteoblasts express NPP1−3, only NPP1 is enriched in membrane-limited vesicles (matrix vesicles), where it plays an important role in the control of mineralization.[16] A loss of functional NPP1 is associated with hypermineralization abnormalities such as osteoarthritis and ossification of the posterior longitudinal ligament of the spine.[17] NPP1 has also been reported to affect insulin signaling via the inhibition of insulin receptor tyrosine kinase activity.[12,18] In addition, NPPs are involved in cell differentiation and motility.[19,20] NPP3 expression is associated with carcinogenesis and metastasis of cancer cells and has been proposed as a tumor marker.[21] As the level of NPP expression is increased in membranes of aged rat brains (NPP1) and in the brain cortex of Alzheimer's disease patients (NPP2), the inhibition of these enzymes has been proposed as a novel alternative for targeting neurodegenerative diseases.[22]

There is currently a lack of specific NPP inhibitors. Due to this state of affairs, the therapeutic potential of NPP inhibition for the treatment of health disorders such as chondrocalcinosis[15] or cancer[23,24] remains virtually unexplored. Indeed, NPP inhibitors have scarcely been reported. Thus, suramin was reported to reduce the level of hydrolysis of *p*-Nph-5′-TMP by NPP by ca. 36% at 250 μM.[25] It is noteworthy that suramin and its derivatives antagonize most P2 receptors and also efficiently inhibit NTPDases and cannot therefore be considered as specific NPP inhibitors.[26] Recently, [3-(*tert*-butyldimethylsilyloxy)phenyl]-1,3,3-oxadiazole-2(3*H*)-thione was reported as an NPP1 inhibitor (K_i = 100 μM).[27] Likewise, biscoumarin derivatives were identified as pure noncompetitive inhibitors of snake venom and human NPP1 enzymes, with K_i and IC_{50} values as low as 50 and 164 μM, respectively, for human NPP1.[28]

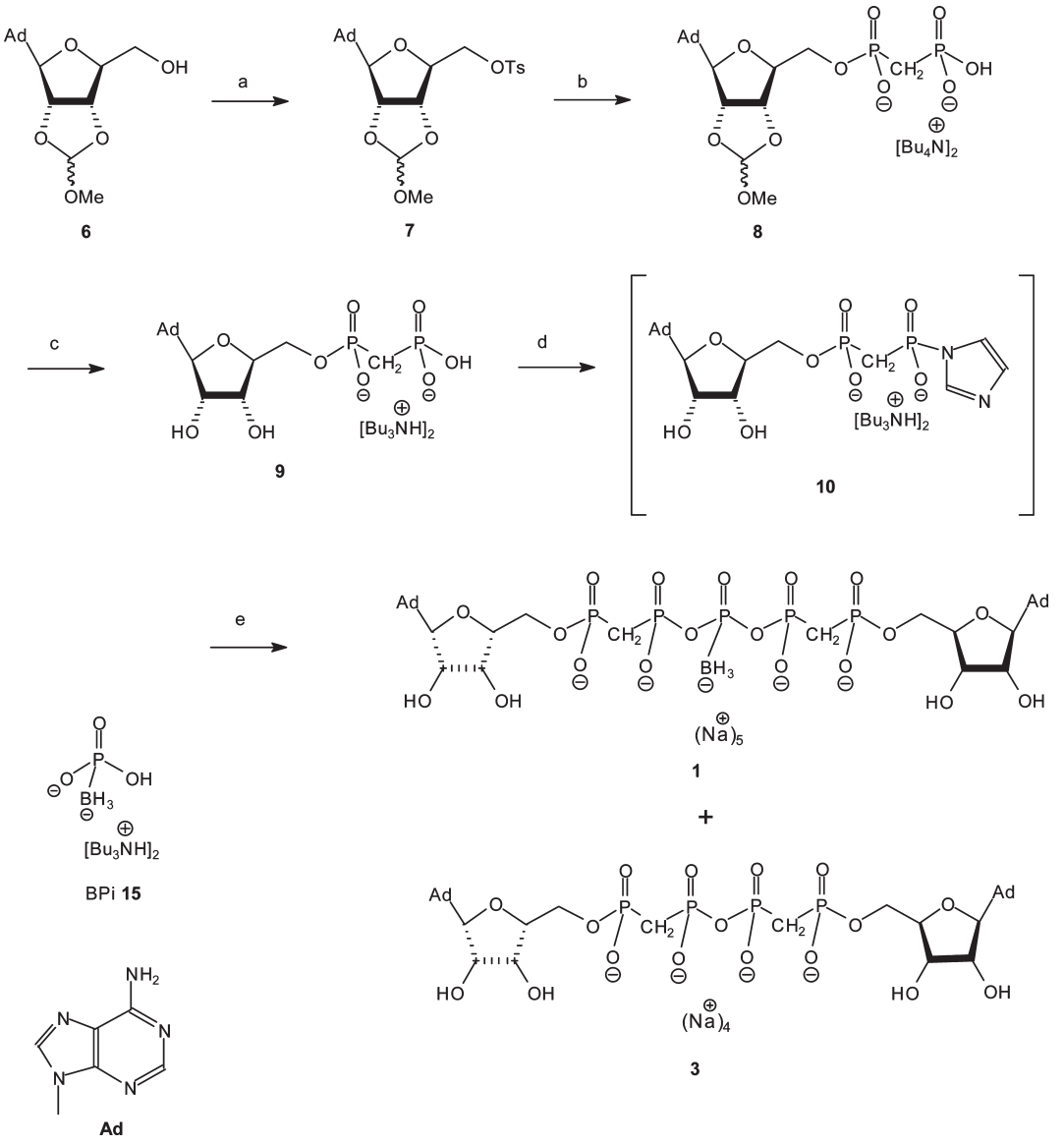
The potential of NPP inhibitors as efficient therapeutic agents, on one hand, and the lack of structure−activity relationship and NPP selectivity data, on the other hand, encouraged us to design and synthesize a series of novel dinucleotide analogues **1**−**4** as potential NPP inhibitors (Figure 1). These analogues were evaluated for their protein selectivity as either agonists of $P2Y_{1,2,11}$ receptors or substrates for the major ectonucleotidases. Furthermore, their inhibitory activity and NPP subtype selectivity were evaluated by comparison of their effects on the other main ectonucleotidases, in the presence of pnp-TMP, Ap_5A, or ATP as the substrate. In addition, dinucleotide analogues **1**−**4** were evaluated as inhibitors of cell surface NPP activity in two cancer cell lines. With this approach, we have been able to identify a most selective NPP inhibitor and establish important structure−activity relationships for such inhibitors.

Results

Synthesis of Dinucleoside Polyphosphonate Analogues 1−4. Dinucleoside polyphosphates are conventionally prepared via the activation of the 5′-terminal phosphate of a nucleotide, thus forming a phosphoryl donor (P-donor), followed by reaction with a nonactivated nucleoside 5′-phosphate, or phosphonate analogue [phosphoryl acceptor (P-acceptor)]. A common method for activation of phosphates/phosphonates uses carbonyldiimidazole (CDI) to form phosphoroimidazolides. The latter may be generated in situ or isolated prior to the reaction with the corresponding nucleotides.[29] We had previously synthesized diadenosine (γ-borano)pentaphosphate (**5**) using an inorganic boranophosphate salt (BP_i)[30] as a P-acceptor and two nucleoside phosphoroimidazolides (NDP-Im) as P-donors.[1] Here, we used the same synthetic method to prepare the corresponding phosphonate analogues, **1** and **2** (Schemes 1 and 2).

Diadenosine $\alpha,\beta-\delta,\varepsilon$-dimethylene-pentaphosphonate, **1**, and di-2′-deoxyadenosine $\alpha,\beta-\delta,\varepsilon$-dimethylene-pentaphosphonate, **2**, were prepared as described above from the α,β-methylene-ADP building blocks, **9**, and α,β-methylene-2′-deoxy-ADP, **13**, respectively. α,β-Methylene-ADP derivatives were synthesized as previously reported.[31] Specifically, adenosine analogue **6**, which is 2′- and 3′-OH-protected, was activated with tosyl chloride to form analogue **7**.[31] The activated nucleoside **7** was then coupled with a tris(tetra-*n*-butylammonium)-methylene diphosphonate salt to form analogue **8**, followed by removal of the protecting group, which provided product **9** (Scheme 1).[31] The related scaffold, α,β-methylene-2′-deoxy-ADP, **13**, was prepared from 2′-deoxyadenosine. A selective tosylation at the 5′-OH position of **11** was conducted at 0 °C to form product **12**,[32] which was then coupled with a tris(tetra-*n*-butylammonium)methylene diphosphonate salt to yield product **13** (Scheme 2).[32]

Scheme 1[a]



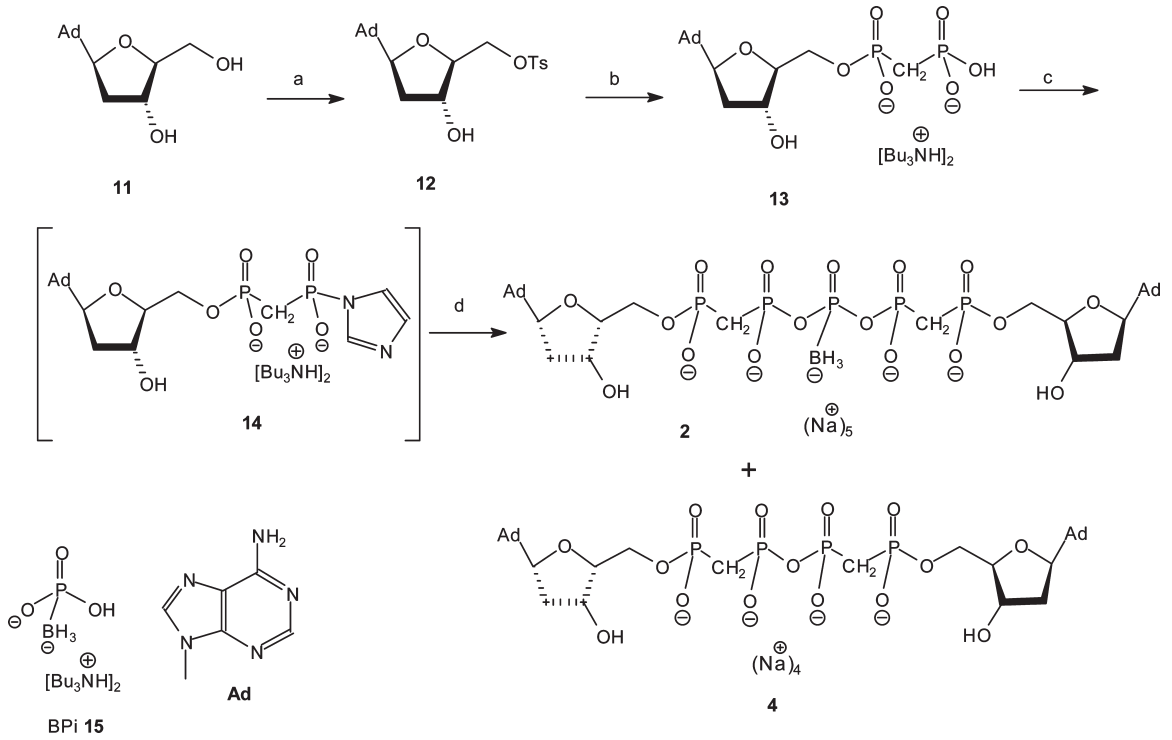
[a] Reaction conditions: (a) CH_2Cl_2, DMAP, TsCl, RT, 12 h, 67%; (b) tetrakis(*n*-butylammonium)methylenediphosphonate, dry DMF, RT, 38 h, 63%; (c) (1) 18% HCl, pH 2.3, RT, 3 h, (2) 23% NH_4OH, pH 9, RT, 35 min, 63%; (d) CDI, DMF, RT, 12 h; (e) BP_i, **15**, $MgCl_2$, RT, 23 h. **1** and **3** obtained in 10 and 20% yields, respectively.

Nucleotides **9** and **13** were activated with CDI in situ to form P-donors **10** and **14**, which were then treated with BP_i, **15**, as a P-acceptor. $MgCl_2$ was added as an activator to overcome the low nucleophilicity of BP_i as a P-acceptor.[33] Products **1** and **2** were obtained in 10 and 21% overall yields, respectively, after LC separation.

Dinucleoside poly(borano)phosphonate products **1** and **2** are probably formed because of the preorganization of a P-acceptor (BP_i) and two P-donors (nucleoside phosphorimidazolides) coordinated with one Mg^{2+} ion (Figure 2). Specifically, the Mg^{2+} ion probably stablizes the folded structure, which involve two molecules of α,β-methylene-ADP-imidazolide (Im) or α,β-methylene-2′-deoxy-ADP-Im and one BP_i ion. This structure provides the correct orientation and proximity for a nucleophilic attack of both nucleoside phosphoroimidazolide reactants by BP_i in an octahedral complex (Figure 2A, paths a and b). Although we expected to obtain analogues **1** and **2** as exclusive products, byproducts **3** and **4** were also isolated with 20 and 28% yields, respectively. The formation of **3** and **4** products is driven by α,β-methylene-ADP, **9**, and α,β-methylene-2′-deoxy-ADP, **13**, which remained in the reaction mixture because of incomplete reaction with CDI. Thus, the activated forms, α,β-methylene-ADP-Im, **10**, and α,β-methylene-2′-deoxy-ADP-Im, **14**, become P-donors, whereas α,β-methylene-ADP, **9**, and α,β-methylene-2′-deoxy-ADP, **13**, rather than BP_i, function as P-acceptors (Figure 2B). Furthermore, because the phosphonate is assumed to be a better nucleophile than BP_i, **15**, byproducts **3** and **4** are obtained (path c) and not adenosine-α,β-CH_2-γ-boranotriphosphate, **16**.

The identity and purity of products **1**−**4** were established by ^1H and ^{31}P NMR, ESI or MALDI negative mass spectrometry, and HPLC in two solvent systems. ^{31}P NMR spectra of products **1** and **2** showed a typical P_γ signal as a multiplet at ~80 ppm in addition to two phosphonate signals at 20 and 10 ppm. ^1H NMR spectra showed borane hydrogen atoms as a very broad signal at ~0.3 ppm and a typical triplet at 2.3 ppm of the bridging methylene group.

Scheme 2[a]



[a] Reaction conditions: (a) pyridine, DMAP, TsCl, 0 °C, 3 h, 62%; (b) tetrakis(*n*-butylammonium)methylenediphosphonate, dry DMF, RT, 38 h, 62%; (c) CDI, DMF, RT, 12 h; (d) BP_i, **15**, $MgCl_2$, RT, 23 h. **2** and **4** obtained in 21 and 28% overall yields in steps c and d, respectively.

Influence of Analogues 1−4 on Ectonucleotidase Activity. (i) Effect of Analogues 1−4 on Recombinant Ectonucleotidases. The experiments were conducted with protein extracts of COS-7 cells transfected with an expression vector encoding one of each ectonucleotidase, or, in a few experiments, with intact cell lines exhibiting NPP activity. In addition, medium from NPP2-transfected cells was assayed for activity to test the secreted form of this enzyme. The protein extracts and media of non-transfected COS-7 cells exhibited a negligible level of NTPDase and NPP activity, thus allowing the analysis of each ectonucleotidase in its native membrane-bound form.[34]

None of the analogues (**1**−**4**) was metabolized by human NTPDases or ecto-5′-nucleotidase (Table 1). These analogues were modestly hydrolyzed by NPPs (Table 1). While compounds **1** and **3** were hydrolyzed by NPP1 and -3 at ∼7.5−13% of the level observed for ATP hydrolysis, analogues **2** and **4** were more resistant to hydrolysis (Table 1). The hydrolysis of ATP by NTPDase1 and -8 was not affected by any of the analogues (**1**−**4**) when used at the same concentration as the substrate (100 μM). NTPDase2 and -3 were modestly inhibited (10−30%) by these analogues (Figure 3). While analogues **3** and **4** inhibited ecto-5′-nucleotidase activity by 90 and 80%, respectively, analogues **1** and **2** did not affect the latter enzymatic activity (Figure 3).

The effect of analogues **1**−**4** on NPP activities was tested using synthetic (pnp-TMP) and natural substrates (Ap_5A and ATP). The level of hydrolysis of pnp-TMP by NPP1 was decreased by more than 90% by all compounds tested (Figure 4A). In the presence of analogues **1**−**4**, the hydrolysis of pnp-TMP by NPP2 was similarly blocked at ∼95% (Figure 4A, inset) and at 60−70% by the secreted form of NPP2 (data not shown). The activity of NPP1 with Ap_5A as the substrate was reduced by 60−80% by analogues **1**, **2**, and **4** and by 20% by analogue **3**. When using ATP as the substrate, NPP1 was inhibited by ∼70−80% in the presence of analogues **2** and **3** and by more than 90% by analogues **1** and **4** (Figure 4B,C). The presence of analogues **1**−**4** reduced the level of hydrolysis of pnp-TMP by NPP3 by ∼30% (Figure 4A) and the level of hydrolysis of Ap_5A by ∼10−60% (Figure 4B). The inhibition of NPP3 activity using ATP as the substrate was more pronounced (∼90%) in the presence of analogues **1** and **4**, and around 65% with analogues **2** and **3** (Figure 4C).

As analogues **1**−**4** significantly reduced the activity of human NPP1, we have tested IC_{50} values as well as some kinetic parameters (K_i and K_i') of the inhibition with pnp-TMP as the substrate. IC_{50} values were similar for all tested analogues, on the order of 10−60 μM, while inhibition constants (K_i) were in the range of 10−50 μM, always smaller than (enzyme−substrate)−inhibitor dissociation constants (K_i') that were in the range of 30−150 μM. The lowest K_i value was observed for analogue **2** (Table 2). Kinetic analysis of K_i and K_i' using the Dixon and Cornish−Bowden methods showed a mixed-type, predominantly competitive, inhibition of NPP1 by all tested analogues (data not shown).

(ii) Effect of Analogues 1−4 on NPP Activity at the Surface of Two Cell Lines. NPP1 is critical in regulating mineralization by generating inorganic pyrophosphate, a potent inhibitor of hydroxyapatite crystal growth. On the other hand, NPP3 is associated with carcinogenesis. Human osteoblastic SaOS-2 cells (HTB-85) are used to investigate the activity of NPP1,[16] as well as HT29, a human colon cancer cell line.[35] HTB-85 and HT29 catabolized pnp-TMP, indicating the presence of NPPs at their surface. As for the enzymes obtained from cell extracts, NPP activity exhibited by both cell lines was blocked by ∼90 and ∼80% by analogues **1** and **2** and by analogues **3** and **4**, respectively (Figure 5).

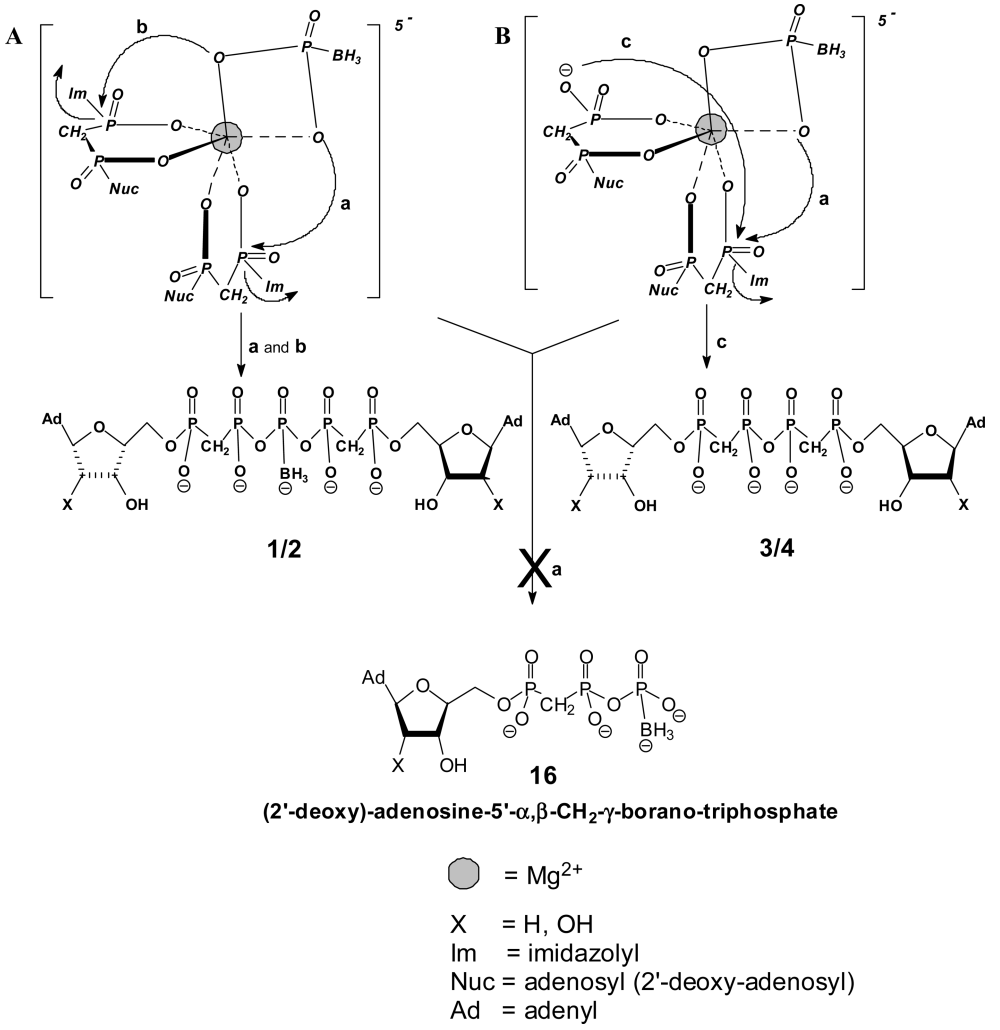


Figure 2. Proposed structures for nucleotide-BP_i Mg^{2+} complexes leading to products **1** and **2** (A) and products **3** and **4** (B).

Table 1. Hydrolysis of Analogues **1**−**4** by Human Ectonucleotidases[a]

	relative activity (% ± SD of ATP, AMP, or Ap_5A hydrolysis)			
human ectonucleotidase	**1**	**2**	**3**	**4**
NTPDase1	0.5 ± 0.02	ND[b]	1.5 ± 0.1	1.1 ± 0.04
NTPDase2	ND[b]	0.2 ± 0.01	0.1 ± 0.01	ND[b]
NTPDase3	ND[b]	ND[b]	5.3 ± 0.2	1.8 ± 0.1
NTPDase8	ND[b]	ND[b]	ND[b]	ND[b]
ecto-5′-nucleotidase	ND[b]	ND[b]	ND[b]	ND[b]
NPP1	7.5 ± 0.3	ND[b]	13 ± 0.6	2.3 ± 0.1
NPP3	12 ± 0.6	5.5 ± 0.2	11 ± 0.6	5.5 ± 0.3

[a] Dinucleotide analogues **1**−**4** (each at 100 μM) were incubated with the indicated ectonucleotidases. The activity with 100 μM ATP (for NTPDases) or AMP (for the ecto-5′-nucleotidase) was set as 100%: 1270 ± 35, 928 ± 55, 202 ± 37, 129 ± 11, and 357 ± 10 nmol of P_i min^{-1} (mg of protein)$^{-1}$ for NTPDase1, -2, -3, and -8 and ecto-5′-nucleotidase, respectively; 100% of the activity with 100 μM Ap_5A as the substrate was 71 ± 5 and 98 ± 9 nmol of nucleotide min^{-1} (mg of protein)$^{-1}$ for NPP1 and NPP3, respectively (n = 3). [b] No hydrolysis detected.

Activity of Analogues 1−4 on the $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ Receptors. GFP (green fluorescent protein) constructs of human $P2Y_1$ and $P2Y_{11}$ receptors were expressed in 1321N1 astrocytoma cells, which lack endogenous expression of both P2X and P2Y receptors. These cells were then incubated with various concentrations of analogues **1**−**4**. The Ca^{2+} response to derivatives **1**−**4** was compared with that due to ATP (Figure 6A,B).

With the exception of derivative **1**, the other analogues (**2**−**4**) were weak agonists of the $P2Y_1$ receptor (Table 3). Compound **1** was 2-fold less potent than the standard agonist ATP (EC_{50} = 0.15 μM) and 30-fold more potent than Ap_4A derivative **3** [EC_{50} = 9 μM (Table 3)]. The 2′-deoxy analogues, **2** and **4**, exhibited comparably weak activities, with EC_{50} values of ≥30 μM for the $P2Y_1$ receptor. No clear plateau was reached up to 100 μM for analogue **2**.

Derivative **1** also exhibited the highest $P2Y_{11}$ receptor agonist potency (EC_{50} = 13 μM) among the diadenosine polyphosphate analogues. The maximal response reached ~80% of that obtained with the standard agonist ATP (EC_{50} = 3.3 μM), but with a 4-fold lower potency. Analogue **3** was found to be a very weak agonist of the $P2Y_{11}$ receptor,

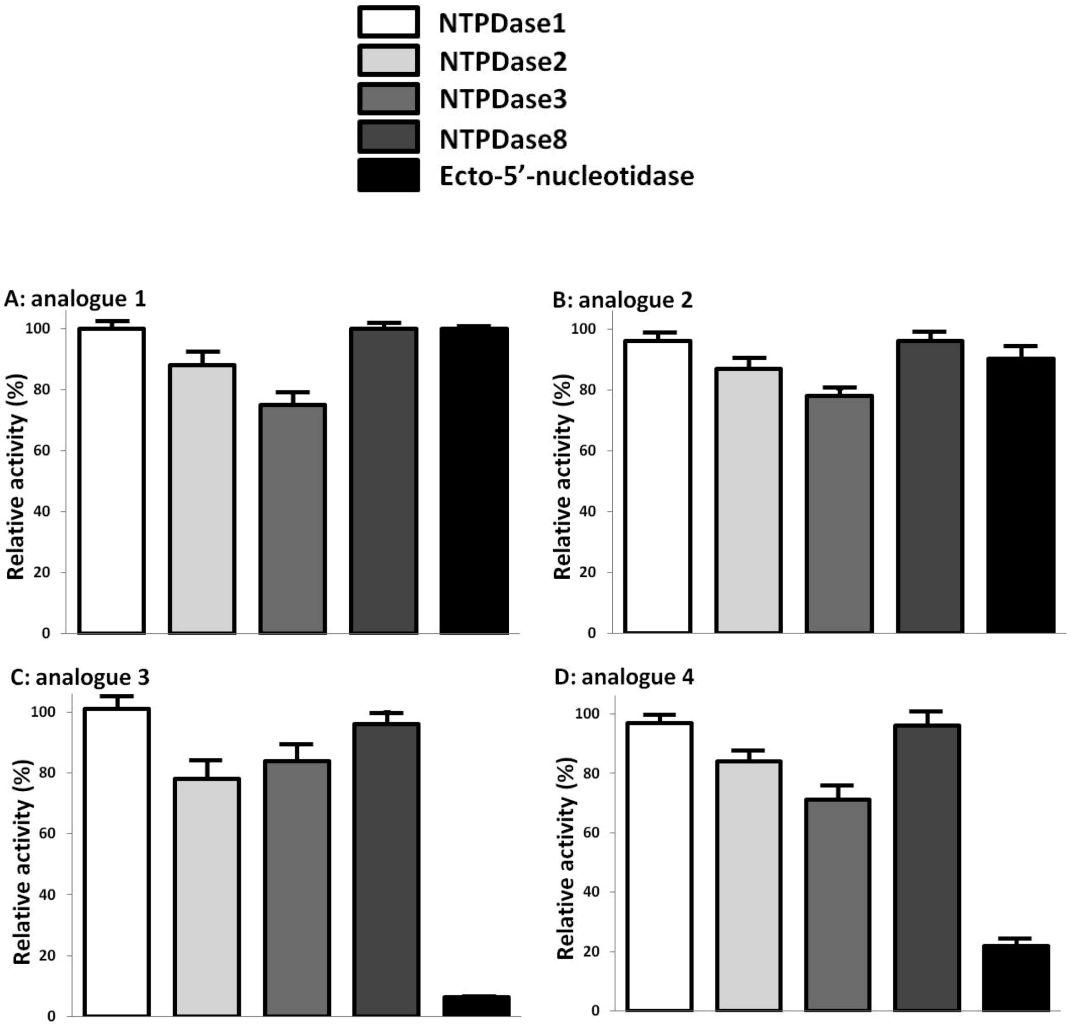


Figure 3. Effects of analogues **1**−**4** on NTPDase and ecto-5′-nucleotidase activity. Either ATP or AMP was used as a substrate in the presence of analogue **1** (A), **2** (B), **3** (C), or **4** (D). Both substrate and analogues **1**−**4** were used at 100 μM. The 100% activity was set with the nucleotide substrate alone (ATP for NTPDases or AMP for the ecto-5′-nucleotidase): 1270 ± 35, 928 ± 55, 202 ± 37, 129 ± 11, and 357 ± 10 nmol of P_i min^{-1} (mg of protein)$^{-1}$ for NTPDase1, -2, -3, and -8 and ecto-5′-nucleotidase, respectively. Data are presented as the mean ± SD of three experiments conducted in triplicate.

with an EC_{50} of ≥40 μM, whose maximal response corresponded to only 15% of that of ATP. The 2′-deoxy analogues **2** and **4** were both inactive at concentrations of ≤50 μM. Analogues **1**−**4** were completely inactive toward the $P2Y_2$ receptor at concentrations of ≤25 μM.

Discussion

Design of NPP Inhibitors. NPP1−3 have a nucleotide pyrophosphatase activity and metabolize NTP directly to NMP and PP_i but may also produce NDP and P_i.[36] These enzymes can be discriminated from other ectonucleotidases by their ability to hydrolyze the diadenosine 5′,5″-polyphosphate analogues, Ap_nA, to AMP and adenosine nucleoside 5′-(n − 1) phosphate.[37] The regulation of the dinucleotide levels by NPPs may be one of the dominant functions exerted by these enzymes.[36] Indeed, the physiological importance of dinucleotides is becoming increasingly evident.[38]

The field of NPP enzymology is still in its infancy. Therefore, NPP specific inhibitors that do not affect other ectonucleotidases, such as NTPDases and 5′-ectonucleotidase and do not trigger or interfere with P2 receptor activation, would be extremely valuable. Furthermore, potent and selective NPP inhibitors could serve as promising therapeutic agents for the treatment of osteoarthritis[39] or cancer.[24]

Nucleotide scaffolds suffer from inherent limitations as therapeutic agents as they interact with numerous proteins[1] and are metabolically unstable.[40] Therefore, here, we selected a dinucleoside polyphosphate scaffold for the development of NPP inhibitors, as this scaffold offers better stability and selectivity than nucleotides.[1] To prevent any activity toward the $P2Y_1$ receptor, we conserved the adenine ring without a methylthio substitution at the C-2 position, as the latter enhances potency toward the $P2Y_1$ receptor.[41] Because NPP1 hydrolyzes the $P_\alpha-P_\beta$ or $P_\delta-P_\varepsilon$ phosphodiester bond in Ap_nAs, as observed in our previous study with analogue **5** (Figure 1),[1] we substituted the oxygen atoms bridging the $P_\alpha-P_\beta$ and $P_\delta-P_\varepsilon$ bonds with methylene groups to obtain a hydrolysis-resistant scaffold, as in analogues **3** and **4**. In addition, because substitution of the nonbridging oxygen atom with a BH_3 group on the central phosphate of Ap_3A conferred resistance to hydrolysis by NPP1 and NPP3,[1] we have introduced a central boranophosphate moiety as in analogue **1**. Moreover, because 2′-deoxyadenosine 5′-(α-thio)triphosphate was shown to be a potent inhibitor of NPPs,[42] we also synthesized 2′-deoxy analogues such as **2**.

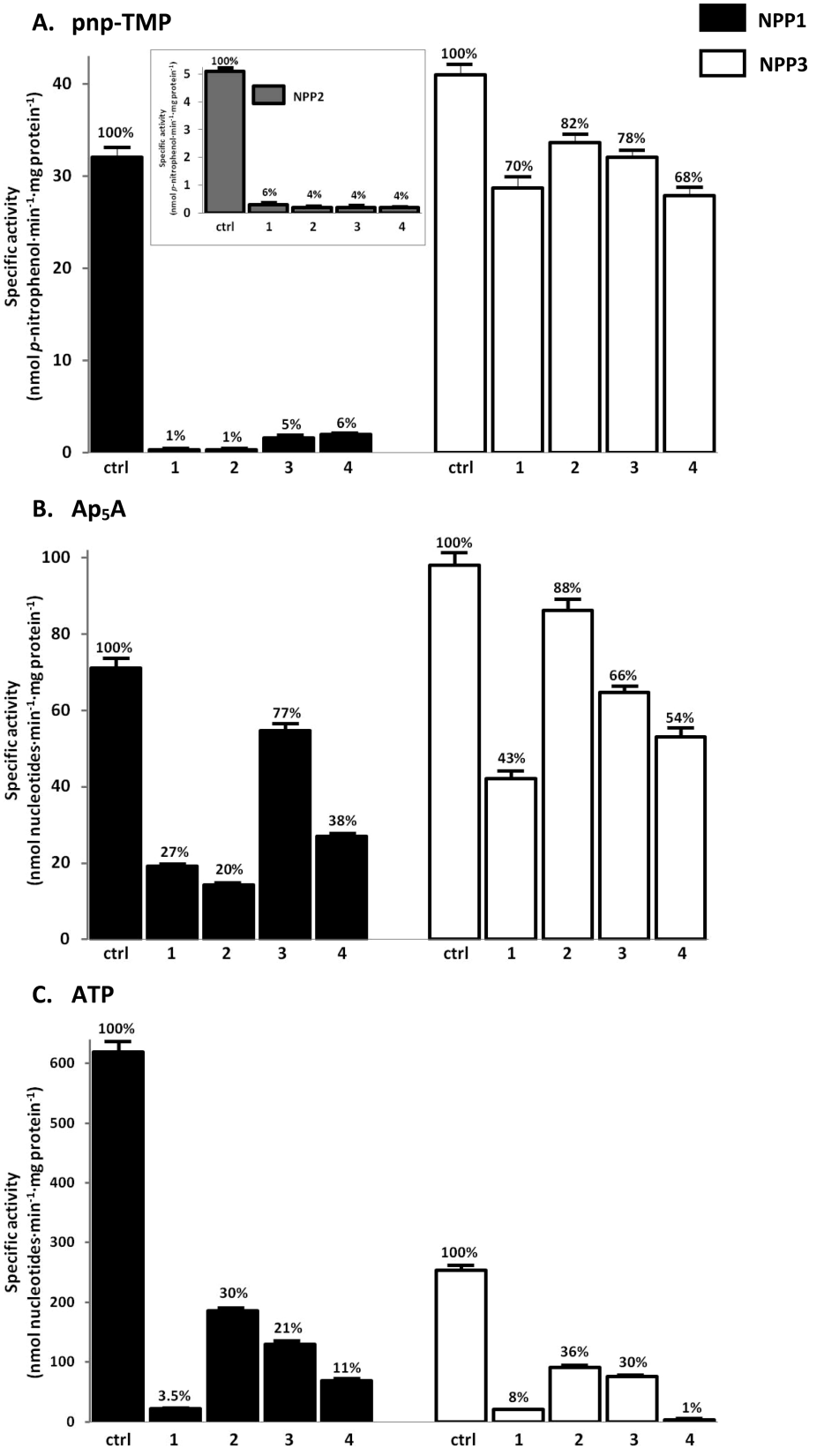


Figure 4. Analogues **1**−**4** inhibit NPP activities. The activity of human NPP1 and NPP3 with either pnp-TMP (A), Ap_5A (B), or ATP (C) as the substrate is shown. NPP2 (membrane-bound form) activity with pnp-TMP as the substrate is presented in the inset of panel A. Substrates and analogues **1**−**4** were studied at a concentration of 100 μM. In the control (ctrl), the substrate was tested in the absence of analogues and was set to 100% of activity. The percentage of residual activity is presented at the top of each bar. Data are presented as the mean ± SD of three to six experiments conducted in triplicate.

Effect of Analogues 1−4 on Ectonucleotidase Activities. All derivatives (**1**−**4**) strongly inhibited the metabolism of both synthetic (pnp-TMP) and natural substrates (Ap_5A and ATP) by NPP1 (Figure 4). Additionally, compounds **1** and **4** inhibited

Table 2. IC_{50}, K_i, and K_i' Analysis of the Inhibition of NPP1 by Analogues **1**−**4**[a]

inhibitor	$IC_{50} \pm$ SD (μM)	$K_i \pm$ SD (μM)	$K_i' \pm$ SD (μM)
1	63 ± 4.4	20 ± 1.4	31 ± 1.5
2	13 ± 0.7	9 ± 0.5	145 ± 6.9
3	33 ± 1.7	13 ± 0.7	147 ± 7.2
4	50 ± 2.0	51 ± 2.5	80 ± 3.8

[a] For the determination of K_i and K_i', pnp-TMP substrate and inhibitors **1**−**4** were used in the concentration range of 2.5×10^{-5} to 1×10^{-4} M. For the determination of IC_{50}, the pnp-TMP concentration was 5×10^{-5} M and the concentrations of inhibitors were in the range of 5×10^{-7} to 2×10^{-4} M. All experiments were performed three times in triplicate.

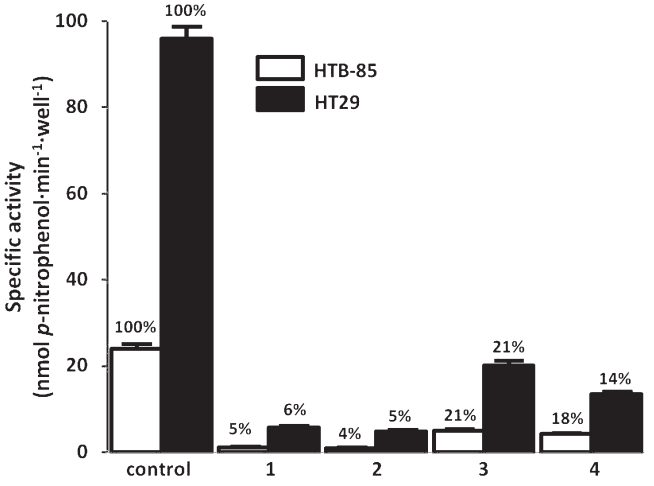


Figure 5. Analogues **1**−**4** inhibit NPP activity at the surface of HTB-85 and HT29 cells. Substrate, pnp-TMP, and analogues **1**−**4** were used at the concentration of 100 μM. In the control, the substrate was tested in the absence of analogues and was set to 100% activity. The percentage of residual activity is presented at the top of each bar. Data are presented as the mean ± SD of three experiments performed in triplicate.

the hydrolysis by NPP3 of pnp-TMP, Ap_5A, and ATP by 30, 50, and >90%, respectively. The kinetic parameters (Table 2) further indicate that analogues **1**−**4** are inhibitors of NPP1. Interestingly, analogues **1**−**4** were not hydrolyzed by NTPDases and also did not affect hydrolysis of ATP by NTPDase1 and -8. Likewise, NTPDase2 and -3 activities were reduced by ≤30% by these derivatives (Figure 3). In addition, analogues **1** and **2** exhibited no inhibitory effect toward ecto-5′-nucleotidase.

From the data given above, the following molecular recognition requirements emerge for NPP1 and -3, for NTPDase1, -2, -3, and -8, and for ecto-5′-nucleotidase. Dinucleotides, having either a tetra- or pentaphosphate linker, do not, or barely, affect NTPDase activity (analogues **1**−**4**). Indeed, such dinucleotides are not recognized as substrates or inhibitors by these enzymes. Furthermore, dinucleotides, having a pentaphosphate linker, were not recognized by ecto-5′-nucleotidase (analogues **1** and **2**). Thus, an Ap_5A scaffold is especially suitable for designing NPP-selective inhibitors.

Analogues **1** and **2** having a pentaphosphate linker inhibited Ap_5A hydrolysis by NPP1 better than analogues **3** and **4** bearing a tetraphosphate chain (Figure 4B), yet analogue **1** inhibited the hydrolysis of ATP by NPP1 better than analogue **2** (Figure 4C), implying that **1** competes with ATP because it has a 2′-OH group, unlike analogue **2**. Namely, recognition of ATP by NPP1 probably involves the 2′-OH group. This requirement

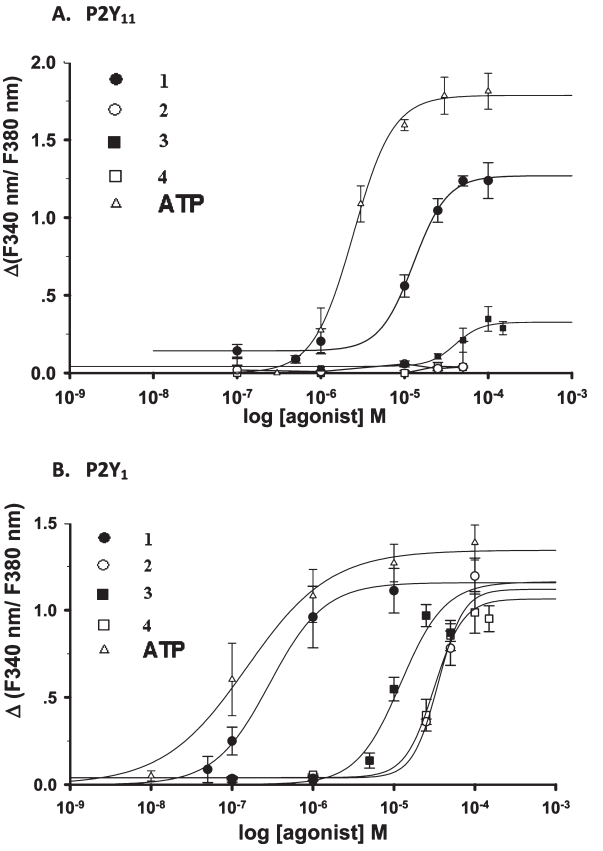


Figure 6. Relative concentration−response plots for dinucleotides **1**−**4** via (A) the $P2Y_{11}$ receptor and (B) the $P2Y_1$ receptor. Data were obtained from 1321N1 cells stably expressing the $P2Y_{11}$GFP receptor (A) or $P2Y_1$GFP receptor (B), triggering the ligand-induced change in $[Ca^{2+}]_i$. Cells were preincubated with 2 μM fura-2 AM for 30 min, and the change in fluorescence ($\Delta F_{340}/F_{380}$) was monitored.

is not important for a NPP1 inhibitor directed against Ap_5A hydrolysis, possibly because recognition of Ap_5A does not involve a 2′-OH group.

NPP3-mediated hydrolysis of ATP is sensitive to inhibition by the dinucleotide analogues [analogues **1**−**4** (Figure 4)]. Apparently, the patterns of recognition of Ap_5A and ATP by NPP3 are different than those for NPP1. Therefore, NPP3 is not affected by analogues **1**−**4** as much as NPP1.

Finally, NPP2 nucleotidase activity for both the membrane-bound forms (Figure 4A) and the secreted forms (data not shown) was highly affected by analogues **1**−**4**. It is noteworthy that in addition to its nucleotidase activity, NPP2 prefers lysophospholipids as substrates. Because the hydrolysis of lysophospholipids and nucleotides is performed by the same catalytic site,[12,43] one can speculate that analogues **1**−**4** might also inhibit the hydrolysis of lysophospholipids by NPP2, and potentially also by NPP4−7. This intriguing possibility will require further investigation.

As for α,β-methylene-ADP, a known ecto-5′-nucleotidase inhibitor,[44] the methylene groups between α,β and γ,δ or δ, ε phosphates conferred strong inhibitory activity to compounds **3** and **4** toward ecto-5′-nucleotidase. In contrast, compounds **1** and **2** had no effect on ecto-5′-nucleotidase activity, thus further emphasizing the specificity of the latter analogues as inhibitors of NPPs.

Activity of Analogues 1−4 at the P2 Receptors. Adenine nucleotide analogues with a methylene group substituting for a bridging oxygen atom and with the replacement of a

Table 3. EC_{50} Values for $[Ca^{2+}]_i$ Elevation by Ligands **1**−**4**, Mediated by the $P2Y_{1,2,11}$ Receptors

receptor subtype	nucleotide analogue	EC_{50} (μM), $[Ca^{2+}]_i$ elevation	reduction in affinity vs ATP
$P2Y_1$	**4**	30	200
	2	≥30	≥200
	3	9	60
	1	0.3	2
	ATP[a]	0.15	1
$P2Y_2$	**1**−**4**	not active up to 25 μM	−
$P2Y_{11}$	**4**	not active up to 50 μM	−
	2	not active up to 50 μM	−
	3	≥40 (∼15% of ATP)	−
	1	13	4
	ATP[a]	3.3	1

[a] ATP was selected as the common reference agonist at both $P2Y_1$ and $P2Y_{11}$ receptors, although ADP is the preferred endogenous $P2Y_1$ receptor agonist.

nonbridging oxygen in P_α with a BH_3 group have proven to be weak agonists of $P2Y_{1,4,6}$ receptors.[25] Likewise, replacing the $P_\alpha-P_\beta$ bond bridging oxygen in the potent $P2Y_1$ receptor agonist (2-MeS-ADP) with a dihalomethylene group (e.g., CCl_2 or CF_2) resulted in reductions in potency of 390- and 1200-fold.[45] In the present work, therefore, we replaced both $P_\alpha-P_\beta$ and $P_\delta-P_\varepsilon$ bond bridging oxygen atoms with methylene groups to prevent activity of the dinucleotide analogues toward the P2Y receptors.

Analogue **5** has been previously shown to be a highly potent $P2Y_1$ receptor agonist (EC_{50} = 63 nM, vs 100 nM for 2-MeS-ADP).[1] Upon replacing both $P_\alpha-P_\beta$ and $P_\delta-P_\varepsilon$ bond bridging oxygen atoms in **5** with methylene groups, yielding analogue **1**, we observed decreased activity toward the $P2Y_1$ receptor. The boranophosphate modification in **1**, which in the case of Ap_nA resulted in significant activity via the $P2Y_1$ receptor,[1] increased the potency of **1** toward the $P2Y_1$ receptor, as compared to **3**. This result might indicate an improved binding to the $P2Y_1$ receptor because of the presence of the relatively lipophilic borane moiety.[46] Analogue **3** was ∼60-fold less potent than ATP, while Ap_4A itself had a potency similar to that of ATP,[47] indicating that replacing the bridging oxygen atom with a methylene group reduces $P2Y_1$ receptor agonist potency. Analogue **2** was >200-fold less potent than ATP, indicating the importance of the 2′-hydroxyl group for molecular recognition by the $P2Y_1$ receptor.

Among natural diadenosine polyphosphates, only Ap_4A may be considered as an agonist of the $P2Y_{11}$ receptor, which is normally activated by ATP derivatives.[48−49] In this study, Ap_4A derivatives **3** and **4** were poor $P2Y_{11}$ receptor agonists or completely inactive, probably resulting from the replacement of the bridging oxygen with a methylene group in the polyphosphate chain, as observed for the $P2Y_1$ receptor. The most potent $P2Y_{11}$ receptor agonist among analogues **1**−**4** was compound **1** (Figure 6A and Table 3). Although a boranophosphate modification increased the potency of analogue **1** as a $P2Y_{11}$ receptor agonist versus the other dinucleotide derivatives tested here, it had a lower potency than ATP (EC_{50} = 13 μM, vs 3.3 μM for ATP). The 2′-deoxy-related Ap_5A scaffold, **2**, was inactive up to 50 μM, thus indicating the essential role of the 2′-hydroxyl group for activity at the $P2Y_{11}$ receptor, as noted above for the $P2Y_1$ receptor.

Conclusion

This study shows that analogues **1**−**4** are moderate but effective inhibitors of NPP1 activity in either cell extracts or intact cells. Analogues **1** and **4** strongly blocked the activity of both NPP1 and -3, yet analogue **1**, found here to be an effective NPP1 inhibitor, exhibited a low activity toward the $P2Y_1$ and $P2Y_{11}$ receptors. On the other hand, analogue **2** did not significantly block NPP3 activity, had no activity on NTPDase1, -2, -3, and -8, as well as ecto-5′-nucleotidase, and virtually no activity toward the $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ receptors, and is therefore the most specific inhibitor of NPP1. Analogue **2** represents a future tool for exploring therapeutic applications such as the development of drugs for the management of cancer, metastasis, and osteoarthritis.

Experimental Section

General. All commercial reagents were used without further purification, unless otherwise noted. All air- and moisture-sensitive reactions were conducted in flame-dried, argon-flushed, two-neck flasks sealed with rubber septa, and the reagents were introduced with a syringe. Progress of reactions was monitored by TLC using precoated Merck silica gel plates (60F-253). Reactants and products were visualized using UV light (Isco, UA-5). Compounds were characterized by NMR using a Bruker AC-200, DPX-300, or DMX-600 spectrometer. ^{1}H NMR spectra were recorded at 200, 300, or 600 MHz. Nucleotides were characterized also by ^{31}P NMR in D_2O, using 85% H_3PO_4 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 by chemical ionization. Nucleotides were analyzed using electron spray ionization (ESI) on a Q-TOF microinstrument (Waters). Primary purification of the nucleotides was achieved on a LC (Isco UA-6) system using a column of Sephadex DEAE-A25, swollen in 1 M $NaHCO_3$ at 3 °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 dinucleotides was achieved on a HPLC (Merck-Hitachi) system using a semipreparative reverse-phase column [Gemini 5u C-18 110A, 250 mm × 10 mm, 5 μm (Phenomenex, Torrance, CA)]. The purity of the dinucleotides was evaluated on an analytical reverse-phase HPLC column system [Gemini 5u C-18 110A, 150 mm × 3.60 mm, 5 μm (Phenomenex)] in two-solvent systems with either solvent systems I and II or solvent system III. Solvent system I consisted of (A) 100 mM triethylammonium acetate (TEAA) (pH 7) and (B) MeOH. Solvent system II consisted of (A) 100 mM TEAA (pH 7) and (B) CH_3CN. Solvent system III consisted of (A) 0.01 M KH_2PO_3 (pH 3.5) and (B) CH_3CN. 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, **6**,[50] α,β-methylene-ADP, **9**, and 2′-deoxy-α,β-methyleme-ADP, **13**,[31] were prepared, as previously described. α,β-Methylene-ADP and 2′-deoxy-α,β-methylene-ADP were separated using a MPLC system [Biotage (Kungsgatan, Uppsala, Sweden)] using an RP-C18 (12+M) column and the following gradient scheme: 3 column volumes of a 100:0 A/B mixture (A being 100 mM TEAA and B being MeOH), 9 column volumes of an A/B gradient from 100:0 to 60:30, and then 5 column volumes of a 60:30 A/B mixture, at a flow rate of 12 mL/min.

Typical Procedure for the Preparation of Diadenosine 5′,5″-(Boranated)polyphosphonate Derivatives (1−4). α,β-Methylene-ADP$(Bu_3NH)^+_2$ and 2′-deoxy-α,β-methylene-ADP$(Bu_3NH)^+_2$ were prepared by applying the corresponding ADP analogues

through a column of activated Dowex 50WX-8 (200 mesh, H^+ form). The eluate was collected in an ice-cooled flask containing tributylamine (2 equiv) and EtOH. The resulting solution was freeze-dried to yield α,β-methylene-ADP$(Bu_3NH)^+_2$, **9**, and 2′-deoxy-α,β-methylene ADP$(Bu_3NH)^+_2$, **13**, as a viscous oil. Bis-(tributylammonium) α,β-methylene-ADP salt (100 mg, 0.16 mmol) was dissolved in dry DMF (2 mL), and CDI (180 mg, 1.11 mmol, 5 equiv) was added. The resulting solution was stirred at room temperature (RT) for 12 h. $BP_i(Bu_3NH^+)_2$, **16** (130 mg, 0.27 mmol, 1.7 equiv), in dry DMF (1.5 mL), and $MgCl_2$ (120 mg, 1.28 mmol, 8 equiv) were added. The resulting solution was stirred at RT for 23 h. The semisolid obtained after evaporation of the solvent was chromatographed at RT on a Sephadex DEAE-A25 column, which was swelled in 1 M $NaHCO_3$ prior to column preparation. The separation was monitored by UV detection (λ = 280 nm). A buffer gradient of water (1 L) to 0.7 M NH_4HCO_3 (1 L) was applied. The relevant fractions were pooled and freeze-dried to yield a white solid. Final purification was achieved on a semipreparative C18 HPLC column. Product **1** was obtained in 10% yield (16 mg). Product **3** was obtained in 20% yield (29 mg).[51] The spectral data for **3** are consistent with the literature.[51] Product **2** was obtained in 21% yield (15 mg). Product **4** was obtained in 28% yield (20 mg) after LC separation.

Purification and Characterization of Diadenosine 5′,5″-P¹,P⁵,α,-β-methylene-δ,ε-methylene-pentaphosphate-γ-borano (1). Product **1** was purified by HPLC on a semipreparative reverse-phase column, using solvent system I, with an A/B gradient from 95:5 to 75:25 over 15 min at a flow rate of 3 mL/min: t_R = 12.88 min; ^{1}H NMR (D_2O, 300 MHz) δ 8.31 (s, H-8, 2H), 8.08 (s, H-2, 2H), 5.99 (d, J = 6.00 Hz, H-1′, 2H), (H2′ signals hidden by the water signal at 3.78 ppm), 3.53 (m, H-3′, 2H), 3.29 (m, H-3′, 2H), 3.13 (m, H-5′, 3H), 2.31 (t, J = 20.71 Hz, CH_2, 3H), 0.50 (m, BH_3, 3H); ^{31}P NMR (D_2O, 81 MHz) δ 78.38 (m,P_γ-BH_3, 1P), 20.55 (d, J = 7.93 Hz, P_α, 2P), 10.77 (m, P_β, 2P); HRMS MALDI (negative) m/z $C_{22}H_{27}D_5N_{10}O_{20}P_5$ calcd 916.0796, found 916.0791 [MD_5]; TLC (2:8:11 NH_4OH/H_2O/2-propanol) R_f = 0.17. The following purity data were obtained on an analytical column: t_R = 1.81 min (99.97% purity) using solvent system II with an A/B gradient from 80:20 to 70:30 over 10 min at a flow rate of 1 mL/min; t_R = 8.00 min (98.31% purity) using solvent system III with an A/B gradient from 100:0 to 70:30 over 10 min at a flow rate of 1 mL/min.

Purification and Characterization of Di-2′-deoxyadenosine 5′,5″-P¹,P⁵,α,β-methylene-δ,ε-methylene-pentaphosphate-γ-borano (2). Product **2** was purified by HPLC, on a semipreparative reverse-phase column, using solvent system I, with an A/B gradient from 95:5 to 70:30 over 20 min at a flow rate of 5 mL/min: t_R = 15.35 min; ^{1}H NMR (D_2O, 300 MHz) δ 8.33 (s, H-8, 1H), 8.33 (s, H-8, 1H), 8.06 (s, H-2, 1H), 8.05 (s, H-2, 1H), 6.32 (t, J = 6.90 Hz, H-1′, 2H) (H2′ and H3′ signals hidden by the water signal at 3.78 ppm), 3.19 (m, H-3′, 2H), 3.07 (m, H-5′, 3H), 2.38 (t, J = 20.70 Hz, CH_2, 3H), 0.50 (m, BH_3, 3H); ^{31}P NMR (D_2O, 81 MHz) δ 76.00 (m, P_γ-BH_3, 1P), 17.82 (s, P_α, 2P), 9.13 (d, J = 32.08 Hz, P_β, 2P); MS ESI m/z 899 (M^-Na^+); TLC (2:8:11 NH_4OH/H_2O/2-propanol) R_f = 0.12. The following purity data were obtained on an analytical column: t_R = 2.18 min (93.1% purity) using solvent system I with an A/B gradient from 70:30 to 50:50 over 10 min at a flow rate of 1 mL/min; t_R = 1.37 min (99.5% purity) using solvent system III with an A/B gradient from 85:15 to 50:50 over 10 min at a flow rate of 1 mL/min.

Purification and Characterization of Di-2′-deoxyadenosine 5′,5″-P¹,P⁵,α,β-methylene-γ,δ-methylene-tetraphosphate (4). Product **4** was purified by HPLC, with a semipreparative reverse-phase column, using solvent system I, with an A/B gradient from 95:5 to 75:25 over 20 min at a flow rate of 5 mL/min: t_R = 16.26 min; ^{1}H NMR (D_2O, 300 MHz) δ 8.33 (s, H-8, 2H), 8.06 (s, H-2, 2H), 6.32 (t, J = 6.30 Hz, H-1′, 2H) (H2′ and H3′ signals hidden by the water signal at 3.78 ppm), 3.18 (m, H-3′, 2H), 3.06 (m, H-5′, 3H), 2.31 (t, J = 2.31 Hz, CH_2, 3H); ^{31}P NMR (D_2O 81 MHz) δ 18.02 (s, P_α, 2P), 8.15 (s, P_β, 2P); HRMS MALDI (negative) m/z $C_{22}H_{31}N_{10}O_{15}P_3$ calcd 799.0920, found 799.0915 [M^{3-}]; TLC (2:8:11 NH_4OH/H_2O/2-propanol) R_f = 0.22. The following purity data were obtained on an analytical column: t_R = 3.39 min (100% purity) using solvent system I with an A/B gradient from 80:20 to 60:30 over 10 min at a flow rate of 1 mL/min; t_R = 1.51 min (98.5% purity) using solvent system III with an A/B gradient from 85:15 to 50:50 over 10 min at a flow rate of 1 mL/min.

Plasmids. Except for human NPP2, the plasmids used in this study have all been described in published reports: human NTPDase1 (GenBank accession no. U87967),[52] human NTPDase2 (GenBank accession no. NM_203368),[53] human NTPDase3 (GenBank accession no. AF033830),[54] human NTPDase8 (GenBank accession no. AY330313),[55] human NPP1 (GenBank accession no. NM_006208),[11] and human NPP3 (GenBank accession no. NM_005021).[56] Human NPP2 (XXU13871, unpublished, submitted to NCBI by J. A. Malone) was subcloned here in expression vector pcDNA3.1.

Cell Culture and Transfection. HTB-85 and HT29 cell lines were grown in 10 cm plates and then transferred into 1 cm plates for activity assays. Cells were incubated in 1 cm plates at 37 °C in α-MEM medium or in Dulbecco's modified Eagle's medium/F-12 nutrient mixture (DMEM/F-12) in the presence of 10% fetal bovine serum (FBS). After reaching full confluence, cells were used in intact cell assays (see below).

Ectonucleotidases were produced by transient transfection of COS-7 cells in 10 cm plates using Lipofectamine (Invitrogen, Burlington, ON), as previously described.[6] Briefly, 80−90% confluent cells were incubated for 5 h at 37 °C in 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. The conditioned medium of NPP2-transfected cells was also collected.

GFP (green fluorescent protein) constructs of the human $P2Y_1$ receptor and $P2Y_{11}$ receptor were expressed in 1321N1 astrocytoma cells, which lack endogenous expression of both P2X and P2Y receptors. The respective cDNA of the given receptor gene was cloned into the pEGFPN1 vector, and after transfection using the FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany), cells were selected with 0.5 mg/mL G318 (Merck Chemicals, Darmstadt, Germany) and grown in DMEM supplemented with 10% fetal calf serum, 100 units/mL penicillin, and 100 units/mL streptomycin at 37 °C and 5% CO_2. The functional expression of the receptor was confirmed by GFP fluorescence and the change in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) upon incubation with the respective standard receptor agonists.

Preparation of Protein Fractions. For the preparation of protein extracts enriched with membrane proteins, transfected cells were washed three times with Tris-saline buffer at 4 °C, collected by being scraped in harvesting buffer [95 mM NaCl, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), and 35 mM Tris (pH 7.5)], and washed twice by 300g centrifugation for 10 min at 3 °C. Cells were resuspended in the harvesting buffer supplemented with 10 mg/mL aprotinin and sonicated. Nucleus 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 secreted form of NPP2 was prepared from the conditioned media of transfected cells that were frozen and stored at −80 °C until they were tested for activity. Protein concentrations were estimated by the Bradford microplate assay using bovine serum albumin (BSA) as the standard.[57]

Enzymatic Activity Assays. (i) NTPDases and Ecto-5′-nucleotidase. Activity was measured as described previously[3] in 0.2 mL of incubation medium Tris-Ringer buffer [120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_3$, 25 mM $NaHCO_3$, 5 mM glucose, and 80 mM Tris (pH 7.3)] at 37 °C with or without analogues **1**−**4** (final concentration of 100 μM), and with or without 100 μM ATP (for NTPDases) or 100 μM AMP (for ecto-5′-nucleotidase) as a substrate. The analogues

were added without ATP or AMP when tested as a potential substrate, and with ATP or AMP when tested for their effect on nucleotide hydrolysis. Either NTPDase or ecto-5′-nucleotidase 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, AMP, or **1**−**4**) and stopped after 15 min with 50 μL of malachite green reagent. The amount of released inorganic phosphate (P_i) was measured at 630 nm using the method of Baykov et al.[58]

(ii) NPPs. Evaluation of the effect of analogues **1**−**4** on human NPP1−3 activity was conducted with either pnp-TMP, ATP, or Ap_5A as the substrate.[11] The reactions were conducted at 37 °C in 0.2 mL of the following incubation mixture: 1 mM $CaCl_2$, 130 mM NaCl, 5 mM KCl, and 50 mM Tris (pH 8.5) with or without analogues **1**−**4** and/or substrates. Substrates and analogues **1**−**4** were all used at a final concentration of 100 μM. Recombinant human NPP1, -2, or -3 cell lysates, as well as soluble proteins containing the secreted form of NPP2, were added to the incubation mixture and preincubated at 37 °C for 3 min. The reaction was initiated by addition of the substrate. For pnp-TMP hydrolysis, the production of *p*-nitrophenol was measured at 405 nm, 15 min after the initiation of the reaction. For Ap_5A and ATP, we stopped the reaction after 30 min by transferring an aliquot of 0.1 mL from the reaction mixture to 0.125 mL of ice-cold 1 M perchloric acid. The samples were centrifuged for 5 min at 13000*g*. Supernatants were neutralized with 1 M KOH (3 °C) and centrifuged for 5 min at 13000*g*. An aliquot of 20 μL was separated by reverse-phase HPLC to evaluate the nucleotide content of each reaction sample (see below). The type of inhibition, IC_{50} (50 μM substrate with the inhibitors at concentrations of 0, 0.5, 5, 25, 50, 75, 100, and 200 μM), K_i, and K_i' (substrate concentrations of 25, 50, 75, and 100 μM and inhibitor concentrations of 0, 25, 50, 75, and 100 μM) were calculated by plotting the data of three independent experiments using pnp-TMP as the substrate according to Dixon and Cornish−Bowden kinetics.

(iii) Separation and Quantification of Nucleotides and Dinucleotides by HPLC. An aliquot of 20 μL of the reaction products (described above) was used for nucleotide analysis by HPLC using a 15 cm × 3.6 mm, 3 μm SUPELCOSIL LC-18-T column (Supelco, Bellefonte, PA). ATP, Ap_5A, analogues **1**−**4**, and their hydrolysis products were separated with a mobile phase consisting of 25 mM TBA, 5 mM EDTA, 100 mM KH_2PO_3/K_2HPO_3 (pH 7.0), and 2% methanol (v/v), at a flow rate of 1 mL/min for 30 min. Separated nucleotides were detected by UV absorption at 260 and 253 nm, identified, and quantified by the comparison of the retention times with those of the appropriate standards.

(iv) Activity Assays with Intact HTB-85 and HT29 Cell Lines. For intact cells, activity assays at the cell surface were conducted in 0.25 mL of the incubation mixture containing 135 mM NaCl in 24-well plates. Reaction was initiated by the addition of pnp-TMP, yielding a final concentration of 100 μM. 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 310 nm.

Calcium Measurements. 1321N1 astrocytoma cells were transfected with the respective plasmid for P2YR-GFP expression, namely pEGFPN1 expression vector plasmids encoding the cDNA for human $P2Y_1$ or $P2Y_{11}$ receptors[59] and the $P2Y_2$ receptor.[60,61] Cells plated on coverslips (22 mm in diameter) and grown to approximately 80% density were incubated with 2 μM fura-2 AM and 0.02% pluronic acid in HEPES-buffered saline [135 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM glucose, and 20 mM HEPES/Tris (pH 7.3)] for 30 min at 37 °C. Cells were superfused (1 mL/min, 37 °C) with different concentrations of dinucleotides **1**−**4**, and the change in $[Ca^{2+}]_i$ was monitored from the respective emission intensity at 510 nm after alternating between 340 and 380 nm as the excitation wavelengths.[62] Concentration−response data were analyzed with SigmaPlot (SPSS Inc., Chicago, IL) using the ratio of the fluorescence intensities with 340 and 380 nm excitation wavelengths.[59,63]

Acknowledgment. This work was supported by grants from the Canadian Institutes of Health Research (CIHR) to J.S., who was also a recipient of a new investigator award from the CIHR and of a Junior 2 Scholarship from the FRSQ, and a grant from Deutsche Forschungsgemeinschaft (DFG) to G.R. (Grant Re 563/15). We thank Dr. Richard Poulin (Scientific Proofreading and Writing Service, CHUQ Research Center) for editing this paper.

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