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Inhibition of human and mouse plasma membrane bound NTPDases by P2 receptor antagonists

Mercedes N. Munkonda, Gilles Kauffenstein, Filip Kukulski, Sébastien A. Lévesque, Charlène Legendre, Julie Pelletier, Élise G. Lavoie, Joanna Lecka, Jean Sévigny *

Centre de Recherche en Rhumatologie et Immunologie, Centre Hospitalier Universitaire de Québec, Université Laval, Québec, QC, Canada

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ABSTRACT

The plasma membrane bound nucleoside triphosphate diphosphohydrolase (NTPDase)-1, 2, 3 and 8 are major ectonucleotidases that modulate P2 receptor signaling by controlling nucleotides' concentrations at the cell surface. In this report, we systematically evaluated the effect of the commonly used P2 receptor antagonists reactive blue 2, suramin, NF279, NF449 and MRS2179, on recombinant human and mouse NTPDase1, 2, 3 and 8. Enzymatic reactions were performed in a Tris/calcium buffer, commonly used to evaluate NTPDase activity, and in a more physiological Ringer modified buffer. Although there were some minor variations, there were no major changes either in the enzymatic activity or in the profile of NTPDase inhibition between the two buffers. Except for MRS2179, all other antagonists significantly inhibited these ecto-ATPases; NTPDase3 being the most sensitive to inhibition and NTPDase8 the most resistant. Estimated IC_{50} showed that human NTPDases were generally more sensitive to the P2 receptor antagonists tested than the corresponding mouse isoforms. NF279 and reactive blue 2 were the most potent inhibitors of NTPDases which almost completely abrogated their activity at the concentration of 100 μM. In conclusion, reactive blue 2, suramin, NF279 and NF449, at the concentrations commonly used to antagonize P2 receptors, inhibit the four major ecto-ATPases. This information may reveal useful for the interpretation of some pharmacological studies of P2 receptors. In addition, NF279 is a most potent non-selective NTPDase inhibitor. Although P2 receptor antagonists do not display a strict selectivity toward NTPDases, their IC_{50} values may help to discriminate some of these enzymes.

1. Introduction

Extracellular nucleotides (e.g. ATP, ADP, UTP and UDP) are signaling molecules that elicit various physiological and pathological responses in virtually every tissue [1]. These effects are mediated by P2 receptors including ion-channel P2X receptors ($P2X_{1-7}$) and G-protein-coupled P2Y receptors ($P2Y_{1,2,4,6,11-14}$) [2]. Two other receptors, CysLT1 and CysLT2, also respond to UDP in addition to cysteinyl leukotrienes [3,4]. More recently, the orphan receptor GPR17 was proposed to display a similar pharmacology responding to both leukotrienes and nucleotides [5].

* *Corresponding author at*: Centre de Recherche en Rhumatologie et Immunologie, Université Laval, 2705, Boulevard Laurier, local T1-49, Québec, QC G1V 4G2, Canada. Tel.: +1 418 654 2772; fax: +1 418 654 2765.
E-mail address: Jean.Sevigny@crchul.ulaval.ca (J. Sévigny).

Abbreviations: MRS2179, 2′-Deoxy-N^6-methyladenosine 3′,5′-bisphosphate; NF279, 8,8′-[Carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)]bis-1,3,5-naphthalenetrisulfonic acid; NF449, 4,4′,4″,4‴-[Carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino))]*tetrakis*-1,3-benzenedisulfonic acid; NTPDase, nucleoside triphosphate diphosphohydrolase.

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The concentrations of extracellular nucleotides at the cell surface are regulated by ectonucleotidases. Nucleoside triphosphate diphosphohydrolases (NTPDases) are prominent members of this family of ectoenzymes. NTPDase1, 2, 3 and 8 are bound to the plasma membrane and appear important for the control of P2 receptor-mediated signaling [6–9]. In contrast, NTPDase4, 5, 6 and 7 are located in the membrane of intracellular organelles. Even though NTPDase5 and 6 may also be found at the plasma membrane and secreted as soluble enzymes, their contribution to the hydrolysis of extracellular nucleotides may be of low importance due to their high K_m values and low specific activities [8].

Plasma membrane-bound NTPDases (members 1, 2, 3 and 8) possess broad substrate specificity towards nucleoside tri- (NTP; e.g. ATP and UTP) and diphosphates (NDP; e.g. ADP and UDP). Individual enzymes, however, differ in regard to NTP/NDP hydrolysis ratios. NTPDase1 hydrolyzes ATP and ADP equally well while NTPDase2 is a preferential triphosphonucleosidase. NTPDase3 and NTPDase8 are functional intermediates between NTPDase1 and NTPDase2. It is also noteworthy that NTPDases require divalent cations (Ca^{2+} or Mg^{2+}) for catalytic activities [8,9].

So far, there is a lack of selective and potent inhibitors of NTPDases. Some polyoxometalate anionic complexes have recently been reported to inhibit rat NTPDases [10]. The only specific commercially available ecto-ATPase inhibitor is 6-*N*,*N*-diethyl-beta, gamma-dibromomethylene-D-ATP (ARL 67156) [11] which displays weak competitive inhibition of NTPDase1 and NTPDase3 [12,13]. Previous works have shown that a few NTPDases can be inhibited by some P2 receptor antagonists [13–18]. Since such inhibition may affect the interpretation of pharmacological assays on P2 receptors, it is of importance to address this issue for all plasma membrane-bound NTPDases. In this work, we systematically tested the effect of some commonly used P2 receptor antagonists on the activity of recombinant plasma membrane-bound NTPDases from human and mouse and show that a few of these molecules can be used as potent NTPDase inhibitors.

2. Materials and methods

2.1. Materials

Aprotinin, nucleotides, phenylmethanesulfonyl fluoride (PMSF) and malachite green were purchased from Sigma–Aldrich (Oakville, ON, Canada). Tris(hydroxymethyl)aminomethane (Tris) was from VWR international (Montreal, QC, Canada). Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen (Burlington, ON, Canada). Fetal bovine serum and antibiotic antimycotic solution were from Wisent (St-Bruno, QC, Canada). P2 receptor antagonists 8,8′-[carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)]bis-1,3,5-naphthalenetrisulfonic acid (NF279), 4,4′,4″,4‴-[carbonylbis(imino-5,1,3-benzenetriyl-*bis*(carbonylimino))]*tetrakis*-1,3-benzenedisulfonic acid (NF449) and 2′-deoxy-N^6-methyladenosine 3′,5′-bisphosphate (MRS2179) were from Tocris Bioscience (Ellisville, MO, USA). Suramin and reactive blue 2 were provided by MP Biomedicals (Solon, OH, USA) (Fig. 1).

2.2. Plasmids

All plasmids used in this report have been described in published reports: human NTPDase1 (GenBank accession no. U87967) [19], human NTPDase2 (NM_203468) [20], human NTPDase3 (AF034840) [21], human NTPDase8 (AY430414) [22], mouse NTPDase1 (NM_009848) [6], mouse NTPDase2 (AY376711) [9], mouse NTPDase3 (AY376710) [23] and mouse NTPDase8 (AY364442) [24].

2.3. Cell transfection and preparation of protein extracts

COS-7 cells were transfected with pcDNA3 expression vectors, each containing the cDNA encoding the indicated NTPDase, using Lipofectamine from (Invitrogen) and harvested 40–72 h later, as previously described [9]. For the preparation of protein extracts, cells were washed three times with Tris-saline buffer (95 mM NaCl and 45 mM Tris, pH 7.5 at 4 °C), collected by scraping in harvesting buffer (95 mM NaCl, 0.1 mM PMSF and 45 mM Tris, pH 7.5) and washed twice by centrifugation (300 × *g*, 10 min, 4 °C). The cells were then resuspended in the harvesting buffer supplemented with 10 μg/ml aprotinin, to prevent proteolysis, and sonicated. Nucleus and cellular debris were discarded by centrifugation (300 *g* for 10 min at 4 °C) and the resulting supernatant (thereafter called protein extract) was aliquoted and stored at −80 °C until use. Protein concentration was estimated by Bradford microplate assay using bovine serum albumin as a standard.

2.4. NTPDase activity assay

Activity of protein extracts from NTPDase transfected COS-7 cells was determined as previously described [23] with some modifications. Enzymatic reaction was performed at 37 °C in 0.2 ml of one of the following three buffers with or without P2 receptor antagonists: Tris/calcium buffer (5 mM $CaCl_2$ and 80 mM Tris–HCl, pH 7.4), Tris/calcium buffer supplemented with 147 mM NaCl and Ringer modified buffer (120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 10 mM dextrose, 80 mM Tris–HCl, pH 7.4). Protein extracts containing human or mouse NTPDases were added to the incubation mixture and preincubated for 3 min at 37 °C. The reaction was initiated by the addition of 0.1 mM substrate (ATP or ADP) and terminated with the addition of 50 μl of malachite green reagent. The inorganic phosphate released was measured as previously described [25]. IC_{50} values for the inhibition of NTPDases by P2 receptor antagonists were calculated using GraphPad Prism software (San Diego, CA, USA) using four to five antagonist concentrations to cover the inhibition curve. For all NTPDase analyzed, the reaction was linear for at least 30 min with either substrate (data not shown). All enzymatic assays were carried out for 10 min.

2.5. Animals and tissue preparation

NTPDase1 deficient (*Entpd1*$^{-/-}$) mice were kindly provided by Dr SC Robson (BIDMC, HMS, Boston) [6]. All procedures were approved by the Canadian Council on Animal Care of the *Université Laval* Animal Welfare Committee. Thoracic aortas were taken on ketamin/xylasin anesthetised mice, embedded

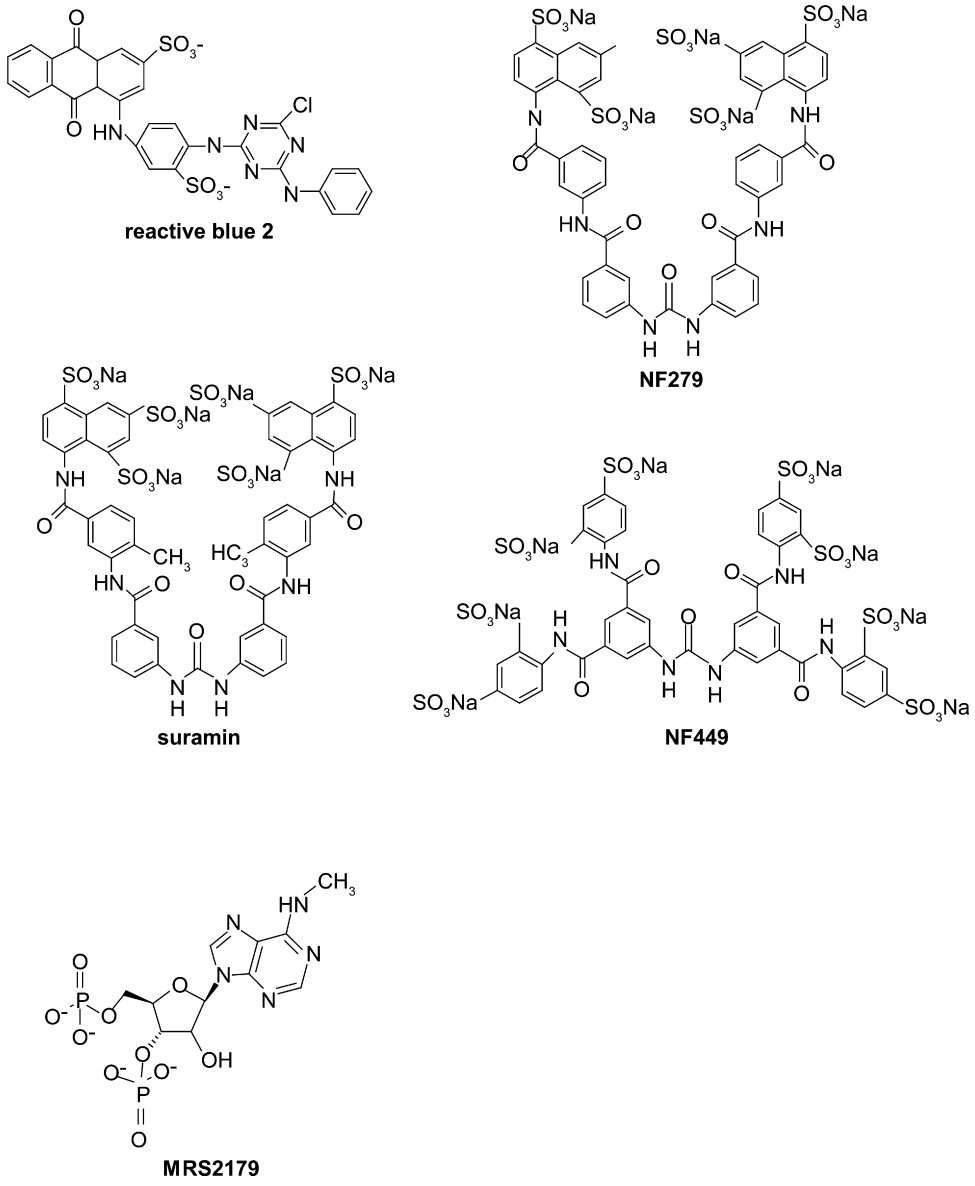

Fig. 1 – Chemical structure of P2 receptor antagonists.

in OCT and frozen in dry ice-cooled isopentane. Sections of 7 μm were cut serially using a cryostat and mounted on glass slides. Frozen sections were fixed in acetone containing 0.5% phosphate buffered-formalin for 2 min at 4 °C.

2.6. *Enzyme histochemistry*

To evaluate the effect of P2 receptor antagonists on ATP hydrolysis by a native NTPDase, a histochemical procedure was performed in mouse aortas, as previously described [26]. Briefly, fixed aorta cryosections were preincubated with or without P2 receptor antagonists for 45 min at room temperature in preincubation buffer (0.25 mM sucrose, 2 mM $CaCl_2$ and 50 mM Tris-maleate, pH 7.4). Enzymatic assays with 500 μM ATP as a substrate were carried out for 2 h at room temperature in incubation buffer (preincubation buffer complemented with 500 μM substrate, 2 mM $Pb(NO_3)_2$, 5 mM $MnCl_2$, 3% dextran T-250). In control experiments, the substrate was omitted. The orthophosphate released from nucleotide hydrolysis is captured by lead and was visualized in situ by precipitation with 1% $(NH_4)_2S$. Afterwards, all sections were counterstained with hematoxylin, mounted with 20% Mowiol 4–88, 50% glycerol and 0.2 M Tris–HCl, pH 8.5, and analyzed with an Olympus BX51 microscope.

2.7. *NTPDase2 immunostaining*

Sectioning and fixation were carried out as described above. After rinsing with PBS, non-specific binding sites were blocked with 7% normal goat serum in PBS for 30 min and the sections incubated overnight with mN2-36$_L$ polyclonal antibody at a 1:2000 dilution, at 4 °C. The specificity of this antibody has previously been described [27]. The staining was performed with Vectastain ABC elite kit (Vector Laboratories; Burlingame, CA) and 3,3′-diaminobenzidine was applied as the chromogen (Sigma–Aldrich) according to the manufacturer's instructions.

After washing with distilled water, sections were counterstained with Harris haematoxylin (Sigma–Aldrich) and mounted in 0.2 M Tris–HCl, 20% Mowiol 4–88, 50% glycerol, pH 8.5. For negative control experiments the primary antibody was replaced by its preimmune serum.

2.8. Statistical analysis

Statistical analysis was done with Student's *t*-test. *p*-values <0.05 were considered statistically significant.

3. Results

The effect of P2 receptor antagonists was tested on human and mouse NTPDase1, 2, 3 and 8. All experiments were carried out with the protein extracts of transfected COS-7 cells, or in a few confirmatory experiments with intact transfected cells. Importantly, the protein extracts of non-transfected COS-7 cells exhibited negligible level of intrinsic nucleotidase activity and allowed the analysis of each NTPDase in its native membrane bound form.

3.1. Effect of buffer composition on ATP and ADP hydrolysis by plasma membrane bound NTPDases

The effect of P2 receptor antagonists on NTPDase activities was tested in two different buffers: a Tris/calcium buffer, generally used in biochemical assays for these enzymes, and a more physiological Ringer modified buffer, commonly used in cellular and pharmacological experiments. We first tested the NTPDase activity in these two buffers. Even if some minor differences could be measured, the activity of most NTPDases was of the same order in both buffers (Table 1). Except for mouse NTPDase1 and NTPDase2, NTPDases were slightly more active in Tris/calcium buffer, and in general by nearly two folds.

The minor differences seen between these two buffers may be due to their different ionic strength. Additional experiments were performed to address this possibility by comparing simultaneously these two buffers plus a third one in which the ionic strength of the Tris/calcium buffer (24 mM) was adjusted to the same one as in the Ringer modified buffer (171 mM) by the addition of 147 mM NaCl. The data obtained are presented in Table 2 and show that the diminution in hydrolytic activity in the Ringer modified buffer, observed for human NTPDase1 and NTPDase2 with both substrates, correlated with an increase in ionic strength (in agreement with unpublished observations on purified enzymes). The situation is not as clear for NTPDase3 and NTPDase8 that may not be affected by the ionic strength (Table 2).

3.2. Inhibition of NTPDases by suramin

Suramin is commonly used as a non-selective P2Y receptor antagonist [28]. We tested the effect of suramin on the activity of human and mouse recombinant NTPDase1, 2, 3 and 8 (Fig. 2) in Tris/calcium and Ringer modified buffers in parallel. In the concentration range commonly used to block P2 receptors (10–100 μM), suramin considerably inhibited all NTPDases tested, except NTPDase8s and mouse NTPDase1. NTPDase1–3 were more sensitive to inhibition by suramin in the Tris/calcium buffer than in the Ringer modified buffer. The differences being statistically significant for NTPDase3s and human NTPDase1 ($p < 0.05$). These differences were also observed in the Tris/calcium buffer supplemented with 147 mM NaCl, suggesting that the ionic strength was responsible for these changes (data not shown). The rank order of potency of suramin inhibition for the human isoforms was NTPDase3 > NTPDase1 ≈ NTPDase2 > NTPDase8 and for mouse isoforms NTPDase2 > NTPDase3 ≫ NTPDase8 (Table 3). It is noteworthy that mouse NTPDase1 was not affected by suramin in the Tris/calcium buffer, even in the presence of 100 μM inhibitor (Fig. 2).

3.3. Inhibition of NTPDases by NF279 and NF449

NF279 and NF449 are suramin derivatives that display potent antagonist activity at P2X receptors ($P2X_{1\gg2>3>4}$ and $P2X_{1>3>7}$, respectively) [29-31]. In addition, NF449 is a weak antagonist

Table 1 – Effect of buffer composition on ATP and ADP hydrolysis by human and mouse plasma membrane bound NTPDases

Enzyme	Reaction medium	Human NTPDases			Mouse NTPDases		
		ATP (μmol P_i min^{-1} mg $protein^{-1}$)	ADP (μmol P_i min^{-1} mg $protein^{-1}$)	ATP/ADP ratio	ATP (μmol P_i min^{-1} mg $protein^{-1}$)	ADP (μmol P_i min^{-1} mg $protein^{-1}$)	ATP/ADP ratio
NTPDase1	Ringer modified	0.23 ± 0.04	0.14 ± 0.03	1.7 ± 0.1	8.3 ± 1.3	5.5 ± 1.1	1.6 ± 0.1
	Tris/calcium	0.40 ± 0.10	0.26 ± 0.07	1.6 ± 0.1	4.2 ± 1.3	2.4 ± 0.7	1.7 ± 0.1
NTPDase2	Ringer modified	0.30 ± 0.03	ND	ND	2.0 ± 0.3	ND	ND
	Tris/calcium	0.74 ± 0.26	ND	ND	1.4 ± 0.7	ND	ND
NTPDase3	Ringer modified	0.20 ± 0.02	0.08 ± 0.01	2.5 ± 0.2	0.29 ± 0.02	0.11 ± 0.01	2.7 ± 0.1
	Tris/calcium	0.40 ± 0.05	0.10 ± 0.01	4.4 ± 0.6	0.34 ± 0.06	0.25 ± 0.06	1.4 ± 0.1
NTPDase8	Ringer modified	0.47 ± 0.06	0.04 ± 0.01	11 ± 1	0.21 ± 0.01	0.06 ± 0.01	3.1 ± 0.1
	Tris/calcium	0.60 ± 0.06	0.17 ± 0.04	4.5 ± 0.9	0.38 ± 0.11	0.26 ± 0.07	1.4 ± 0.1

Activities of protein extracts from NTPDases transfected COS-7 cells with ATP and ADP as substrates were determined as detailed in Section 2. Results are expressed as the mean ± S.E.M. of at least two independent experiments performed in triplicate. ND: ADP is a poor substrate of NTPDase2 and has not been tested.

Table 2 – Influence of the ionic strength on ATP and ADP hydrolysis by human NTPDases

Enzyme	Reaction medium	ATP (μmol P_i min^{-1} mg $protein^{-1}$)	ADP (μmol P_i min^{-1} mg $protein^{-1}$)	ATP/ADP ratio
NTPDase1	Ringer modified	0.34	0.27	1.3
	Tris/calcium	0.43	0.36	1.2
	Tris/calcium + NaCl	0.30	0.24	1.2
NTPDase2	Ringer modified	0.67	0.05	13.6
	Tris/calcium	1.02	0.06	16.7
	Tris/calcium + NaCl	0.53	0.04	14.1
NTPDase3	Ringer modified	0.22	0.12	1.9
	Tris/calcium	0.67	0.16	4.0
	Tris/calcium + NaCl	0.53	0.16	3.2
NTPDase8	Ringer modified	0.35	0.08	4.3
	Tris/calcium	0.44	0.09	4.7
	Tris/calcium + NaCl	0.43	0.07	6.4

The activity of protein extracts from NTPDase transfected cells was assessed in three different buffers: a ''Ringer modified'' buffer (ionic strength 171 mM); a ''Tris/calcium'' buffer (ionic strength 24 mM); and a ''Tris/calcium + NaCl'' buffer in which the ionic strength of the Tris/calcium buffer was adjusted to 171 mM by adding 147 mM NaCl. Experiments were performed once in triplicate with the three buffers tested simultaneously.

for $P2Y_1$, $P2Y_2$ and $P2Y_{12}$ receptors [32,33]. As shown by IC_{50} values (Table 3), NF279 inhibited all NTPDases stronger than suramin but in the same order of potency: NTPDase3 > NTPDase2 ≈ NTPDase1 > NTPDase8 for human isoforms and NTPDase2 > NTPDase3 > NTPDase8 ≈ NTPDase1 for mouse isoforms. The magnitude of inhibition was similar in both buffers with the exception that 10 μM NF279 inhibited both ATPase and ADPase activities of mouse NTPDase1 much more potently in the Ringer modified buffer: 11 ± 2% and 4 ± 2% in the Tris/calcium buffer compared to 44 ± 10% and

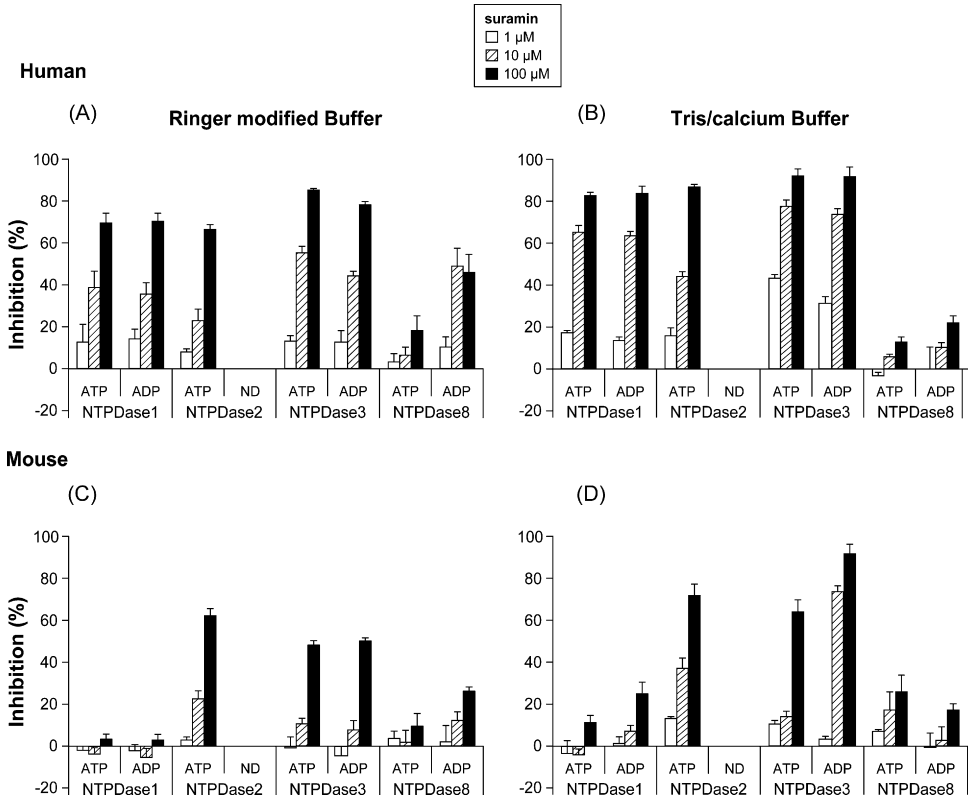

Fig. 2 – Effect of suramin on ATP and ADP hydrolysis by plasma membrane bound NTPDases. Enzymatic activity assays were carried out with protein extracts obtained from the indicated NTPDase transfected COS-7 cells as described in Section 2. The hydrolysis of 100 μM substrate was measured in the presence of 1, 10 or 100 μM of P2 receptor antagonist. (A and B) Human NTPDases; (C and D) mouse NTPDases; (A and C) assays carried out in Ringer modified buffer; (B and D) assays performed in Tris/calcium buffer. Results represent the mean ± S.E.M. of three to five independent experiments performed in triplicate.

Table 3 – IC_{50} (μM) values for selected P2 receptor antagonists

Enzymes	suramin		NF279		NF449		reactive blue 2	
	ATP	ADP	ATP	ADP	ATP	ADP	ATP	ADP
Human								
NTPDase1	16 ± 1	18 ± 4	5.2 ± 0.4	2.7 ± 0.5	>100	>100	53 ± 10	32 ± 4
NTPDase2	24 ± 3	ND	4.2 ± 0.1	ND	>100	ND	12 ± 2	ND
NTPDase3	4.3 ± 1.4	12 ± 5	0.6 ± 0.2	0.3 ± 0.1	9.6 ± 2.1	12 ± 2	3.3 ± 0.7	2.1 ± 0.1
NTPDase8	>100	>100	36 ± 2*	3.1 ± 1.1*	>100	>100	>100	>100
Mouse								
NTPDase1	>100	>100	52 ± 3	50 ± 2	>100	>100	>100	>100
NTPDase2	21 ± 2	ND	3.3 ± 0.5	ND	>100	ND	22 ± 2	ND
NTPDase3	31 ± 2	32 ± 2	9.8 ± 1.2	4.5 ± 0.5	82 ± 22	89 ± 24	5.4 ± 0.3	3.8 ± 0.1
NTPDase8	>100	>100	45 ± 9*	14 ± 2*	>100	>100	>100	>100

Enzymatic reactions were carried out for 10 min in Ringer modified buffer in the presence of 100 μM ATP or ADP. IC_{50} were calculated from a dose–response regression analysis using GraphPad Prism software and are presented in μM. Results are expressed as the mean ± S.E.M. of three to four independent experiments performed in triplicate. ND: ADP is a poor substrate of NTPDase2 and was not determined.

* Student's *t*-test analysis between IC_{50} for ATP and ADP. *p*-value < 0.05 was considered statistically different.

67 ± 3% in the Ringer modified buffer, respectively ($p < 0.01$). Mouse NTPDase1 was slightly less inhibited by NF279 than the human isoform whereas 100 μM NF279 nearly fully inhibited NTPDase2 and 3 of both species. Of the enzymes tested, human NTPDase3 was the most sensitive to NF279. Indeed, both ATPase and ADPase activities of this enzyme were inhibited by over 50% (over 85% in Tris/calcium buffer) in the presence of 1 μM NF279 and were nearly fully inhibited in the presence of 10 μM NF279 in both reaction buffers. NTPDase8s were slightly more sensitive to NF279 in Ringer modified buffer compared to Tris/calcium buffer, especially with ADP as substrate (Fig. 3). In comparison to NF279, NF449 was a weaker inhibitor of all NTPDases examined (Fig. 4 and Table 3). The rank order of potency of NF449 inhibition was similar for human and mouse isoforms: NTPDase3 > NTPDase1 ≈ NTPDase2 ≥ NTPDase8. Human NTPDase3 was the most sensitive NTPDase to inhibition by NF449. ADPase activity of human NTPDase3 was differently inhibited in the two media, as 1 μM NF449 decreased it by 79 ± 7% in the Tris/calcium buffer and only by 14 ± 4% in the Ringer modified buffer; 100 μM NF449 completely abrogated ADP hydrolysis by human NTPDase3 in Tris/calcium buffer. A weaker inhibition

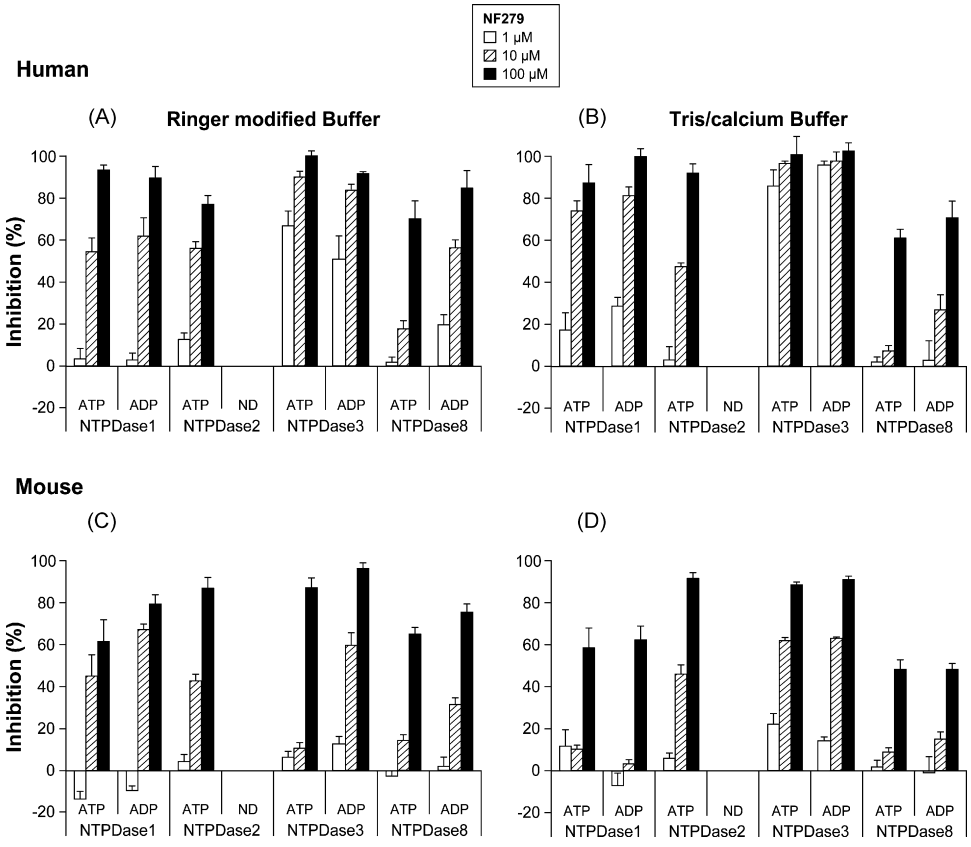

Fig. 3 – Effect of NF279 on ATP and ADP hydrolysis by NTPDases. See the description of Fig. 2.

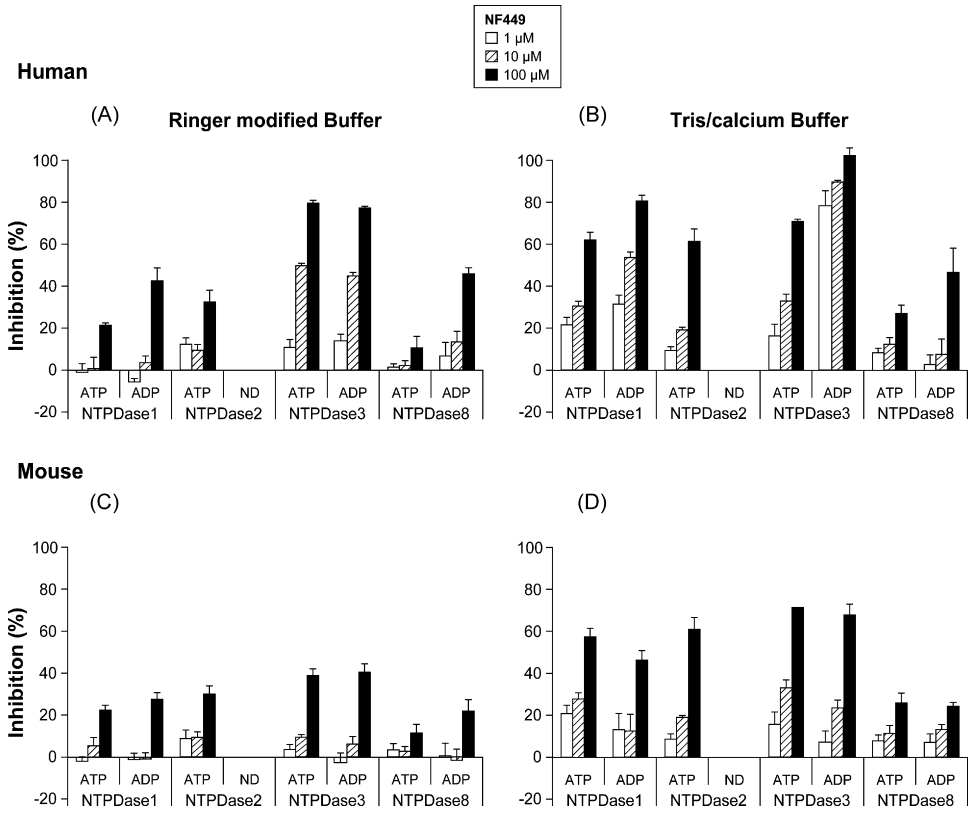

Fig. 4 – Effect of NF449 on ATP and ADP hydrolysis by NTPDases. See the description of Fig. 2.

was also observed in the Tris/calcium + NaCl buffer suggesting again that the ionic strength was responsible for these changes (data not shown). In contrast, the inhibition of ATPase activity of human NTPDase3 was similar in both buffers (Fig. 4). Overall, the inhibition of all NTPDases caused by NF449 was weaker in the Ringer modified buffer compared to the Tris/calcium buffer (Fig. 4, Table 3).

3.4. *Inhibition of NTPDases by reactive blue 2*

Reactive Blue 2 is a non-selective P2Y receptor antagonist that inhibits virtually all P2Y receptors [28]. Reactive blue 2 displays also $P2X_{3-4}$ antagonist activity in the micromolar range [34,35]. Reactive blue 2 turned out to be a very potent inhibitor of all plasma membrane-bound NTPDases. This inhibition was generally greater in the Tris/calcium buffer than in the Ringer modified buffer (Fig. 5). In Tris/calcium buffer, 100 μM reactive blue 2 completely inhibited human NTPDase1, 2, 3 as well as mouse NTPDase2 and 3. Human and mouse NTPDases were inhibited with the following rank order of potency: NTPDase3 ≥ NTPDase2 ≥ NTPDase1 ≥ NTPDase8 (Table 3, Fig. 5). The most potent inhibition was observed for NTPDase3s with estimated IC_{50} values in the low micromolar range in Ringer modified buffer (Table 3).

3.5. *Effect of MRS2179*

MRS2179 is an AMP analogue displaying a highly selective antagonist activity at the $P2Y_1$ receptor [36]. At the concentration range commonly used to inhibit $P2Y_1$ (10–30 μM) MRS2179 had a very limited effect on either human or mouse NTPDases (Fig. 6). This $P2Y_1$ receptor antagonist inhibited half of the ATPase and ADPase activities of human NTPDase3 at 100 μM, only in the Tris/calcium reaction medium. This enzyme, in Ringer modified buffer, or other NTPDases in either media, remained mainly unaffected by MRS2179 up to 100 μM (Fig. 6). The same range of inhibition was obtained with 500 μM AMP (data not shown).

3.6. *In situ inhibition of NTPDase2 in Entpd1$^{-/-}$ mouse aortas*

We evaluated whether the inhibition of NTPDases by P2 receptor antagonists would also apply to native enzymes in situ. For this, we used the enzyme histochemistry technique previously described [26]. This technique allows the detection of nucleotidase activity on tissue sections by the formation of a brown-colored lead precipitate associated with the free phosphate released from nucleotide hydrolysis. As previously described [7] two NTPDases are expressed in blood vessels: NTPDase1 is highly expressed on the endothelium and smooth muscle cells and NTPDase2 in the surrounding adventitial layer. We used *Entpd1*$^{-/-}$ mice aortas as an NTPDase2 exclusively expressing tissue. NTPDase2 activity was strongly diminished in the presence of suramin, NF279, and reactive blue 2, while NF449 inhibited the enzyme less efficiently (Fig. 7). These observations are in agreement with the results obtained with the protein extracts from COS-7

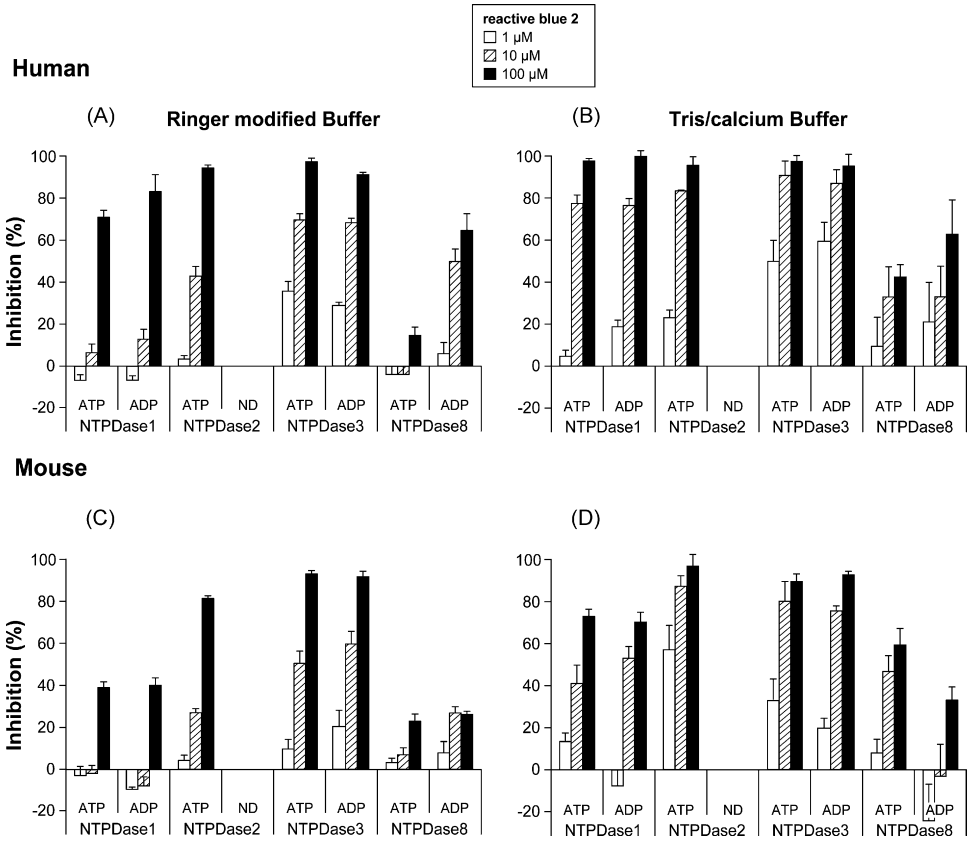

Fig. 5 – Effect of reactive blue 2 on ATP and ADP hydrolysis by NTPDases. See the description of Fig. 2.

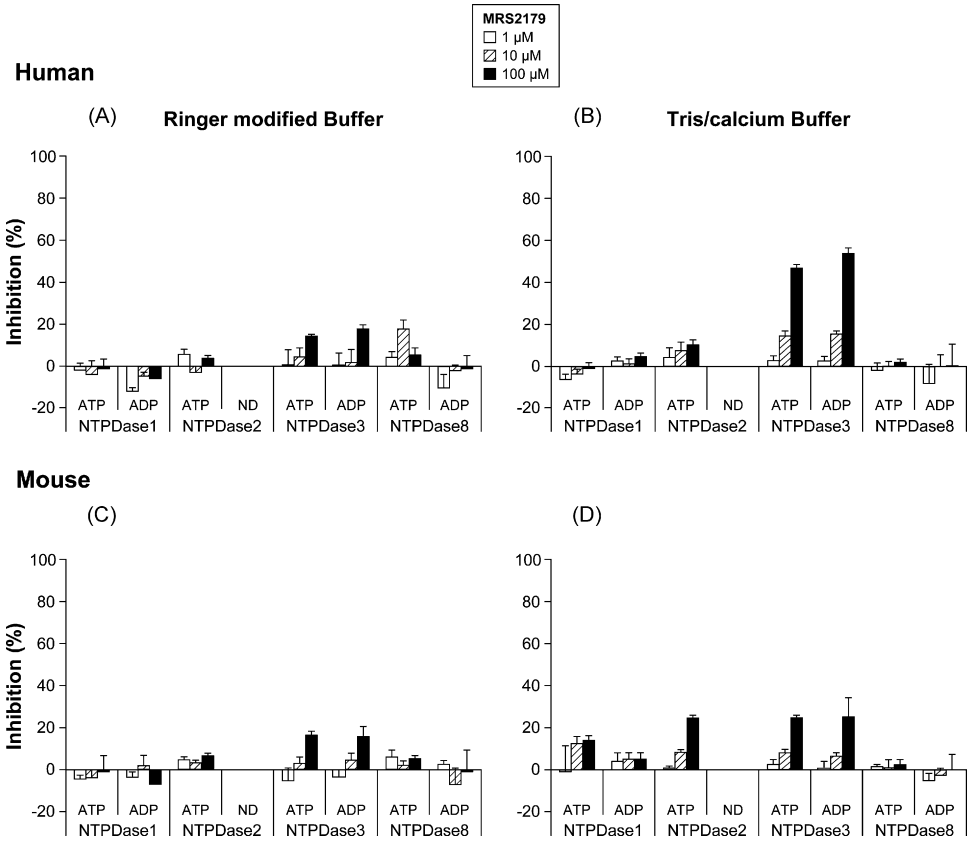

Fig. 6 – Effect of MRS2179 on ATP and ADP hydrolysis by NTPDases. See the description of Fig. 2.

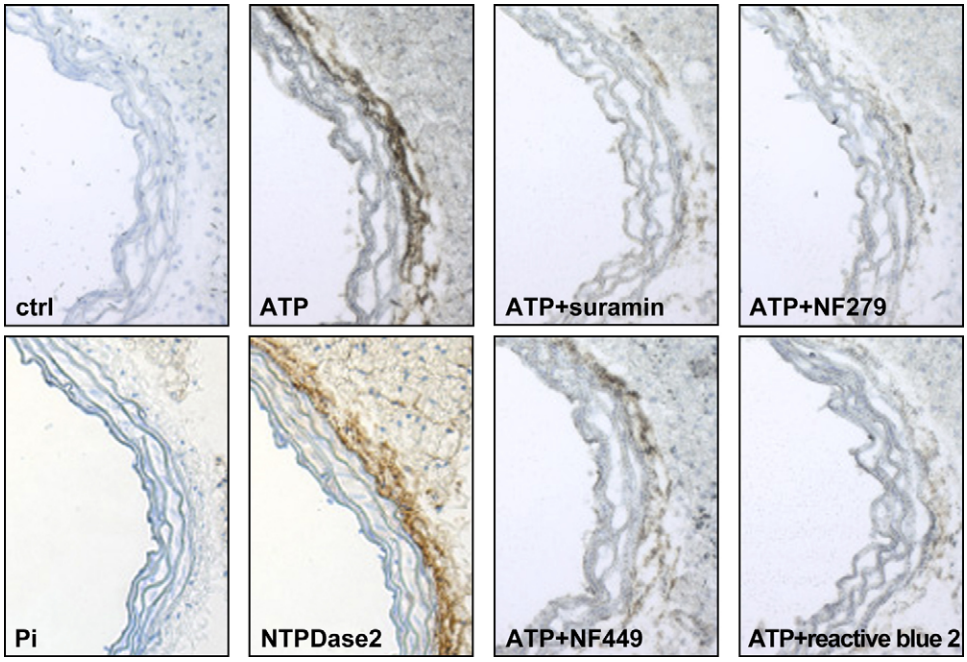

Fig. 7 – In situ inhibition of mouse aorta NTPDase2. NTPDase2 activity was visualised in an *Entpd1*$^{-/-}$ mouse aorta by the lead precipitation method. The experiment was performed with 500 μM ATP as substrate for 2 h at 37 °C with or without the indicated P2 antagonists (100 μM). Control (ctrl) refers to the experiment conducted without ATP. NTPDase2 was immunolocalised in an *Entpd1*$^{-/-}$ mouse aorta using the mN2-36$_L$ antibody. "Pi" indicates the corresponding preimmune antibody. The ATPase activity matches the NTPDase2 immunolocalisation. The enzyme activity is inhibited to different extent by P2 receptors' antagonists, in agreement with the data presented in Figs. 2–5 and Table 3.

cells transfected with mouse NTPDase2 (Figs. 2–5 and Table 3).

4. Discussion

The regulation of functions exerted by nucleotide signalling is complex due to the presence of various P2 receptors and other factors including the regulation of the concentration of nucleotides at the cell surface by ectonucleotidases. To further document the functions of extracellular nucleotides it is relevant, among other things, to identify and locate the ectonucleotidases involved in the systems of interest, to determine the expression levels of these enzymes and to define their biochemical properties. NTPDase1, 2, 3 and 8 are key ectonucleotidases. As nucleotides represent both the agonists of P2 receptors as well as the substrates of NTPDases it is conceivable that some antagonists of P2 receptors bear some effects on NTPDases. The identification of some P2 receptor antagonists as inhibitors of NTPDases may be of interest as there are no good specific inhibitors of these enzymes so far. In addition, effects on NTPDases by P2 receptor antagonists may also complicate the analysis of pharmacological assays where these molecules are used, and this must therefore be further documented. In this work we have investigated the effect of some commonly used P2 receptor antagonists on the activity of recombinant plasma membrane NTPDases from human and mouse species.

With the exception of MRS2179, all P2 receptor antagonists tested displayed inhibitory effects towards human and mouse plasma membrane NTPDases. Overall, we found that human recombinant NTPDases were more sensitive to P2 receptor antagonists than the corresponding mouse isoforms. In both species the NTPDases were generally affected by the tested P2 antagonists with the following rank starting with the most sensitive NTPDase to inhibition: NTPDase3 > NTPDase2 > NTPDase1 > NTPDase8.

NF279 was a very potent inhibitor of all NTPDases, inhibiting most enzymes completely and of over 60% for the remaining more resistant NTPDases, and that in both conditions tested either in Tris/calcium or in Ringer modified buffers. Reactive blue 2 was another very potent inhibitor of NTPDases. The latter was more potent in the Tris/calcium buffer. Suramin also potently inhibited NTPDases but to a lower extent than the two above P2 antagonists. Interestingly, suramin was not an inhibitor of mouse NTPDase1 (Fig. 2). It can therefore be used, with for example NF279, to discriminate an effect of the latter enzyme. NF449, a derivative of suramin as for NF279, also inhibited NTPDase activities but less efficiently than the above three other antagonists. Nevertheless, NF449 displayed some selectivity as an inhibitor of NTPDase3. In addition, we also confirmed that some of these P2 receptor antagonists could fully inhibit an NTPDase in situ, in the occurrence mouse NTPDase2, as expected from the biochemical assays.

The effects of a few P2 receptor antagonists on ectonucleotidases have previously been reported [13–18]. However,

most of these studies were conducted with tissue preparations from different sources/species and most often did not formally address the identity of the ectonucleotidase involved. Moreover, a comparison of the effect of P2 receptor antagonists on ectonucleotidases/NTPDases was not possible due to differences in experimental conditions and measurement techniques in all these papers. These reported data are nevertheless mainly consistent with the data presented here. For example, the purified smooth muscle chicken gizzard ecto-ATPase was inhibited by several P2 receptor antagonists, reactive blue 2 being the most potent with an IC_{50} of 44 μM [37]. The ecto-ATPase of the bovine pulmonary artery endothelium, rat C6 glioma cells and mouse RAW 264.7 cells were inhibited by suramin and reactive blue 2 with IC_{50} values of 4, 4.4 and 4 μM for suramin, and 4.5, 4.7 and 4.7 μM for reactive blue 2, respectively [14].

Few papers also reported the inhibition of recombinant NTPDases. Iqbal et al. reported that suramin and reactive blue 2 were potent inhibitors of rat NTPDases [13]. In this study, suramin inhibited rat recombinant NTPDases in the following order, NTPDase3 > NTPDase2 ≫ NTPDase1 with respective K_i values of 13 μM, 65 μM and 300 μM. The sensitivity to inhibition by reactive blue 2 was NTPDase3 > NTPDase1 ≥ NTPDase2 with respective K_i values of 1 μM, 24 μM and 20 μM [13]. Rat NTPDase3 expressed in *Xenopus laevis* oocytes, was also weakly inhibited by 300 μM NF449 (25 ± 4%) [38]. In agreement with our results, in these works NTPDase3 was the most sensitive NTPDase to inhibition by P2 receptor antagonists.

MRS2179 is an analogue of AMP displaying selective antagonistic activity at the $P2Y_1$ receptor and is not a substrate for NTPDases [36]. MRS2179 did not affect human and mouse NTPDase activities in the concentration range commonly used to inhibit $P2Y_1$. The highest concentration of MRS2179 tested (100 μM) partially (50%) inhibited only human NTPDase3 activity in Tris/calcium buffer (Fig. 6). Taken together, the data presented here indicate that MRS2179 is not an inhibitor of NTPDases, especially of NTPDase1. This is an important information as this molecule has been proposed in antithrombotic therapies [39]. Indeed, the inhibition of NTPDase1 activity at the surface of the vascular endothelium leads to ADP accumulation in the blood which induce platelet aggregation and thrombosis [6,40].

In previous work, we showed similar ATP:ADP hydrolysis ratios in a Tris/calcium buffer for human and mouse NTPDases [9] as what was observed here (Tables 1 and 2). In general, these hydrolysis ratios were similar in the Ringer modified buffer with some modest variations that may be due, to some extent, to the ionic strength. We, and others, have previously observed that the ATP:ADP hydrolysis ratio was also affected by the pH [9]. Different experimental or physiological conditions where pH, divalent cations (identity and concentrations), and ionic strength vary may affect the hydrolysis of P2 receptor agonists by NTPDases which may in turn shape the biological functions played by these enzymes. In this work we have also observed that the NTPDases' activities were in general inhibited slightly more by the P2 receptor antagonists in the Tris/calcium buffer and that this correlated with a lower ionic strength. Increasing the ionic strength also appeared to reduce slightly the biochemical activity of a few NTPDases (Tables 1 and 2).

In conclusion, NF279, reactive blue 2 and suramin are potent inhibitors of human and mouse NTPDase1, 2 and 3. NTPDase8 is the most resistant isoform to inhibition by the P2 receptor antagonists tested here while NTPDase3 is the most sensitive. To our knowledge, together with few polyoxometalate anionic complexes, NF279 is among the most potent NTPDase inhibitor identified so far. On the one hand, these inhibitions may complicate the interpretation of the pharmacological experiments using suramin, NF279, NF449 and reactive blue 2. On the other hand, these P2 receptor antagonists can be used as potent inhibitors of NTPDases and may allow the discrimination of a few of these enzymes. These molecules may also constitute a basic scaffold to design new and potentially specific inhibitors of NTPDases.

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