Novel Inhibitors of Nucleoside Triphosphate Diphosphohydrolases: Chemical Synthesis and Biochemical and Pharmacological Characterizations

Fernand-Pierre Gendron,[†] Efrat Halbfinger,[‡] Bilha Fischer,[‡] Martine Duval,[§] Pédro D'Orléans-Juste,[§] and Adrien R. Beaudoin*,[†]

Départment de Biologie, Université de Sherbrooke, Sherbrooke, QC J1K 2R1, Canada, Department of Chemistry, Gonda-Goldschmied Center, Bar-Ilan University, Ramat-Gan 52900, Israel, and Institute de Pharmacologie de Sherbrooke, Université de Sherbrooke, Sherbrooke, QC J1H 5N4, Canada

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To elucidate the physiological role played by nucleoside triphosphate diphosphohydrolase (NTPDase; EC 3.6.1.5), adenine nucleotide analogues, modified on the purine ring, have been synthesized and tested as potential inhibitors. Resistance of ATP analogues to hydrolysis and their potency as NTPDase inhibitors were evaluated. For this purpose, a particulate fraction isolated from bovine spleen was used as the enzyme source. Among the synthesized analogues, 8-thiobutyladenosine 5′-triphosphate (8-BuS-ATP) was found to be the most effective nonhydrolyzable competitive inhibitor, with an estimated K_i of 10 μM. This nonhydrolyzable analogue did not exert any P2X-receptor-mediated effect on endothelium-denuded blood vessels, from the guinea pig mesenteric bed. In agreement with this observation, infusion of the analogue did not cause any significant blood pressure variations of the precontracted vessel. Because in previous studies on isolated turkey erythrocytes and rat astrocytes 8-BuS-ATP was not able to trigger any $P2Y_1$-receptor-mediated effect, it therefore appears that this NTPDase inhibitor does not interfere with purinergic receptors.

Introduction

In the past decade, evidence has grown in support of the concept that extracellular ATP and its dephosphorylated metabolites are involved in cell signaling in the different physiological systems of vertebrates.[1−5] The biological effects elicited by extracellular purine nucleotides are determined by their rate of release in the extracellular medium, the activity of ectonucleotidases, and their binding affinity for ATP-receptors (P2-Rs). ATP and ADP are released by both exocytotic and nonexocytotic mechanisms, which are still poorly defined.[6−8] These nucleotides and the pyrimidine nucleotide UTP exert their effects through purinergic type-2 receptors (P2) activation. Receptors of this class are designated either P2Y for those coupled to G-protein or P2X for those which are ligand-gated ion channels.[1,9−13] Adenosine, the end product of ATP dephosphorylation, can interact with P1-purinoceptors (G-protein coupled). It often elicits a cellular response antagonistic to the one triggered by P2 receptor activation.[1,14]

Extracellular concentrations of ATP and its metabolites are modulated primarily by ectonucleotidases, namely ecto-nucleotide triphosphate diphosphohydrolase (NTPDase; EC 3.6.1.5), ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP; no EC) and 5′ nucleotidase (EC 3.1.3.5).[15−19] NTPDase, formerly known as ATP diphosphohydrolase (ATPDase), catalyses the sequential hydrolysis of β- and γ-phosphate of nucleoside di- and triphosphate, producing the corresponding nucleoside monophosphate derivative.[20−24] Several NTPDases have been recently sequenced and their encoding gene shows that it corresponds to CD39, a marker of activated lymphocytes.[25−27] NTPDase assays on tissues of "knock out" and "wild-type" mice have demonstrated the major role of this enzyme in the extracellular metabolism of purine nucleotides.[28] Two main types of NTPDase have been found in rat, pig, bovine, and human tissues (78 and 54 kDa).[20,24,29−30] In pig and bovine organs NTPDase, the 78 kDa isoform is predominant with one exception, the pancreas, where the 54 kDa type is the main type, as previously shown.[24] The latter is a truncated fragment of the 78 kDa isoform.[24] Recent data show that the other fragment (27 kDa) is still associated with the cellular membrane.

The rapid breakdown of ATP to adenosine is a variable generally ignored in purinoceptor studies.[18,31−33] This can be partially circumvented either by the use of nonhydrolyzable ATP analogues or by introducing specific inhibitors of ectonucleotidases, provided that the latter are not P2R-ligands. In this respect, we have previously shown that P2-R agonists, modified on the phosphate chain e.g., ADP-β-S, α,β-CH_2-ATP, β,γ-CH_2-ATP, and β,γ-imido-ATP, **1a**−**d** (Chart 1), are not hydrolyzed by NTPDase while acting as NTPDase inhibitors.[34] On one hand various 2-thioether adenine nucleotides exhibit relative enzymatic stability regarding dephosphorylation by ectonucleotidases in various preparations: bovine "brain membrane", chick skeletal muscle derived myotubes, and C6 glioma cell.[35] On the other hand, these compounds are potent P2Y-R ligands whereas no activity was observed with P2X-R subtypes.[36−39] Likewise, 2-thioether-ATP-α-S derivatives, **3**, which are potent insulin secretagogues acting through P2-Rs, exhibited relative stability in the presence of the 54 kDa

* Corresponding author. Tel: (819) 821-7946. Fax: (819) 821-8049. E-mail: abeaudoi @ courrier.usherb.ca.
[†] Départment de Biologie, Université de Sherbrooke.
[‡] Bar-Ilan University.
[§] Institute de Pharmacologie de Sherbrooke, Université de Sherbrooke.

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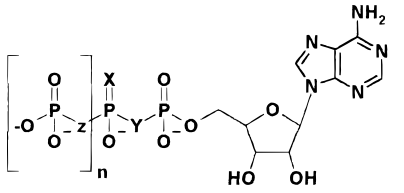
Chart 1. Structures of Different ATP Analogues

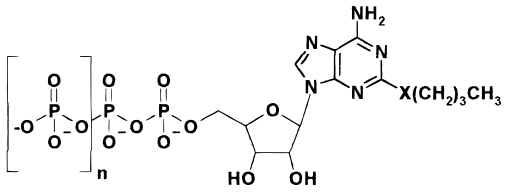


1 **a** : X = S, Y = O, Z = O, n = 0

b : X = O, Y = CH_2, Z = O, n = 1

c : X = O, Y = O, Z = CH_2, n = 1

d : X = S, Y = CH_2, Z = NH, n = 1

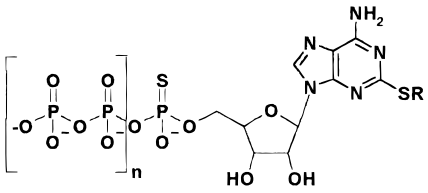


2 **a** : n = 1, X = S

b : n = 0, X = S

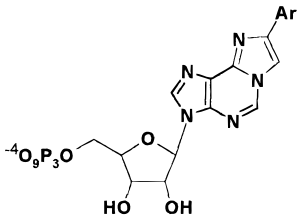
c : n = 1, X = NH

d : n = 1, X = O



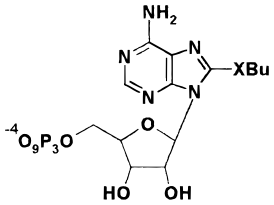
3. n = 1, **a** :R = hexyl, **b** :R = benzyl

4. n = 0, **a** :R = hexyl, **b** :R = benzyl



5 a : Ar = p-NO_2-C_6H_4

b : Ar = p-NH_2-C_6H_4



6. X = S

7. X = NH

8. X = O

NTPDase.[40] We have also shown that some 2-thioether adenosine-5′-monophosphorothioate (2-thioether-AMP-S), **4**, derivatives exert an inhibitory effect on the same enzyme.[40] In a previous study we have also shown that etheno-ATP derivatives, **5**, were hydrolyzed at 47% (**5a**) and 22% (**5b**), the rate of ATP. From this study it was concluded that the relative stability of these molecules could be attributed either to the bulky arylethenо moiety, which turns these derivatives into poor substrates, or might indicate that a free N1, N6 region is required for H bonding.[41]

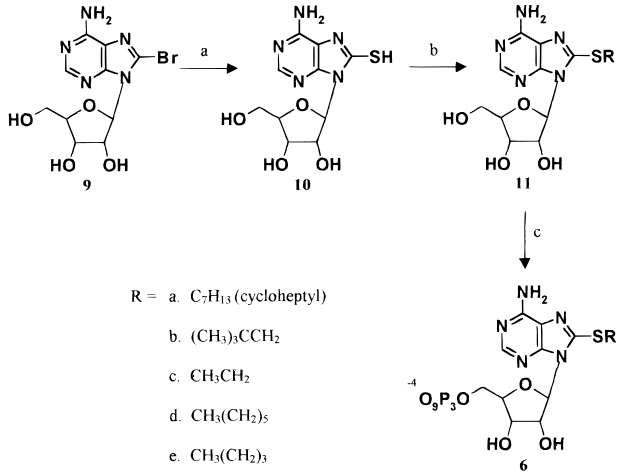
A specific NTPDase inhibitor could potentially be a valuable tool to amplify the biological effects induced by endogenous ATP and ADP and to define the role of NTPDase in the extracellular metabolism of these purines. Ideally, this inhibitor should not interact with either P2Y or P2X receptor subtypes. For this purpose we synthesized 8-substituted ATP derivatives which we have recently identified as very poor P2Y-R ligands in turkey erythrocyte membranes and rat astrocytes.[39] These derivatives included 8-butyl thioether-, 8-butylamino-, and 8-butyl ether-ATP derivatives, **6**−**8**.

In this paper we present what appears to be the first NTPDase inhibitor that does not interfere with purinoceptors. Indeed, this inhibitor binds neither the P2Y nor the P2X receptor subtypes tested so far. In this work, resistance to hydrolysis and inhibitory properties of new ATP analogues, bearing a substitution at either position C2 or C8 of the purine ring, were evaluated. Kinetic studies carried out with a NTPDase particulate preparation put in evidence a new family of ATP analogues that act as NTPDase inhibitors.

Results

Selection and Synthesis of ATP Analogues for Evaluation as NTPDase Inhibitors. Compounds **2a**−**d** and **6**−**8** were prepared as described before.[39] On the basis of the promising hydrolytic stability of compounds **6**−**8**, a new series of 8-thioether ATP analogues were synthesized for structure activity relationship (SAR) studies (Scheme 1). The desired derivatives, **6a**−**e**, were obtained in three steps from 8-Br-adenosine in good yields. 8-Mercaptoadenosine, **10**, was obtained in a quantitative yield from 8-Br-adenosine upon treatment with 10 equiv of NaSH in wet DMF at 100 °C overnight. The corresponding dry sodium thiolate salt,

Scheme 1. Synthesis of 8-Thioether-ATP Derivatives[a]



[a] (a) 10 equiv NaSH, wet DMF, 100 °C overnight, 100% yield. (b) (1) compound **10**, MeOH, 0.25 M NaOH, RT, freeze-drying, (2) alkyl bromide, DMF, 60 °C overnight, 83−93% yield; (c) (1) $POCl_3$, Proton Sponge, (2) $P_2O_7H_2(Bu_3NH^+)_2$, (3) 0.2 M TEAB.

obtained upon dissolution of **10** in MeOH/0.25 M NaOH and subsequent freeze-drying, was treated with the appropriate alkyl bromide in DMF at 60 °C to yield compounds **11** in high yields. Finally these compounds were 5′-triphosphorylated,[39] to give nucleotides **6** in reasonable yields.

The thioether side chains varied in length (e.g., from ethyl to butyl and hexyl, **6c**, **6e**, **6d**), and in bulkiness (from ethyl to cycloheptyl and *tert*-butylmethylene, **6c**, **6a**, **6b**), for evaluation of the effect of these parameters on the inhibitory potential of these new derivatives. For the sake of comparison, we also included 8-Br-ATP in the above series, for studying possible electronic effects of an electron-withdrawing group at the C8 position on the affinity to NTPDase.

Hydrolysis of ATP Analogues. Hydrolysis rates of ATP analogues **2a**−**d** and **6**−**8** as well as 8-Br-ATP were compared with ATP. As shown in Figure 1, NTPDase hydrolyzes all the analogues bearing a substitution at C2 of the purine ring, but the rate was slower than that obtained with ATP. Hydrolysis rates of 2-BuS-ATP (**2a**) and 2-BuS-ADP (**2b**) were 53% and 50% of that of ATP hydrolysis, respectively, as compared to 65% and 75% for 2-BuNH-ATP (**2c**) and 2-BuO-ATP (**2d**), respectively (Figure 1). In contrast, rates of hydrolysis of C8-substituted analogues were much lower, some of them being almost totally resistant. Indeed, 8-EtS-ATP (**6c**), 8-hexS-ATP (**6d**), 8-BuNH-ATP (**7**), and 8-BuO-ATP (**8**), were hydrolyzed at 15%, 11.6%, 23%, and 11% the rate of ATP, whereas 8-BuS-ATP (**6e**), 8-CH_2tBuS-ATP (**6b**), and 8-cycloheptylS-ATP (**6a**) were almost completely resistant to NTPDase hydrolysis, with rates of hydrolysis 1,4%, 7.2%, and 6.6% the rate obtained with ATP, respectively. For comparison, 8-Br-ATP was tested and, as shown in Figure 1, it was hydrolyzed at about 83% the rate of ATP. From these data, it appears that both the position and nature of the substituents are important to confer resistance to enzymatic hydrolysis. Thus, analogues with electron-donating (ED) groups at C8, but not 8-Br-ATP, are hydrolyzed at a lower rate than their corresponding C2 analogues.

Kinetic Parameters of Hydrolyzed Analogues. Apparent K_m and V_{max} of ATP were estimated at 18 μM and 1.65 μmol of P_i released/min/mg of protein, respectively (Table 1). With ADP as the substrate, the calculated apparent K_m and V_{max} were 33 μM and 1.3 μmol of P_i released/min/mg of protein (Table 1), respectively. These K_m values are comparable to those previously reported.[34,42] As summarized in Table 1, ATP, 2-BuS-ATP (**2a**), 2-BuO-ATP (**2d**), 2-BuNH-ATP (**2c**), 8-Br-ATP, 8-EtS-ATP (**6c**), 8-BuNH-ATP (**7**), and 8-BuO-ATP (**8**) show about the same affinities for the enzyme, their K_m values ranging between 12 and 36 μM. In contrast, the estimated V_{max} for these C2-substituted analogues are about one-half the value obtained with ATP. With the C8-substituted analogues, 8-Br-ATP, 8-EtS-ATP (**6c**), 8-BuNH-ATP (**7**), and 8-BuO-ATP (**8**), V_{max} values were estimated at 38%, 18%, 17%, and 12% the value obtained with ATP, respectively. As for the ADP analogue, 2-BuS-ADP (**2b**), apparent K_m and V_{max} were estimated at 63 ± 14 μM and 0.94 ± 0.1 μmol of P_i released/min/mg of protein, respectively (Table 1).

Inhibition by C-8-Substituted Analogues. Inhibition and K_i of nonhydrolyzable analogues, **6a**,**b**,**d**,**e**, were investigated with ATP as the substrate at two concentrations (three in the case of 8-BuS-ATP, **6e**). Figures S1 and S2 (see the Supporting Information) represent rates of ATP catalysis in the presence of the different inhibitors. With 8-BuS-ATP (**6e**), the Lineweaver−Burk linear representation shows intersection on the ordinate axis, indicative of competitive inhibition (Figure S1A, Supporting Information).[43] Dixon replots of the data (Figure S1B, Supporting Information) allowed us to estimate a K_i of 10 ± 2 μM (Table 1). The same protocol was applied to determine K_i and the type of inhibitions for 8-CH_2tBuS-ATP (**6b**), (Figure S2A, Supporting Information), 8-cycloheptylS-ATP (**6a**) (Figure 3B), and 8-hexylS-ATP (**6d**) (Figure S3, Supporting Information), which were estimated at 45, 31, and 16 μM, respectively (Table 1). In contrast, to analogues **6a** and **6b** (Figure S2, Supporting Information), which produced a mixed type of inhibition, 8-hexylS-ATP (**6d**) behaves as a noncompetitive inhibitor (Figure S3, Supporting Information).

Evaluation of 8-BuS-ATP Potency as a P2X-Receptor Agonist. It is well-known that P2X-R activation, at the surface of vascular smooth muscle cells, causes vasoconstriction.[1,14] In this respect, 8-BuS-ATP (**6e**) was evaluated for P2X-R activity using the model of the guinea pig mesenteric bed, treated with CHAPS to remove the endothelium. Response to 200 μM of noradrenaline was set at 100% (37 mmHg), corresponding to the baseline used for the calculation of perfusion pressure variations. Intra-arterial bolus administration of increasing concentrations of 8-BuS-ATP (**6e**) (0.1−1000 pmol) did not induce any significant variations of perfusion pressure (Figure 2A). Effective removal of the endothelium was shown by a lack of response to NK-1 ([Sar^9,$Met(O_2)^{11}$]SP), as compared to intact mesentery. Denuded vessels were still responsive to NaNP (sodium nitroprussiate) induced vasodilatation (Figure 2C), indicating the integrity of the smooth muscle layer and thereby confirming that 8-BuS-ATP did not interact with the P2X purinoceptor associated to these cells.

Influence of 8-BuS-ATP on the Vasodilatation Induced by ATP on an Intact Mesenteric Bed. It was shown, in a previous study, that 8-BuS-ATP (**6e**)

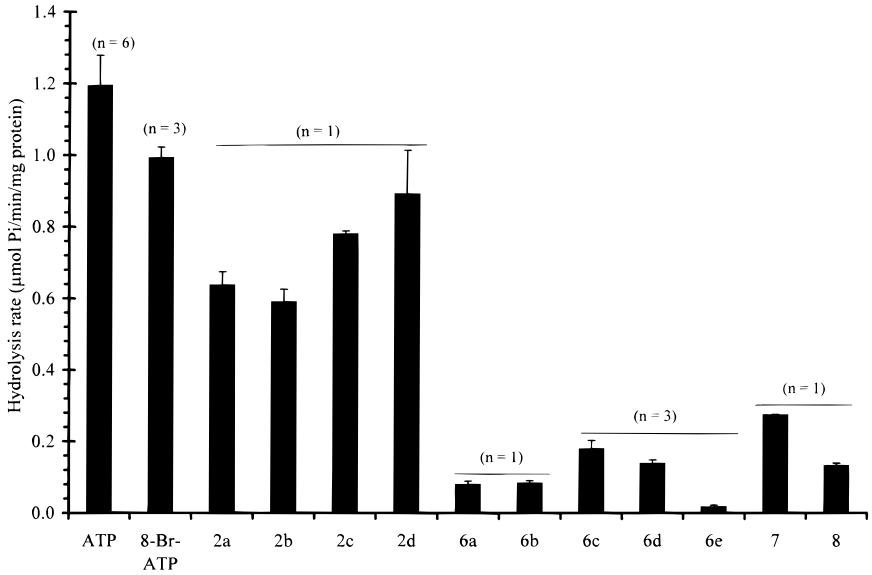


Figure 1. Hydrolysis of ATP and analogues by NTPDase. ATP and analogues were used at a concentration of 100 μM. Hydrolysis was carried out at 37 °C for 7 min in the presence of 1.9 μg of protein. C2-Substituted analogues (**2a**−**d**) are all hydrolyzed by the enzyme, whereas C8-substituted analogues (**6**−**8**) are more or less resistant to NTPDase hydrolysis. Results are expressed as the mean ± SEM of *n* replicates (see figure) carried out in triplicate.

Table 1. Kinetic Parameters of Bovine Spleen NTPDase for ATP, ADP, and Purine Analogues[a]

substrates		K_m (μM)	V_{max} (μmol/min/mg of protein)	inhibitors		K_i (μM)
ATP		18 ± 1	1.65 ± 0.10	8-cycloheptylS-ATP	**6a**	31 ± 2.5
ADP		33 ± 1	1.30 ± 0.08	8-CH_2tBuS-ATP	**6b**	45 ± 2.5
2-BuS-ATP	**2a**	36 ± 6	0.83 ± 0.05	8-hexylS-ATP	**6d**	16 ± 2.0
2-BuS-ADP	**2b**	63 ± 14	0.94 ± 0.10	8-BuS-ATP	**6e**	10 ± 2.0
2-BuNH-ATP	**2c**	32 ± 8	0.99 ± 0.10			
2-BuO-ATP2d	**2d**	28 ± 8	0.82 ± 0.09			
8-Br-ATP		22 ± 5	0.63 ± 0.04			
8-EtS-ATP	**6c**	12 ± 5	0.30 ± 0.03			
8-BuNH-ATP	**7**	20 ± 7	0.28 ± 0.03			
8-BuO-ATP	**8**	26 ± 5	0.20 ± 0.01			

[a] Experiments were carried out in triplicate and results are expressed as the mean ± SEM of the best fit obtained with GraFit 4 software (Erithacus, UK). Apparent K_m and V_{max} were estimated from the Eadie and Hofstee representation and K_i values were from Dixon plots.

did not activate the $P2Y_1$ receptor, in vitro.[39] We showed here that 8-BuS-ATP (7 μM) did not modify the response of the isolated mesenteric bed to ATP. From these experiments it appears that this analogue is also devoid of any agonistic effect on the P2Y-receptor (Figure 2B). The integrity of the endothelium was assessed and confirmed with NK-1 (100 pmol) and NaNP (3 nmol) (Figure 2C).

Discussion

Extracellular purines, namely ATP and ADP, are involved in many physiological processes.[1−5] These molecules are rapidly dephosphorylated by ectonucleotidases, mainly by the ecto-nucleotide triphosphate diphosphohydrolase.[16−19,44] To define the role played by extracellular nucleotides and to develop drugs based on P2-R ligands, it is desirable to block ectonucleotidases activity or alternatively to use nonhydrolyzable analogues of these nucleotides as agonists.[37,45−46] In previous studies, several analogues of ATP were found to be hydrolyzed by ectonucleotidases.[32,47−49] However, P2-purinoceptors agonists modified on the phosphate chain, such as ADP-β-S, α,β-, or β,γ-methylene-ATP, and β,γ-imido-ATP (**1a**−**d**), were generally resistant to hydrolysis by NTPDase and were also inhibitors of ectonucleotidases to a certain extent.[34] Recently, we demonstrated that ecto-NTPDase (EC 3.6.1.5) was involved in the hydrolysis of some purine nucleotide analogues, e.g., 2-thioether-ATP-α-S derivatives, 2-Cl-ATP, and 2-CH_3S-ATP.[34,40] In contrast, 2-thioether-AMP−S (**4**) was resistant to hydrolysis and was an inhibitor of NTPDase.[40]

To reduce potential artifacts resulting from the solubilization of NTPDase by detergents, a particulate fraction was used. It is important to mention that there was no other detectable level of ATPase or ADPase, in the preparation, than those attributable to NTPDase. Also, possible alkaline phosphatase activity was inhibited by tetramisole added to our assays. In view of finding a specific and potent NTPDase inhibitor, in the present study we examined two series of ATP analogues substituted at positions C2 and C8, respectively. First, we tested their resistance to NTPDase hydrolysis and it clearly appeared that analogues substituted with electron-donating groups at C8 were more resistant to hydrolysis than the corresponding C2-substituted analogues. However, a significant level of hydrolysis was found with 8-Br-ATP, 8-BuNH-ATP (**7**), 8-BuO-ATP (**8**), and 8-EtS-ATP (**6c**), whereas 8-cycloheptylS-ATP (**6a**), 8-CH_2tBuS-ATP (**6b**), 8-hexylS-ATP (**6d**), and 8-BuS-ATP (**6e**), were resistant to hydrolysis by NTPDase. It

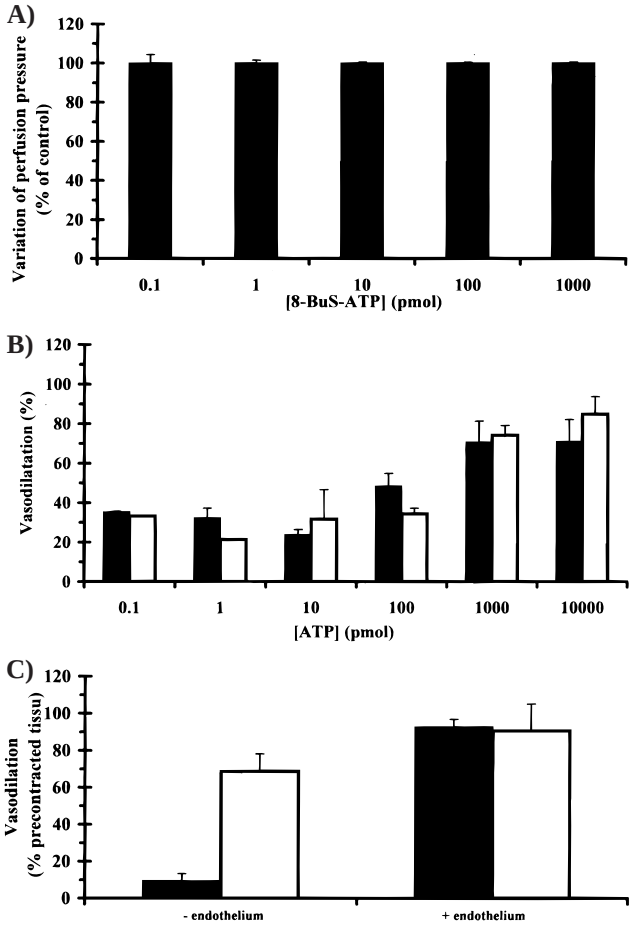


Figure 2. Purinergic activity of 8-BuS-ATP (**6e**). Effect of 8-BuS-ATP (0.1−1000 pmol) on denuded mesenteric bed of guinea pig. Results are expressed as a percentage of the control (control = 37 mmHg). No variations of perfusion pressure were measured. Results are the mean ± SEM of at least three experiments. Effect of 8-BuS-ATP on the relaxing effect of ATP on intact mesenteric bed. ATP (0.1−10000 pmol) in the presence (open bars) or in the absence (control, closed bars) of 7 μM of 8-BuS-ATP. Results are expressed as a percentage of vasodilatation measured from a precontracted vessel (200 μM of noradrenaline). No significant difference. Results are the mean ± SEM of at least three experiments. Vascular responses with or without endothelium. Endothelium integrity was tested with 100 pmol of NK-1 (closed bar) and blood vessels responsiveness was evaluated by 3 nmol of NaNP (open bar). Results are expressed as a percentage of vasodilatation measured from a precontracted vessels (200 μM of noradrenaline). In denuded mesentery there is significant response to NK-1. Results are the mean of three experiments or more.

is noteworthy that all the C2-substituted molecules displayed K_m in range found for ATP and ADP. This indicates that affinity for the catalytic site is equivalent for all these analogues. Hence, the position and nature of the substituent is clearly important for resistance to the catalytic activity of the enzyme.

Hydrophobic interactions and H-bonds of the C2 substituent appear to be important determinants for P2Y-R ligand affinity. The conformational preference of the ligands in solution, determined by NMR experiments, may explain in part the differences in P2Y-R potency between the 2- and 8-substituted compounds. All 2-substituted derivatives possess an anti conformation, whereas the 8-ether and thioether analogues are in the syn conformation.[39] The latter are apparently not tolerated by the tentative $P2Y_1$-R binding site.[39,50,51] In contrast, the NTPDase active site can accommodate 8-thioether-ATP analogues, and even derivatives bearing large or bulky substituents (8-CH_2tBuS-ATP, and 8-cycloheptylS-ATP, and 8-hexylS-ATP), probably in their expected syn conformation. This conformation is likely unfavorable for catalytic activity, namely, the orientation of the triphosphate chain in the syn conformation is probably shifted away from the catalytic amino acid residues.

Having established that these 8-thioether-ATP derivatives were not significantly hydrolyzed, we then investigated their potency as NTPDase inhibitors. One striking feature is that the four derivatives, **6a**,**b**,**d**,**e** are good inhibitors. Again the affinity (K_i) falls in the same range of affinity as that of ATP and ADP, with one exception, 8-CH_2tBuS-ATP, which has a slightly higher K_i. However, even if these four analogues all display efficient NTPDase inhibition, we consider 8-BuS-ATP, **6e**, as the inhibitor of choice, because it exhibits competitive inhibition with a K_i lower than those measured for compounds **6a**,**b**,**d**, which are less stable analogues toward NTPDase hydrolysis. The fact that this inhibitor interacts specifically with the binding site of the enzyme potentially reduce the risk of interference with other ATP-binding enzymes or receptors. 8-CH_2-tBuS-ATP (**6b**) and 8-cycloheptylS-ATP (**6a**) showed mixed types of inhibition, thereby complicating the interpretation of their interaction with NTPDase. The mixed type of inhibition obtained with these two inhibitors may be explained by a new theoretical mechanism derived from a mechanism proposed by Westley.[52] We know that the enzyme could exist in either a monomer, dimer, and tetramer.[53,54] Polymerization would modify the protein in such a way to unmask the hidden catalytic site. In the case mentioned above, the inhibitor would favor a dissociation of the polymer and on the other hand would also interact with the catalytic site, thereby explaining the mixed type of inhibition. In agreement with this hypothesis, recent studies of Kirley[53] and Guidotti[54] have shown that polymerization may be an important modulator of NTPDase catalytic activity. In this context, an inhibitor that favors a dissociation of the enzyme polymers would reduce its activity while competing at the same time with ATP for the catalytic site of either the polymeric form of the NTPDase or for the catalytic site of subunits. This new theoretical model could also explain the characteristic sensitivity of the NTPDase to detergents. In the case of the analogue **6d**, it gives rise to noncompetitive inhibition, thereby suggesting that its inhibitory effects are the results of an interaction with another part of the enzyme distinct from the catalytic site. From these results, the catalytic site of NTPDase would apparently tolerate long and bulky substituents at the C8 position and also tolerate nucleotides in syn and anti conformations. The compounds with the same electronic nature of the modified purine ring have the same affinity for the catalytic site, since thioether, amino and ether-ATP derivatives show similar apparent affinity (K_m or K_i) for the enzyme. Moreover, we could hypothesize that there is a hydrophobic pocket of limited volume which interacts favorably with the hydrophobic side chain at C8 position. Furthermore, the sulfur atom is capable of

H-bonding with nearby H-bond donors in the catalytic site of NTPDase. These factors may explain the differences between 8-Br-ATP, 8-BuNH-ATP (**7**), 8-BuO-ATP (**8**), and 8-BuS-ATP (**6e**). Studies are currently carried out to map the NTPDase binding site and to identify the major binding determinants of this enzyme.

The second aspect considered in this study was the interference with purinoceptors. Recent studies have indicated that 8-BuS-ATP was a poor agonist for $P2Y_1$ receptor. In rat astrocytes this compound has no effect on the $[Ca^{2+}]_i$ level, whereas the corresponding 2-substituted ATP analogues potently increased the $[Ca^{2+}]_i$ level. Similar results were obtained with turkey erythrocytes membranes.[39] Since this molecule qualifies as a potent inhibitor, we tested its influence in the isolated mesenteric bed of the guinea pig for potential P2X and P2Y purinergic effects. Isolated guinea pig mesenteric artery and vein have been shown to respond to ATP via a P2X-purinoceptor located at the surface of the smooth muscle cells.[55,56] This latter purinoceptor was sensitive to α,β-CH_2-ATP (**1b**), a P2X agonist.[57,58] CHAPS was used to remove the blood vessel endothelium, which gives rise to endothelium denuded vessels. Nonfunctional endothelium has been shown by a lack of response to 100 pmol of NK-1.[59] Our data show that 8-BuS-ATP was not able to initiate any P2X-R effect at concentrations up to 1000 pmol, indicating that it does not interact with P2X-R. We also evaluated the effect of an infusion of 8-BuS-ATP on the vasodilatory response induced by administration of increasing ATP concentrations (0.1−10 000 pmol), in intact mesenteric bed. Even in the presence of 7 μM 8-BuS-ATP, the response to ATP was unmodified, confirming that 8-BuS-ATP did not interact with P2Y-R.

In conclusion, our study demonstrates the potential of a new class of ATP analogues as inhibitors (C8 substituted) of this novel family of proteins (NTPDases) that play a key role in the extracellular metabolism of nucleotides in the different physiological systems of vertebrates.

Experimental Section

Chemistry. General. New compounds were characterized by proton and carbon nuclear magnetic resonance using a Bruker AC-200 or DPX-300 spectrometer. The chemical shifts are reported in ppm relative to TMS as an internal standard. Nucleotides were characterized also by ^{31}P NMR in D_2O using 85% H_3PO_4 as an external reference on a Bruker AC-200 spectrometer. Mass spectra were recorded on an AutoSpec-E-FISION VG high-resolution mass spectrometer. Nucleotides were characterized by FAB (fast atom bombardment) and high-resolution FAB using a glycerol matrix under FAB negative conditions on an AutoSpec-E-FISION VG high-resolution mass spectrometer. Separation of the newly synthesized nucleotides was achieved on an LC (Isco UA-6) using DEAE A-25 Sephadex (HCO_3^- form) anion exchanger as described below. Final purification was done on an HPLC (Merck−Hitachi) system using a semipreparative LiChroCART LiChrospher 60 RP-select B column (1 × 25 cm, Merck KgaA) and a linear gradient of 0.1 M triethylammonium acetate buffer (TEAA, pH 7.5) and methanol (see below) at 6 mL/min flow rate. For analytical purposes, a LiChroCART LiChrospher 60 RP-select B column (250 mm × 4.6 mm, Merck KGaA) was used by applying the same gradient as above at 1 mL/min flow rate. The purity of the nucleotides described below was evaluated on an analytical column in two different solvent systems. One solvent system (I) was 0.1 TEAA/CH_3OH, 80:20 to 20:80 in 20 min. The second solvent system (II) was (A) 5 mM tetrabutylammonium phosphate (TBAP) in methanol and (B) 60 mM ammonium phosphate and 5 mM TBAP in 90% water/10% methanol, applying a gradient of 25% A to 75% A in 20 min.

8-Mercaptoadenosine (10). NaSH (0.8 g, 10 equiv) was added to a solution of 8-bromoadenosine (0.5 g, 1.44 mmol) in DMF (7 mL). The mixture was warmed to 100 °C and a few drops of water were added to improve solubility. The mixture was stirred at 100 °C overnight. The solvent was evaporated under high vacuum and the residue was coevaporated repeatedly with MeOH, until the residue turned into a solid. The residue was dissolved in water and neutralized with NaOH. After freeze-drying, the product was purified on a silica gel column ($CHCl_3$:MeOH 10:1). The product was obtained as a yellowish powder (100% yield, mp 169−170 °C). ^1H NMR (CD_3OD, 200 MHz) 8.09 (s, 1H, H-2), 6.65 (d, J = 7 Hz, 1H, H-1′), 5.01 (dd, J = 7, 5.5 Hz,1H, H-2′), 4.39 (dd, J = 5.5, 2.5 Hz, 1H, H-3′), 4.13 (q, J = 2.5 Hz, 1H, H-4′), 3.87 (dd, J = 12.5, 2.5 Hz, 1H, H-5′), 3.71 (dd, J = 12.5, 3 Hz, 1H, H-5′). ^{13}C NMR (CD_3OD, 300 MHz) δ 167.88 (C-6), 151.92 (C-2), 148.12 (C-4), 147.88 (C-8), 107.00 (C-5), 88.62 (C-1′), 85.59 (C-4′), 70.70 (C-2′), 70.62 (C-3′), 62.13 (C-5′). MS (CI/NH_3): m/z 317 M + NH_4^+.

8-(Thiocycloheptyl)adenosine (11a). A suspension of 8-mercaptoadenosine (75 mg, 0.25 mmol) in MeOH (2 mL) was dissolved in 0.25 M NaOH (1 mL). The clear, yellow solution was stirred at room temperature for 1 h. After freeze-drying, the thiolate sodium salt, obtained as a yellowish solid, was dissolved in dry DMF (2 mL) and bromocycloheptane (38 μL, 1.1 equiv) was added. The solution was stirred overnight under nitrogen at 60 °C. The solvent was evaporated under high vacuum and the yellow residue was coevaporated repeatedly with MeOH, until the residue turned into a yellow solid. The solid was triturated with petroleumether/ether 1:1, and then separated on a silica gel column ($CHCl_3$:MeOH 20:1). Product **11a** was obtained as a white solid in 83% yield (82 mg) after evaporation and drying, mp 205−6 °C. ^1H NMR (DMSO-d_6, 200 MHz): 8.07 (s, 1H, H-2), 7.36 (br s, 2H, NH_2), 5.84 (d, J = 7 Hz, 1H, H-1′), 5.00 (dd, J = 7, 5 Hz, 1H, H-2′), 4.16 (dd, J = 5, 2 Hz, 1H, H-3′), 4.07−3.91 (m, 2H, H-4′ & SCH), 3.68 (dd, J = 12, 4 Hz, 1H, H-5′), 3.51 (dd, J = 12.5, 4 Hz, 1H, H-5′), 2.19−1.93 (m, 2H), 1.84−1.42 (m, 11H). ^{13}C NMR (DMSO-d_6, 300 MHz): δ 154.80 (C-6), 151.51 (C-2), 150.03 (C-4), 147.94 (C-8), 119.75 (C-5), 88.96 (C-1′), 86.64 (C-4′), 71.31 (C-2′), 71.08 (C-3′), 62.27 (C-5′), 48.42 (SCH), 34.51 (CH_2), 34.14 (CH_2), 27.77 (CH_2), 27.76 (CH_2), 25.14 (CH_2), 25.04 (CH_2). FAB (positive): m/z 396 MH^+.

8-(Thio-2,2-dimethylpropyl)adenosine (11b). The compound was prepared as described for 8-(thiocycloheptyl)adenosine and obtained in 79% yield (73 mg) as a yellowish solid, mp 141−2 °C. ^1H NMR (CD_3OD, 200 MHz) δ 8.06 (s, 1H, H-2), 6.02 (d, J = 7 Hz, 1H, H-1′), 4.99 (dd, J = 7, 5 Hz, 1H, H-2′), 4.33 (dd, J = 5, 2 Hz, 1H, H-3′), 4.17 ("q", J = 2 Hz, 1H, H-4′), 3.88 (dd, J = 12.5, 2.5 Hz, 1H, H-5′), 3.72 (dd, J = 12.5, 3 Hz, 1H, H-5′), 3.42 and 3.35 (AB, J = 7 Hz, 2H, SCH), 1.09 (s, 9H, $SCH_2(CH_3)_3$). ^{13}C NMR (CD_3OD, 300 MHz) 155.92 (C-6), 152.91 (C-4), 152.11 (C-2), 151.69 (C-8), 110.64 (C-5), 91.19 (C-1′), 88.89 (C-4′), 74.13 (C-2′), 73.20 (C-3′), 64.17 (C-5′), 47.69 (SCH_2), 29.07 (3C, $SCH_2(CH_3)_3$). MS (CI/NH_3) m/z: 368 $(M-H)^+$.

8-(Thioethyl)adenosine (11c). A suspension of 8-mercaptoadenosine (270 mg, 0.9 mmol, in 7 mL MeOH) was dissolved in 0.25 M NaOH (3.6 mL). The clear, yellow solution was stirred at room temperature for 1 h. After freeze-drying, the thiolate sodium salt, obtained as a yellowish solid, was dissolved in dry DMF (3 mL) and bromoethane (101 μL, 1.5 equiv) was added. The solution was stirred under nitrogen at room temperature for 3 h. The solvent was evaporated under high vacuum and the yellow residue was coevaporated repeatedly with MeOH, until the residue turned into a yellow solid. The solid was separated on a silica gel column ($CHCl_3$:MeOH 15:1). Product **11c** was obtained as a white solid in 93% yield after evaporation and drying (273 mg), mp 176 °C. ^1H NMR (DMSO-d_6, 200 MHz): 8.05 (s, 1H, H-2), 7.31 (br s, 2H, NH_2), 5.76 (d, J = 7 Hz, 1H, H-1′), 5.66 (dd, J = 8.5, 3.5 Hz, 1H,

OH-5′), 5.42 (d, J = 6 Hz, 1H, OH-2′), 5.21 (d, J = 4 Hz, 1H, OH-3′), 4.99 (br q, J = 6 Hz, 1H, H-2′), 4.15 (br s, 1H, H-3′), 3.96 (br s, 1H, H-4′), 3.68 (dt, J = 12, 3.5 Hz, 1H, H-5′), 3.60−3.44 (m, 1H, H-5′), 3.43−3.14 (m, 1H, SCH_2CH$_3$), 1.36 (t, J = 7 Hz, 3H, SCH$_2$CH_3). ^{13}C NMR (DMSO-d_6, 300 MHz): 154.49 (C-6), 151.30 (C-2), 150.40 (C-4), 148.53 (C-8), 119.66 (C-5), 88.86 (C-1′), 86.63(C-4′), 71.29 (C-2′), 70.01 (C-3′), 62.24 (C-5′), 26.77 (SCH_2CH$_3$), 14.85(SCH$_2$$CH_3$). MS (CI/CH$_4$): m/z 328 MH$^+$; High-resolution MS: calcd for $C_{12}H_{18}N_5O_4S$ 328.1079, found 328.1069.

8-(Thio-*n*-hexyl)adenosine (11d). The compound was prepared as described for 8-(thioethyl)adenosine and obtained in 91% yield (314 mg) as a white solid, mp 169−171 °C. ^{1}H NMR (DMSO-d_6, 200 MHz): δ 8.05 (s, 1H, H-2), 7.29 (br s, 2H, NH$_2$), 5.77 (d, J = 7 Hz, 1H, H-1′), 5.67 (dd, J = 9, 3.5 Hz, 1H, OH-5′), 5.42 (d, J = 6 Hz, 1H, OH-2′), 5.21 (d, J = 4 Hz, 1H, OH-3′), 4.99 (“q”, J = 6 Hz, 1H, H-2′), 4.15 (br s, 1H, H-3′), 3.96 (br s, 1H, H-4′), 3.67 (dt, J = 12, 3.5 Hz, 1H, H-5′), 3.60−3.43 (m, 1H, H-5′), 3.42−3.18 (m, 1H, SCH_2), 1.69 (quint, J = 7 Hz, 2H, SCH$_2$CH_2), 1.50−1.18 (m, 6H, CH_2CH_2CH_2CH$_3$), 0.86 (t, J = 7 Hz, 3H, CH$_3$). ^{13}C NMR (DMSO-d_6, 300 MHz): 154.55 (C-6), 151.27 (C-2), 150.38 (C-4), 148.71 (C-8), 119.61 (C-5), 88.85 (C-1′), 86.63(C-4′), 71.28 (C-2′), 71.02 (C-3′), 62.23 (C-5′), 32.37 (SCH_2), 30.70 (CH$_2$), 28.80 (CH$_2$), 27.71 (CH$_2$), 21.99 (CH$_2$), 13.88 (CH$_3$). MS (CI/CH$_4$): m/z 384 MH$^+$. High-resolution MS: calcd for $C_{16}H_{26}N_5O_4S$ 384.1705, found 384.1696.

Nucleoside 5′-Triphosphorylation. Nucleosides **11a−d** were 5′-triphosphorylated according to a literature procedure.[38]

8-(Thiocycloheptyl)adenosine 5′-triphosphate (6a) was obtained in 60% yield (79 mg). Final separation was achieved via HPLC by applying a linear gradient of TEAA/CH$_3$OH 70:30 to 20:80 in 20 min (6 mL/min), t_R 9.53 min. ^{1}H NMR (D$_2$O, 200 MHz): δ 8.17 (s, 1H, H-2), 6.09 (d, J = 6 Hz, 1H, H-1′), 5.15 (t, J = 6 Hz, 1H, H-2′), 4.62−4.51 (m, 1H, H-3′), 4.37−4.14 (m, 3H, H-4′ & H-5′), 3.93−3.75 (m, 1H, SCH), 2.15−1.90 (m, 2H),1.80−1.36 (m, 11H). ^{31}P NMR (D$_2$O, 200 MHz, pH 9) δ −5.34 (d), −10.37 (d), −21.32 (t). UV: λ_{max} 282 nm. HRFAB: calcd for $C_{17}H_{27}N_5O_{13}P_3S$ 634.0539, found 634.0540. t_R: 14.99 min (95% purity) using solvent system I, 13.19 min (97% purity) using solvent system II.

8-(Thio-2,2-dimethylpropyl)adenosine 5′-triphosphate (6b) was obtained in 65% yield (77 mg). Final separation was achieved via HPLC by applying a linear gradient of TEAA/CH$_3$OH 70:30 to 20:80 in 20 min (6 mL/min), t_R 7.51 min. ^{1}H NMR (D$_2$O, 200 MHz): δ 8.21 (s, 1H, H-2), 6.13 (d, J = 6 Hz, 1H, H-1′), 5.20 (t, J = 6 Hz, 1H, H-2′), 4.62 (dd, J = 6 Hz, 1H, H-3′), 4.42−4.25 (m, 3H, H-4′ & H-5′), 3.29 and 3.35 (ABq, J = 12 Hz, 2H, SCH$_2$), 1.06 (s, 3H, SCH$_2$(CH_3)$_3$). ^{31}P NMR (D$_2$O, 200 MHz, pH 9) δ −10.23 (d), −10.79 (d), −22.61 (t). UV: λ_{max} 282 nm. HRFAB: calcd for $C_{15}H_{25}N_5O_{13}P_3S$ 608.0382, found 608.0360. t_R: 12.71 min (96% purity) using solvent system I, 11.31 min (95% purity) using solvent system II.

8-(Thioethyl)adenosine 5′-triphosphate (6c) was obtained in 43% yield (84 mg). Final separation was achieved via HPLC by applying a linear gradient of TEAA/CH$_3$OH 90:10 to 20:80 in 20 min (6 mL/min), t_R 8.11 min. ^{1}H NMR (D$_2$O, 200 MHz): 8.15 (s, 1H, H-2), 6.10 (d, J = 6.5 Hz, 1H, H-1′), 5.17 (t, J = 6.5 Hz, 1H, H-2′), 4.65−4.55 (m, 1H, H-3′), 4.40−4.16 (m, 3H, H-4′ & H-5′), 3.30 and 3.26 (ABq of t, J = 11.5, 7 Hz, 1H each, SCH$_2$), 1.39 (t, J = 7 Hz, 3H, CH$_3$). ^{31}P NMR (D$_2$O, 200 MHz, pH 9) −5.12 (d), −10.31 (d), −20.98 (t). UV: λ_{max} 282 nm. HRFAB: calcd for $C_{12}H_{18}N_5O_{13}NaP_3S$ 587.9732, found 587.9650. t_R: 7.16 min (96% purity) using solvent system I, 3.55 min (94% purity) using solvent system II.

8-(Thio-*n*-hexyl)adenosine 5′-triphosphate (6d) was obtained in 58% yield (111 mg). Final separation was achieved via HPLC by applying a linear gradient of TEAA/CH$_3$OH 70:30 to 20:80 in 20 min (6 mL/min), t_R 10.73 min. ^{1}H NMR (D$_2$O, 200 MHz): 8.17 (s, 1H, H-2), 6.01 (d, J = 6.5 Hz, 1H, H-1′), 5.19 (t, J = 6.5 Hz, 1H, H-2′), 4.64−4.55 (m, 1H, H-3′), 4.40−4.14 (m, 3H, H-4′ & H-5′), 3.32 and 3.24 (ABq of t, J = 14, 7 Hz, 1H each, SCH$_2$), 1.73 (“quint”, J = 7 Hz, 2H, SCH$_2$CH_2), 1.52−1.09 (m, 6H), 0.82 (t, J = 7 Hz, 3H, CH$_3$); ^{31}P NMR (D$_2$O, 200 MHz, pH 9) −5.12 (d), −10.25 (d), −21.03 (t). UV: λ_{max} 282 nm. t_R: 15.25 min (>97% purity) using solvent system I, 15.52 min (>97% purity) using solvent system II.

Enzymology. Reagents and Solutions. ATP, tetramisole, malachite green, bovine serum albumin fraction V (BSA), CHAPS, sodium nitroprussiate (NaNP), (−)-arterenol bitartrate (noradrenaline), heparin, and indomethacin were obtain from Sigma Chemical Co. (St. Louis, MO). ADP was obtained from Roche (Laval, QC, Canada), and Bradford reagent was purchased from Bio-Rad Laboratories (Mississauga, Ontario, Canada). [Sar^9,Met(O$_2$)^{11}]SP (NK-1) was synthesized by Dr. W. Neugebauer from the Université de Sherbrooke. Preparations of Krebs and phosphate-buffered saline (PBS) were as followed: Krebs solution, 5.5 mM glucose, 117.5 mM NaCl, 1.2 mM MgSO$_4$, 1.2 mM KH$_2$PO$_4$, 4.7 Mm KCl, 2.5 Mm CaCl$_2$, 25 Mm NaHCO$_3$, pH 7.4; PBS, 137 mM NaCl, 3 mM KCl, 10 mM Na$_2$HPO$_4$ and 1.7 mM KH$_2$PO$_4$, pH 7.4. All the other reagents were of analytical grade and bought from Sigma Chemical Co. (St. Louis, MO).

Enzymatic Assays. Isolation of Particulate Fractions. Bovine spleens were obtained from a local slaughterhouse, and isolation of the particulate fraction was carried out as previously described.[23]

NTPDase Assays. Enzyme activity was routinely measured by the release of inorganic phosphorus with the malachite green colorimetric assay.[60] Resistance to hydrolysis was measured at 37 °C in 1 mL of the following incubation medium: 8mM CaCl$_2$, 5 mM tetramisole, 50 mM Tris base, 50 mM imidazole, buffered at pH 7.6, and 100 μM of either ATP or its analogues. Apparent K_m and V_{max} values for ATP, ADP, and each of the hydrolyzable purine nucleotide analogues were derived from Eadie and Hofstee plots, with substrate concentrations ranging between 10 and 300 μM for ATP and ADP, and between 15 and 100 μM for the analogues, unless stated otherwise. In both cases the reaction was started by the addition of 1.9 μg of the enzyme preparation and stopped after 7 min with 250 μL of the malachite green reagent. Apparent K_i values for nonhydrolyzable purine nucleotide analogues were derived from Dixon replots, using inhibitor concentrations ranging from 0 to 100 μM. Reactions were performed in the same incubation buffer, as previously described, and were started by the addition of nonsaturating ATP concentrations. Protein concentration was determined with the Bradford microplate assay using bovine serum albumin as a standard of reference.[61]

P2-Receptors Assays. Surgical Procedures. Dunkin−Hartley guinea pig (300−350 g) of either sex were sacrificed by cervical dislocation following the Canadian Council on Animal Care. The guinea pig mesentery was prepared as described by Berthiaume et al.[59] Briefly, the colic and ileocolic branches of the superior mesenteric artery were tied and the superior mesenteric artery cannulated (Portex size tube 3FG). To isolate the mesenteric bed from the intestine, the mesentery was perfused (2 mL/min, for 5 min) via the mesenteric artery with a Krebs solution containing heparin (100 U/mL). The mesentery was then separated by cutting close to the intestine. A resting period of 60 min was then allowed during which time the guinea pig mesenteric bed was perfused (2 mL/min) with a warmed (37 °C) and gassed Krebs solution (95% O$_2$ and 5% CO$_2$) containing indomethacin (5 μM), as described earlier. In all the assays, the perfusion pressure was increased to obtain a flow rate of 6 mL min^{-1}. The response of mesenteric bed, precontracted with noradrenaline (200 μM) in 0.9% saline solution, to the different drugs was measured with a pressure transducer (Statham, model P-23AC) and recorded on a Grass physiograph (model 79D).

P2X-Receptor Assays. Guinea pig mesenteric bed was denuded from its endothelium layer by using 20 mM of CHAPS in PBS.[59] Briefly, the CHAPS solution was infused for 45 s, followed by a resting period of 30 min. Finally, a second 45 s infusion of 20 mM CHAPS was carried out. Blood vessels were then precontracted as earlier described. The efficiency of the endothelium removal technique was assessed by an intra-arterial bolus injection of 100 pmol of NK-1 in PBS. Reactivity of the media layer was confirmed by bolus a injection of 3 nmol

of NaNP (sodium nitroprussiate) in PBS. Bolus injections of increasing concentrations of 8-BuS-ATP (0.1 to 1000 pmol) in PBS were administered. Variations of perfusion pressure were measured. Between each injection of 8-BuS-ATP a resting period allowed the return of pressure to baseline (i.e. precontracted pressure).

P2Y-Receptor Assays. Intact mesenteric bed vessels were precontracted with noradrenaline (200 μM). Mesentery was infused for 7 min with 7 μM of 8-BuS-ATP or PBS (control), followed by intra-arterial bolus injection of increasing concentrations of ATP (0.1−10 000 pmol). A resting period between each ATP injection allowed return to baseline, as earlier described. The reactivity of the blood vessels was assessed by a bolus injection of 3 nmol of NaNP. Response of the endothelium layer was confirmed by injection of NK-1 (100 pmol).

Statistics. Data are expressed as mean ± SEM, and the number of replicates is noted in the figure legends. Kinetic studies have been performed using Grafit software version 4 (Erithacus, UK). Unless stated otherwise, comparison between data was performed by one-way ANOVA test. Probability values of less than 0.05 were considered significant.

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Supporting Information Available: Figure S1, the inhibitory effect of 8-BuS-aTP (**6e**) on ATP hydrolysis by NTPDase; Figure S2, Dixon representations of 8-CH2tBuS-ATP (**6b**) and 8-cycloheptylS-ATP (**6a**) inhibition; and Figure S3, Dixon representation of 8-thiohexyl-ATP (**6d**) inhibition. This material is available free of charge via the Internet at http://pubs.acs.org.

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