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Mechanisms of cell death induced by 2-chloroadenosine in leukemic B-cells

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ARTICLE INFO

Article history:
Received 2 November 2007
Accepted 17 December 2007

Keywords:
2-Chloroadenosine
Mitochondrial pathway
ATP
Mcl-1
B-CLL

ABSTRACT

2-Chloroadenosine (2-CAdo) is an adenosine deaminase-resistant analogue of adenosine, widely used as an adenosine receptor agonist. This compound has been shown to induce apoptosis in several cell types either via activation of adenosine receptors or via intracellular metabolism. However, the molecular mechanisms of 2-CAdo-induced apoptosis are unclear. Here, we analyzed the effects of 2-CAdo in the leukemia cell line EHEB. 2-CAdo was found to induce apoptosis in EHEB cells, as shown by caspase-3 activation, DNA fragmentation, poly(ADP-ribose) polymerase (PARP) cleavage and phosphatidylserine exposure. Cytotoxicity of 2-CAdo was completely suppressed by 5-iodotubercidin, an adenosine kinase inhibitor, indicating that apoptosis induced by 2-CAdo was the result of its intracellular metabolism. Accordingly, we found that 2-CAdo was efficiently converted into 2-chloroATP. In parallel, a decrease of intracellular ATP concentration as well as a general inhibition of macromolecular synthesis, involving DNA, RNA and protein synthesis, was observed. Moreover, 2-CAdo induced cytochrome *c* release into the cytosol, indicating activation of the intrinsic pathway of apoptosis. This was found associated with a decline in Mcl-1 protein level and p53-independent. Inhibition of AMP deaminase by coformycin markedly prevented ATP depletion, and also significantly reduced 2-CAdo cytotoxicity and caspase-3 activation. In conclusion, our data show that intracellular metabolism of 2-CAdo can lead to activation of the intrinsic pathway of apoptosis and that ATP depletion, in addition to the accumulation of the triphosphate analogue, contributes to 2-CAdo-induced apoptosis.

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1. Introduction

2-Chloroadenosine (2-CAdo) is an adenosine deaminase-resistant analogue of adenosine [1], widely used as an adenosine receptor agonist [2]. However, studies have shown that 2-CAdo may be phosphorylated intracellularly by adenosine kinase and converted to 5′-phosphorylated derivatives [3,4]. This implies that biological effects of 2-CAdo could result not only from activation of extracellular receptors, but also from intracellular uptake.

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Abbreviations: 2-CAdo, 2-chloroadenosine; CdA, 2-chloro-2′-deoxyadenosine; 2-CATP, 2-chloroATP; CLL, chronic lymphocytic leukaemia; HPLC, high-performance liquid chromatography; ITu, 5-iodotubercidin; NBTI, nitrobenzylthioinosine; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide.

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doi:10.1016/j.bcp.2007.12.007

2-CAdo has been shown to induce apoptosis in several cell types including mouse [5] and human thymocytes [6,7], rat primary astrocytes [8], human astrocytoma cells [9], human peripheral blood mononuclear cells [10], human T-lymphocytes [11], myoblastic cells and myotubes [12,13], rheumatoid synoviocytes [14] and a prostate cancer cell line [15]. Depending on cell type, 2-CAdo-induced apoptosis has been attributed to activation of extracellular adenosine (A_{2A} or A_3) receptors [5–8], to intracellular metabolism [9,12,14,15], or both [10]. Nevertheless, little is known about the ensuing molecular mechanisms by which 2-CAdo induces apoptosis. In T-lymphocytes, DNA cleavage following activation of adenosine receptors was reported to be mediated by cyclic AMP-dependent mechanisms [5] or to result primarily from an elevation of intracellular Ca^{2+} [6]. A role of caspase-3 in 2-CAdo-induced apoptosis has also been demonstrated [14,16], but the process initiating the activation of caspase-3 is not yet well understood. In astrocytoma cells, an atypical pathway involving caspase-2, as an initiator caspase, and effector caspase-3 has been observed, whereas mitochondrial depolarization, release of cytochrome *c* and activation of caspase-9 or caspase-8 were not detected [16]. In prostate cancer cells, cytotoxicity of 2-CAdo was explained by an arrest of DNA synthesis due to an inhibition of deoxynucleoside triphosphate synthesis [15].

A number of nucleoside analogues (in particular 2′-deoxyderivatives) are apoptosis-inducing agents, and used clinically as anti-cancer drugs. Among the purine analogues, fludarabine and 2-chloro-2′-deoxyadenosine (CdA, cladribine) have important therapeutic activity in indolent lymphoid malignancies and particularly in B-cell chronic lymphocytic leukaemia (B-CLL) [17]. Although the mechanism of action of the deoxyadenosine analogue CdA has been thoroughly analyzed, the potential apoptotic effect of the related analogue 2-CAdo has not been investigated in leukemic cells. In this study, we investigated the effects of 2-CAdo in EHEB cells, a B-CLL cell line [18], and in B-CLL lymphocytes isolated from patients. Results show that 2-CAdo is cytotoxic for leukemic B-cells and induces apoptosis after intracellular metabolism. We further examined the molecular mechanisms at the basis of this uptake-mediated effect.

2. Materials and methods

2.1. Reagents

2-CAdo, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), nitrobenzylthioinosine (NBTI), horseradish peroxidase conjugated anti-rabbit (or anti-mouse) antibodies and anti-β-actin antibody were purchased from Sigma–Aldrich. CdA was synthesized and supplied by Prof. J. Marchand (Laboratory of Organic Chemistry, Université catholique de Louvain, Louvain-la-Neuve). [Methyl-^{3}H]-thymidine (76 Ci/mmole), [5-^{3}H]uridine (28 Ci/mmole) and L-[4,5-^{3}H]leucine (160 Ci/mmole), Ficoll-Paque PLUS (density: 1.077), Hybond C-extra membranes, ECL enhanced chemiluminescence kit were from GE Healthcare. FCS and penicillin-streptomycin were purchased from BioWhittaker Europe. RPMI 1640 and all tissue culture reagents were from Gibco/Invitrogen. Coformycin was from Calbiochem. Ac-DEVD-AMC (Ac-Asp-Glu-Val-Asp-AMC) and AMC (7-Amino-4-methylcoumarin) were purchased from Alexis Biochemicals. The annexin V-FITC apoptosis detection kit was from Pharmingen. 5-Iodotubercidin (ITu) was from Research Biochemicals Inc. Other chemicals, materials and reagents were from Sigma, Calbiochem or Bio-Rad Laboratories.

2.2. Cell preparation and incubation

EHEB cells, a B-cell line established from B-CLL lymphocytes [18], were cultured in RPMI-1640 with Glutamax, supplemented with 10% heat-inactivated FCS, at 37 °C in an atmosphere of 5% CO_2 in air. Peripheral blood from B-CLL patients was obtained after written informed consent in accordance with the hospital ethics committee. All B-CLL patients had a diagnosis confirmed by cytological and immunological studies. Mononuclear cells were isolated by Ficoll-Paque sedimentation, washed with PBS, resuspended in RPMI-1640 supplemented with 10% FCS and 1% penicillin-streptomycin, and cultured like EHEB cells. Hydrophobic inhibitors were dissolved in DMSO and equal amount of DMSO was added in control cells.

2.3. Analysis of cytotoxicity

EHEB cells (4×10^4 cells/well) and B-CLL cells (2×10^6 cells/well) were incubated with or without the indicated drugs in 96-well plates. After 24 or 72 h, cell viability was measured using the MTT assay as described in Ref. [19]. Controls and 2-CAdo concentrations were set up in triplicate. The absorbance of each well was measured at 540 nm with a Multiwell Scanning Spectrophotometer (Molecular Devices). Cell viability, expressed as a percentage, was calculated by the equation: (mean absorbance of treated well/mean absorbance of control wells) $\times$ 100. The IC_{50} was determined graphically.

2.4. Analysis of apoptosis by flow cytometry

Apoptosis was investigated by dual annexin V and propidium iodide (PI) staining using a FACSCalibur (Beckton Dickinson) flow cytometer. After incubation, 5×10^6 cells were collected, washed twice with PBS and processed according to the annexin V-FITC apoptosis detection kit (Beckton Dickinson), followed by flow cytometer analysis. Annexin V binds to phosphatidylserine that becomes exposed on the plasma membrane of cells undergoing apoptosis. This allows for the discrimination of living cells (unstained with either fluorochrome) from early apoptotic cells (stained only with annexin V) and late apoptotic cells (stained with both annexin V and PI). Flow cytometric two-parameter dot plots and quadrant statistics were generated by CellQuest software (Beckton Dickinson).

2.5. Assay for caspase-3 activity

After incubation, 4×10^6 EHEB cells were washed twice in ice-cold PBS. Cell pellets were resuspended in 125 μl of ice-cold buffer containing 10 mM Tris–HCl, pH 7.4, 10 mM EGTA, 1 mM EDTA, 2 mM dithiothreitol, 1% Nonidet-P40, protease inhibitors (5 mM benzamidine, 1 mM *p*-toluenesulfonyl fluoride, 5 μg/ml leupeptin and antipain) and lysed for 20 min on ice.

Cell lysates were centrifugated at 16,100 × g for 10 min at 4 °C and the supernatants were used to determine the activity of caspase-3 by fluorometric assay. Cell extracts (100 μl, containing ~250 μg of protein) were incubated at 37 °C in 50 mM Tris–HCl, pH 7.4, dithiothreitol 5 mM and 37.5 μM Ac-DEVD-AMC, used as a fluorogenic substrate. Release of AMC was measured using a spectrofluorometer (Bio-Rad) with a pair of excitation/emission wavelength of 360/460 nm. Caspase-3 activity was calculated by converting fluorescence units into micromoles of AMC released per min per mg of protein using a standard curve obtained from free AMC.

2.6. Measurement of intracellular nucleotides

After incubation in the presence of increasing concentrations of 2-CAdo, EHEB cells (1×10^7 cells/condition) were washed twice in ice-cold PBS before addition of 350 μl of 1 N $HClO_4$ to the cell pellet. The supernatant obtained after centrifugation was neutralized with 3 M K_2CO_3 and used for nucleotide analysis. Nucleotides were separated by high-performance liquid chromatography as described previously [19]. The nucleotides were quantified by electronic integration with reference to external standards. The nucleotide analogue 2-chloroATP (2-CATP) was identified by comparing its retention profile and absorption spectrum with those of an authentic standard. The intracellular concentration of nucleotides was calculated using a cellular volume of 1.91 pl for EHEB cells, assuming that the nucleotides were uniformly distributed in a total cell volume. The volume of cells, assumed to be spherical, was calculated on the basis of a mean diameter of 14.4 ± 1.21 μm ($n = 10$), which was determined with an optical microscope (Zeiss, West Germany) and a micrometric screw.

2.7. Measurement of DNA, RNA and protein synthesis

DNA, RNA or protein synthesis was investigated by measuring the incorporation of [methyl-^{3}H]-thymidine, [5-^{3}H]-uridine and L-[4,5-^{3}H]leucine, respectively, into the acid-insoluble cellular fraction. After pre-incubation with 2-CAdo for the indicated time, cells ($1,6 \times 10^5$ cells in 800 μl) were incubated for 60 min with 4 μCi of the appropriate labeled precursor. Subsequently, 200 μl of cell suspension were taken in triplicate, harvested on a multiscreen assay system 96-well filtration plate (Millipore; catalogue number MAGV N22), lysed with 200 μl of TCA 8% and washed successively with 2 × 200 μl of TCA 8%, 3 × 100 μl of H_2O and finally with 2 × 100 μl of 70% ethanol. Remaining radioactivity associated with filters was counted. Results are expressed in percentage of radioactivity incorporated in untreated cells.

2.8. Western blot analysis

PARP cleavage analysis was performed as described in Ref. [20]. For the analysis of p53 and of pro- or anti-apoptotic proteins, 1×10^7 cells were washed twice with ice-cold PBS and lysed on ice for 20 min in 450 μl of a lysis buffer containing 20 mM HEPES, pH 7.9, 150 mM NaCl, 1 mM $MgCl_2$, 5 mM EDTA, pH 8, 1% Nonidet P-40, 0.1% SDS, 50 mM NaF, protease inhibitors (5 mM benzamidine, 1 mM *p*-toluenesulfonyl fluoride, 5 μg/ml leupeptin and antipain) and 1 mM orthovanadate freshly added. Lysates were centrifuged at 16,100 × g for 10 min at 4 °C, and the supernatants were stored at −80 °C until use. Aliquots of cell extracts (50–80 μg) were subjected to SDS-PAGE in gels containing 12% (w/v) acrylamide and transferred to Hybond C-extra membranes. Membranes were blocked in PBS with 5% (w/v) fat-free milk powder for 1 h at room temperature and then probed with primary antibodies in PBS-T (Tween 0.1%, w/v) for 1 h at room temperature or overnight at 4 °C. After extensive washing in PBS-T to remove the primary antibody, the membranes were incubated for 1 h at room temperature with the appropriate secondary antibody coupled to horseradish peroxidase (1/10,000). After further extensive washing in PBS-T and a last in PBS, the blots were developed using enhanced chemiluminescence. The primary antibodies used were from pro-survival Bcl-2 family and pro-apoptosis bcl-2 family antibody sampler kit (Cell Signaling). Equal loading of protein was assessed using β-actin.

For cytochrome *c* analysis, 1×10^7 cells were washed in ice-cold PBS and lysed for 2.5 min in 100 μl of ice-cold lysis buffer (25 mM Tris–HCl pH 6.8, 250 mM sucrose, 1 mM EDTA and 200 μg/ml digitonin). Then, cell lysates were centrifuged for 10 min at 16,100 × g at 4 °C and proteins from the supernatant (60 μg) were resolved by SDS-PAGE (12%). Cytochrome *c* was analyzed by Western blot using a mouse anti-cytochrome *c* antibody, as described above.

2.9. Miscellaneous

High molecular weight DNA fragmentation was analyzed by pulsed field gel electrophoresis as described in Ref. [21]. The protein content of cell extracts was measured by the method of Bradford [22], using BSA as standard. All results of repeated experiments are given as the means ± S.E.M. Significance was estimated by the paired two-tailed Student's *t*-test.

3. Results

3.1. 2-CAdo induces apoptotic cell death in EHEB cells

EHEB cells were incubated for 72 h with increasing concentrations of 2-CAdo and cell survival was evaluated by the MTT reduction assay. As illustrated in Fig. 1A, 2-CAdo decreased cell viability with a half-maximal inhibitory concentration (IC_{50}) of 10.7 ± 0.6 μM ($n = 3$). To understand the mode of cell death induced by 2-CAdo in EHEB cells, we first measured activity of caspase-3, a key mediator of apoptosis in mammalian cells. We found that treatment with 2-CAdo led to a time- and dose-dependent activation of caspase-3, which was already detectable after 6 h and reached a maximal value after 24 h (Fig. 1B). These results were confirmed by immunoblotting that showed cleavage of the enzymatically inactive procaspase-3 into the active caspase-3 (results not shown). High molecular weight DNA fragments of 50 kb (Fig. 1C) and PARP cleavage (Fig. 1D), two other specific manifestations of apoptosis, were also detected after a 24 h-incubation with 2-CAdo. Finally, analysis of EHEB cells for annexin V and PI staining by flow cytometry showed a significant increase of the percentage of both early apoptotic (annexin V positive, PI negative) and late apoptotic (annexin V positive, PI positive)

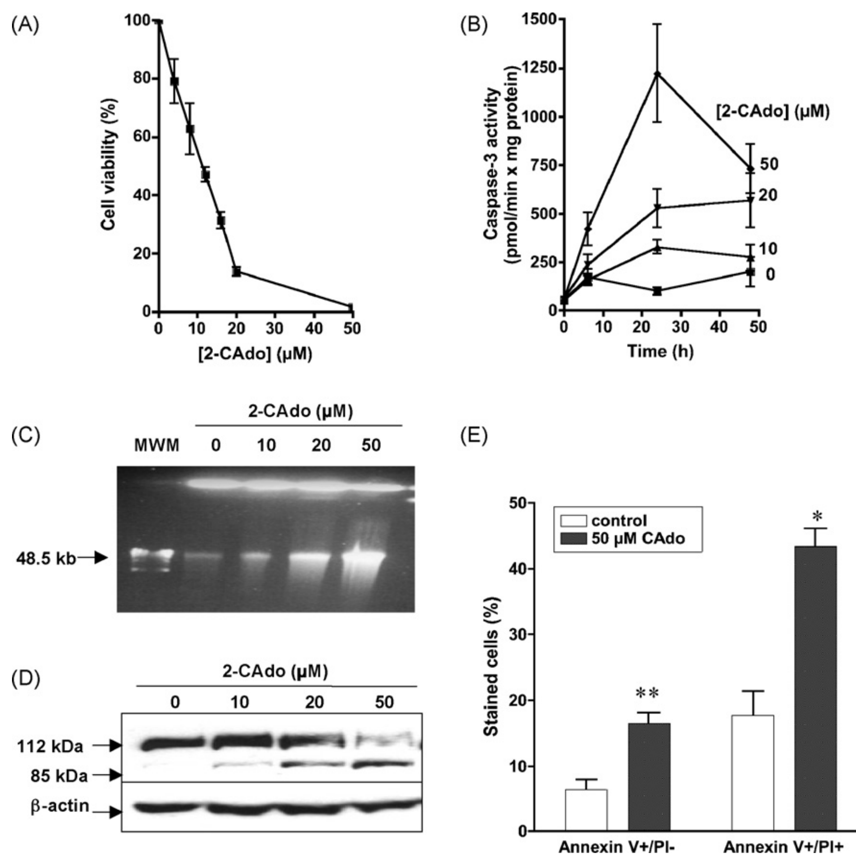

Fig. 1 – Characteristics of 2-CAdo-induced cell death in EHEB cells. (A) Cell viability measured by the MTT assay after a 72 h-incubation with increasing concentrations of 2-CAdo. Results are means ± S.E.M. of three separate experiments. (B) Caspase-3 activity determined after 6, 24 and 48 h of incubation with the indicated concentrations of 2-CAdo. Results are means ± S.E.M. of three separate experiments. (C) High molecular weight DNA fragmentation. EHEB cells were incubated for 24 h with the indicated concentrations of 2-CAdo. MWM, molecular weight markers. (D) PARP cleavage. EHEB cells were incubated for 24 h with the indicated concentrations of 2-CAdo. β-Actin was used as a loading control. (E) Annexin V and PI staining. EHEB cells were incubated for 24 h with or without 50 μM 2-CAdo. Results are means ± S.E.M. of three separate experiments. Annexin V+/PI− cells and annexin V+/PI+ cells represent early apoptotic cells and late apoptotic cells, respectively. Significance relative to the absence of 2-CAdo: *P < 0.05; **P < 0.01.

cells after a 24 h-incubation with 50 μM 2-CAdo (Fig. 1E). Taken together, these results demonstrate that 2-CAdo-induced cell death in EHEB cells involves apoptosis.

3.2. *Cytotoxicity of 2-CAdo requires 2-CAdo metabolism*

In order to elucidate whether the cytotoxic effect of 2-CAdo was mediated by activation of adenosine receptors or by intracellular metabolism, we analyzed the effect of 50 μM 2-CAdo in EHEB cells incubated in the presence of NBTI, a nucleoside transport inhibitor [23], or in the presence of ITu, a strong inhibitor of adenosine kinase [24]. As shown in Fig. 2A, 2-CAdo cytotoxicity was strongly reduced by NBTI, although the latter displayed slight toxicity by itself ($P < 0.001$), and was completely suppressed by ITu. Similarly, caspase-3 activation induced by 50 μM 2-CAdo was strongly reduced in the presence of NBTI and fully prevented by ITu (Fig. 2B). These results indicate that the induction of cell death by 2-CAdo is related to its phosphorylation within the cells rather than to its interaction with extracellular adenosine receptors.

3.3. *2-CAdo is phosphorylated intracellularly and provokes ATP depletion*

Since cytotoxicity of 2-CAdo requires 2-CAdo metabolism, we examined the ability of EHEB cells to accumulate phosphorylated derivatives of 2-CAdo. EHEB cells were incubated for various times with 50 μM 2-CAdo and their nucleotide content was analyzed by HPLC. As depicted in Fig. 3A, 2-CATP time-dependently accumulated inside the cells, reaching a maximal concentration after 8 h, close to 1 mM. Subsequently, the level of 2-CATP slowly decreased to reach a concentration of ~600 μM after 24 h. Mono- and diphosphates of 2-CAdo were below detection limit (~15 μM). Concomitantly with the accumulation of 2-CATP, a time-dependent decline in intracellular ATP content was observed. Both ADP and AMP decreased in parallel with ATP (results not shown), indicating that 2-CAdo induced a depletion of the total adenine nucleotide pool. The latter was reduced by ~80% after a 24 h-incubation with 50 μM 2-CAdo. Fig. 2B shows the dose-dependent accumulation of 2-CATP and depletion of ATP upon

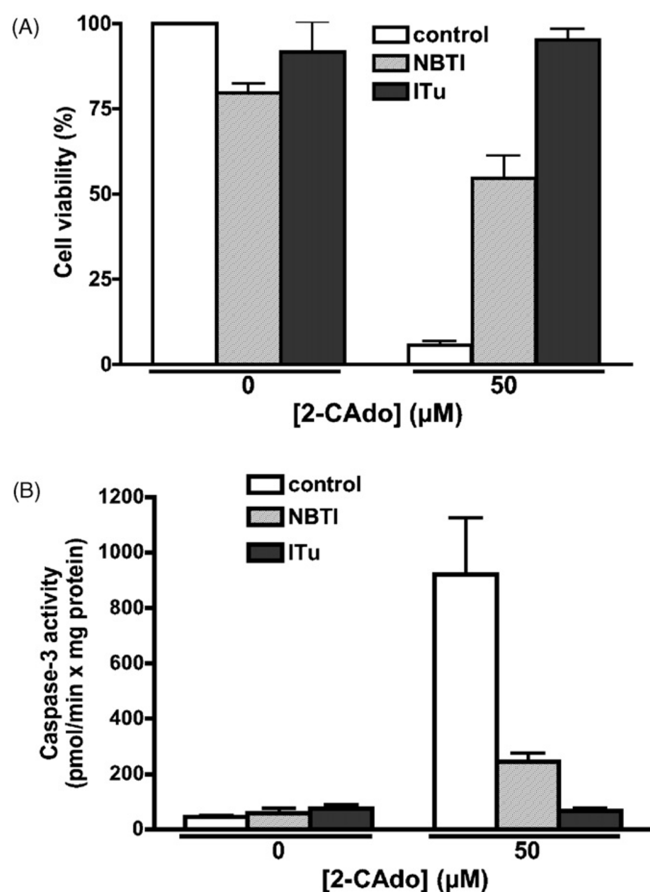

Fig. 2 – Protection of EHEB cells from 2-CAdo by NBTI and ITu. Cells were incubated in the absence or in the presence of 50 μM 2-CAdo with or without 10 μM NBTI or 0.1 μM ITu. (A) Cell viability measured by the MTT assay after a 72 h-incubation. Results are means ± S.E.M. of six (for NBTI) or four (for ITu) separate experiments. (B) Caspase-3 activity determined after a 24 h-incubation. Results are means ± S.E.M. of three separate experiments.

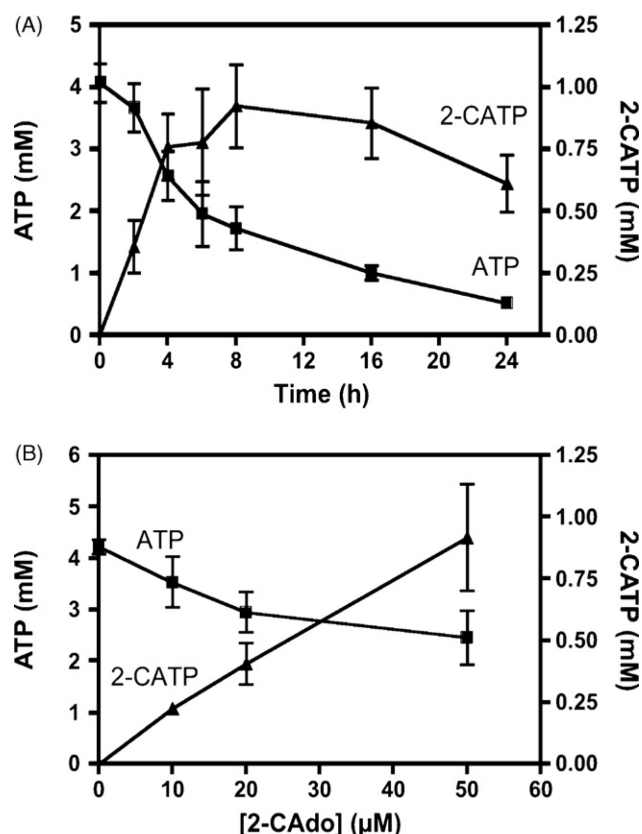

Fig. 3 – Accumulation of 2-CATP and depletion of ATP in EHEB cells incubated with 2-CAdo. (A) Cells were incubated with 50 μM 2-CAdo for the indicated times. (B) Cells were incubated for 8 h in the presence of the indicated concentrations of 2-CAdo. Nucleotides were analyzed by HPLC. Results are means ± S.E.M. of three separate experiments.

incubation with increasing concentration of 2-CAdo. Additional experiments showed that ITu, which suppressed 2-CAdo cytotoxicity, completely suppressed 2-CATP accumulation and ATP depletion (not shown).

3.4. *2-CAdo inhibits nucleic acid and protein synthesis*

Since 2-CATP is an analogue of ATP, we investigated whether it had any effect on nucleic acid synthesis. EHEB cells were incubated with 50 μM 2-CAdo for 7 and 15 h before a 1-h pulse with [^{3}H]-thymidine or [^{3}H]-uridine. We observed a time-dependent inhibition of both DNA and RNA synthesis, reaching about 40 and 70% after 8 and 16 h of incubation, respectively (Fig. 4A). Additional experiments showed that inhibition of nucleic acid synthesis was not only time-dependent, but also dose-dependent (not shown).

Inhibition of RNA synthesis should logically lead to a decrease of protein synthesis. Therefore, we investigated the effect of 2-CAdo on protein synthesis by measuring its effect on the incorporation of L-[4,5-^{3}H]leucine into protein. EHEB cells were incubated for 9 h before a 1 h pulse with the labeled precursor. As expected (Fig. 4B), incubation with 2-CAdo led to a dose-dependent inhibition of protein synthesis.

3.5. *2-CAdo activates the intrinsic pathway of apoptosis*

Death of leukemic cells in response to nucleoside analogues, including CdA and fludarabine, usually involves activation of the intrinsic pathway of apoptosis, which is controlled by the Bcl-2 family proteins [17]. Global inhibition of RNA and protein synthesis induced by 2-CAdo could result in a decrease of the level of key survival proteins belonging to this family, allowing permeabilization of mitochondria, release of cytochrome *c* and hence caspase-3 activation. First, we investigated whether cytochrome *c* was released into the cytosol of cells incubated with 2-CAdo. As illustrated in Fig. 5A, a dose- and time-dependent release of cytochrome *c* was indeed induced by 2-CAdo treatment, this release being already observed after 6–8 h with 50 μM 2-CAdo. These results indicate that 2-CAdo activated the intrinsic pathway of apoptosis in EHEB cells and suggest that activation of caspase-3 resulted from the assembly of apoptosome and

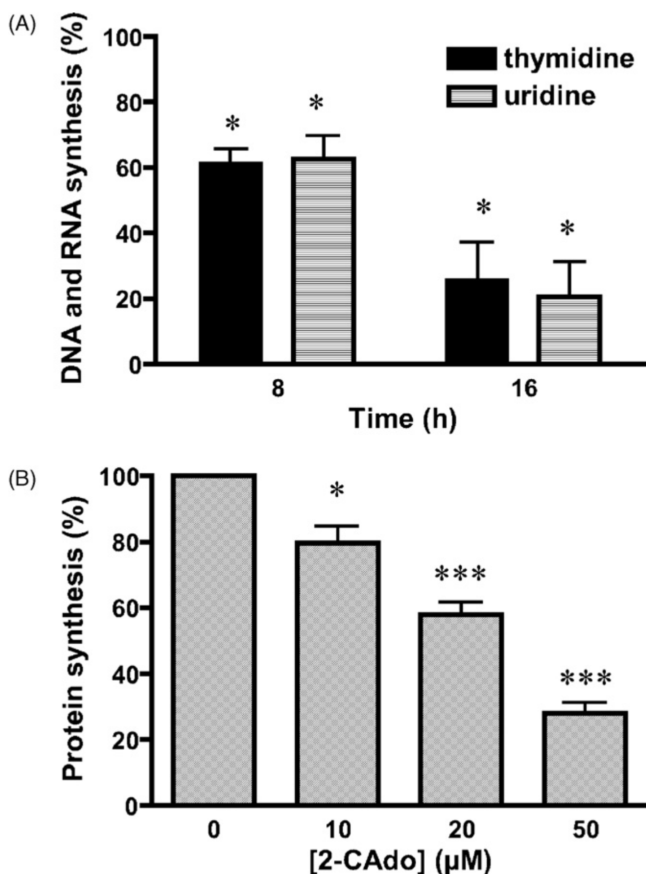

Fig. 4 – Effect of 2-CAdo on DNA, RNA and protein synthesis. (A) EHEB cells were incubated for 7 and 15 h without or with 50 μM 2-CAdo and then pulsed with [^{3}H]thymidine or [^{3}H]uridine for 60 min. Results are expressed in percentage of incorporation measured in untreated cells and are means ± S.E.M. of three separate experiments. (B) EHEB cells were incubated for 9 h with increasing concentrations of 2-CAdo and then pulsed with L-[4,5-^{3}H]leucine for 60 min. Results are means ± S.E.M. of five experiments. Significance relative to the absence of 2-CAdo: $^*P < 0.05$; $^{***}P < 0.001$.

from the activation of caspase-9 [25]. Accordingly, Western blot analysis showed cleavage of procaspase-9, whereas processing of caspase-8, the apical caspase of the extrinsic pathway, could not be detected (results not shown). We next investigated the level of Bcl-2 family members, some of them acting as inhibitors of apoptosis and others promoting cell death [26]. Fig. 5B shows that the levels of the pro-apoptotic proteins Bax, Bak, Bad and Puma were not modified after 8 h of incubation in the presence of 50 μM 2-CAdo and rather decreased after 24 h, precluding a role of these proteins in the release of cytochrome *c*. The anti-apoptotic proteins Bcl-2, Bcl_{xl}, and XIAP were also not modified by 2-CAdo (Fig. 5C). In contrast, a decrease in Mcl-1 protein level was noted in EHEB cells incubated with 50 μM 2-CAdo, being already present at 8 h. A more detailed analysis (Fig. 5D) showed that the level of Mcl-1 began to decrease after 6 h with 50 μM 2-CAdo, when a release of cytochrome *c* was detected, and that the decrease in the level of Mcl-1 upon incubation with 2-CAdo was dose-dependent (Fig. 5D). These results suggest that activation of the intrinsic pathway of apoptosis induced by 2-CAdo could be related to down-regulation of Mcl-1.

A number of chemotherapeutic drugs, including nucleoside analogues, activate the intrinsic pathway of apoptosis as a result of the accumulation of the p53 tumor suppressor [17]. However, incubation of cells with 50 μM 2-CAdo for 8 or 24 h induced no changes in p53 levels or p53 phosphorylation at Ser-15, although the related nucleoside analogue CdA, used as a positive control, provoked p53 accumulation and phosphorylation (Fig. 5E).

3.6. *ATP depletion plays a role in 2-CAdo-induced cell death*

As explained above, 2-CAdo induced a loss of ATP without concomitant elevation of ADP and AMP, which indicated that 2-CAdo treatment provoked an increase of the catabolism of adenine nucleotides into nucleosides and bases. The limiting enzyme in this process is AMP deaminase that converts AMP to IMP. In most cells, this enzyme is activated during metabolic stress, such as phosphate trapping due to the accumulation of a phosphorylated metabolite, and serves, in part, to maintain the equilibrium of the adenylate kinase reaction by removing accumulating AMP from the adenylate pool [27,28]. To evaluate the role of ATP depletion in 2-CAdo cytotoxicity, we investigated whether pharmacological inhibition of AMP deaminase could diminish or prevent adenine nucleotide catabolism induced by 2-CAdo. For this purpose, we used the inosine analogue coformycin, a potent inhibitor of adenosine deaminase, but also of AMP deaminase although at higher concentrations (more than two order of magnitudes) [29]. As shown in Fig. 6A, 10 μM coformycin strongly reduced the decline of ATP induced by a 24 h-incubation with 50 μM 2-CAdo, confirming that an increased AMP deaminase activity was indeed responsible for the loss of ATP induced by 2-CAdo. As also illustrated in Fig. 6A, coformycin did not prevent phosphorylation of 2-CAdo into 2-CATP that even accumulated at a higher concentration. Despite this accumulation, coformycin significantly reduced 2-CAdo cytotoxicity (Fig. 6B), even if we take into account that coformycin alone displayed a slight protective effect ($P < 0.01$). Indeed, 2-CAdo-induced loss of cell viability was reduced from 56.5 ± 7.2% in the absence of coformycin to 36.3 ± 5.5% in its presence ($n = 5$; $P < 0.01$). Coformycin also diminished 2-CAdo-induced apoptosis, assessed by caspase-3 activation (Fig. 6C) or by annexin V binding (not shown). Taken together, these results indicate that ATP depletion contributes to 2-CAdo-induced apoptosis.

3.7. *2-CAdo induces apoptosis in B-CLL lymphocytes*

Because EHEB cells constitute a model for B-CLL, we investigated the cytotoxicity of 2-CAdo in lymphocytes isolated from B-CLL patients. We found that 2-CAdo decreased viability of B-CLL cells with an IC_{50} of 4.82 ± 0.36 μM ($n = 8$). Like in EHEB cells, 2-CAdo induced caspase-3 activation in B-CLL cells, independently of a p53 elevation (results not shown).

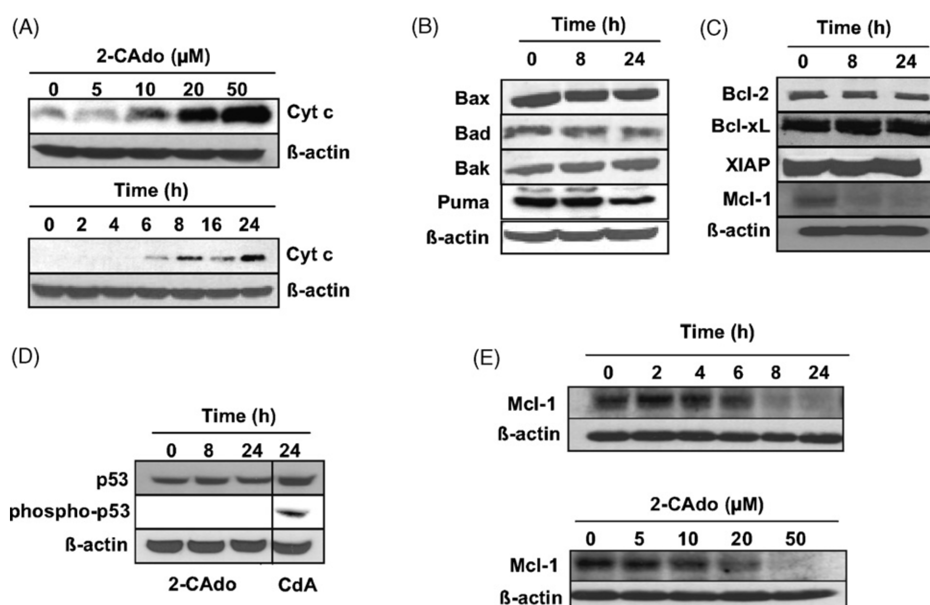

Fig. 5 – Analysis of the intrinsic pathway of apoptosis in EHEB cells incubated with 2-CAdo. (A) Release of cytochrome *c* (Cyt *c*) in the cytosol. EHEB cells were incubated for 24 h with various concentrations of 2-CAdo or for various times with 50 µM 2-CAdo. (B) Pro-apoptotic proteins and (C) anti-apoptotic proteins. Cells were incubated for the indicated times with 50 µM 2-CAdo. (D) Time- and dose-effect of 2-CAdo on Mcl-1 protein level. Cells were incubated for various times with 50 µM 2-CAdo or for 24 h with various concentrations of 2-CAdo. (E) p53 level and phosphorylation. Cells were incubated for various times with 50 µM 2-CAdo, or for 24 h with 10 µM CdA, used as a positive control. Total levels of p53 and p53 phosphorylated at Ser-15 were analyzed. In each experiment, β-actin was used as a loading control. Representative results are shown.

4. Discussion

The present study shows that 2-CAdo is cytotoxic toward leukemic B-cells, including B-CLL lymphocytes, as already observed for several other cell types. Since 2-CAdo induces activation of caspase-3, high molecular weight DNA fragmentation, PARP cleavage and externalization of phosphatidylserine (Fig. 1), we conclude that 2-CAdo-induced cell death implies apoptosis. As explained above, 2-CAdo can exert its action via two different mechanisms: activation of extracellular adenosine receptors, or intracellular transport and metabolism. Our data strongly support an intracellular action. Indeed, both cytotoxicity and caspase-3 activation induced by 2-CAdo were markedly decreased by the nucleoside transport inhibitor NBTI, and moreover completely abolished by the adenosine kinase inhibitor ITu (Fig. 2). Incomplete protection by NBTI is probably due to a residual 2-CAdo uptake independent of ENT1 and ENT2 equilibrative nucleoside transporters, which are both inhibited at a 10 µM NBTI concentration [30].

Intracellular metabolism of 2-CAdo was proven by HPLC analysis showing that 2-CATP time- and dose-dependently accumulated following treatment of cells with 2-CAdo, whereas endogenous ATP decreased (Fig. 3). The triphosphate derivative, which was the only UV-detectable metabolite formed from 2-CAdo and thus most probably the toxic metabolite, reached concentrations up to 1 mM, which is about 100-fold higher than those achieved in leukemic cells after treatment with the deoxynucleoside analogue CdA [31]. The consequence of this extensive intracellular metabolism of 2-CAdo is a general inhibition of macromolecular synthesis, involving DNA, RNA and protein synthesis (Fig. 4). Inhibition of DNA synthesis is probably not the result of an incorporation of 2-CATP into DNA, since this triphosphate analogue is a ribonucleotide, but could be secondary to a depletion of deoxynucleoside triphosphates as observed in prostate cancer cells treated with 2-CAdo [15]. Alternatively, inhibition of DNA synthesis could be the result of the inhibition of RNA synthesis and of the ensuing cell death, as proposed by Krett et al. [32] who also found inhibition of both RNA and DNA synthesis after treatment of cells with the ribonucleoside analogue 8-aminoadenosine. On the other hand, 2-CATP, being an analogue of ATP, might be incorporated into RNA, as demonstrated for other nucleoside analogues, such as fludarabine [33] and 8-chloroadenosine [34]. In both cases, these nucleoside analogues were preferentially incorporated into mRNA, resulting in inhibition of RNA synthesis due to premature termination of the RNA transcripts. Thus, our observation of an inhibition of RNA synthesis by 2-CAdo could be explained at least partially, by misincorporation of 2-CAdo into RNA, as well as by the decline in ATP, a direct precursor for RNA synthesis.

Inhibition of DNA synthesis and/or RNA synthesis are frequently reported mechanisms of cytotoxicity of chemotherapeutic drugs and are also potential mechanisms of 2-CAdo cytotoxicity, by delaying cell growth and/or by reducing the expression of proteins that are important for cell survival, like that of anti-apoptotic Bcl-2 family proteins. Our observation that 2-CAdo induced a release of cytochrome *c* into cytosol (Fig. 5A) and thus an activation of the intrinsic

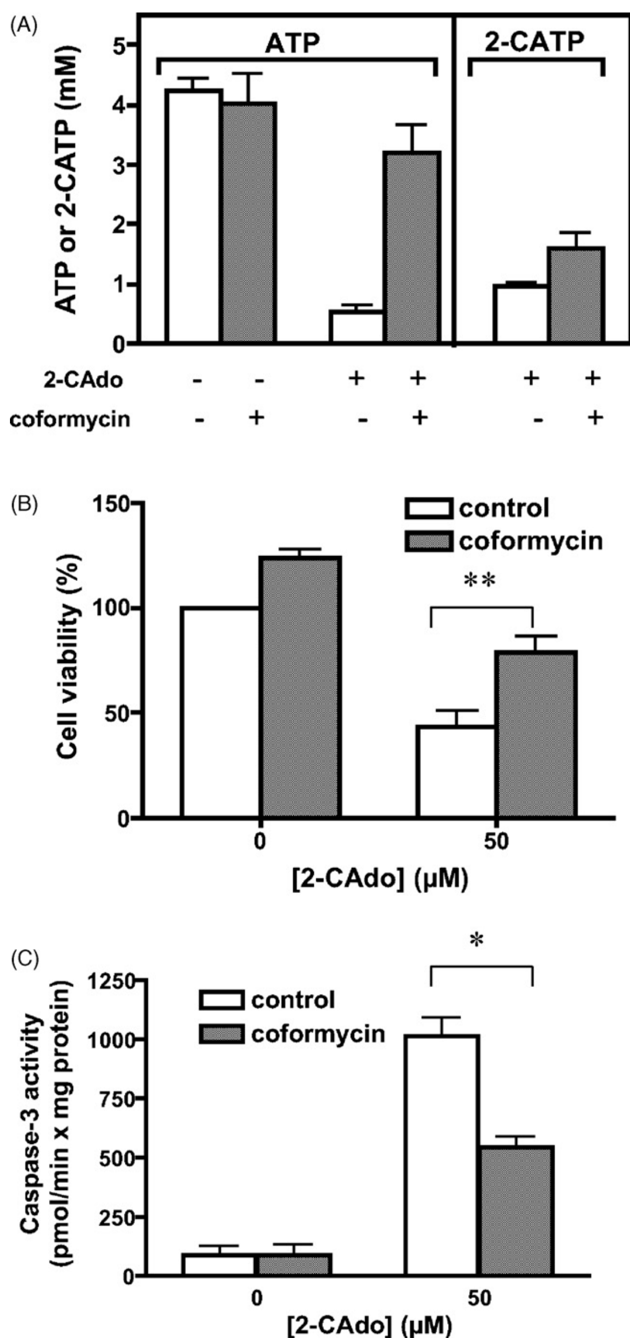

Fig. 6 – Effect of coformycin on cytotoxic effects of 2-CAdo. EHEB cells were incubated for 24 h without or with 50 μM 2-CAdo in the absence or in the presence of 10 μM coformycin. (A) ATP and 2-CATP concentration determined by HPLC. (B) Cell viability measured by the MTT assay. (C) Caspase-3 activity. Results are means ± S.E.M. of three (A and C) or five (B) separate experiments. *$P < 0.05$; **$P < 0.01$.

pathway of apoptosis indicated mitochondrial disruption and supported the hypothesis that 2-CAdo could indirectly target anti-apoptotic protein(s). Accordingly, our study showed that 2-CAdo induced an early and profound decrease of the level of the anti-apoptotic protein Mcl-1 (Fig. 5C and D), whereas it did not affect the level of Bcl-2, Bcl-xl and XIAP. Mcl-1 mRNA and protein are characterized by a very short half-life [35], so that inhibition of RNA and protein synthesis is expected to induce a rapid decrease of Mcl-1 protein level. Our results are consistent with other studies showing that inhibition of RNA synthesis could lead to down-regulation of Mcl-1. Particularly, in CLL cells, the nucleoside analogues 8-aminoadenosine [36], 8-chloroadenosine [37], and cytarabine [38], have been shown to inhibit RNA synthesis and decrease Mcl-1 level, which has been related to entry into apoptosis. Seliciclib [39] and flavopiridol [40,41], two cyclin-dependent kinase inhibitors found to inhibit transcription, have also been reported to initiate apoptosis by the same mechanism. The critical role of Mcl-1 in cell survival has been demonstrated by several approaches, including down-regulation of Mcl-1 by small interfering RNA (siRNA) or Mcl-1 antisense oligonucleotides, which can be sufficient to initiate apoptosis [39,42–44]. Moreover, enhanced expression of Mcl-1 has been shown to protect B-CLL cells from chemotherapy-induced apoptosis [45]. Our results showing that 2-CAdo activates the intrinsic pathway of apoptosis contrast with those obtained in human astrocytoma cells, where an atypical pathway involving caspase-2 as an initiator caspase, and effector caspase-3 has been reported, whereas release of cytochrome *c* was not detected [16].

Activation of the intrinsic pathway of apoptosis by chemotherapeutic drugs is often related to phosphorylation and accumulation of p53, followed by up-regulation of its transcriptional targets, such as Bax, able to form pores in the mitochondrial membrane. Nevertheless, there were no changes in p53 level or phosphorylation upon treatment by 2-CAdo and no increase in the level of Bax or other pro-apoptotic proteins. These data show that 2-CAdo triggers apoptosis in a p53-independent manner, an important difference with respect to other nucleoside analogues including CdA and fludarabine, which induce p53 stabilization [17].

Depletion of intracellular ATP in parallel with the accumulation of 2-CATP is similar to what was observed with other adenosine analogues, such as 8-chloroadenosine [37,46], 8-aminoadenosine [32] and several N6-substituted derivatives [47], all being efficiently phosphorylated by adenosine kinase. Decline in cellular ATP has been associated with cell death involving either necrosis or apoptosis [48,49] and with drug-induced cytotoxicity [50,51]. To elucidate the role of ATP depletion in 2-CAdo induced cell death, we used coformycin that allowed substantial maintaining of ATP (Fig. 6A), without preventing 2-CAdo phosphorylation. That ATP depletion by itself plays a role in 2-CAdo cytotoxicity was indicated by our observation that cell viability (Fig. 6B) was increased by two-fold when ATP loss was hindered by coformycin. Moreover, our observation that protection of ATP by coformycin reduced caspase-3 activation and phosphatidylserine externalization indicates a decrease of apoptosis. Thus ATP depletion induced by 2-CAdo appears to be an event that strengthens the cytotoxic action of 2-CATP. The exact mechanism by which ATP depletion contributes to apoptosis induced by 2-CAdo has still to be clarified. Decline in ATP could confer a competitive advantage to 2-CATP, raising the likelihood for its misincorporation into RNA. Or, as suggested in a recent study [52], intracellular nucleotides, and particularly ATP, could

represent physiological pro-survival factors, functioning as natural inhibitors of apoptosome, which implies that a reduction of their concentration would contribute to the apoptotic process.

In summary, we have shown that 2-CAdo induces apoptosis in leukemic B-cells as a result of its intracellular metabolism, causing inhibition of DNA and RNA synthesis, depletion of the survival protein Mcl-1, and activation of the intrinsic pathway of apoptosis in a p53-independent way. In addition, we have emphasized the role of ATP depletion in 2-CAdo-induced apoptosis, showing that the decline in cellular bioenergy potentiates the cytotoxic action of 2-CATP. We postulate that depletion of ATP plays a similar role in apoptosis induced by other adenosine analogues, also substrates of adenosine kinase, such as 8-chloroadenosine [37,46], 8-aminoadenosine [32,36] or N6-substituted derivatives [47] in various types of cells, including B-CLL lymphocytes. To conclude, our data provide additional arguments for developing adenosine analogues, as an alternative to deoxyadenosine analogues, as chemotherapeutic agents for B-CLL patients with resistance to conventional agents, particularly with chemoresistance resulting from the loss of p53 function.

Acknowledgements

This work was supported by grants from the Belgian Fonds National de la Recherche Scientifique (Télévie, FRSM, Crédit au Chercheur), by the Interuniversity Attraction Poles Program-Belgian Science Policy (Network P6/5), and by private foundations: Bauchau, Maisin, Goor and Salus Sanguinis. We thank Professor G. Van den Berghe for helpful comments.

REFERENCES

[1] Clarke DA, Davoll J, Philips FS, Brown GB. Enzymatic deamination and vasodepressor effects of adenosine analogs. J Pharmacol Exp Ther 1952;106:291–302.

[2] Daly JW. Adenosine receptors: targets for future drugs. J Med Chem 1982;25:197–207.

[3] Yamanaka H, Kamatani N, Nishida Y, Nishioka K, Mikanagi K. Relationship between phosphorylation and cytotoxicity of 2-chloroadenosine and 6-methylmercaptopurine riboside in human cells. Biochim Biophys Acta 1984;798:291–4.

[4] Claeyssens S, Hamet M, Chedeville A, Basuyau JP, Lavoinne A. Influence of 2-chloroadenosine on the nucleotide content of isolated rat hepatocytes. FEBS Lett 1988;232: 317–22.

[5] Kizaki H, Suzuki K, Tadakuma T, Ishimura Y. Adenosine receptor-mediated accumulation of cyclic AMP-induced T-lymphocyte death through internucleosomal DNA cleavage. J Biol Chem 1990;265:5280–4.

[6] Szondy Z. Adenosine stimulates DNA fragmentation in human thymocytes by Ca(2+)-mediated mechanisms. Biochem J 1994;304:877–85.

[7] Szondy Z. The 2-chlorodeoxyadenosine-induced cell death signalling pathway in human thymocytes is different from that induced by 2-chloroadenosine. Biochem J 1995;311:585–8.

[8] Abbracchio MP, Ceruti S, Barbieri D, Franceschi C, Malorni W, Biondo L, et al. A novel action for adenosine: apoptosis of astroglial cells in rat brain primary cultures. Biochem Biophys Res Commun 1995;213:908–15.

[9] Ceruti S, Franceschi C, Barbieri D, Malorni W, Camurri A, Giammarioli AM, et al. Apoptosis induced by 2-chloro-adenosine and 2-chloro-2′-deoxy-adenosine in a human astrocytoma cell line: differential mechanisms and possible clinical relevance. J Neurosci Res 2000;60: 388–400.

[10] Barbieri D, Abbracchio MP, Salvioli S, Monti D, Cossarizza A, Ceruti S, et al. Apoptosis by 2-chloro-2′-deoxy-adenosine and 2-chloro-adenosine in human peripheral blood mononuclear cells. Neurochem Int 1998;32:493–504.

[11] Szondy Z. Methylprednisolone and 2-chloroadenosine induce DNA fragmentation at different stages of human T-lymphocyte development. Immunol Lett 1997;58:59–65.

[12] Ceruti S, Giammarioli AM, Camurri A, Falzano L, Rufini S, Frank C, et al. Adenosine- and 2-chloro-adenosine-induced cytopathic effects on myoblastic cells and myotubes: involvement of different intracellular mechanisms. Neuromuscul Disord 2000;10:436–46.

[13] Frank C, Giammarioli AM, Falzano L, Rufini S, Ceruti S, Camurri A, et al. 2-Chloro-adenosine induces a glutamate-dependent calcium response in C_2C1_2 myotubes. Biochem Biophys Res Commun 2000;277:546–51.

[14] Koshiba M, Kosaka H, Nakazawa T, Hayashi N, Saura R, Kitamura N, et al. 2-Chloroadenosine but not adenosine induces apoptosis in rheumatoid fibroblasts independently of cell surface adenosine receptor signalling. Br J Pharmacol 2002;135:1477–86.

[15] Minelli A, Bellezza I, Agostini M, Bracarda S, Culig Z. Mechanism of 2-chloroadenosine toxicity to PC3 cell line. Prostate 2006;66:1425–36.

[16] Ceruti S, Beltrami E, Matarrese P, Mazzola A, Cattabeni F, Malorni W, et al. A key role for caspase-2 and caspase-3 in the apoptosis induced by 2-chloro-2′-deoxy-adenosine (cladribine) and 2-chloro-adenosine in human astrocytoma cells. Mol Pharmacol 2003;63:1437–47.

[17] Van Den Neste E, Cardoen S, Offner F, Bontemps F. Old and new insights into the mechanisms of action of two nucleoside analogs active in lymphoid malignancies: fludarabine and cladribine (review). Int J Oncol 2005;27:1113–24.

[18] Saltman D, Bansal NS, Ross FM, Ross JA, Turner G, Guy K. Establishment of a karyotypically normal B-chronic lymphocytic leukemia cell line; evidence of leukemic origin by immunoglobulin gene rearrangement. Leuk Res 1990;14:381–7.

[19] Bontemps F, Delacauw A, Cardoen S, Van Den Neste E, Van Den Berghe G. Metabolism and cytotoxic effects of 2-chloroadenine, the major catabolite of 2-chloro-2′-deoxyadenosine. Biochem Pharmacol 2000;59:1237–43.

[20] Smal C, Lisart S, Maerevoet M, Ferrant A, Bontemps F, Van Den Neste E. Pharmacological inhibition of the MAPK/ERK pathway increases sensitivity to 2-chloro-2′-deoxyadenosine (CdA) in the B-cell leukemia cell line EHEB. Biochem Pharmacol 2007;73:351–8.

[21] Huang P, Robertson LE, Wright S, Plunkett W. High molecular weight DNA fragmentation: a critical event in nucleoside analogue-induced apoptosis in leukemia cells. Clin Cancer Res 1995;1:1005–13.

[22] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.

[23] 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.

[24] Henderson JF, Paterson AR, Caldwell IC, Paul B, Chan MC, Lau KF. Inhibitors of nucleoside and nucleotide metabolism. Cancer Chemother Rep 1972;2(3):71–85.
[25] Hengartner MO. The biochemistry of apoptosis. Nature 2000;407:770–6.
[26] Borner C. The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. Mol Immunol 2003;39:615–47.
[27] Chapman AG, Atkinson DE. Stabilization of adenylate energy charge by the adenylate deaminase reaction. J Biol Chem 1973;248:8309–12.
[28] Van den Berghe G, Bronfman M, Vanneste R, Hers HG. The mechanism of adenosine triphosphate depletion in the liver after a load of fructose. A kinetic study of liver adenylate deaminase. Biochem J 1977;162:601–9.
[29] Van den Berghe G, Bontemps F, Hers HG. Purine catabolism in isolated rat hepatocytes. Influence of coformycin. Biochem J 1980;188:913–20.
[30] King AE, Ackley MA, Cass CE, Young JD, Baldwin SA. Nucleoside transporters: from scavengers to novel therapeutic targets. Trends Pharmacol Sci 2006;27:416–25.
[31] Albertioni F, Lindemalm S, Reichelova V, Pettersson B, Eriksson S, Juliusson G, et al. Pharmacokinetics of cladribine in plasma and its 5′-monophosphate and 5′-triphosphate in leukemic cells of patients with chronic lymphocytic leukemia. Clin Cancer Res 1998;4:653–8.
[32] Krett NL, Davies KM, Ayres M, Ma C, Nabhan C, Gandhi V, et al. 8-Amino-adenosine is a potential therapeutic agent for multiple myeloma. Mol Cancer Ther 2004;3:1411–20.
[33] Huang P, Plunkett W. Action of 9-beta-D-arabinofuranosyl-2-fluoroadenine on RNA metabolism. Mol Pharmacol 1991;39:449–55.
[34] Stellrecht CM, Rodriguez Jr CO, Ayres M, Gandhi V. RNA-directed actions of 8-chloro-adenosine in multiple myeloma cells. Cancer Res 2003;63:7968–74.
[35] Yang-Yen HF. Mcl-1: a highly regulated cell death and survival controller. J Biomed Sci 2006;13:201–4.
[36] Balakrishnan K, Wierda WG, Keating MJ, Gandhi V. Mechanisms of cell death of chronic lymphocytic leukemia lymphocytes by RNA-directed agent, 8-NH2-adenosine. Clin Cancer Res 2005;11:6745–52.
[37] Balakrishnan K, Stellrecht CM, Genini D, Ayres M, Wierda WG, Keating MJ, et al. Cell death of bioenergetically compromised and transcriptionally challenged CLL lymphocytes by chlorinated ATP. Blood 2005;105:4455–62.
[38] de Vries JF, Falkenburg JH, Willemze R, Barge RM. The mechanisms of Ara-C-induced apoptosis of resting B-chronic lymphocytic leukemia cells. Haematologica 2006;91:912–9.
[39] MacCallum DE, Melville J, Frame S, Watt K, Anderson S, Gianella-Borradori A, et al. Seliciclib (CYC202, R-Roscovitine) induces cell death in multiple myeloma cells by inhibition of RNA polymerase II-dependent transcription and down-regulation of Mcl-1. Cancer Res 2005;65:5399–407.
[40] Gojo I, Zhang B, Fenton RG. The cyclin-dependent kinase inhibitor flavopiridol induces apoptosis in multiple myeloma cells through transcriptional repression and down-regulation of Mcl-1. Clin Cancer Res 2002;8:3527–38.
[41] Chen R, Keating MJ, Gandhi V, Plunkett W. Transcription inhibition by flavopiridol: mechanism of chronic lymphocytic leukemia cell death. Blood 2005;106:2513–9.
[42] Zhang B, Gojo I, Fenton RG. Myeloid cell factor-1 is a critical survival factor for multiple myeloma. Blood 2002;99:1885–93.
[43] Moulding DA, Giles RV, Spiller DG, White MR, Tidd DM, Edwards SW. Apoptosis is rapidly triggered by antisense depletion of MCL-1 in differentiating U937 cells. Blood 2000;96:1756–63.
[44] Hussain SR, Cheney CM, Johnson AJ, Lin TS, Grever MR, Caligiuri MA, et al. Mcl-1 is a relevant therapeutic target in acute and chronic lymphoid malignancies: down-regulation enhances rituximab-mediated apoptosis and complement-dependent cytotoxicity. Clin Cancer Res 2007;13:2144–50.
[45] Petlickovski A, Laurenti L, Li X, Marietti S, Chiusolo P, Sica S, et al. Sustained signaling through the B-cell receptor induces Mcl-1 and promotes survival of chronic lymphocytic leukemia B cells. Blood 2005;105:4820–7.
[46] Gandhi V, Ayres M, Halgren RG, Krett NL, Newman RA, Rosen ST. 8-Chloro-cAMP and 8-chloro-adenosine act by the same mechanism in multiple myeloma cells. Cancer Res 2001;61:5474–9.
[47] Mlejnek P, Dolezel P. Apoptosis induced by N6-substituted derivatives of adenosine is related to intracellular accumulation of corresponding mononucleotides in HL-60 cells. Toxicol In Vitro 2005;19:985–90.
[48] Skulachev VP. Bioenergetic aspects of apoptosis, necrosis and mitoptosis. Apoptosis 2006;11:473–85.
[49] Izyumov DS, Avetisyan AV, Pletjushkina OY, Sakharov DV, Wirtz KW, Chernyak BV, et al. Wages of fear: transient threefold decrease in intracellular ATP level imposes apoptosis. Biochim Biophys Acta 2004;1658:141–7.
[50] Seto S, Carrera CJ, Kubota M, Wasson DB, Carson DA. Mechanism of deoxyadenosine and 2-chlorodeoxyadenosine toxicity to nondividing human lymphocytes. J Clin Invest 1985;75:377–83.
[51] Martin DS, Spriggs D, Koutcher JA. A concomitant ATP-depleting strategy markedly enhances anticancer agent activity. Apoptosis 2001;6:125–31.
[52] Chandra D, Bratton SB, Person MD, Tian Y, Martin AG, Ayres M, et al. Intracellular nucleotides act as critical prosurvival factors by binding to cytochrome *c* and inhibiting apoptosome. Cell 2006;125:1333–46.