

## RESEARCH PAPER

# 8-BuS-ATP derivatives as specific NTPDase1 inhibitors

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## BACKGROUND AND PURPOSE

Ectonucleotidases control extracellular nucleotide levels and consequently, their (patho)physiological responses. Among these enzymes, nucleoside triphosphate diphosphohydrolase-1 (NTPDase1), -2, -3 and -8 are the major ectonucleotidases responsible for nucleotide hydrolysis at the cell surface under physiological conditions, and NTPDase1 is predominantly located at the surface of vascular endothelial cells and leukocytes. Efficacious inhibitors of NTPDase1 are required to modulate responses induced by nucleotides in a number of pathological situations such as thrombosis, inflammation and cancer.

## EXPERIMENTAL APPROACH

Here, we present the synthesis and enzymatic characterization of five 8-BuS-adenine nucleotide derivatives as potent and selective inhibitors of NTPDase1.

## KEY RESULTS

The compounds 8-BuS-AMP, 8-BuS-ADP and 8-BuS-ATP inhibit recombinant human and mouse NTPDase1 by mixed type inhibition, predominantly competitive with  $K_i$  values  $<1 \mu\text{M}$ . In contrast to 8-BuS-ATP which could be hydrolyzed by other NTPDases, the other BuS derivatives were resistant to hydrolysis by either NTPDase1, -2, -3 or -8. 8-BuS-AMP and 8-BuS-ADP were the most potent and selective inhibitors of NTPDase1 expressed in human umbilical vein endothelial cells as well as *in situ* in human and mouse tissues. As expected, as a result of their inhibition of recombinant human NTPDase1, 8-BuS-AMP and 8-BuS-ADP impaired the ability of this enzyme to block platelet aggregation. Importantly, neither of these two inhibitors triggered platelet aggregation nor prevented ADP-induced platelet aggregation, in support of their inactivity towards P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors.

## CONCLUSIONS AND IMPLICATIONS

The 8-BuS-AMP and 8-BuS-ADP have therefore potential to serve as drugs for the treatment of pathologies regulated by NTPDase1.

## Abbreviations

8-BuS-ADP, 8-Thiobutyladenosine 5'-diphosphate; 8-BuS-AMP, 8-Thiobutyladenosine 5'-monophosphate; 8-BuS-ATP, 8-Thiobutyladenosine 5'-triphosphate; ARL 67156,  $N^6,N^6$ -diethyl-D- $\beta$ - $\gamma$ -dibromomethylene-ATP; DMF, Dimethylformamide; EGM, Endothelial Cell Growth; E-NPP, ecto-nucleotide pyrophosphatase/phosphodiesterase; E-NTPDase, ecto-nucleoside triphosphate diphosphohydrolase; HRMS, high-resolution mass spectrometry;  $P_i$ , inorganic phosphate; pnp-TMP, *p*-nitrophenyl thymidine 5'-monophosphate; POM-1, polyoxometalates; PPP, platelet-poor plasma; TEAA, triethylammonium acetate; TBAP, tetrabutylammonium phosphate

## Introduction

Extracellular nucleotides modulate a variety of functions, including development (Zimmermann, 2006), platelet aggregation (Stafford *et al.*, 2003), pain perception (Burnstock, 2006a; Zylka, 2011), cardiovascular regulation (Erlinge and Burnstock, 2008), airway epithelial transport (Fausther *et al.*, 2010), control of endocrine gland secretion (Stojilkovic and Koshimizu, 2001), neurotransmission and neuromodulation (Burnstock, 2007), inflammation as well as immune reactions (Atkinson *et al.*, 2006; Robson *et al.*, 2006; Borsellino *et al.*, 2007). The effects of these nucleotides are mediated by the activation of several receptors, namely P2X1-7, P2Y<sub>1,2,4,6,11-14</sub>, and perhaps also cysLT1R, cysLT2R and/or GPR17 (Ciana *et al.*, 2006; Burnstock, 2006b). Three enzyme families have the ability to hydrolyze ATP and ADP at the medium/membrane interface (Zimmermann, 2001; Robson *et al.*, 2006; Zimmermann *et al.*, 2012), thus keeping free nucleotides at a low extracellular concentration, namely the ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) family, the ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) family (Goding *et al.*, 2003) and non-specific alkaline phosphatases (Picher *et al.*, 2003). The eight members of the E-NTPDase family include four true ectonucleotidases, namely NTPDase1 (also known as CD39, ATPase, ecto-apyrase, ecto-ADPase), NTPDase2 (ecto-ATPase, CD39L1), NTPDase3 (CD39L3, HB6), and NTPDase8 (Bigonnesse *et al.*, 2004; Kukulski *et al.*, 2011b). The latter ectonucleotidases represent the main enzymes responsible for the hydrolysis of nucleotides in the cellular environment at physiological pH. These subtypes have two plasma membrane-spanning domains and an active site facing the extracellular milieu. These enzymes hydrolyze the  $\gamma$ - and  $\beta$ -phosphates of nucleotides in the presence of divalent cations ( $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ ) (Kukulski *et al.*, 2005).

NTPDase1 is the main ectonucleotidase in both vascular endothelial and smooth muscle cells, as well as in blood cells that regulate associated biological responses. NTPDase1 has been shown so far to be involved in acute immune responses (Deaglio and Robson, 2011; Kukulski *et al.*, 2011b) and leukocyte migration (Kukulski *et al.*, 2011a), to contribute to the antithrombotic property of endothelial cells (Marcus *et al.*, 1991; Kaczmarek *et al.*, 1996; Enjyoji *et al.*, 1999) and to modulate angiogenesis, vascular permeability (Guckelberger *et al.*, 2004; Yegutkin *et al.*, 2011), and vasoconstriction (Kauffenstein *et al.*, 2010; Li *et al.*, 2011). Also of interest, NTPDase1 expression in sinusoidal endothelial cells from mice livers stimulates tumour cell proliferation and limits cell death triggered by extracellular ATP (Feng *et al.*, 2011). NTPDase1-specific inhibitors would be extremely useful for elucidating the physiological roles of this enzyme and might also be assessed as promising drug candidates for the treatment of some cardiovascular diseases (Sévigny *et al.*, 2002; Marcus *et al.*, 2003; Atkinson *et al.*, 2006; Robson *et al.*, 2006), immune diseases (Borsellino *et al.*, 2007; Moncrieffe *et al.*, 2010) and cancer (Müller *et al.*, 2006; Mandapathil *et al.*, 2009). Therefore, NTPDase1 inhibitors may be valuable to potentiate the effect of P2 receptor agonists present in the vicinity of NTPDase1 by extending the half-life of their ligands.

No specific inhibitors of NTPDase1 have yet been reported to this day. The only NTPDase inhibitors known so far are ATP analogues [8-BuS-ATP, **1** (Gendron *et al.*, 2000) and  $\text{N}^6, \text{N}^6$ -diethyl-D- $\beta$ - $\gamma$ -dibromomethylene-ATP (ARL 67156), **2** (Crack *et al.*, 1995; Lévesque *et al.*, 2007)], suramin, **3** and related compounds, polyoxometalates (POM-1), **4** (Müller *et al.*, 2006), sulfonate dyes such as reactive blue 2, **5** and pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS), **6** (Bultmann *et al.*, 1996; Chen *et al.*, 1996; Munkonda *et al.*, 2007; Wittenburg *et al.*, 1996) (Figure 1). We have developed 8-BuS-ATP a decade ago as the first potent NTPDase inhibitor ( $K_i \sim 10 \mu\text{M}$ ) (Gendron *et al.*, 2000), but its subtype selectivity had not been determined thus far. ARL 67156 was found to be a weak and non-selective NPP1, NTPDase1 and NTPDase3 inhibitor. POM-1 is more effective than ARL 67156 at blocking NTPDase1-dependent ATP breakdown but its usefulness is limited by off-target actions on synaptic transmission (Lévesque *et al.*, 2007; Wall *et al.*, 2008). More recently, anthraquinone derivatives have been tested as NTPDase inhibitors. 1-Amino-2-sulfo-4-(2-naphthylamino) anthraquinone, **7** (Figure 1) was shown to be a potent inhibitor of NTPDase1 and NTPDase3 ( $K_i$  of 0.33 and 2.22  $\mu\text{M}$ , respectively) (Baqi *et al.*, 2009). Interestingly, monoclonal antibodies have been obtained that specifically inhibit human NTPDase3 (Munkonda *et al.*, 2009) and NTPDase2 (<http://ectonucleotidases-ab.com>) but to our knowledge, no inhibitory antibody to NTPDase1 has yet been reported thus far.

The need for potent and subtype-specific NTPDase inhibitors prompted us to perform a structure-activity relationship study using the natural substrate ATP. For this purpose, we synthesized and evaluated a series of 8-BuS-ATP analogues, **8–11**, towards more stable and specific inhibitors of NTPDase1.

## Methods

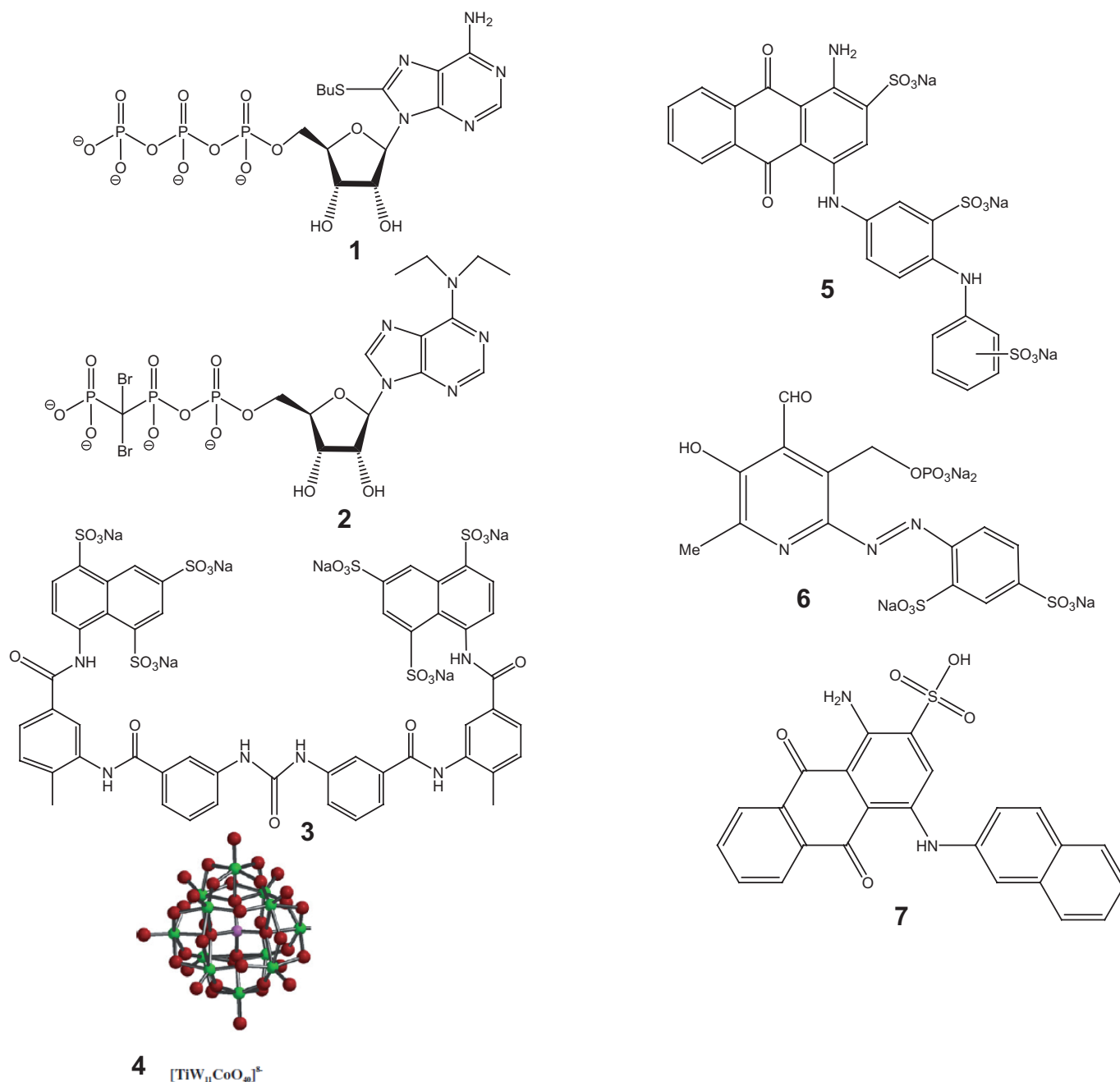
### Enzymatic assays

**Materials.** Aprotinin, nucleotides, PMSF and Malachite green were purchased from Sigma-Aldrich (Oakville, ON, Canada). Tris was obtained from VWR (Montreal, QC, Canada). DMEM was obtained from Invitrogen (Burlington, ON, Canada). FBS and antibiotics-antimycotics solution were from Wisent (St-Bruno, QC, Canada). 8-Thiobutyladenosine 5'-triphosphate (8-BuS-ATP) was synthesized as previously described (Gendron *et al.*, 2000).

**Plasmids.** Plasmids used in this study have all been described previously: human NTPDase1 (GenBank accession no. U87967) (Kaczmarek *et al.*, 1996), human NTPDase2 (NM\_203468) (Knowles and Chiang, 2003), human NTPDase3 (AF034840) (Smith and Kirley, 1998), human NTPDase8 (AY430414) (Fausther *et al.*, 2007), human NPP1 (NM\_006208) (Belli and Goding, 1994) and human NPP3 (NM\_005021) (Jinhua *et al.*, 1997).

**Cell transfection and protein preparation.** COS-7 cells (in 10 or 15 cm dishes) were transfected with an expression vector (pcDNA3) incorporating the cDNA that encodes each ecto-





**Figure 1**

NTPDase inhibitors: **(1)**, 8-BuS-ATP; **(2)**,  $N^6,N^6$ -diethyl-D- $\beta$ - $\gamma$ -dibromomethylene-ATP (ARL 67156); **(3)**, suramin; **(4)**, polyoxometalates (POM-1); **(5)**, reactive blue 2; **(6)**, pyridoxal phosphate-6-azophenyl-2',4'-disulfonic acid (PPADS); **(7)**, 1-amino-2-sulfo-4-(2-naphthylamino)anthraquinone.

nucleotidase using Lipofectamine (Invitrogen) and harvested 40–72 h later, as described (Kukulski *et al.*, 2005). For the preparation of protein extracts, transfected cells were washed three times with 95 mM NaCl and 45 mM Tris, pH 7.5, at 4°C, collected by scraping using the same buffer supplemented with 0.1 mM PMSF and washed twice by centrifugation (300× *g*, 10 min, 4°C). The cells were then resuspended in harvesting buffer supplemented with 10  $\mu\text{g ml}^{-1}$  aprotinin and sonicated. Nucleus and cellular debris were discarded

after another centrifugation (300× *g*, 10 min, 4°C) and the resulting supernatant (hereinafter called protein extract) was aliquoted and stored at –80°C until use. Protein concentration was estimated by a Bradford microplate assay using bovine serum albumin as a standard (Bradford, 1976).

**Enzymatic reactions.** Experiments were carried out with protein extracts of COS-7 cells transfected with an expression vector encoding each ectonucleotidase (1–2  $\mu\text{g}$  proteins per

assays in 200  $\mu$ L reaction buffer), or with intact human umbilical vein endothelial cells (HUVECs), which express NTPDase1. Protein extracts from non-transfected COS-7 cells showed negligible ATPase and ADPase activities, representing less than 5 and 1%, respectively, of those found in extracts from COS-7 cells transfected with NTPDase1.

**NTPDases (EC 3.6.1.5):** Enzyme activity was measured as described (Kukulski *et al.*, 2005) in 0.2 mL of incubation medium (5 mM  $\text{CaCl}_2$  and 80 mM Tris, pH 7.4) or Tris-Ringer buffer (in mM: 120 NaCl, 5 KCl, 2.5  $\text{CaCl}_2$ , 1.2  $\text{MgSO}_4$ , 25  $\text{NaHCO}_3$ , 5 D-glucose, 80 Tris, pH 7.4) at  $37^\circ\text{C} \pm 8$ -BuS analogues. NTPDase protein extracts were added to the incubation mixture and pre-incubated at  $37^\circ\text{C}$  for 3 min. The reaction was initiated by the addition of 10–100  $\mu$ M ATP and stopped after 10–15 min with 50  $\mu$ L of Malachite green reagent. Membrane-bound activity was measured with confluent HUVECs at passage two attached to 24-well plates (about 200 000 cells per well). Cells were maintained in endothelial cell growth (EGM) medium supplemented with 10% FBS until the activity assay that was performed in the buffers indicated above supplemented with 125 mM NaCl. The reactions were initiated as above and stopped by transferring an aliquot of the reaction mixture to a tube containing the Malachite green reagent. Net cell-bound enzyme activity was calculated after subtracting the value measured in the control cell reaction mixture where the substrate was added after the Malachite green reagent. Released inorganic phosphate ( $\text{P}_i$ ) was measured at 630 nm according to Baykov *et al.*, (1988). All experiments were performed in triplicate. Note that the presence of 100  $\mu$ M 8-BuS-ATP, **1**, 8-BuS-AMP, **10**, or 8-BuS-ADP, **11**, did not affect the linearity of the  $\text{P}_i$  determination up to 40  $\mu$ M (data not shown) which was accordingly never exceeded in the assays. One unit of enzymatic activity corresponds to the release of 1  $\mu$ mol  $\text{P}_i$ ·(min·mg of protein) $^{-1}$  or 1  $\mu$ mol  $\text{P}_i$  min $^{-1}$ ·well $^{-1}$  at  $37^\circ\text{C}$  for protein extracts and intact cells, respectively. The  $\text{IC}_{50}$  parameters were determined from a dose-response non-linear regression analysis. The  $K_{i,\text{app}}$  for 8-BuS-AMP, **10** and 8-BuS-ADP, **11**, was performed according to Dixon and Cornish-Bowden method (Dixon, 1953; Cornish-Bowden, 1974) and the  $K_{i,\text{app}}$  for 8-BuS-ATP was calculated from its  $\text{IC}_{50}$  using the Cheng-Prusoff equation. All kinetic values were calculated using the GraphPad Prism software (GraphPad Software Inc. San Diego, CA, USA).

Note that for all NTPDases, the reaction was linear for at least the first 30 min with either ATP or ADP as a substrate. Therefore, all enzymatic assays in this work were carried out for 10–15 min.

**NPPs (EC 3.1.4.1; EC 3.6.1.9):** Evaluation of the effect of analogues **1** and **8–11** on human NPP1 and NPP3 activity was carried out with *p*-nitrophenyl thymidine 5'-monophosphate (pnp-TMP) as substrate (Belli and Goding, 1994; Vollmayer *et al.*, 2003). Reactions were carried out at  $37^\circ\text{C}$  in 0.2 mL of the incubation mixture (in mM: 1  $\text{CaCl}_2$ , 140 NaCl, 5 KCl and 50 Tris, pH 8.5,  $\pm$ test analogues). Substrates and inhibitors were all used at a final concentration of 100  $\mu$ M. Human NPP1 or NPP3 extract was added to the incubation mixture and pre-incubated at  $37^\circ\text{C}$  for 3 min. The reaction was initiated by addition of the substrate. For pnp-TMP, the production of *p*-nitrophenol was measured at 405 nm, 15 min after initiation of the reaction.

**Enzyme histochemistry assays:** For histochemical studies, freshly dissected tissues were embedded in O.C.T. freezing medium (Tissue-Tek<sup>®</sup>; Sakura Finetek, Torrance, CA, USA) and snap-frozen in isopentane in dry ice and stored at  $-80^\circ\text{C}$  until use. Sections of 6  $\mu$ m were obtained and fixed in 10% phosphate-buffered formalin mixed with cold acetone (Fisher Scientific, Ottawa, ON, Canada).

Localization of ectonucleotidase activities was determined using the Wachstein/Meisel lead phosphate method (Braun *et al.*, 2003). Fixed slices were pre-incubated for 30 min at room temperature (RT) in 50 mM Tris-maleate buffer, pH 7.4, containing 2 mM  $\text{CaCl}_2$ , 250 mM sucrose and 2.5 mM levamisole as an inhibitor of alkaline phosphatases. Enzymatic reaction was performed for 1 h at  $37^\circ\text{C}$  in the same buffer supplemented with 5 mM  $\text{MnCl}_2$  to inhibit intracellular staining (Dahl and Pratley, 1967), 2 mM  $\text{Pb}(\text{NO}_3)_2$ , 3% Dextran T-250 and in the presence of 200–500  $\mu$ M ATP with or without 8-BuS derivatives. For control experiments, substrate was either omitted or added in the absence of divalent cations, which are essential for NTPDase activity. The reaction was revealed by incubation with 1%  $(\text{NH}_4)_2\text{S}$  v/v for exactly 1 min. Samples were counterstained with aqueous haematoxylin or DAPI, mounted with Mowiol mounting medium, and observed and photographed under a BX51 Olympus microscope.

### Platelet aggregation assays

The investigation standards conformed to principles outlined in the Helsinki declaration. Platelet-rich plasma (PRP) was prepared from the blood of healthy volunteers according to standard procedures (Sévigny *et al.*, 2002). Briefly, whole blood was collected in heparinized tubes, centrifuged at room temperature at 300 $\times g$  for 12 min and the upper layer, consisting of the platelet-rich plasma (PRP) fraction, was collected. Platelets were used from 1.5 to 2 h after collection from the volunteers. Platelet aggregation was measured in an AggRAM aggregometer. The extent of platelet aggregation corresponded to the decrease in  $\text{OD}_{600}$  observed with a 0.6 mL PRP sample maintained at  $37^\circ\text{C}$ . PPP, obtained after centrifugation of the PRP for 3 min at 15 000 $\times g$ , was used as the control of reference. Platelet aggregation was initiated with the addition of 8  $\mu$ M ADP. Where indicated, NTPDase1-transfected COS-7 cell lysates (6  $\mu$ g protein diluted in incubation buffer A with 145 mM NaCl) with or without test drugs, were added to PRP. Note that appropriate control experiments contained either incubation buffer with intact COS-7 cells, protein extracts from non-transfected COS-7 cells, or an equivalent amount of water as a control for the tested compounds as they were all diluted in water.

For parallel assays using light microscopy, 100  $\mu$ L of the above reaction mixture was placed on microscope slides 10 min after initiation of platelet aggregation. Slides were then air-dried at  $37^\circ\text{C}$  and stained using Diff-Quick kit (Dade Behring Inc., Newark, DE, USA). The remainder of the reaction mixture was spun at 300 $\times g$  for 3 min, and free platelets in the supernatant were counted.

### Nucleotide synthesis

**General.** All air- and moisture-sensitive reactions were carried out in flame-dried, argon-flushed, two-necked flasks

sealed with rubber septa, and reagents were introduced with a syringe. TLC analysis was performed on pre-coated Merck silica gel plates (60F-254). Visualization was accomplished using a UV light. Nucleosides were separated on a medium pressure liquid chromatography system (Biotage, Uppsala, Sweden) using a silica gel column (12" M or 25" M); separation conditions are indicated below for each compound. New compounds were characterized (and resonances assigned) by NMR using Bruker DMX-600, DPX-300, or AC-200 spectrometers. Nucleoside  $^1\text{H}$  NMR spectra were recorded in  $\text{CD}_3\text{OD}$  or in  $\text{D}_2\text{O}$ , and the chemical shifts are reported in ppm relative to HDO (4.78 ppm) as an internal standard. Nucleotides were characterized also by  $^{31}\text{P}$  NMR in  $\text{D}_2\text{O}$  with an AC-200 spectrometer at pH 8, using 85%  $\text{H}_3\text{PO}_4$  as an external reference. All final products were characterized by chemical ionization and high-resolution mass spectrometry (HRMS) using an AutoSpec-E Fision VG high-resolution mass spectrometer. Nucleotides were desorbed from a glycerol matrix by fast atom bombardment at low and high resolution. Primary purification of nucleotides was achieved on an LC system (Isco UA-6) using a DEAE Sephadex A-25 column that had been swelled in 1 M  $\text{NaHCO}_3$  at RT overnight. Final purification of nucleotides was achieved on a HPLC system (Hitachi) with a semipreparative reverse-phase (Gemini 5  $\mu\text{m}$  C-18 110 Å, 250  $\times$  10 mm, 5  $\mu\text{m}$ ; Phenomenex, Torrance, CA, USA). For analytical purposes, a microcolumn (Gemini 5  $\mu\text{m}$ , C-18 110 Å, 150  $\times$  4.60 mm, 5  $\mu\text{m}$ ; Phenomenex) was used. The purity of the nucleotides was evaluated on an analytical column using two different solvent systems. Peaks were detected by UV absorption at 253 nm. Solvent system I was (A)  $\text{CH}_3\text{CN}$  and (B) 0.1 M triethylammonium acetate (TEAA) (pH 7). Solvent system II was (A) 5 mM tetrabutylammonium phosphate (TBAP) in MeOH and (B) 60 mM ammonium phosphate and 5 mM TBAP in 90% water/10% MeOH.

#### 8-Butylthio- $\beta$ , $\gamma$ -imido – adenosine 5' – triphosphate (**8**)

8-Butylthio-adenosine (**12**) (Halbfinger *et al.*, 1999) (100 mg, 0.28 mmol) was dissolved in dry trimethyl phosphate (724  $\mu\text{L}$ ), cooled to  $-15^\circ\text{C}$  and a proton sponge (94 mg, 0.44 mmol, 1.6 eq) was added. After stirring for 20 min, phosphoryl chloride ( $\text{POCl}_3$ ) (76.7  $\mu\text{L}$ , 0.36 mmol, 1.3 eq) was added dropwise and the reaction was stirred for 2 h. A 1 M solution of  $[\text{Bu}_3\text{NH}^+]_2[\text{CH}_2(\text{PO}_3)_2]^{2-}$  in dimethylformamide (DMF; 2.1 mL, 2.1 mmol, 7.5 eq) and  $\text{Bu}_3\text{N}$  (275  $\mu\text{L}$ , 1.15 mmol, 4 eq) was added at once. After stirring for 1 min, triethylammonium bicarbonate buffer (pH 7) TEAB (5 mL, 0.2 M) was added and the clear solution was stirred for 45 min at RT. The latter solution was freeze-dried overnight. The semisolid obtained was then purified by chromatography on an activated DEAE Sephadex A-25 column using a  $\text{NH}_4\text{HCO}_3$  gradient (0–0.48 M). The relevant fractions were freeze-dried repeatedly to yield the product **8** as a white solid in a 35% yield (58 mg). Final separation was achieved on HPLC applying a linear gradient of TEAA/ $\text{CH}_3\text{CN}$  80:20 to 55:45 in 12 min (flow rate, 6 mL  $\text{min}^{-1}$ ), with a measured retention time ( $t_R$ ) of 12 min (>95% purity) using solvent system II (60 mM ammonium phosphate (A) and 5 mM TBAP in 90% water/10% methanol (B), applying a gradient of 25% A to 75% A in 20 min),  $t_R$  = 6 min (>97% purity).  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 200 MHz)  $\delta$ : 8.17 (s, 1H, H2), 6.12 (d,  $J$  = 7 Hz, 1H, H1'), 5.19 (t,  $J$  = 7 Hz, 1H, H2'), 4.64–4.55 (m, 1H, H3'), 4.40–4.14

(m, 3H, H4', H5', H5''), 3.60 (m, 2H,  $\text{SCH}_2$ ), 1.73 (m, 2H,  $\text{SCH}_2\text{CH}_2$ ), 1.40 (m, 2H,  $\text{SCH}_2\text{CH}_2\text{CH}_2$ ), 0.90 (t,  $J$  = 6.5 Hz, 3H,  $\text{CH}_3$ ) ppm;  $^{31}\text{P}$  NMR  $\delta$ :  $-10.1$  (d),  $-9.9$  (d),  $-0.2$  (s) ppm; MS TOF ES $^+$ : 593 ( $\text{M}^+\text{H}$ ); HRMS: calculated for  $\text{C}_{14}\text{H}_{21}\text{N}_6\text{O}_{12}\text{P}_3\text{S}$ , 593.0173; found, 593.0163.

#### 8-Butylthio-2',3'-isopropylidene – adenosine (**13**)

Tosylic acid (*p*-TsOH) (285 mg, 1.5 mmol) was added to dry 8-butylthioadenosine (**12**) (Halbfinger *et al.*, 1999) (266.5 mg, 0.75 mmol) in a two-neck flask under Ar, followed by the addition of dry DMF (4 mL). 2,2-Dimethoxypropane (2.7 mL) was then added and the clear solution was stirred for 24 h at RT. Volatiles were evaporated and the residue was coevaporated at  $40^\circ\text{C}$  with EtOH. After separation on a silica gel column and upon elution with 30% ethyl acetate in ether, product **13** was obtained as a white solid in a 46% yield (100 mg).  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 200 MHz)  $\delta$ : 8.35 (s, 1H, H2), 6.22 (d,  $J$  = 4 Hz, 1H, H1'), 5.35 (t,  $J$  = 5 Hz, 1H, H2'), 5.14 (m, 1H, H3'), 4.32 (m, 1H, H4'), 3.90–3.62 (m, 2H, H5', H5''), 3.42–3.20 (m, 2H,  $\text{SCH}_2$ ), 2.93 (s, 3H,  $\text{CH}_3\text{C}$ ), 2.80 (s, 3H,  $\text{CH}_3\text{C}$ ), 1.80–1.22 (m, 4H,  $\text{SCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ ), 0.91 (t,  $J$  = 6 Hz, 3H,  $\text{CH}_3$ ) ppm; MS TOF ES $^+$ : 395 ( $\text{M}^+\text{H}$ ). HRMS: calculated for  $\text{C}_{17}\text{H}_{25}\text{N}_5\text{O}_4\text{S}$ : 395.1627; found: 395.1635.

#### 8-Butylthio-2',3'-isopropylidene – adenosine- $\beta$ , $\gamma$ -methylene 5'-triphosphate (**14**)

8-Butylthio-2',3'-isopropylidene-adenosine (**13**) (100 mg, 0.25 mmol) was dissolved in dry trimethyl phosphate (1.5 mL), cooled to  $-15^\circ\text{C}$ , and proton sponge was added (178.6 mg, 0.83 mmol, 3.3 eq). After stirring for 20 min,  $\text{POCl}_3$  (27.4  $\mu\text{L}$ , 0.3 mmol, 1.2 eq) was added dropwise and the reaction was stirred for 2 h at  $-15^\circ\text{C}$ . A 1 M solution of the bis(tributyl)ammonium salt of methylene diphosphonic acid in DMF (1.7 mL, 1.7 mmol, 6.8 eq) and  $\text{Bu}_3\text{N}$  (170  $\mu\text{L}$ , 0.7 mmol, 2.8 eq) were added at once. After stirring for 6 min at  $-15^\circ\text{C}$ , 1 M TEAB (5 mL) was added and the clear solution was stirred for 45 min at RT. The latter was freeze-dried overnight. The semisolid obtained then purified by chromatography on an activated DEAE Sephadex A-25 column (diameter 1.6 cm and length 15 cm) using a two-step gradient of  $\text{NH}_4\text{HCO}_3$  (400 mL for a 0–0.2 M gradient and then 700 mL for a 0.2–0.4 M gradient). The relevant fractions were freeze-dried repeatedly to generate the product **14** as a white solid in a 60% yield. This residue was used without further purification.  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 200 MHz)  $\delta$ : 8.40 (s, 1H, H2), 6.20 (d,  $J$  = 7 Hz, 1H, H1'), 5.61 (t,  $J$  = 8.5 Hz, 1H, H2'), 5.25 (m, 1H, H3'), 4.20 (m, 1H, H4'), 3.40–3.20 (m, 2H, H5', H5''), 2.4 (t,  $J$  = 14 Hz, 2H,  $\text{SCH}_2$ ), 1.90–1.40 (m, 10H,  $\text{SCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$ ,  $\text{CH}_3\text{C}$ ,  $\text{CH}_3\text{C}$ ), 1.0 (t,  $J$  = 7.5 Hz, 3H,  $\text{CH}_3$ ) ppm;  $^{31}\text{P}$  NMR ( $\text{D}_2\text{O}$ , pH 8.5)  $\delta$ : 18.0 (m), 10.0 (m),  $-10.1$  (m) ppm; MS TOF ES $^+$ : 629 ( $\text{M}^+\text{H}$ ).

#### 8-Butylthio – 5'- $\beta$ , $\gamma$ -methylene-adenosine 5'-triphosphate (**9**)

8-Butylthio-2',3'-isopropylidene- $\beta$ , $\gamma$ -methylene-triphosphate (**14**) (42 mg, 0.66 mmol) was dissolved in 2 mL of trifluoroacetic acid (TFA). The reaction was stirred at RT for 10 min. The volatiles were evaporated. The semisolid obtained was purified by chromatography on an activated DEAE Sephadex A-25 column using a two-step gradient of  $\text{NH}_4\text{HCO}_3$  (700 mL for a 0–0.15 M gradient followed by 700 mL for a 0.15–0.3 M

gradient). The relevant fractions were freeze-dried repeatedly to yield the product **9** as a white solid in a 70% yield (27.3 mg). Final separation was achieved on HPLC applying a linear gradient of TEAA/CH<sub>3</sub>CN 86:14 to 78:22 in 25 min (flow rate, 6 mL min<sup>-1</sup>),  $t_R$  = 12.4 min (99.4% purity). Purity was also evaluated using a linear gradient of TEAA/CH<sub>3</sub>CN (80:20 to 55:45 in 15 min), with a  $t_R$  = 4.3 min (98% purity) using a linear gradient of phosphate buffer/CH<sub>3</sub>CN (90:10 to 80:20; flow rate, 1 mL min<sup>-1</sup>). <sup>1</sup>H NMR (D<sub>2</sub>O, 300 MHz)  $\delta$ : 8.22 (s, 1H, H2), 6.15 (d,  $J$  = 6.5 Hz, 1H, H1'), 5.23 (t,  $J$  = 6.5 Hz, 1H, H2'), 6.13 (m, 1H, H3'), 4.32 (m, 3H, H4', H5', H5''), 2.26 (m, 2H, SCH<sub>2</sub>), 1.74 (m, 2H, SCH<sub>2</sub>CH<sub>2</sub>), 1.50 (m, 2H, CH<sub>2</sub>CH<sub>3</sub>), 0.90 (t,  $J$  = 7 Hz, 3H, CH<sub>3</sub>) ppm; <sup>31</sup>P NMR (D<sub>2</sub>O, 200 MHz, pH 8.5)  $\delta$ : 15.0 (d), 9.0 (d), -10.1 (d) ppm; MS TOF ES<sup>+</sup>: 590 (M<sup>+</sup>H<sup>+</sup>); HRMS calcd. for C<sub>15</sub>H<sub>22</sub>N<sub>5</sub>O<sub>12</sub>P<sub>3</sub>S, 589.0199; found, 589.0220.

8-Butylthio – adenosine 5'-triphosphate (**1**),  
8-butylthio – adenosine 5'-diphosphate (**10**),  
8-butylthio – adenosine 5'-monophosphate (**11**)

Compounds **1** and **12** were synthesized according to our previously published procedure (Halbfinger *et al.*, 1999). Compound **10** was a by-product of the triphosphorylation of 8-BuS-adenosine (**12**) and finally, compound **11** was obtained as a result of hydrolysis upon prolonged incubation in water solution at RT of 8-BuS-ATP, **1** (Figure 2).

## Statistical analysis

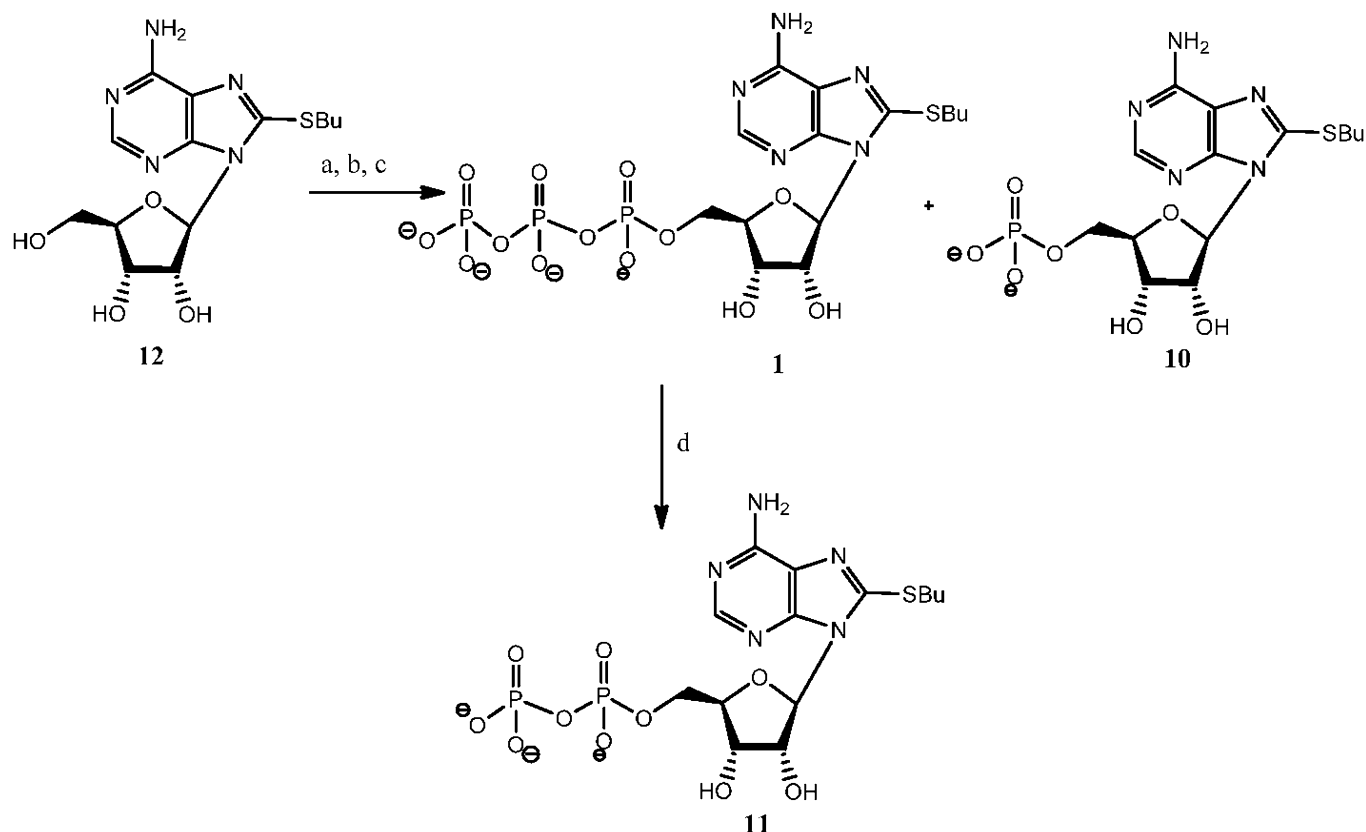
Statistical analysis was done using Student's *t*-test. *P*-values below 0.05 were considered statistically significant.

## Results

### Rational design of potential NTPDase inhibitors

To study the structure-activity relationships of ATP analogues towards NTPDases and identify more potent and enzyme subtype-selective inhibitors we synthesized and evaluated a series of adenine nucleotide derivatives, analogues **8–11**. Compound **1**, 8-BuS-ATP, (Gendron *et al.*, 2000) was reported previously as a potent NTPDase inhibitor. As we show in Table 1 the  $\beta$ ,  $\gamma$ -phosphoester bond in **1** is susceptible to hydrolysis by NTPDases. Indeed NTPDase2, -3 and -8 hydrolyzed this compound and ATP with comparable efficacy (Table 1). To prevent hydrolysis of the terminal,  $\beta$ ,  $\gamma$ -phosphoester bond, we introduced an imido (**8**) or a methylene (**9**) group in the phosphate chain of 8-BuS-ATP. We also generated derivatives with a shorter phosphate chain, namely 8-BuS-AMP (**10**) and 8-BuS-ADP (**11**).

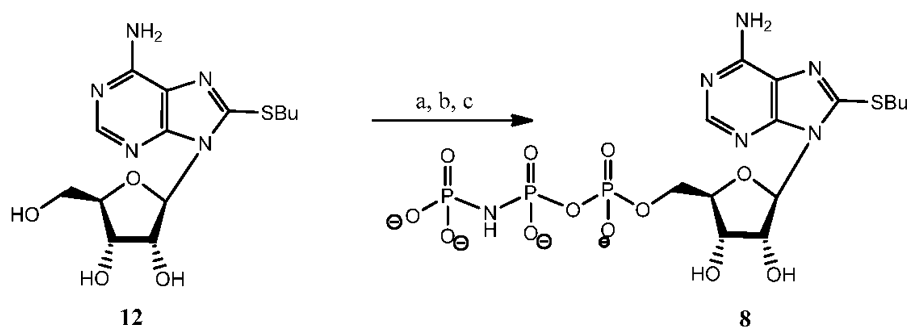
The 8-BuS- $\beta$ , $\gamma$ -imido-adenosine-5'-triphosphate analogue, **8**, was prepared using imidodiphosphate instead of



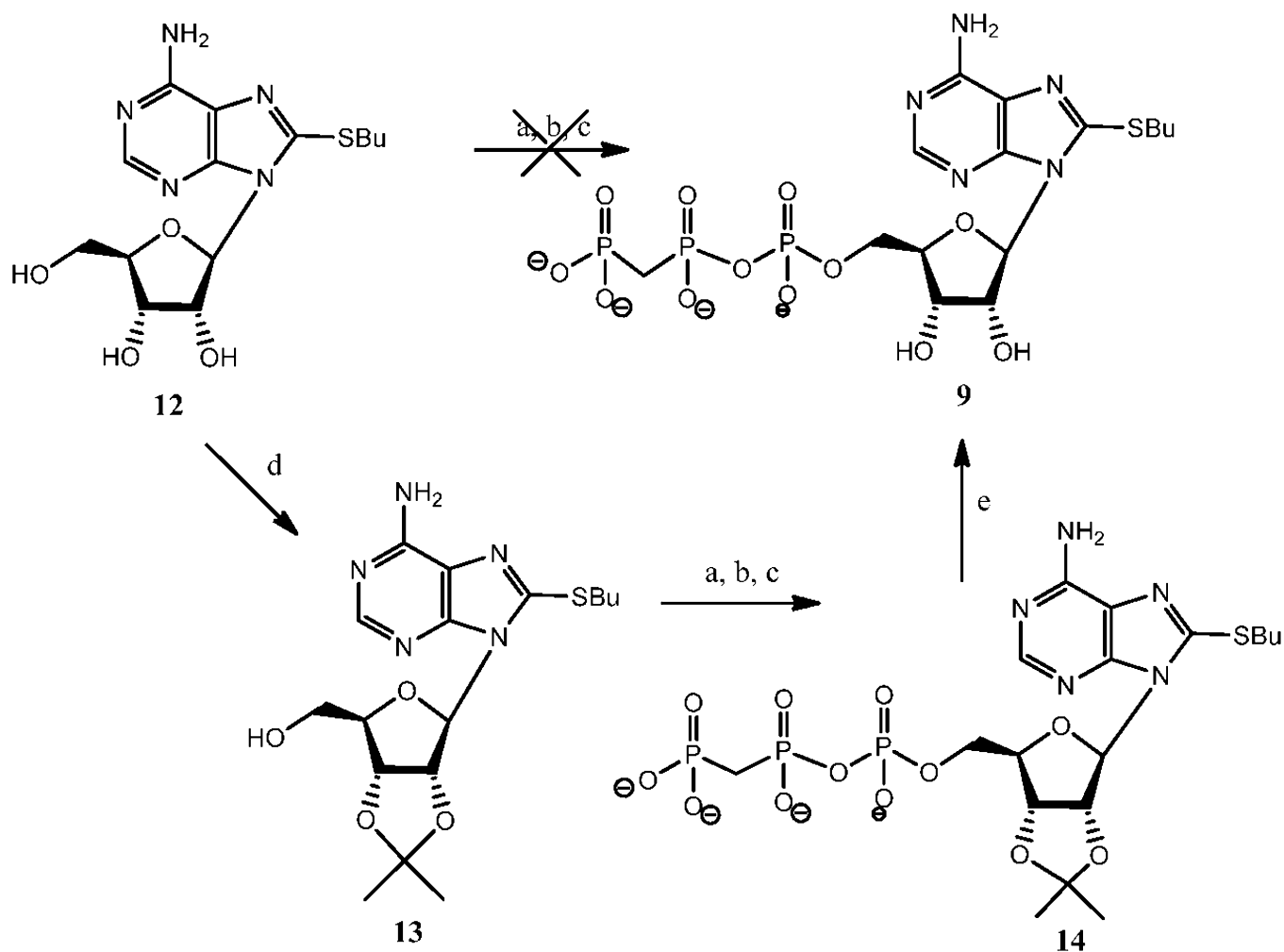
**Figure 2**

Synthesis of 8-BuS-AMP **10** and 8-BuS-ADP, **11**. <sup>a</sup> Reagents and conditions: a, POCl<sub>3</sub>, proton sponge, -15°C, 2 h; b, [Bu<sub>3</sub>NH<sup>+</sup>]<sub>2</sub>[(P<sub>2</sub>O<sub>5</sub>)<sub>2</sub>]<sup>2-</sup>/NBu<sub>3</sub>, -10°C, 6 min; c, 1M TEAB, RT, 45 min, 50% overall yield for **1** and 35% for **10**; d, H<sub>2</sub>O, 10 d, RT.



**Figure 3**

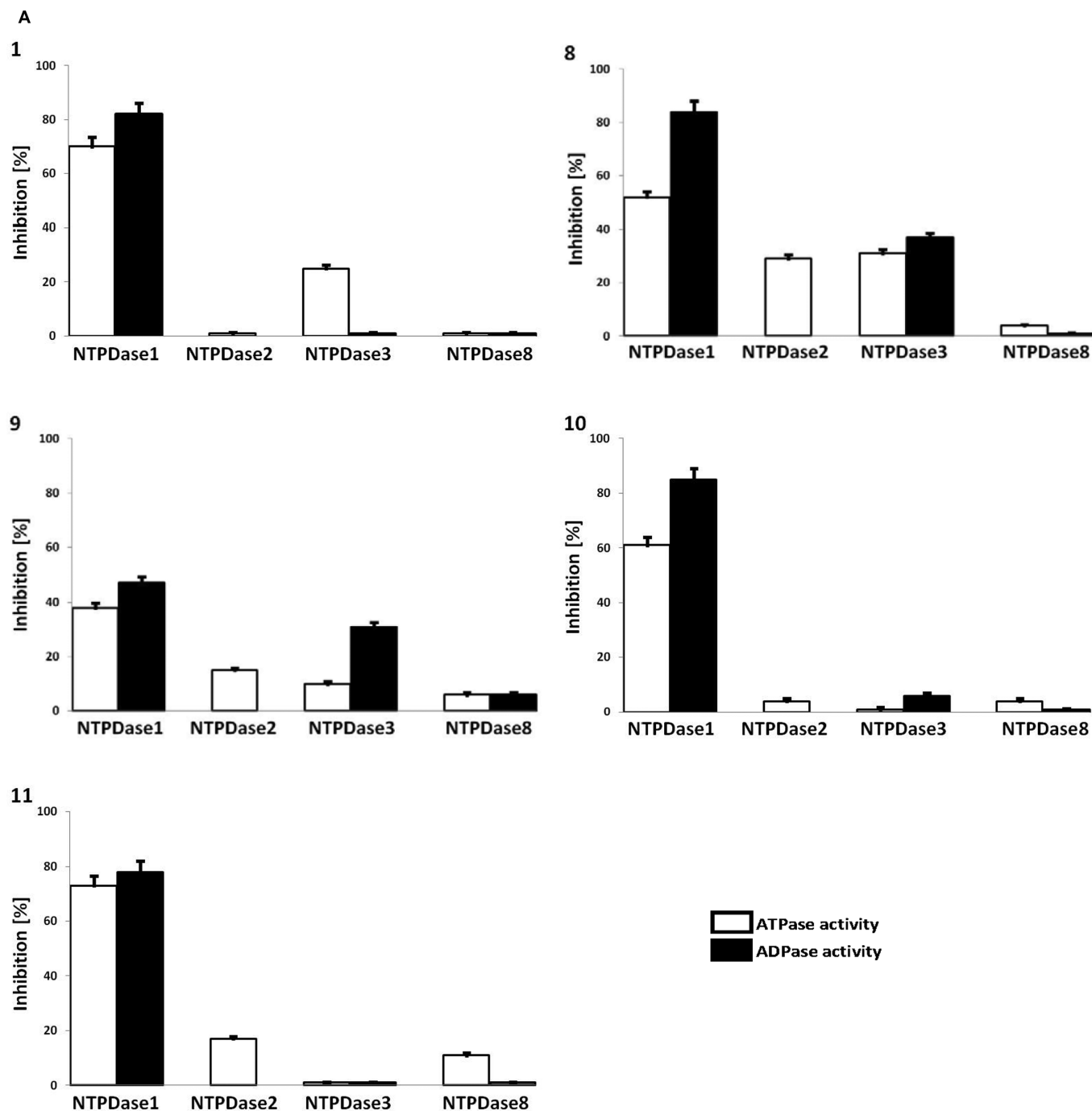
Synthesis of 8-BuS-AMP-PNP, **8**. <sup>a</sup> Reagents and conditions: a, POCl<sub>3</sub>, proton sponge, -15°C, 2 h; b, [Bu<sub>3</sub>NH<sup>+</sup>]<sub>2</sub>[NH(PO<sub>3</sub>)<sub>2</sub>]<sup>2-</sup>/NBu<sub>3</sub>, -10°C, 6 min; c, 1M TEAB, RT, 45 min, overall yield 35%.

**Figure 4**

Synthesis of 8-BuS-AMP-PCP, **9**. <sup>a</sup> Reagents and conditions: a, POCl<sub>3</sub>, proton sponge, -15°C, 2 h; b, [Bu<sub>3</sub>NH<sup>+</sup>]<sub>2</sub>[CH<sub>2</sub> PO<sub>3</sub>]<sub>2</sub><sup>2-</sup>/NBu<sub>3</sub>, -10°C, 6 min; c, 1M TEAB, RT, 45 min; d, 2,2-dimethoxypropane, *p*-TsOH, DMF, RT, 24 h; e, TFA, RT, 10 min, 60% overall yield.

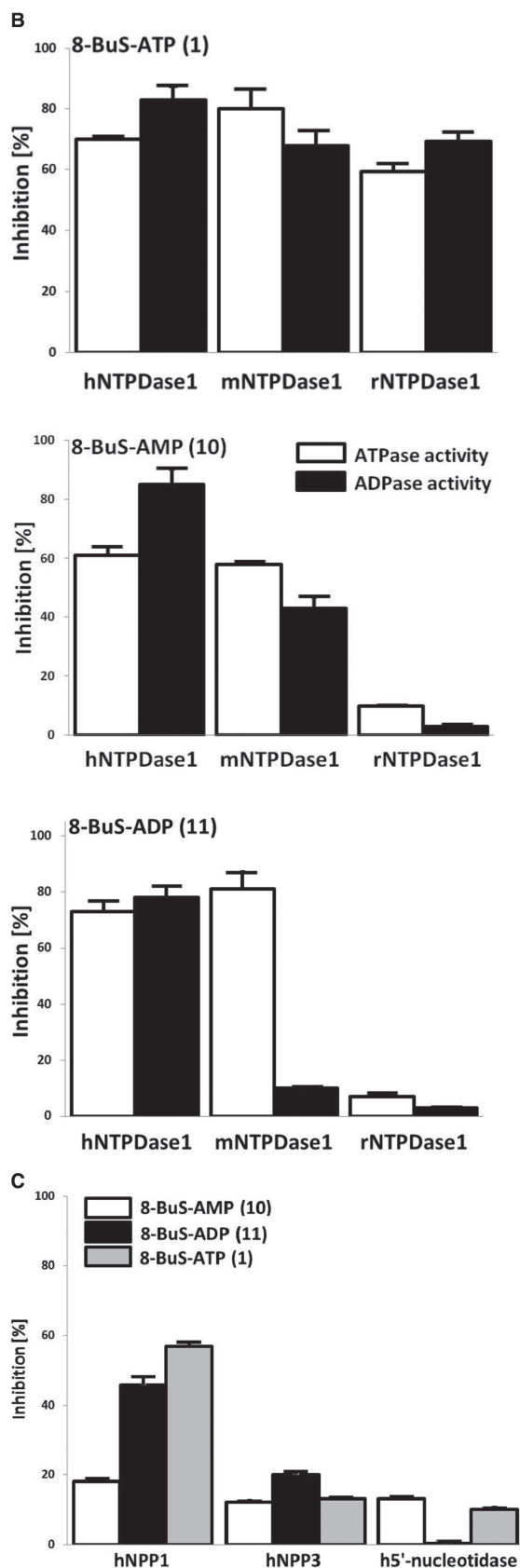
pyrophosphate in the second step of the Ludwig's 5'-triphosphorylation reaction (Figure 3) (Ludwig, 1981). Our attempt to synthesize **9** directly under 5'-triphosphorylation conditions using methylene diphosphonic acid instead of

pyrophosphate resulted in a complex mixture of by-products, making the isolation of the desired product nearly impossible. Thus, we implemented a different strategy (Figure 4): we first protected 2',3'-hydroxyl groups with an isopropylidene



**Figure 5**

Influence of adenine nucleotide analogues **1** and **8–11** on recombinant ectonucleotidase activities. Enzymatic assays were carried out with protein extracts from COS-7 cells transfected with an expression vector encoding the respective enzyme. The substrate (ATP, ADP, AMP or pnp-TMP) was added together with each BuS derivative, both at 100  $\mu$ M. The 8-BuS derivatives are indicated in the *top left* of each panel or in the legend. (A) The activity (without inhibitor) with the substrate ATP corresponded to  $670 \pm 35$ ,  $928 \pm 55$ ,  $202 \pm 37$ , and  $129 \pm 11$  nmol  $P_i \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$  for human NTPDase1, –2, –3 and –8, respectively, and with ADP to  $530 \pm 22$ ,  $110 \pm 10$ , and  $30 \pm 5$  nmol  $P_i \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$  for NTPDase1, –3 and –8, respectively. Data are presented as the mean  $\pm$  SD of 3 experiments carried out in triplicate. (B) Comparative effect of 8-BuS-ATP (**1**; top panel), 8-BuS-AMP (**10**; middle panel) and 8-BuS-ADP (**11**; lower panel) on the ATPase and ADPase activities of human, mouse and rat NTPDase1. Relative activities are expressed as the mean  $\pm$  SD of 3 independent experiments, each performed in triplicate. The activity with ATP without inhibitors was  $670 \pm 35$ ,  $990 \pm 39$  and  $750 \pm 32$  nmol  $P_i \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$  for human, mouse and rat NTPDase1, respectively, and with ADP,  $530 \pm 22$ ,  $878 \pm 35$  and  $690 \pm 25$  nmol  $P_i \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$  for human, mouse and rat NTPDase1, respectively. (C) Compounds **10** and **11** modestly affect NPP and ecto-5'-nucleotidase activities. The activity without inhibitors with pnp-TMP as substrate were  $30 \pm 2$  and  $61 \pm 4$  nmol p-nitrophenol  $\text{min}^{-1} \cdot \text{mg protein}^{-1}$  for NPP1 and NPP3, respectively. The activity without inhibitors of ecto-5'-nucleotidase was  $1.7 \pm 0.1$   $\mu$ moles  $P_i \text{ min}^{-1} \cdot \text{mg protein}^{-1}$ . Data are presented as the mean  $\pm$  SD of 3 experiments carried out in triplicate.

**Figure 5**

Continued

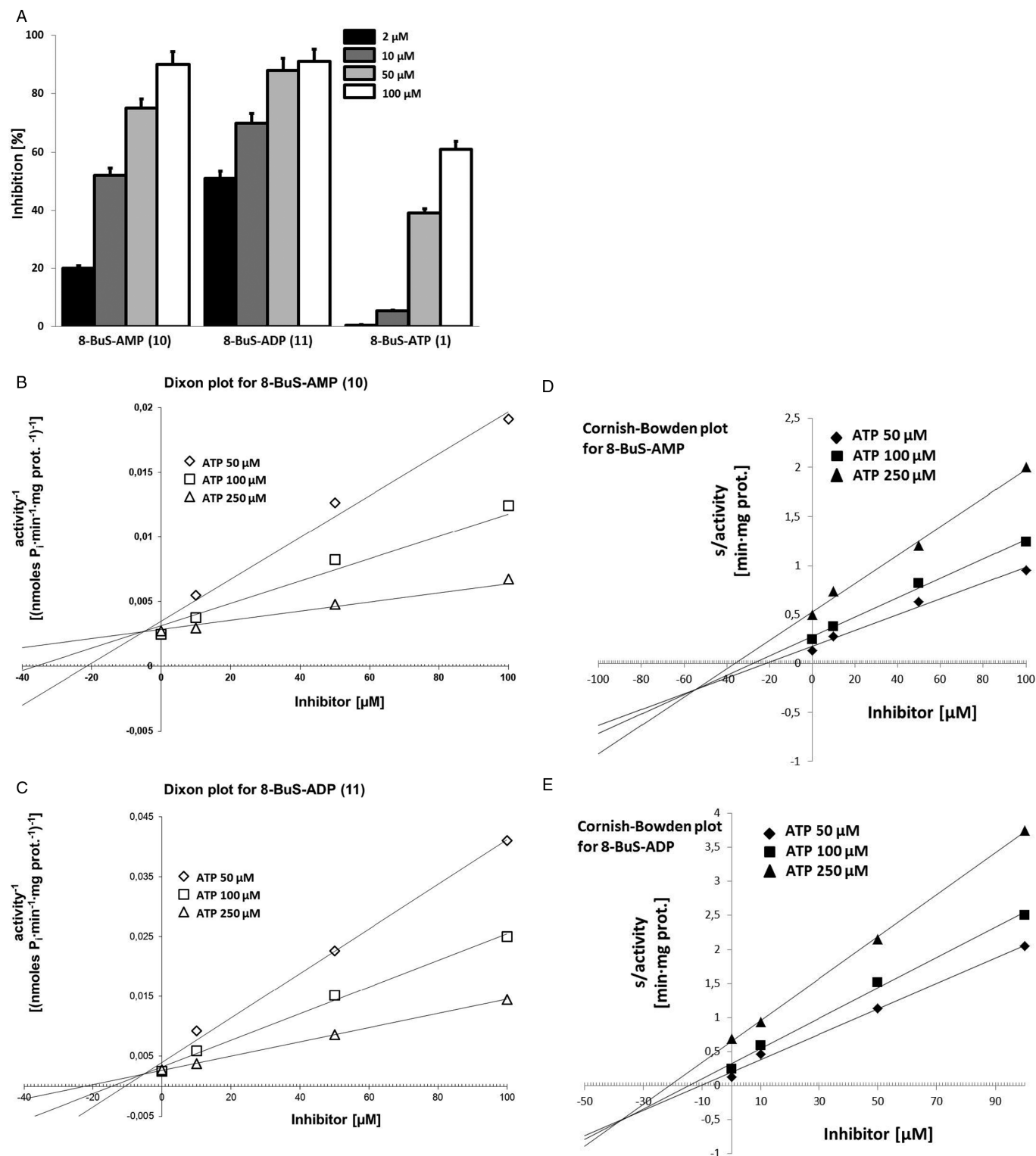
protective group, and then applied our triphosphorylation procedure (Gillerman and Fischer, 2010) with methylene diphosphonic acid. Compound **13**, 8-butylthio-2',3'-isopropylidene-adenosine, was obtained by treatment of 8-BuS-adenosine (**12**) (Halbfinger *et al.*, 1999) with 2,2-dimethoxypropane in DMF in the presence of *p*-toluene sulphonic acid for 24 h at RT. In the next step, monophosphorylation with POCl<sub>3</sub> at -15°C for 2 h and subsequent triphosphorylation using tetrabutylammonium methylene diphosphonic acid resulted in product **14**. Thus, product **9** was obtained in a 60% yield, after deprotection of hydroxyl groups with TFA for 10 min at RT and liquid chromatographic separation.

### *Influence of analogues 8–11 on ectonucleotidase activity*

The low intrinsic nucleotidase activity found in control COS-7 cell extracts allowed the analysis of each ectonucleotidase in its native membrane-bound form. All tested compounds, substrates and inhibitors were tested at equimolar concentrations (100 µM) unless otherwise indicated.

First, we evaluated the stability of the analogues towards hydrolysis. While 8-BuS-ATP (**1**) was hydrolyzed quite well by human NTPDase2, -3 and -8 (Table 1), 8-BuS-β,γ-methylene-ATP (**9**) 8-BuS-AMP (**10**) and 8-BuS-ADP (**11**) were stable to hydrolysis by human NTPDase1-3 and -8. Analogue **8** was also resistant to hydrolysis by NTPDase1, -2 and -3 but could be modestly hydrolysed by NTPDase8 (Table 1).

NTPDase1 ATPase activity was inhibited weakly (38–52%) and non-selectively by 8-BuS-ATP derivatives **8** and **9**, whereas **1**, **10** and **11** were strong inhibitors of NTPDase1 ATPase activity in an exclusive fashion (70, 61 and 73%, respectively) (Figure 5A). The ADPase activity of NTPDase1 was inhibited by these analogues in a manner similar to ATPase activity (Figure 5A). Increasing the pre-incubation time of **1** and **11** with human NTPDase1 for 2, 10 and 20 min did not improve the inhibition (data not shown) suggesting that this effect was rapid. The inhibition of NTPDase1 by **1**, **10** and **11** was effective for human and mouse but not for rat NTPDase1 (Figure 5B). These compounds inhibited mouse NTPDase1 activity, with one exception, mouse NTPDase1 ADPase activity was not affected by **11** (Figure 5B). While molecule **11** was specific to mouse NTPDase1, as it did not affect the ATPase activity of mouse NTPDase2, -3 and -8, the activity of the later NTPDases were modestly inhibited by **1** and **10** (data not shown). As analogues **1**, **10** and **11** significantly and specifically reduced the activity of human NTPDase1, we tested the kinetic parameters (*K<sub>i</sub>* and *IC<sub>50</sub>*) of inhibition with ATP as substrate. The *K<sub>i</sub>*s (evaluated by Dixon plot for **10** and **11** (Figure 6), and Cheng-Prusoff equation for **1**) were similar for these three analogues, which were in the order of 0.8–0.9 µM, while the *IC<sub>50</sub>* values were in the 6–35 µM range, with the lowest value evaluated for 8-BuS-ATP (Table 2). The type of inhibition was evaluated for the best 2 compounds (**10** and **11**). Using both methods, Dixon (Figure 6B,C) and Cornish-Bowden (Figure 6D,E), we determined that our inhibitors present mixed type inhibition, predominantly competitive (Dixon, 1953; Cornish-Bowden, 1974).



**Figure 6**

Concentration dependent inhibition (A) and  $K_i$  determination (B) of ATPase activity of human NTPDase1 by 8-BuS-AMP (**10**), 8-BuS-ADP (**11**) and 8-BuS-ATP (**1**). (A) The activity with 100  $\mu\text{M}$  ATP without inhibitors was  $650 \pm 33 \text{ nmol P}_i \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ .  $K_i$  determination for (B, D) 8-BuS-AMP (**10**) and (C, E) 8-BuS-ADP (**11**) by Dixon (B, C) and Cornish-Bowden (D, E) plot. ATP concentration range was 50, 100 and 250  $\mu\text{M}$ , and the inhibitor's range was 0, 10, 50 and 100  $\mu\text{M}$ . The data of one representative experiment out of 3 is shown.



**Table 1**

Stability of analogues **1** and **8–11** to hydrolysis by human NTPDases

Compound	NTPDase1	NTPDase2	NTPDase3	NTPDase8
<b>1</b>	5 ± 3	55 ± 13	36 ± 4	64 ± 18
<b>8</b>	2.0 ± 0.1	1.0 ± 0.1	3.0 ± 0.1	14 ± 1
<b>9</b>	1.0 ± 0.1	ND	2.0 ± 0.1	4.0 ± 0.2
<b>10</b>	ND	ND	ND	2.0 ± 0.1
<b>11</b>	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	3.0 ± 0.1

Each analogue was tested as a substrate for each NTPDase and was compared with the activity obtained with ATP which was set as 100% and which corresponds to  $670 \pm 35$ ,  $928 \pm 55$ ,  $202 \pm 37$ , and  $129 \pm 11$  nmol  $P_i \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$  for NTPDase1, -2, -3 and -8, respectively. Results are expressed in %  $\pm$ SD of three independent experiments, each performed in duplicate. ND, not detected.

**Table 2**

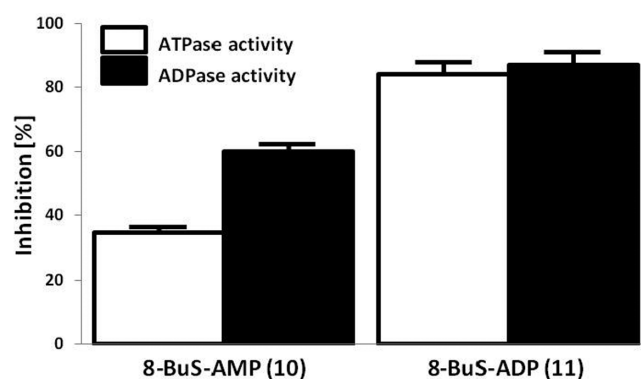
Kinetic parameters of 8-BuS-adenine nucleotide derivatives for human NTPDase1 inhibition

	8-BuS-ATP ( <b>1</b> )	8-BuS-AMP ( <b>10</b> )	8-BuS-ADP ( <b>11</b> )
$I_{C_{50}}$ [ $\mu\text{M}$ ]	$5.6 \pm 1.2$	$35 \pm 2$	$28 \pm 2$
$K_i$ [ $\mu\text{M}$ ]	$0.8 \pm 0.2$	$0.8 \pm 0.1$	$0.9 \pm 0.2$

Results are expressed as [ $\mu\text{M}$ ]  $\pm$  SEM of three independent experiments, each performed in duplicate.

We then tested the influence of the two most stable and potent inhibitors of NTPDase1, 8-BuS-AMP (**10**) and 8-BuS-ADP (**11**) on the activity of other ectonucleotidases, namely NPPs and ecto-5'-nucleotidase. Compound **11**, 8-BuS-ADP, inhibited weakly (<50%) the activity of human NPP1 as measured with pnp-TMP (Figure 5C). These derivatives did not, or only modestly, inhibit human ecto-5'-nucleotidase (Figure 5C).

**8-BuS derivatives inhibit NTPDase1 activity in tissues and cells.** Endothelial cells express high levels of NTPDase1. Both analogues **10** and **11** inhibited the ATPase and ADPase activity of HUVEC cells, with 8-BuS-ADP reducing this activity by >80% (Figure 7) and 8-BuS-AMP by 35 and 60%, respectively (Figure 7). These results were confirmed by cytochemistry with HUVECs (Figure 8A). Analogues **10** and **11** also inhibited the ATPase activity of NTPDase1 *in situ* in human pancreas and liver (Figure 8B,C) which was mainly found in blood vessels and capillaries/sinusoids. Importantly, analogue **10** showed strong specificity in histochemistry assays, exclusively inhibiting NTPDase1 activity in liver and pancreas tissues (Figure 8B,C). In mouse liver, pancreas and kidney analogues **1** and **11** also inhibited ATPase activity of NTPDase1 that is exhibited in vascular elements, arteries, veins and capillaries/sinusoids (Figure 8D,E and data not shown). The ATPase activity due to NTPDase3 (Vekaria *et al.*, 2006) and/or NTPDase8 (Sévigny *et al.*, 2000) in kidney tubules remained (Figure 8E). This experiment showed the specificity of the inhibition by these molecules.

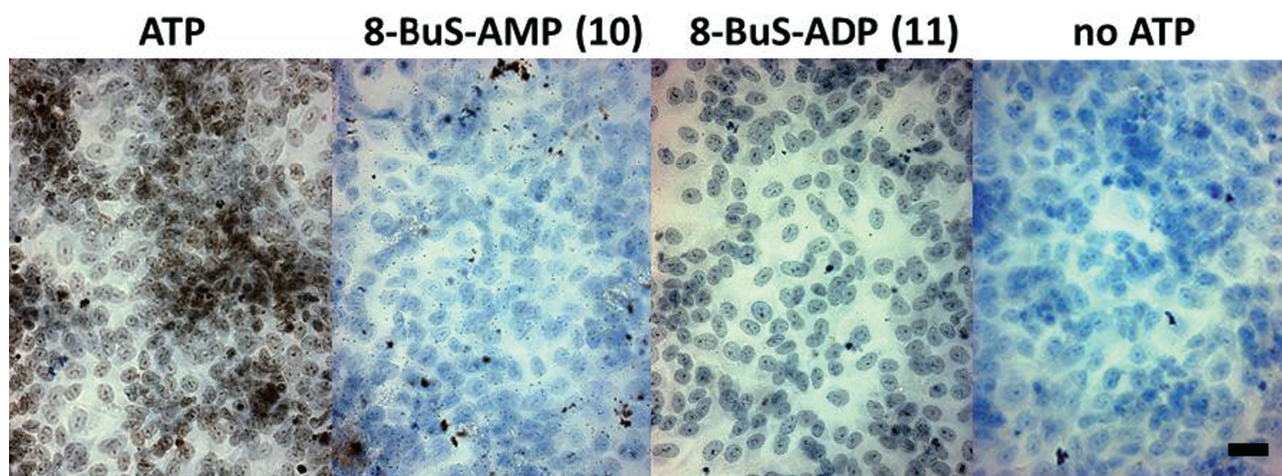

**Figure 7**

Compounds **10** and **11** inhibit the ectonucleotidase activity of intact HUVECs. Hydrolysis of 100  $\mu\text{M}$  ATP (open bars) and 100  $\mu\text{M}$  ADP (solid bars) was evaluated in the presence of 100  $\mu\text{M}$  8-BuS-AMP (**10**) or 8-BuS-ADP (**11**). The activity without inhibitors were  $2.25 \pm 0.01$  and  $3.31 \pm 0.02$  nmol  $P_i \cdot \text{min}^{-1} \cdot \text{well}^{-1}$  for ATP and ADP respectively. Activities are expressed as the mean  $\pm$  SD of 3 independent experiments with confluent cells from different donors at passage 2, each performed in triplicate, mean cell number in one well was in the order of 250 000.

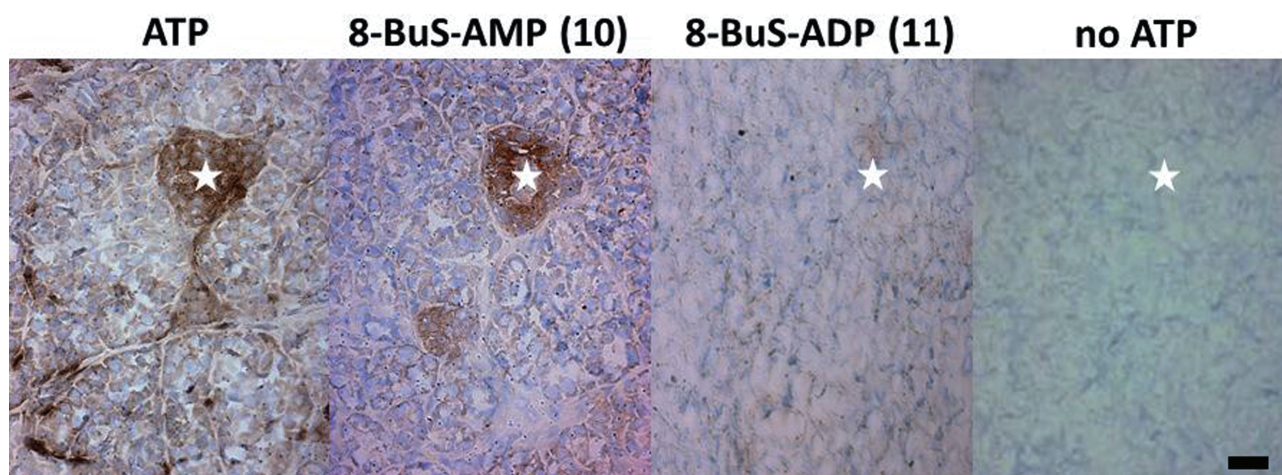
**8-BuS-AMP and 8-BuS-ADP inhibit NTPDase1 function in platelet aggregation.** We next estimated how the inhibition of NTPDase1 by 8-BuS-adenine nucleotide affects platelet aggregation using the recombinant enzyme. As expected, the inhibition of recombinant NTPDase1 by either **10** or **11** resulted in an impaired ability of this enzyme to prevent platelet aggregation (Figure 9A,B). These analogues did not *per se* initiate platelet aggregation (Figure 9A,B and data not shown), despite the fact that 8-BuS-ADP is an analogue of ADP that could in principle have activated P2Y<sub>1</sub> and P2Y<sub>12</sub> in platelets (Gachet, 2012).

Light microscopy further confirmed the above observation with a more systematic quantification of these effects. This method showed that in the presence of **10** or **11**, human NTPDase1 could not prevent the formation of platelet aggregates initiated by ADP (Figure 9C) and that the number of non-aggregated platelets in the mixture containing human

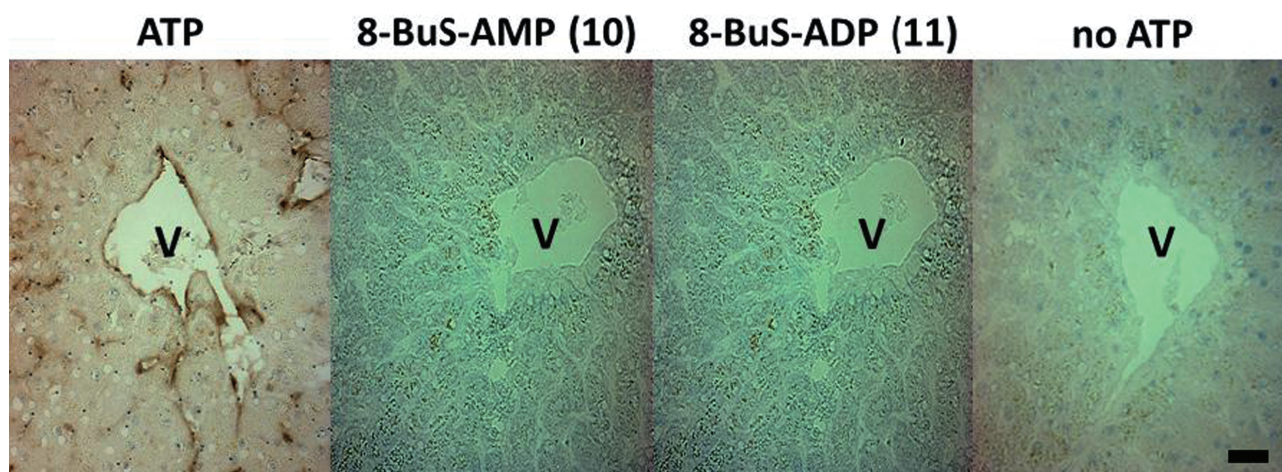
## A HUVEC



## B Human pancreas

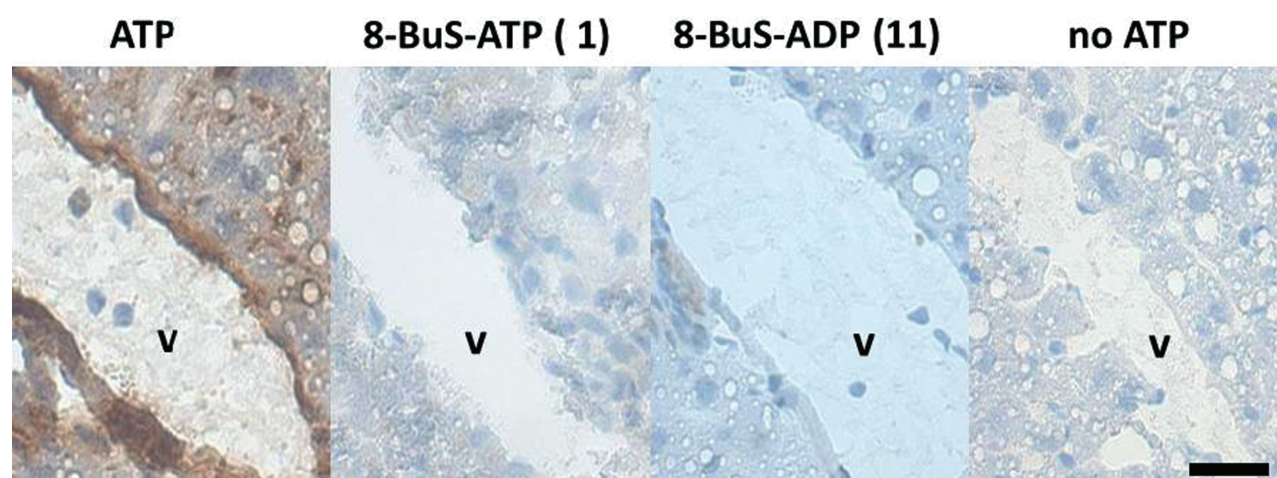


## C Human liver

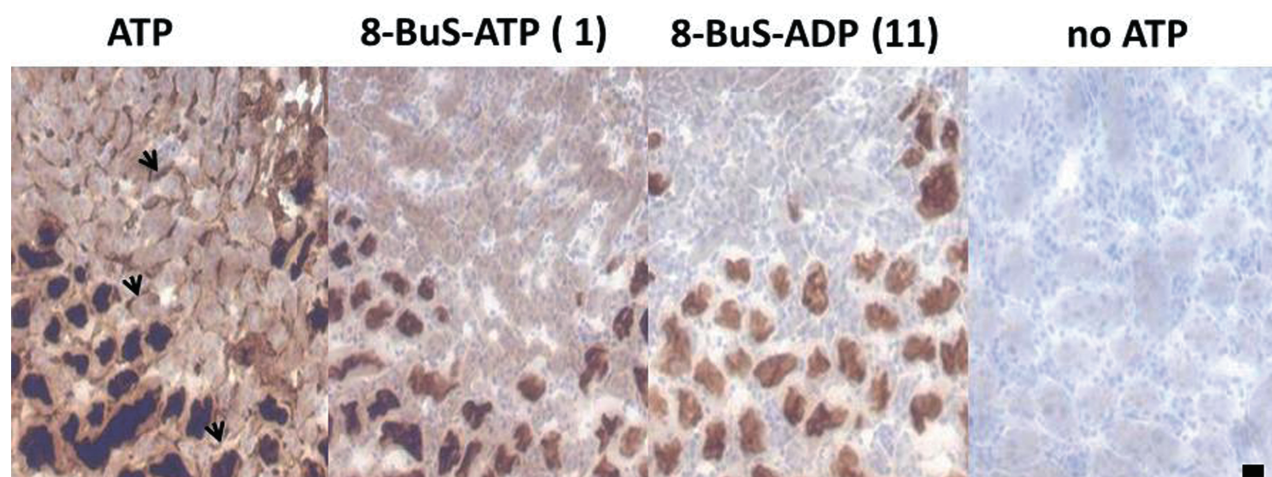




## D Mouse liver



## E Mouse kidney



### Figure 8

Inhibition of ATPase activity in human and mouse cells and tissues by compounds **1**, **10** and **11** using enzyme histochemistry. Substrate, ATP, was used at a final concentration of 200 and 500  $\mu$ M for human and mouse, respectively, and inhibitors at 100 and 500  $\mu$ M for human and mouse, respectively. (A) ATPase activity is present at the surface of HUVEC due to NTPDase1. (B) In pancreas, ATPase activity is seen as a brown precipitate in Langerhans islets cells due to NTPDase3, and in blood vessels, in the luminal membrane of acinar cells and in zymogen granules due to NTPDase1. (C) In the liver (D for mouse), ATPase activity due to NTPDase1 is present in vascular endothelium, central vein and sinusoids, as well as in K  pffer cells. Inhibition of ATPase activity in mouse liver (D) and kidney (E) by compounds **1** and **11** using enzyme histochemistry. ATPase activity due to NTPDase1 is seen as a brown precipitate in vascular elements, veins and capillaries, which is inhibited by **1** and **11**. In contrast, the activity due to NTPDase3 and/or NTPDase8 in kidney tubules remains. Serial sections were used for all tissues. Nuclei were counterstained with Haematoxylin. Scale bar = 50  $\mu$ m; \* = Langerhans islet; V = vein; black arrows = capillaries.

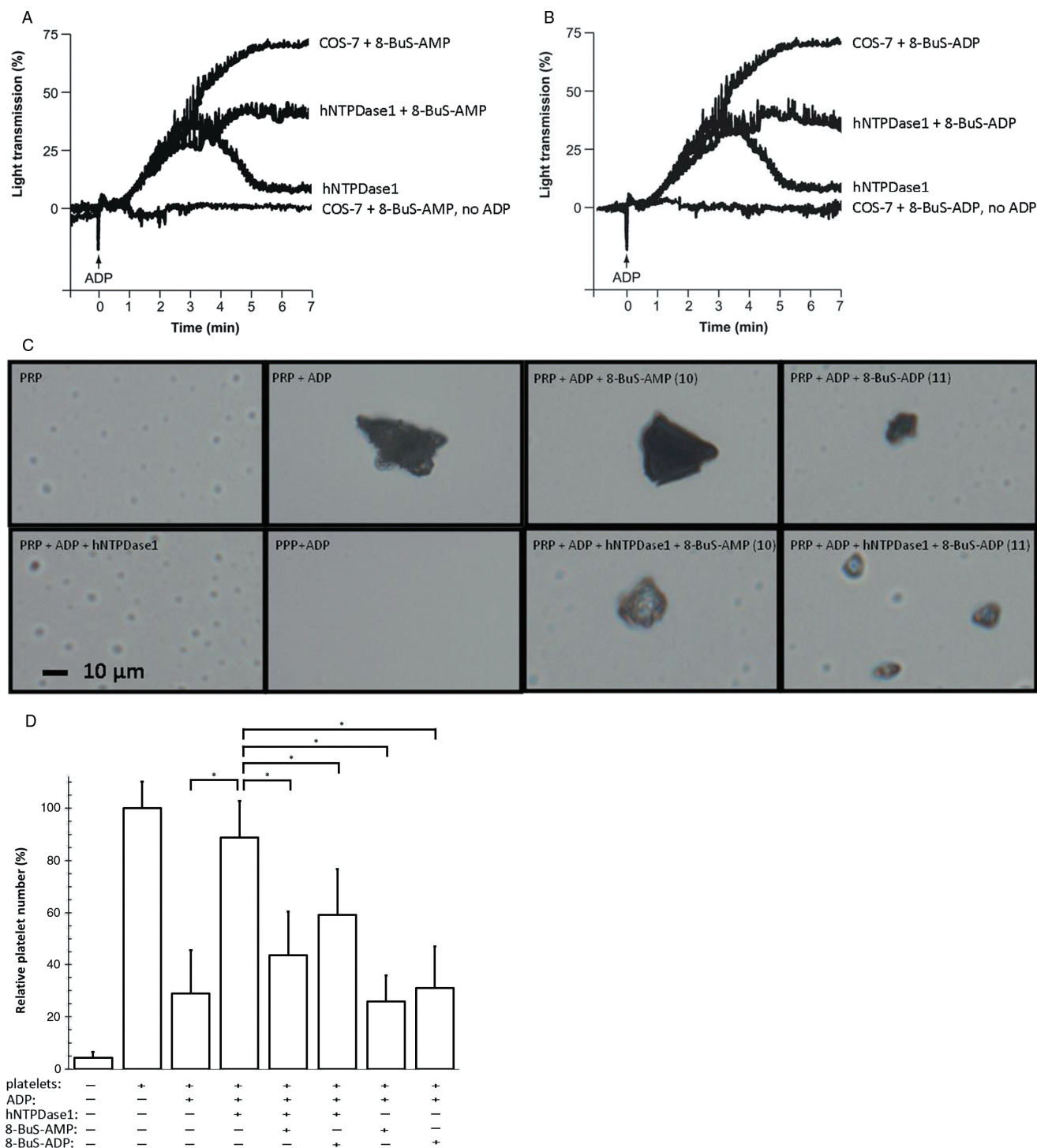
NTPDase1 was reduced to levels observed in absence of the enzyme (Figure 9D).

## Discussion

We previously reported 8-BuS-ATP (**1**) as the first inhibitor of bovine spleen NTPDase activity (Gendron *et al.*, 2000). In the current study, we show that 8-BuS-ATP inhibits NTPDase1

(Figure 5A). Unfortunately, our data also show that this molecule is a substrate for human NTPDase2, -3 and -8 (Table 1), thus limiting the use of 8-BuS-ATP as an NTPDase1 inhibitor in situations where other ectonucleotidase are present. Therefore, we have synthesized four non-hydrolyzable 8-BuS-ATP analogues, **8-11**, and tested their potential effect on NTPDase activity.

Two of the stable analogues, namely **8** and **9**, decreased the activity of NTPDase1 (Table 1). However, the substitution



**Figure 9**

Compounds **10** and **11** inhibit recombinant human NTPDase1 and its ability to block platelet aggregation induced by ADP. Human NTPDase1 was obtained from a protein extract from transfected COS-7 cells (6  $\mu$ g protein). Proteins from non-transfected COS-7 cells (6  $\mu$ g) were used as controls. The controls with protein extract from non-transfected COS-7, with and without ADP, were similar to the assays with inhibitors. They were omitted for clarity. Platelet aggregation in the presence or absence of the indicated protein extract and nucleotide analogue was initiated with 8  $\mu$ M ADP. (A) Platelet aggregation curves of one out of 3 representative experiments with 8-BuS-AMP are shown. (B) Platelet aggregation curves of one out of 3 representative experiments with 8-BuS-ADP are shown. (C) Qualitative assessment of the effect of 8-BuS-AMP (100  $\mu$ M) and 8-BuS-ADP (100  $\mu$ M) on platelet aggregation  $\pm$  hNTPDase1, by light microscopy (40 $\times$ ). The reaction was performed for 10 min. Pictures of one out of 3 representative experiments are shown. The control without platelets is represented by platelet poor plasma (PPP) and with non-aggregated platelets by platelet-rich plasma (PRP). (D) Number of non-aggregated platelets after 10 min of reaction  $\pm$  nucleotide analogues and hNTPDase1. The 100% value was set at  $3.15 \times 10^6$  platelets in 1 mL. The significant statistical differences are indicated as  $*P < 1 \cdot 10^{-3}$ .



of a  $p_s$ ,  $p_\delta$ -bridging atom oxygen with an imido group in **8** and by a methylene group in **9** led to a weak and non-specific inhibition (Figure 5A). The next three compounds [8-BuS-ATP (**1**), 8-BuS-AMP (**10**) and 8-BuS-ADP (**11**)] specifically inhibited the activity of NTPDase1, while **10** and **11** were stable to hydrolysis by NTPDases. Indeed, analogues **10** and **11** did not affect the activity of the other ectonucleotidases, namely NTPDase2, -3, -8 and CD73, and only modestly inhibited NPP1 and NPP3 (Figure 5). The inhibition of NTPDase1 by **10** and **11** was effective for the human and mouse enzymes, but not for rat NTPDase1 (Figure 5B). The  $K_i$  value of analogues **1**, **10** and **11** towards human NTPDase1 was estimated at 0.8, 0.8 and 0.9  $\mu$ M, respectively (Figure 6 and Table 2). The  $K_i$  for 8-BuS-ATP was about 10-fold lower than what we reported before for a particulate fraction from bovine spleen (Gendron *et al.*, 2000). Although NTPDase1 was likely the major NTPDase in this tissue, the relative contribution of other nucleotidase in that fraction was not known. Kinetic analysis, as performed using Dixon and Cornish-Bowden methods (Dixon, 1965; Cornish-Bowden, 1974), revealed that **1**, **10** and **11** display a mixed-type, predominantly competitive, inhibition towards recombinant human NTPDase1 (Table 2 and Figure 6B-E).

The kinetic parameters of the present series of NTPDase1 inhibitors are more interesting than those already reported for ARL 67156, a commercial NTPDase inhibitor (Iqbal *et al.*, 2005; Lévesque *et al.*, 2007) and POM-1 (Müller *et al.*, 2006; Yegutkin *et al.*, 2012) as both are less potent inhibitors of NTPDase1 than 8-BuS-derivatives. The  $K_{i,app}$  for ARL 67156 was reported to be  $11 \pm 5$   $\mu$ M and for POM-1 2.6  $\mu$ M. Both are also not specific inhibitors for NTPDase1 (Müller *et al.*, 2006; Lévesque *et al.*, 2007).

The most active NTPDase inhibitors described in the present study might reveal to be valuable tools for the treatment of certain cardiovascular and immunological disorders. Indeed, recent studies described adenosine and nucleotides as modulators of the development of cardiovascular and immunological complications (Hourani, 1996; Hechler *et al.*, 2005; Waehre *et al.*, 2006). In this study, we found that both derivatives **10** and **11** facilitate platelet aggregation by inhibiting NTPDase1 activity (Figure 9). In a perspective of drug development, such an ability might well be of key importance for patients with haemophilia A, which is associated with low levels of ATP and ADP in thrombocytes (Mareeva *et al.*, 1987). Increases in ADP levels may be also crucial for the therapy of several platelet dysfunctions such as those found in hereditary giant platelet syndrome (Walsh *et al.*, 1975), Noonan's syndrome (Flick *et al.*, 1991), hypothermia (Straub *et al.*, 2011) or thrombocytopenia (Oliveira *et al.*, 2011).

The data presented here suggest that both compounds **10** (especially) and **11** might be used as selective inhibitors of human and mouse NTPDase1 since they are devoid of activity towards other plasma membrane-bound ectonucleotidases (Figures 5, 7 and 8). It can be noted that all three compounds showed variable inhibition of NTPDases from different species as tested in human, mouse and rat (Figure 5B and data not shown). Species specificity has often been observed and this is why one should always be cautious about the molecule used and in which species. For example, other ectonucleotidase inhibitors such as ARL 67156 (Lévesque *et al.*, 2007) and

clopidogrel (J Lecka and J Sévigny, unpubl. obs.) show important species differences. The human NTPDases are more sensitive to P2 antagonists than the murine forms (Munkonda *et al.*, 2007) etc.

Inhibition by the BuS derivatives was efficient not only with recombinant NTPDase1, but also when the enzyme was expressed by vascular endothelial cells, as seen with intact HUVECs in culture as well as *in situ* with different organs from both human and mouse (Figures 5A,B, 7 and 8). Also of interest, neither **10** nor **11** *per se* triggered platelet aggregation nor prevented ADP-induced platelet aggregation, suggesting that they are inactive towards P2Y<sub>1</sub> and P2Y<sub>12</sub> receptors. These data are in keeping with the previous observation that compound **1** (8-BuS-ATP) did not affect P2 receptors (Gendron *et al.*, 2000).

In summary, analogues 8-BuS-AMP (**10**) and 8-BuS-ADP (**11**) may be used to specifically address the function of NTPDase1 or merely block its ectonucleotidase activity for various *in vitro*, and potentially, *in vivo* purposes. Through the specific blockade of NTPDase1, these novel inhibitors also open potential clinical avenues regarding platelet haemostasis, immune reactions and cancer. They may also serve as prototypical structures for the development of even more potent NTPDase1-specific inhibitors.

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## Conflict of interest

None.

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