Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 621–630
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

2-ClATP exerts anti-tumoural actions not mediated by P2 receptors in neuronal and glial cell lines

Nadia D'Ambrosi[a,b], Stefano Costanzi[c], Daniela F. Angelini[d], Rosaria Volpini[c], Giuseppe Sancesario[b], Gloria Cristalli[c], Cinzia Volonté[a,e,*]

[a]*Fondazione Santa Lucia, Cellular Neurobiology Unit, Rome, Italy*
[b]*Department of Neuroscience, University of Rome Tor Vergata, Rome, Italy*
[c]*Department of Chemical Sciences, University of Camerino, Camerino, Italy*
[d]*Fondazione Santa Lucia, Neuroimmunology Unit, Rome, Italy*
[e]*C.N.R. Institute of Neurobiology and Molecular Medicine, Rome, Italy*

Received 14 May 2003; accepted 17 September 2003

Abstract

We investigated the effects of the ATP analogue and P2 receptor agonist 2-ClATP on growth and survival of different neuronal (PC12, PC12nnr5 and SH-SY5Y) and glial (U87 and U373) cell lines, by the use of direct count of intact nuclei, fluorescence microscopy, fluorescence-activated cell sorter analysis (FACS) and high pressure liquid chromatography (HPLC). 2-ClATP lowered the number of cultured PC12nnr5, SH-SY5Y, U87 and U373 cells to almost 5%, and of PC12 cells to about 35% after 3–4 days of treatment. EC_{50} was in the 5–25 μM range, with 2-ClATP behaving as a cytotoxic or cytostatic agent. Analysis of the biological mechanisms demonstrated that pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (P2 receptor antagonist and nucleotidases inhibitor), but not Caffeine or CGS-15493 (P1 receptor antagonists) effectively prevented 2-ClATP-induced toxicity. 2-ClATP metabolic products (2-ClADP, 2-ClAMP, 2-Cladenosine) and new synthesis derivatives (2-CldAMP, 2-Cldadenosine-3′,5′-bisphosphate and 2-CldATP) exerted similar cytotoxic actions. Inhibition of both serum nucleotidases and purine nucleoside transporters strongly reduced 2-ClATP-induced cell death, which was conversely increased by the nucleotide hydrolyzing enzyme apyrase. The adenosine kinase inhibitor 5-iodotubericidin totally prevented 2-ClATP or 2-Cladenosine-induced toxicity.

In summary, our findings indicate that 2-ClATP exerts either cell cycle arrest or cell death, acting neither on P2 nor on P1 receptors, but being extracellularly metabolized into 2-Cladenosine, intracellularly transported and re-phosphorylated.

Keywords: PC12 cells; SH-SY5Y cells; U87, U373 cells; Cell death; Adenosine kinase

1. Introduction

Nucleotides and nucleosides represent a class of signaling molecules in essentially all tissues. They can exert different biological functions in various systems, according to the extracellular environment of each target cell and to the molecular repertoire of the plasma membrane. In particular, significant are the presence of P1-adenosine receptors [1], P2-ATP receptors [2], nucleoside transporters [3] and degrading enzymes, such as ecto-nucleotidases [4] or nucleoside-deaminases [5]. The variety and interplay of these activities contribute to render the extracellular milieu a very dynamic and heterogeneous source of signaling inputs. As a matter of fact, ATP is a very unstable compound, being metabolized by ecto-nucleotidases to generate several intermediate metabolites to the final product adenosine [6]. ATP and adenosine have been in turn recognized as molecules with both trophic and toxic actions in different cell types. It was actually reported that extracellular ATP and its analogs can support cell survival and neurite regeneration in PC12 cells [7],

* Corresponding author. Tel.: +39-06-5150-1557; fax: +39-06-5150-1556.
E-mail address: cinzia@in.rm.cnr.it (C. Volonté).

Abbreviations: 2-ClAdo, 2-Cladenosine; FCS, fetal calf serum; HS, horse serum; 5′IT, 5-iodotubericidin; NBTI, nitrobenzyl-6-thioinosine; NGF, nerve growth factor; PBS, phosphate-buffered saline; PF1, 2-ClAMP; PF3, 2-CldAMP; PF4, 2-CldAdo-3′,5′-bisphosphate; PF5, 2-CldATP; PF10, 2-ClADP; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; PI, propidium iodide.

0006-2952/$ – see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2003.09.015

regulate the differentiation of skeletal muscle cells [8], and induce proliferation of different cell types [9–14]. While these trophic effects seem to be mediated by P2 receptors, the toxic actions exerted by extracellular ATP can be due to P2 receptor activation [15,16] and/or additional pathways [17–20]. For instance, $P2X_7$ is mainly characterized as a cytolytic pore in macrophages and lymphocytes [21], while P2 receptor-mediated toxicity generally involves the activation of multiple receptor subclasses. Cell death induced by P2 receptors can also result from indirect mechanisms, such as release of pro-inflammatory cytokines in macrophages and microglia [22,23]. On the other hand, adenosine can induce cell death both by binding to P1 receptors [24–26] or being intracellularly transported to produce ATP through the salvage pathway [27,28].

Although more stable than ATP itself, 2-ClATP can be degraded by ATP-diphosphohydrolases to finally generate the deaminase-resistant compound, 2-Cladenosine (2-ClAdo) [29]. This last, in contrast to the trophic actions exerted by 2-ClATP, is known to possess anti-tumoural and cytotoxic effects in several cells [30,31]. In addition, the 2′-deoxyderivative of 2-ClAdo (cladribine) is clinically used for the treatment of indolent lymphoid malignancies and, in particular, against hairy cell leukemia [32]. The molecular mechanisms responsible for the antiproliferative effects induced by 2-chloroderivatives of adenosine are generally mediated by intracellular pathways [33], albeit the activation of extracellular adenosine receptors has also been reported [34].

In this work, we studied the actions of extracellular nucleotides on the growth and survival of phaeochromocytoma PC12 and variant PC12nnr5, on neuroblastoma SH-SY5Y and on glioma U87 and U373 cell lines. In particular, we analyzed the effects induced by the ATP analogue 2-ClATP, previously shown to induce differentiation and survival of PC12 cells [7] mainly acting on P2 receptors. We now demonstrate that 2-ClATP induces cytostaticity or cytotoxicity not via P2 or P1 receptors, but through the extracellular formation of 2-ClAdo, its consequent intracellular transport and re-phosphorylation.

2. Materials and methods

2.1. Materials

All drugs used in this work were purchased from Sigma–Aldrich, except XAMR 0721, which was acquired from Calbiochem.

2.2. Synthesis of 2-chloroderivatives of adenosine phosphates

PF1 [35] and PF4 [36] were synthesized following procedures similar to those described in the literature. The analytical data are consistent with those reported. Here we describe the synthesis of PF3, PF5, and PF10. Briefly, the nucleoside monophosphates were synthesized from the corresponding nucleosides by means of the Yoshikawa reaction [37], utilizing $POCl_3$ as phosphorylating agent (Fig. 1). On the other hand, the synthesis of the nucleosides polyphosphates was performed treating the corresponding monophosphates, previously activated by reaction with 1-1′-carbonyldiimidazole, with ammonium monophosphate or pyrophosphate (Fig. 1) [38].

2.2.1. General synthetic procedures

^1H and ^{31}P nuclear magnetic resonance (NMR) spectra were obtained with Varian VXR 300 MHz spectrometer; δ in ppm, J in Hz. All exchangeable protons were confirmed by addition of D_2O. The reactions were monitored by thin layer chromatography (TLC), using pre-coated TLC plates with silica gel 60 F-254 (Merck) and iC_3H_7OH–H_2O–NH_4OH (30%) (5.5/1/3.5) as mobile phase. The nucleotides were purified by means of ionic exchange chromatography on a Sephadex DEAE A-25 column (HCO_3^- form) equilibrated with H_2O and eluted with a linear gradient of H_2O/0.5 M NH_4HCO_3.

2.2.2. 2-Chloro-2′-deoxyadenosine-5′-monophosphate (2-CldAMP, PF3)

To 86 mg of 2-Cl-2′-deoxyadenosine (0.30 mmol) dissolved in 1.5 mL of trimethyl phosphate were added 4 equivalents of $POCl_3$ (112 μL, 184 mg, 1.2 mmol). The solution was stirred 45 min at 0°, H_2O was added (1.5 mL) and the solution was neutralized by adding triethylamine dropwise.

After ion exchange chromatography, 72 mg of PF3 (0.19 mmol, 63%) were obtained. The reaction gave also 34 mg of PF4 (0.07 mmol, 23%) as secondary product.

^1H NMR (D_2O) δ 2.54 and 2.72 (1H each, m, H-2′), 3.97 (2H, m, H-5′), 4.22 (1H, m, H-4′), 4.66 (1H, m, H-3′), 6.35 (1H, t, H-1′, $J = 6.96$ Hz), 8.38 (1H, s, H-8).

^{31}P NMR (D_2O) δ 1.83 (s).

2.2.3. 2-Chloro-2′-deoxyadenosine-5′-trisphosphate (2-CldATP, PF5)

To 56 mg of PF3 (0.15 mmol), dissolved in 1 mL of dry dimethylformamide (DMF), were added 36 μL of tri-*n*-butylamine (28 mg, 0.15 mmol). The solution was stirred for 20 min at room temperature and then evaporated to dryness under anhydrous conditions. After resuspension in 1.4 mL of dry DMF, 1-1′-carbonyldiimidazole (122 mg, 0.75 mmol) was added and the mixture was stirred for 3 hr at room temperature. Methanol (49 μL, 38.5 mg 1.2 mmol) was added and left for 30 min. Then 6 mL of 0.5 M bis(tri-*n*-butylammonium)pyrophosphate in DMF (3 mmol) was added and the mixture was left for 14 hr at room temperature. The solvent was removed *in vacuo*, the mixture dissolved in H_2O and purified by means of ion exchange chromatography to obtain 44 mg of PF5 (0.07 mmol, 47%).

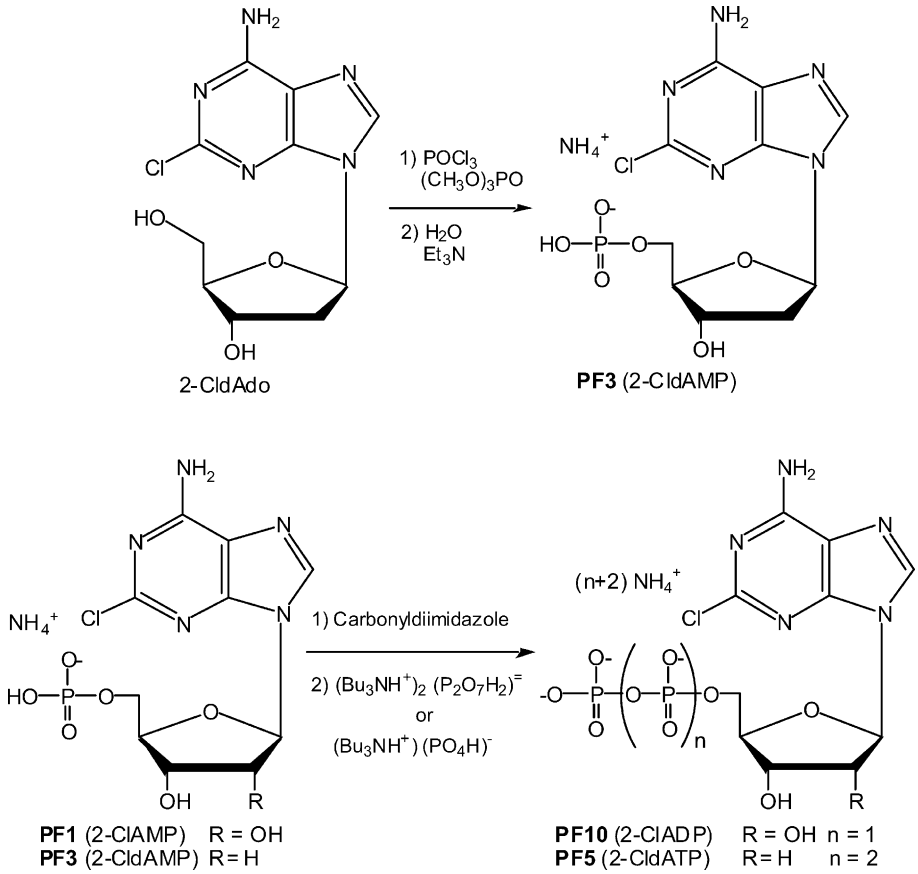


Fig. 1. Schematic view of new-synthesis 2-chloroderivatives of adenosine phosphate. The compounds were obtained with synthesis procedures described in Section 2.

^{1}H NMR (D_2O) δ 2.76 (2H, m, H-2′), 4.23 (2H, m, H-5′), 4.53 (1H, m, H-4′), 5.48 (1H, m, H-3′), 6.40 (1H, t, H-1′, $J = 6.46$ Hz), 8.47 (1H, s, H-8).

^{31}P NMR (D_2O) δ −22.42 (m, Pβ), −10.81 (d, Pγ), −10.02 (d, Pα).

2.2.4. 2-Chloroadenosine-5′-diphosphate (2-ClADP, PF10)

To 60 mg of 2-ClAMP (0.15 mmol), dissolved in 1 mL of dry DMF, were added 36 μL of tri-*n*-butylamine (28 mg, 0.15 mmol). The solution was stirred for 20 min at room temperature and then evaporated to dryness under anhydrous conditions. After resuspension in 1.4 mL of dry DMF, 1-1′-carbonyldiimidazole (122 mg, 0.75 mmol) was added and the mixture was stirred for 3 hr at room temperature. Methanol (49 μL, 38.5 mg, 1.2 mmol) was added and left for 30 min. Then 6 mL of 0.5 M (tri-*n*-butylammonium)phosphate in DMF (3 mmol) was added and the mixture was left for 14 hr at room temperature. The solvent was removed *in vacuo*, the mixture dissolved in H_2O and purified by means of ion exchange chromatography to obtain 30 mg of 2-ClADP (PF10, 0.058 mmol, 39%).

^{1}H NMR (D_2O) δ 4.07 (2H, m, H-5′), 4.24 (1H, m, H-4′), 4.40 (1H, m, H-3′), 4.59 (1H, t, H-2′, $J = 4.58$ Hz), 6.00 (1H, m, H-1′, $J = 5.60$ Hz), 8.34 (1H, s, H-8).

^{31}P NMR (D_2O) δ −10.63 (m, Pβ), −10.12 (m, Pα).

2.3. Cell cultures

Rat phaeochromocytoma PC12 and PC12nnr5 cell lines were cultured on collagen-coated dishes in RPMI 1640 medium supplemented with 10% heat-inactivated HS and 5% FCS; human neuroblastoma SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12, supplemented with 15% FCS; human glioma U87 and U373 cell lines were grown in 10% FCS in DMEM. All culture media were supplemented with glutamine (2 mM), penicillin (50 U/mL), and streptomycin (50 μg/mL) and all cell lines grown at 37° in 5% CO_2.

2.4. Cell survival assay

Cells were grown in 24-well flat bottom plates at a density of 3×10^5 per well, in the presence of the appropriate medium (15% sera), as cited above, or 1.5% sera (1% heat-inactivated HS, 0.5% FCS) or inactivated 15% sera (56° for 90 min). Drugs were added 4 hr after seeding and the cell number was evaluated after various lengths of times by direct count of intact nuclei [39]. Briefly, the method is based on the solubilization of cellular membranes by a mild detergent, followed by direct count of all

intact nuclei. In all cases, triplicate wells were scored and counts represent means ± SEM.

2.5. Chromatin staining by Höechst 33258

PC12 and PC12nnr5 cells seeded on 35 mm plates were kept with or without 100 μM 2-ClATP for 24 hr. Cells were then fixed for 15 min with 4% paraformaldehyde in PBS, washed three times with PBS and incubated for 10 min with 1 μg/mL Höechst 33258. After three final washes in PBS, nuclear condensation was visualized under UV light, using fluorescence microscopy.

2.6. Propidium iodide staining and FACS analysis

PC12 and PC12nnr5 cells, cultured on 24-well plates, were treated with or without 100 μM 2-ClATP for different times and were then collected and centrifuged for 10 min at 200 *g*. The pellet was resuspended in 1 mL PBS/5 mM EDTA, dissociated thoroughly and centrifuged. The cells were fixed in ice-cold 70% ethanol in PBS, kept at −20° for more than 30 min, spun for 10 min at 200 *g* and incubated for 30 min at room temperature with 1 mg/mL PI in PBS, also containing 0.1% (v/v) Triton X-100 and 2 mg/mL DNase-free RNase A (Sigma–Aldrich). Flow cytometry was then performed with FACSCalibur (BD Biosciences) flow cytometer. The data were analyzed using CellQuest 3.3 software. For the purpose of analysis, acquired events were gated to eliminate cell aggregates.

2.7. [^{3}H]thymidine incorporation

PC12 cells in complete medium were seeded on 96-well plate (5×10^4 cells/well) in the presence or absence of 100 μM 2-ClATP. [^{3}H]thymidine (25 μCi/mL, final concentration 10 μM) was added to the cultures 8 hr after plating. At different times thereafter, the cells were washed, harvested and DNA isolated on fiberglass filters (Wallac) using a Tomtek 96-well harvester. The filters were then air-dried and assessed for specifically bound radioactivity, by liquid scintillation counting.

2.8. HPLC analysis

For the measurement of 2-ClATP breakdown, PC12nnr5 cells cultured on 90 mm dishes in the presence of 15% sera, were exposed to 1 mM 2-ClATP. At different times, samples from the culture medium were collected, filtered on 0.2 μm NY membranes and analyzed by HPLC with a HP 1090 Liquid Chromatograph series II. Separations were carried out on a 150 mm × 4.60 mm Phenomenex Sinergy 4 μm Polar-RP 80 Å column. The elution solvent was CH_3COOH 2% in H_2O:CH_3OH 75:25, the injection volume was 20 μL, the flow rate was 700 μL/min and the detector was DAD $\lambda_1 = 265$ nm, $\lambda_2 = 254$ nm, $\lambda_3 = 275$ nm, $\lambda_r = 550$ nm.

2.9. Statistical analysis

Statistical differences were verified by one-way analysis of variance and $P < 0.05$ was considered significant: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3. Results

3.1. Extracellular 2-ClATP reduces phaeochromocytoma, neuroblastoma and glioma cell number in culture

To study the biological effects of the slowly hydrolysable ATP analogue 2-ClATP on different models of neuronal and glial tumoural cells, a single dose of 100 μM was added to the phaeochromocytoma PC12 and PC12nnr5 cells, to the neuroblastoma SH-SY5Y and to the glioma U87 and U373 cells, in the presence of optimal sera concentrations. As shown in Fig. 2A, 2-ClATP always inhibited cell growth, as detected by count of viable, intact nuclei after 4 days of treatment. Maximal effect (90–100% inhibition) was obtained with PC12nnr5, SH-SY5Y, U87 and U373 cells, while about 65% inhibition was observed with PC12 cells.

3.2. 2-ClATP exerts a cytotoxic effect in PC12nnr5 and a cytostatic action in PC12 cells

We further analyzed the inhibition of cell growth induced by 2-ClATP and compared PC12 to PC12nnr5 cells. The two cell lines are apparently similar, being PC12nnr5 a PC12 variant mostly lacking the Nerve Growth Factor (NGF)-high affinity TrkA receptor [40,41]. In the presence of sera, 2-ClATP inhibited cell growth in 3 days, with EC_{50} at about 5 μM (for PC12 cells) or 25 μM (for PC12nnr5 cells) and with maximal effects at 100 μM (data not shown). Time-course experiments showed that 2-ClATP reduced PC12nnr5 cells of about 20% in 24 hr, 60% in 48 hr and 100% in 3 days (Fig. 2B). Conversely, the number of PC12 cells was maintained constant up to at least 7 days (Fig. 2C). These results were confirmed by morphological observations: while 2-ClATP-treated PC12nnr5 cultures showed cellular debris, shrunken, phase-dark and floating cells, PC12 cells remained instead round and phase-bright (data not shown). In order to assess whether the effect of 2-ClATP was to induce cell death, to arrest cell cycle or both, we performed the following experiments. PC12nnr5 and PC12 cells were incubated with 100 μM 2-ClATP and stained with Höechst 33258 to analyze nuclear DNA condensation (Figs. 3A and 4A). Typical apoptotic nuclei (indicated by arrows) with DNA at different levels of condensation were observed in PC12nnr5 (Fig. 3A) but not in PC12 cells (Fig. 4A). We further assessed DNA fragmentation and ploidy, by staining with PI and performing subsequent cytofluorimetric analysis. As indicated in Fig. 3B, control

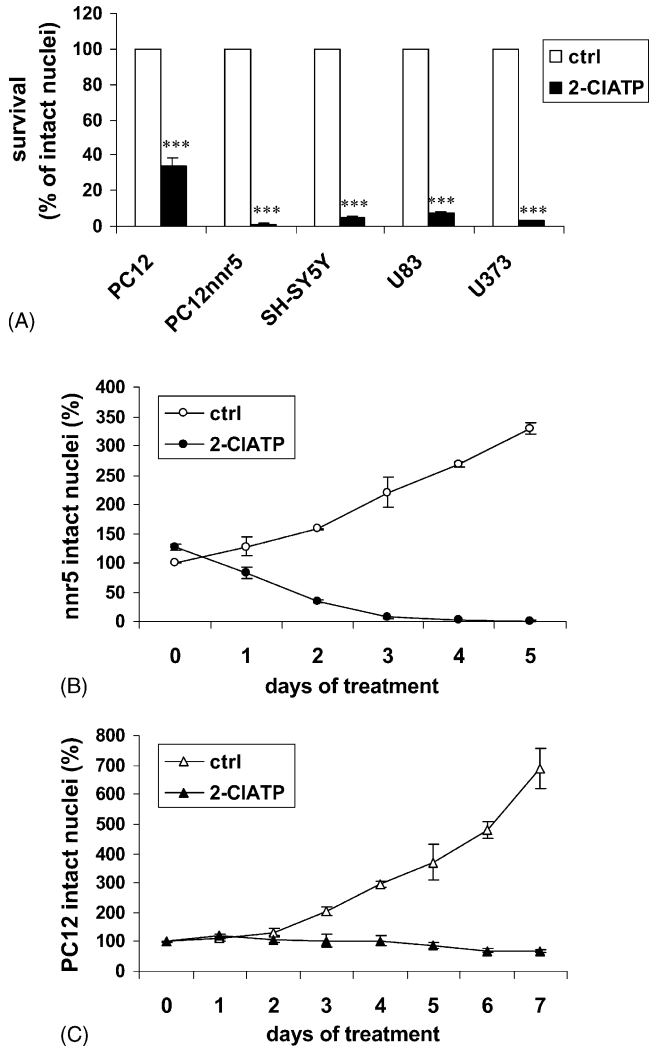


Fig. 2. 2-ClATP reduces the number of neuronal and glial cells in culture. All cells maintained under proliferating conditions were treated with a single dose of 100 μM 2-ClATP. Cell survival was assessed 4 days later (A) or as indicated (B, C) by direct count of intact nuclei. Counts represent means ± SEM (N = 3) and 100% survival is referred to untreated cultures. In (A), $^{***}P < 0.001$ with respect to corresponding control, one-way variance analysis.

PC12nnr5 cells displayed about 21% of cells in $S + G_2$ phases and only 0.93% in the apoptotic sub G_1/G_0 fraction. The treatment with 2-ClATP for 30 hr increased the sub G_1/G_0 fragmented DNA peak to 44% of the entire DNA population, while the $S + G_2$ populations were reduced to about 7%. The cytofluorimetric analysis revealed no significant difference between controls and 2-ClATP-treated PC12 cells up to 3 days (data not shown). [^{3}H]Thymidine incorporation into PC12 cells exposed to 2-ClATP for 36 and 60 hr showed instead that DNA duplication was reduced of 40 and 50%, with respect to untreated cultures (Fig. 4B).

Once established that 2-ClATP was cytostatic with PC12 cells (Figs. 2A–C and 4), but cytotoxic with PC12nnr5 (Figs. 2A–B and 3), we further pursued the analysis of the toxic effect of 2-ClATP mainly in PC12nnr5 cells.

Table 1
Action of P2X (*) and P2Y (°) antagonists on 100 μM 2-ClATP-induced toxicity in PC12nnr5 cells

Antagonist	Percent of intact nuclei
(*) oATP 50 μM	3 ± 1
(*) DIDS 200 μM	5 ± 2
(*) Brilliant Blue G 30 μM	7 ± 3
(*) Calmidazolium 1 μM	1 ± 0.5
(*) TNP-ATP 120 μM	8 ± 0.5
(*) MRS 2159 100 μM	9 ± 3
(*) NF 023 100 μM	4 ± 3
(*) PPNDS 300 μM	47 ± 1***
(°) PIT 10 μM	4 ± 2
(°) MRS 2179 10 μM	8 ± 0.5
(°) XAMR 0721 100 μM	7 ± 1
(*,°) Suramine 300 μM	10 ± 2
(*,°) P5P 200 μM	9 ± 1
(*,°) PPADS 300 μM	74 ± 7.5***
(*,°) RB-2 300 μM	9 ± 3

PC12nnr5 cells were incubated in the simultaneous presence of 100 μM 2-ClATP and the above indicated P2 receptor antagonists. Cell survival was assessed 4 days later, by direct count of intact nuclei. Counts represent means ± SEM (N = 3).

Abbreviations: oATP, ATP-2′,3′-dialdehyde; DIDS, 4,4′-diisothiocyanatostilbene-2,2′disulphonic acid; TNP-ATP, trinitrophenyl-ATP; PIT, 2,2′-pyridylsatogen; P5P, pyridoxal-5′-phosphate; RB-2, reactive blue-2.

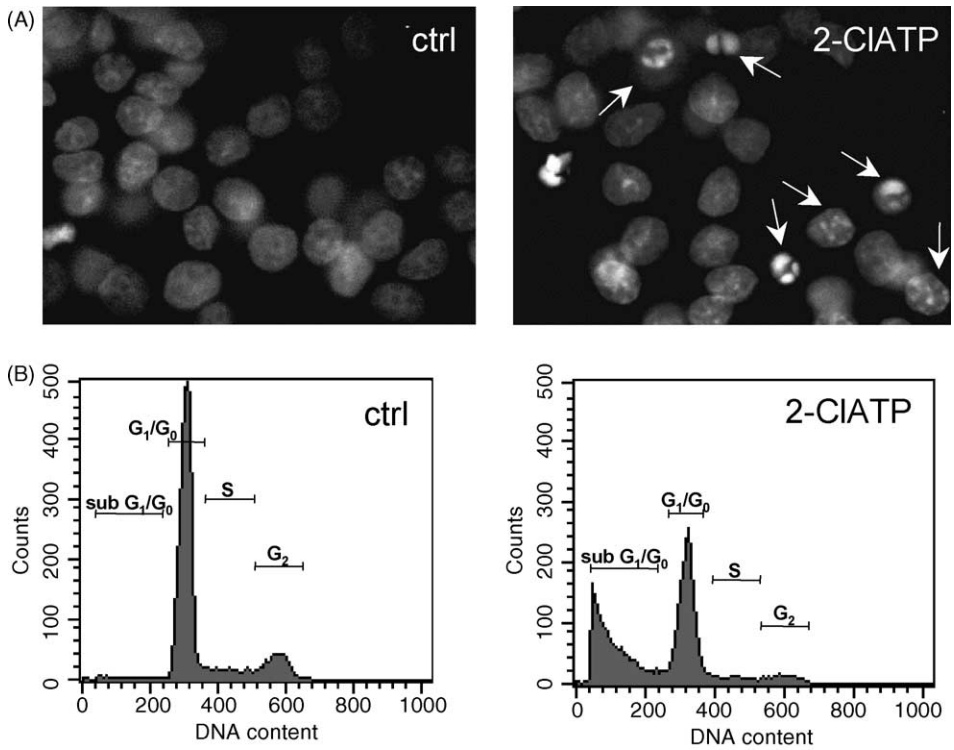
$^{***}P < 0.001$ with respect to 2-ClATP, one-way variance analysis.

3.3. Cytotoxicity induced by 2-ClATP is mediated by neither P2 nor P1 receptors

The toxic effect induced by 2-ClATP in PC12nnr5 cells was tested in the presence of several P2 antagonists with different specificities towards P2X and P2Y receptors (Table 1). Only pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate) (PPNDS) and PPADS (both used at 300 μM) sustained, respectively, 47 and 74% survival in 3 days of 2-ClATP exposure (Table 1). We then tested 2-ClATP metabolites and derivatives. As shown in Fig. 5A, extracellular PF10 (Fig. 1), PF1 (Fig. 1) and 2-ClAdo (100 μM) were able to induce complete cell death in 3 days. Similarly effective (Fig. 5A) were also the newly synthesized derivatives PF3, PF4 and PF5 (Fig. 1). In order to investigate the potential involvement of P1 receptors, the non-selective adenosine antagonists Caffeine and CGS-15493 were tested, respectively at 100 μM and 250 nM. None reverted the toxic action exerted by 2-ClATP or 2-ClAdo (Fig. 5B).

3.4. Extracellular 2-ClATP is toxic only when metabolized into 2-ClAdo mainly by serum ATPases

Having assessed that 2-ClAdo can exactly mimic the toxic effect of 2-ClATP, we investigated the possible extracellular degradation of the nucleotide. It was reported that PPADS can block ecto-ATPases in various cell types, acting therefore not only as a P2 receptor antagonist, but also as an inhibitor of adenosine poly-phosphates hydrolyzing enzymes [42]. PC12nnr5 cells treated, respectively



Marker	Events	% Gated
All	21856	100.00
sub G_1/G_0	204	0.93
G_1/G_0	17385	79.54
S	1645	7.53
G_2	2648	12.12

Marker	Events	% Gated
All	21158	100.00
sub G_1/G_0	9290	43.91
G_1/G_0	10361	48.97
S	750	3.54
G_2	782	3.70

Fig. 3. 2-ClATP induces apoptosis in PC12nnr5 cells. PC12nnr5 cells were exposed to 100 μM 2-ClATP for 24 hr (A) or 30 hr (B) and stained with Höechst 33258 (1 μg/mL) (A) or PI (1 mg/mL) (B). Condensed nuclei stained with Höechst 33258 (indicated by arrows) were visualized using Zeiss fluorescent microscope (A), while DNA ploidy was measured by PI incorporation and cytofluorimetric analysis (B). In both cases, Ctrl represents untreated cultures. Results are representative of three independent experiments.

with 2-ClATP or PF10 in the presence of 300 μM PPADS showed a remarkable (80%) or partial (50%) reduction of cell death (Fig. 6A). On the other hand, 300 μM PPADS was only slightly (35%) or no effective on PF1- or 2-ClAdo-induced toxicity (Fig. 6A). This indicates selectivity of PPADS towards ATPases and ADPases, but not 5′-nucleotidases. In order to identify if mainly sera or cellular ecto-enzymes were responsible for the potential 2-ClATP hydrolysis, we either inactivated or lowered the sera in the culture media. Cell death caused by 2-ClATP in PC12nnr5 was completely prevented by heat-inactivating the sera (Fig. 6B). Moreover, 2-ClATP in 1.5% sera (instead of 15%) reduced its toxic effect from 95 to 60% (data not shown). Consistently, the simultaneous addition of the ATP-hydrolyzing enzyme apyrase (5 unit/mL), increased cell death by 2-ClATP of nearly 50 and 80%, respectively in the presence of active or inactivated sera (Fig. 6B). To further confirm the extracellular degradation of 2-ClATP into 2-ClAdo, we performed HPLC analysis on the culture media from PC12nnr5 cells treated with 1 mM 2-ClATP. The extracellular pool of halogenated nucleotides (tri-, di- and mono-phosphates) decreased as a function of time in culture, while 2-ClAdo progressively augmented (Fig. 6C). The calculated 2-ClAdo peak areas expressed in mAbsorbance Units × second were 122.48, 183.43, 658.6, respectively, in 3, 6 and 24 hr, also confirming the extracellular formation and accumulation of 2-ClAdo.

3.5. Cytotoxicity is dependent on 2-ClAdo intracellular phosphorylation

We finally investigated the effects of 2-ClATP/2-ClAdo in the presence of inhibitors of nucleoside transport and intracellular metabolism. When 2-ClATP or 2-ClAdo were challenged with the purine transport inhibitor NBTI (20 μM), survival increased from 20 to about 50% (Fig. 7). Moreover, the adenosine kinase inhibitor 5′IT, used at 20 nM, completely reverted the toxic action of 2-ClATP and 2-ClAdo (Fig. 7). Conversely and as expected, the adenosine deaminase inhibitor erythro-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA) didn't influence cell survival (Fig. 7), being 2-ClAdo a deaminase-resistant molecule [30].

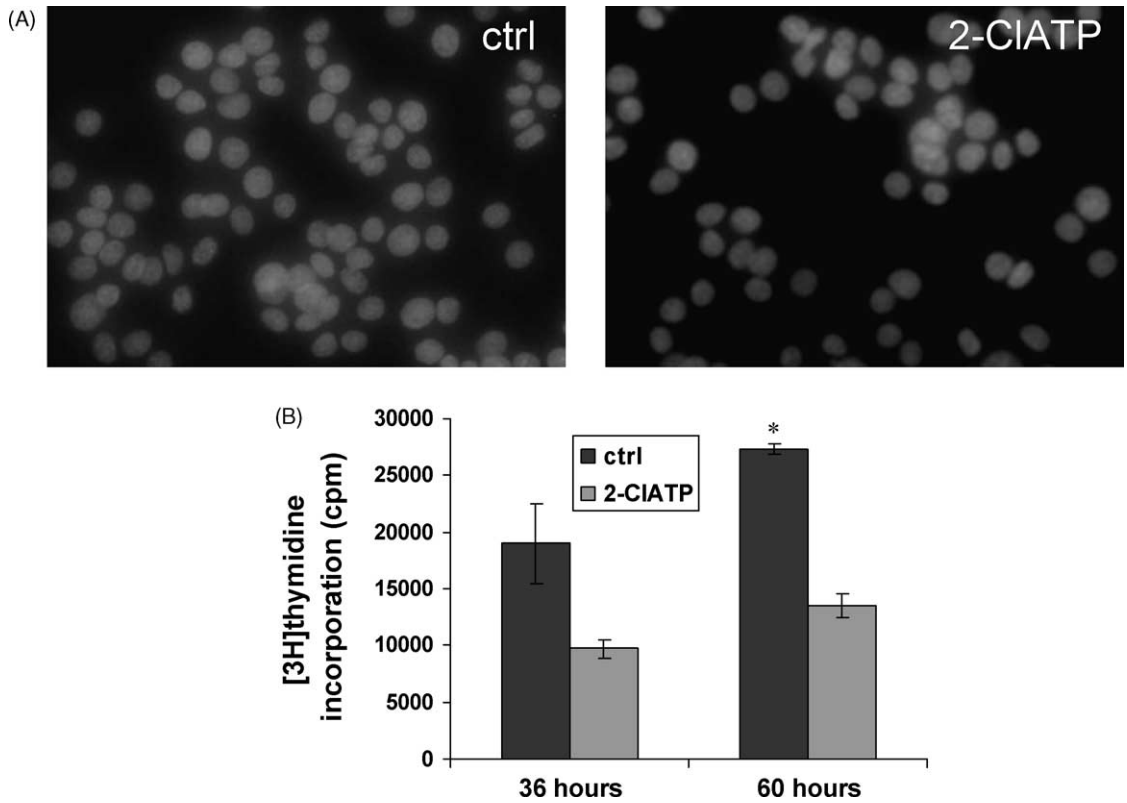


Fig. 4. 2-ClATP reduces proliferation but doesn't induce apoptosis in PC12 cells. PC12 cells were exposed to a single dose of 100 μM 2-ClATP. After 48 hr, untreated and drug-exposed cells were stained with Höechst 33258 (1 μg/mL). Nuclear chromatin condensation was visualized using Zeiss fluorescent microscope (A). Images are representative of three independent experiments. In (B) untreated and 2-ClATP-treated PC12 cells were incubated with [^{3}H]thymidine (25 μCi/mL) and after DNA isolation the incorporation of [^{3}H]thymidine was measured by liquid scintillation counting. Counts represent means ± SEM (N = 3). $^*P < 0.05$ with respect to corresponding 36 hr value, one-way variance analysis.

4. Discussion

Many recent findings reported new roles for extracellular purines and pyrimidines, acting either as growth factors to induce cell proliferation [9,10], survival [7,43] and differentiation [7,13,44,45], or as toxic agents to mediate cell death [46]. This variety of effects can be sustained by several different means. It often requires intense signaling, sometimes is dependent on multiple reinforcing ligands and it can very often be triggered by the coordination of partially dependent, overlapping or even contrasting processes, rather then by all-or-none responses. Given the ability of 2-ClATP to sustain PC12 cell differentiation in the complete absence of sera or NGF [7], we analyzed the effect of 2-ClATP on growth and survival of additional neuronal and glial cells. We have shown here that 2-ClATP reduces the number of PC12, PC12nnr5, SH-SY5Y, U87 and U373 cells in the presence of sera and this occurs according to multiple features and through several independent mechanisms. Differently from the pro-survival and neuritogenic action [7], cell death induced by 2-ClATP is P2 receptor independent. This was established by the lack of effect exerted by various P2 receptor antagonists, once established that PPADS was indirectly protective by inhibiting ecto-ATPase activities [42]. Nevertheless, the lack of effect by additional ATPases inhibitors such as Suramin and NF-023 could be explained by their restricted efficacy with all cell lines [20]. Moreover, inactivation or reduction of sera respectively prevented or attenuated cell death, while increased toxicity was instead obtained with apyrase. Finally, HPLC analysis proved the extracellular breakdown of 2-ClATP and the consequent accumulation of 2-ClAdo. Consistently with a P2 receptor-independent mechanism, several 2-ClATP derivatives and metabolites (PF5, PF10, PF4, PF1, PF3 and 2-ClAdo) also induced cell death. Moreover, toxicity of 2-ClATP was due to its hydrolysis into 2-ClAdo mainly mediated by serum ATPases, while cellular ecto-nucleotidases seemed to play only a minor role, although well expressed and characterized in PC12 cells [47].

Once generated, 2-ClAdo is known to exert toxic actions via two different mechanisms: acting on P1 receptors [25], or being intracellularly transported and metabolized by the nucleotide salvage pathway enzymes. In this last case, the overall effect is inhibition of DNA synthesis and thus cell cycle arrest and death [48]. All our data strongly support the intracellular pathway: (1) the P1 antagonists Caffeine and CGS-15493 did not prevent 2-ClATP- or 2-ClAdo-induced cell death. (2) NBTI, inhibiting the intracellular adenosine transport, was instead partially protective. This

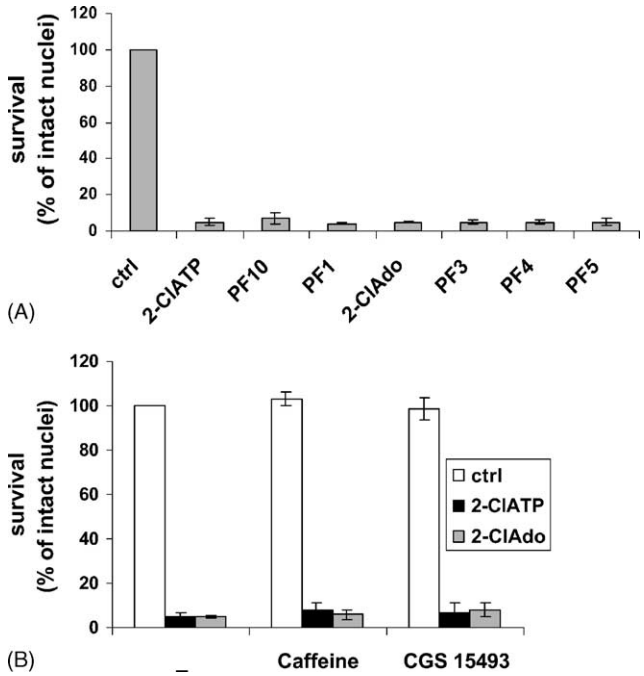


Fig. 5. 2-ClATP metabolites exert toxicity not mediated by P1 receptors. PC12nnr5 cells were exposed to the indicated purinergic compounds, all used at 100 μM, in the absence (A) or in the simultaneous presence of Caffeine (100 μM) or CGS-15493 (250 nM) (B). After 3 days of treatment, cell survival was measured by direct count of intact nuclei. Counts represent means ± SEM (N = 4) and 100% survival is referred to untreated cultures.

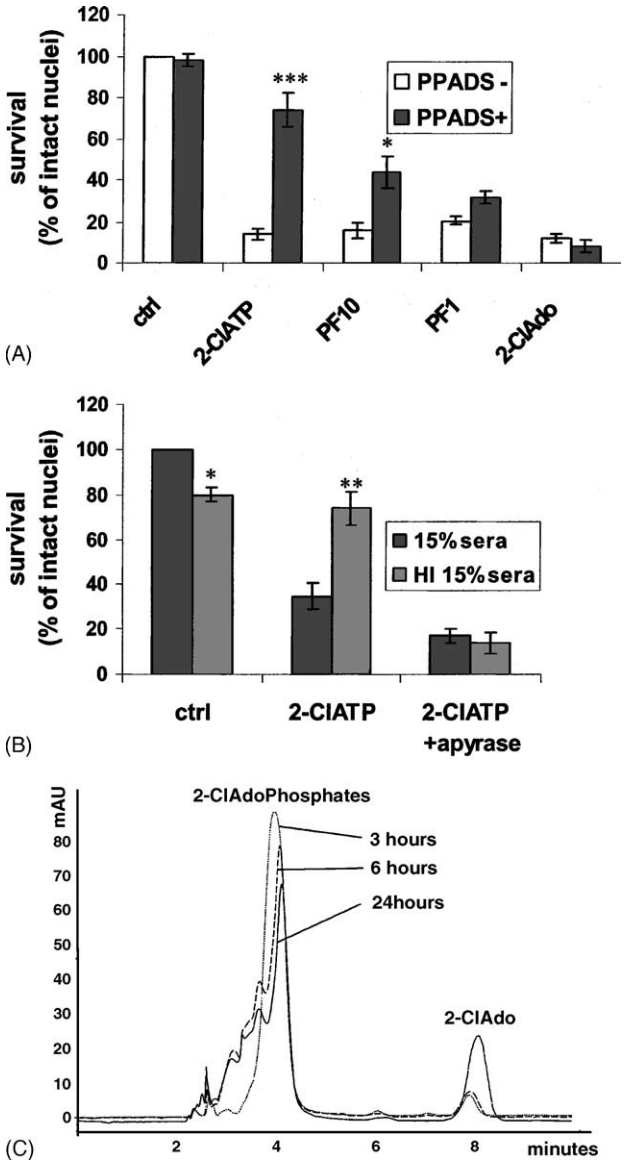


Fig. 6. 2-ClATP-toxicity is dependent on the activity of extracellular ATPases. PC12nnr5 cells were exposed to the indicated purinergic compounds, all used at 100 μM, in the presence of 15% sera (A, B), or heat-inactivated sera (HI 15% sera) (B). In (A and B), the cells were simultaneously incubated with or without 300 μM PPADS or with 5 U/mL apyrase and survival was assessed 60 hr (A) or 48 hr (B) later, by direct count of intact nuclei. Counts represent means ± SEM (N = 3). In (A), $^{***}P < 0.001$, $^{*}P < 0.05$ with respect to corresponding PPADS; in (B), $^{**}P < 0.005$, $^{*}P < 0.05$ with respect to corresponding 15% sera, one-way variance analysis. In (C), PC12nnr5 cells were exposed to 1 mM 2-ClATP and the culture media was collected after 3, 6 and 24 hr and analyzed for nucleotide breakdown by HPLC, all as described in Section 2. Values are expressed as mAbsorbance Units (mAU).

incomplete effect is probably due to the presence on PC12 cells of transporters both sensitive (*es*-type or ENT1) and insensitive (*is*-type or ENT2) to NBTI [49] and to the fact that nucleosides can be simultaneously internalized by these molecules [50]. Nevertheless, mechanisms conjoint or additional to nucleoside transport may still be involved in cell death. (3) The adenosine kinase inhibitor 5′IT prevented cell death, further confirming the intracellular mechanism of 2-ClATP and 2-ClAdo. Antiproliferative drugs are generally known to elicit toxic actions by interfering with DNA synthesis directly through the polymerases or the enzyme ribonucleotide reductase [33], causing in turn a nucleotide disequilibrium called ''pyrimidine starvation'' [48]. This last mechanism, however, seemed to play a minor role in 2-ClATP/2-ClAdo-induced toxicity, since the direct addition of uridine as an extracellular pyrimidine source didn't rescue PC12nnr5 cells from death (data not shown).

A peculiarity emerging from our data is the cytostatic effect of 2-ClATP/2-ClAdo in PC12 cells, compared to the cytotoxicity induced in PC12nnr5 cells (demonstrated by the presence of chromatin condensation and DNA fragmentation). Although highly speculative, this might rely on the main difference between the two cell variants, that is the absence of TrkA from PC12nnr5 cells. Indeed, in the absence of TrkA, the low affinity p75 NGF receptor often behaves as a pro-cell death effector [51] and its activation (by growth factors present in sera and/or by P2 receptors [52]) could therefore sustain apoptotic signals and reinforce 2-ClATP/2-ClAdo-induced toxicity. In Trk-containing PC12 cells, 2-ClATP might instead exert trophic roles through either P2 receptors [7] or TrkA trans-activation by its adenosine-metabolites [53]. This would occur simultaneously to the toxicity induced by intracellular 2-ClAdo and the overall effect would be cell cycle arrest, but not cell death.

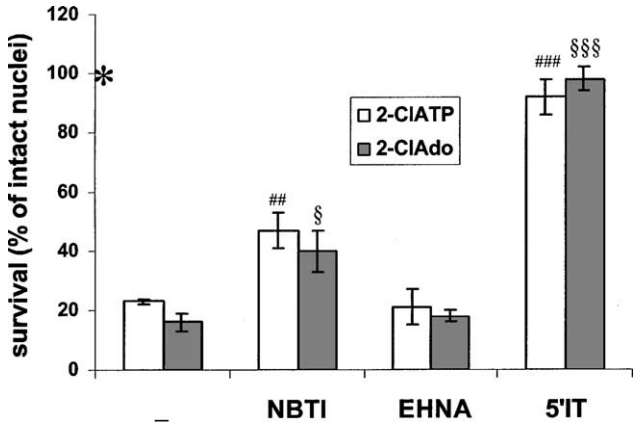


Fig. 7. The toxic action of 2-ClATP and 2-ClAdo depends on nucleoside intracellular transport and re-phosphorylation. PC12nnr5 cells were cultured with 2-ClATP or 2-ClAdo (both used at 100 μM) in the absence or the presence of 20 μM NBTI, 10 μM EHNA or 20 nM 5′IT. Cell survival was assessed 2–3 days later, by direct count of intact nuclei. Counts represent means ± SEM (N = 3) and 100% survival, indicated by the asterisk, is referred to untreated cultures. $^{###}P < 0.001$, $^{##}P < 0.005$ with respect to 2-ClATP, $^{§§§}P < 0.001$, $^{§}P < 0.05$ with respect to 2-ClAdo, one-way variance analysis.

In conclusion, from the present study we evince that the proliferative (with sera) or quiescent (without sera) cellular phase, the specific receptor repertoire (presence or absence of TrkA), pattern of nucleoside transporters (NBTI sensitive or insensitive) and degrading enzymes and, lastly, the peculiar extracellular environment are all parameters contributing to the final outcome of extracellular purines administration, in terms of either cell death, survival or differentiation. This unpredictability of effects is particularly noteworthy *in vivo*, when all the biological parameters concerned with extracellular purines cannot be easily known or controlled.

Acknowledgments

The research presented was supported by Progetto Coordinato CNR Agenzia 2000 and by Cofinanziamento MURST 2001/2002. The useful suggestions and critical reading of the manuscript by Prof. Sauro Vittori and the professional style editing by Mrs. Catherine Wrenn are gratefully acknowledged.

References

[1] Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 2001;53:527–52.
[2] Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev 1998;50:415–92.
[3] Hyde RJ, Cass CE, Young JD, Baldwin SA. The ENT family of eukaryote nucleoside and nucleobase transporters: recent advances in the investigation of structure/function relationships and the identification of novel isoforms. Mol Membr Biol 2001;18:53–63.
[4] Zimmermann H, Braun N. Ecto-nucleotidases—molecular structures, catalytic properties, and functional roles in the nervous system. Prog Brain Res 1999;120:371–85.
[5] Cristalli G, Costanzi S, Lambertucci C, Lupidi G, Vittori S, Volpini R, Camaioni E. Adenosine deaminase: functional implications and different classes of inhibitors. Med Res Rev 2001;2:105–28.
[6] Zimmermann H. Biochemistry, localization and functional roles of ecto-nucleotidases in the nervous system. Prog Neurobiol 1996;49: 589–618.
[7] D'Ambrosi N, Murra B, Cavaliere F, Amadio S, Bernardi G, Burnstock G, Volontè C. Interaction between ATP and nerve growth factor signalling in the survival and neuritic outgrowth from PC12 cells. Neuroscience 2001;108:527–34.
[8] Ryten M, Dunn PM, Neary JT, Burnstock G. ATP regulates the differentiation of mammalian skeletal muscle by activation of a P2X5 receptor on satellite cells. J Cell Biol 2002;158:345–55.
[9] Sanches G, de Alencar LS, Ventura AL. ATP induces proliferation of retinal cells in culture via activation of PKC and extracellular signal-regulated kinase cascade. Int J Dev Neurosci 2002;20:21–7.
[10] Rathbone MP, Middlemiss PJ, Gysbers JW, Andrew C, Herman MA, Reed JK, Ciccarelli R, Di Iorio P, Caciagli F. Trophic effects of purines in neurons and glial cells. Prog Neurobiol 1999; 59:663–90.
[11] Lee WK, Choi SW, Lee HR, Lee EJ, Lee KH, Kim HO. Purinoceptor-mediated calcium mobilization and proliferation in HaCaT keratinocytes. J Dermatol Sci 2001;25:97–105.
[12] Chung T, Crilly KS, Anderson WH, Mukherjee JJ, Kiss Z. ATP-dependent choline phosphate-induced mitogenesis in fibroblasts involves activation of pp70 S6 kinase and phosphatidylinositol 3′-kinase through an extracellular site. Synergistic mitogenic effects of choline phosphate and sphingosine 1-phosphate. J Biol Chem 1997; 272:3064–72.
[13] Batra S, Fadeel I. Release of intracellular calcium and stimulation of cell growth by ATP and histamine in human ovarian cancer cells (SKOV-3). Cancer Lett 1994;77:57–63.
[14] Burnstock G. Purinergic signaling and vascular cell proliferation and death. Arterioscler Thromb Vasc Biol 2002;22:364–73.
[15] Amadio S, D'Ambrosi N, Cavaliere F, Murra B, Sancesario G, Bernardi G, Burnstock G, Volontè C. P2 receptor modulation and cytotoxic function in cultured CNS neurons. Neuropharmacology 2002;42:489–501.
[16] Ryu JK, Kim J, Choi SH, Oh YJ, Lee YB, Kim SU, Jin BK. ATP-induced *in vivo* neurotoxicity in the rat striatum via P2 receptors. Neuroreport 2002;13:1611–5.
[17] Wiendl HS, Schneider C, Ogilvie A. Nucleotide metabolizing ectoenzymes are upregulated in A431 cells periodically treated with cytostatic ATP leading to partial resistance without preventing apoptosis. Biochim Biophys Acta 1998;1404:282–98.
[18] Schneider C, Wiendl H, Ogilvie A. Biphasic cytotoxic mechanism of extracellular ATP on U-937 human histiocytic leukemia cells: involvement of adenosine generation. Biochim Biophys Acta 2001;1538: 190–205.
[19] Bradley KK, Bradley ME. Purine nucleoside-dependent inhibition of cellular proliferation in 1321N1 human astrocytoma cells. J Pharmacol Exp Ther 2001;299:748–52.
[20] Schrier SM, Florea BI, Mulder GJ, Nagelkerke JF, IJzerman AP. Apoptosis induced by extracellular ATP in the mouse neuroblastoma cell line N1E-115: studies on involvement of P2 receptors and adenosine. Biochem Pharmacol 2002;63:1119–26.
[21] Di Virgilio F, Chiozzi P, Falzoni S, Ferrari D, Sanz JM, Venketaraman V, Baricordi OR. Cytolytic P2X purinoceptors. Cell Death Differ 1998;5:191–9.
[22] Ferrari D, Chiozzi P, Falzoni S, Dal Susino M, Melchiorri L, Baricordi OR, Di Virgilio F. Extracellular ATP triggers IL-1 beta release by activating the purinergic P2Z receptor of human macrophages. J Immunol 1997;159:1451–8.

[23] Inoue K. Microglial activation by purines and pyrimidines. Glia 2002;40:156–63.
[24] Merighi S, Mirandola P, Milani D, Varani K, Gessi S, Klotz KN, Leung E, Baraldi PG, Borea PA. Adenosine receptors as mediators of both cell proliferation and cell death of cultured human melanoma cells. J Invest Dermatol 2002;119:923–33.
[25] Di Iorio P, Kleywegt S, Ciccarelli R, Traversa U, Andrew CM, Crocker CE, Werstiuk ES, Rathbone MP. Mechanisms of apoptosis induced by purine nucleosides in astrocytes. Glia 2002;38:179–90.
[26] Kohno Y, Sei Y, Koshiba M, Kim HO, Jacobson KA. Induction of apoptosis in HL-60 human promyelocytic leukemia cells by adenosine A (3) receptor agonists. Biochem Biochem Biophys Res Commun 1996;219:904–10.
[27] Wakade TD, Palmer KC, McCauley R, Przywara DA, Wakade AR. Adenosine-induced apoptosis in chick embryonic sympathetic neurons: a new physiological role for adenosine. J Physiol 1995;488: 123–38.
[28] Rounds S, Yee WL, Dawicki DD, Harrington E, Parks N, Cutaia MV. Mechanism of extracellular ATP- and adenosine-induced apoptosis of cultured pulmonary artery endothelial cells. Am J Physiol 1998; 275:L379–88.
[29] Picher M, Sevigny J, D'Orleans-Juste P, Beaudoin AR. Hydrolysis of P2-purinoceptor agonists by a purified ectonucleotidase from the bovine aorta, the ATP-diphosphohydrolase. Biochem Pharmacol 1996;51:1453–60.
[30] Koshiba M, Kosaka H, Nakazawa T, Hayashi N, Saura R, Kitamura N, Kumagai S. 2-Chloroadenosine but not adenosine induces apoptosis in rheumatoid fibroblasts independently of cell surface adenosine receptor signalling. Br J Pharmacol 2002;135:1477–86.
[31] Ceruti S, Giammarioli AM, Camurri A, Falzano L, Rufini S, Frank C, Fiorentini C, Malorni W, Abbracchio MP. Adenosine- and 2-chloroadenosine-induced cytopathic effects on myoblastic cells and myotubes: involvement of different intracellular mechanisms. Neuromuscul Disord 2000;10:436–46.
[32] Mey U, Strehl J, Gorschluter M, Ziske C, Glasmacher A, Pralle H, Schmidt-Wolf I. Advances in the treatment of hairy-cell leukaemia. Lancet Oncol 2003;4:86–94.
[33] Iwasaki H, Huang P, Keating MJ, Plunkett W. Differential incorporation of ara-C, gemcitabine, and fludarabine into replicating and repairing DNA in proliferating human leukemia cells. Blood 1997;90:270–8.
[34] Dubey RK, Gillespie DG, Zacharia LC, Mi Z, Jackson EK. A(2b) receptors mediate the antimitogenic effects of adenosine in cardiac fibroblasts. Hypertension 2001;37:716–21.
[35] Ravi RG, Kim HS, Servos J, Zimmermann H, Lee K, Maddileti S, Boyer JL, Harden TK, Jacobson KA. Adenine nucleotide analogues locked in a Northern methanocarba conformation: enhanced stability and potency as P2Y(1) receptor agonists. J Med Chem 2002;45: 2090–100.
[36] Camaioni E, Boyer JL, Mohanram A, Harden TK, Jacobson KA. Deoxyadenosine bisphosphate derivatives as potent antagonists at P2Y1 receptors. J Med Chem 1998;41:183–90.
[37] Yoshikawa M, Kato T, Takenishi T. A novel method for phosphorylation of nucleosides to 5′-nucleotides. Tetrahedron Lett 1967;50:5065–8.
[38] Cristalli G, Mills DC. Identification of a receptor for ADP on blood platelets by photoaffinity labelling. Biochem J 1993;291:875–81.
[39] Rukenstein A, Rydel RE, Greene LA. Multiple agents rescue PC12 cells from serum-free cell death by translation- and transcription-independent mechanisms. J Neurosci 1991;11:2552–63.
[40] Green SH, Rydel RE, Connolly JL, Greene LA. PC12 cell mutants that possess low- but not high-affinity nerve growth factor receptors neither respond to nor internalize nerve growth factor. J Cell Biol 1986;102: 830–43.
[41] Loeb DM, Maragos J, Martin-Zanca D, Chao MV, Parada LF, Greene LA. The trk proto-oncogene rescues NGF responsiveness in mutant NGF-nonresponsive PC12 cell lines. Cell 1991;66:961–6.
[42] Chen BC, Lee CM, Lin WW. Inhibition of ecto-ATPase by PPADS, suramin and reactive blue in endothelial cells, C6 glioma cells and RAW 264.7 macrophages. Br J Pharmacol 1996;119:1628–34.
[43] Fujita N, Kakimi M, Ikeda Y, Hiramoto T, Suzuki K. Extracellular ATP inhibits starvation-induced apoptosis via P2X2 receptors in differentiated rat pheochromocytoma PC12 cells. Life Sci 2000;66:1849–59.
[44] Gysbers JW, Guarnieri S, Mariggio MA, Pietrangelo T, Fano G, Rathbone MP. Extracellular guanosine 5′triphosphate enhances nerve growth factor-induced neurite outgrowth via increases in intracellular calcium. Neuroscience 2000;96:817–24.
[45] D'Ambrosi N, Cavaliere F, Merlo D, Milazzo L, Mercanti D, Volontè C. Antagonists of P2 receptors prevent NGF-dependent neuritogenesis in PC12 cells. Neuropharmacology 2000;39:1083–94.
[46] Agteresch HJ, Dagnelie PC, van den Berg JW, Wilson JH. Adenosine triphosphate: established and potential clinical applications. Drugs 1999;58:211–32.
[47] Vollmayer P, Koch M, Braun N, Heine P, Servos J, Israr E, Kegel B, Zimmermann H. Multiple ecto-nucleotidases in PC12 cells: identification and cellular distribution after heterologous expression. J Neurochem 2001;78:1019–28.
[48] Pastor-Anglada M, Felipe A, Casado FJ. Transport and mode of action of nucleoside derivatives used in chemical and antiviral therapies. Trends Pharmacol Sci 1998;19:424–30.
[49] Kobayashi S, Zimmermann H, Millhorn DE. Chronic hypoxia enhances adenosine release in rat PC12 cells by altering adenosine metabolism and membrane transport. J Neurochem 2000;74:621–32.
[50] Baldwin SA, Mackey JR, Cass CE, Young JD. Nucleoside transporters: molecular biology and implications for therapeutic development. Mol Med Today 1999;5:216–24.
[51] Casaccia-Bonnefil P, Carter BD, Dobrowsky RT, Chao MV. Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature 1996;383:716–9.
[52] Cavaliere F, Sancesario G, Bernardi G, Volonté C. Extracellular ATP and nerve growth factor intensify hypoglycemia-induced cell death in primary neurons: role of P2 and NGFRp75 receptors. J Neurochem 2002;83:1129–38.
[53] Lee FS, Chao V. Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proc Natl Acad Sci USA 2001;98:3555–60.