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Metabolism and Cytotoxic Effects of 2-Chloroadenine, the Major Catabolite of 2-Chloro-2′-deoxyadenosine

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ABSTRACT. EHEB cells, a continuous cell line derived from a patient with B cell chronic lymphocytic leukemia (B-CLL), synthesized, when incubated with tritiated 2-chloro-2′-deoxyadenosine (CdA), labeled mono-, di-, and triphosphate ribonucleosides at a much higher rate than CdA deoxyribonucleotides. Further analysis revealed that these ribonucleotides were formed from labeled 2-chloroadenine (CAde), which contaminated commercial tritiated CdA at a proportion of 2–3%. Since CAde is the major catabolite of CdA measured in plasma after oral or intravenous administration of CdA to patients, its metabolism and in particular its potential cytotoxicity were investigated both in EHEB cells and in B-CLL lymphocytes. Phosphorylation of CAde was inhibited by adenine, indicating that its initial metabolism most probably proceeds via adenine phosphoribosyltransferase (EC 2.4.2.7). In both cell types, chloro-ATP was the major metabolite formed from CAde and its concentration increased proportionally at least up to 50 μM CAde. At high concentration, CAde metabolism was accompanied by a decrease in intracellular ATP. Cytotoxicity of CAde, evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, showed an IC_{50} of 16 μM in EHEB cells and 5 μM in B-CLL lymphocytes. At cytotoxic concentrations, apopain/caspase-3 activation and high molecular weight DNA fragmentation were observed, indicating that CAde cytotoxicity results from induction of apoptosis. However, since CAde cytotoxicity requires higher concentrations than CdA, it probably does not play a role in the therapeutic effect of CdA in the treatment of hematologic malignancies. BIOCHEM PHARMACOL **59**;10:1237–1243, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. 2-chloro-2′-deoxyadenosine, 2-chloroadenosine, 2-chloroadenine, EHEB cells, CLL lymphocytes, apopain/caspase-3

CdA§, an analogue of deoxyadenosine which is resistant to degradation by adenosine deaminase, is highly toxic to both non-dividing and proliferating lymphocytes [1–3]. CdA has shown activity in the treatment of several hematologic malignancies, including hairy cell leukemia and CLL (for a review, see Ref. 4). Intracellularly, CdA is phosphorylated by deoxycytidine kinase (EC 2.7.1.74) to CdAMP, and thereafter successively converted to CdADP and CdATP. The cytotoxicity of CdA results from the inhibitory effect of CdATP on various enzymes involved in DNA replication and repair, including ribonucleotide reductase (EC 1.17.4.1) and DNA polymerase (EC 2.7.7.7) [5]. An inhibitory effect of CdA on dCMP deaminase (EC 3.4.5.12), mediated by CdAMP, has also been proposed [6]. While it is clear that CdA phosphorylation is a prerequisite for CdA toxicity in various cancer cell lines *in vitro* and that the level of this phosphorylation may be correlated with the response to CdA in the treatment of B-CLL patients [7], it may not be the sole explanation for CdA toxicity [8]. In particular, very little has been reported on the metabolism and potential cytotoxicity of CAde, the major catabolite of CdA measured in plasma of patients after oral or intravenous administration of CdA [9].

Upon incubation with [8-^{3}H]CdA of EHEB cells, a continuous cell line derived from a patient with B-CLL [10], we fortuitously observed that these cells utilized a contaminant present in the commercial preparation of labeled CdA at a much higher rate than CdA itself. After we had identified this contaminant as CAde, we investigated its fate and cytotoxicity in EHEB cells and in lymphocytes isolated from patients with B-CLL.

MATERIALS AND METHODS

Materials

Unlabeled CdA (>99.9% purity) was synthesized and supplied by Professor L. Ghosez, Laboratory of Organic Chemistry, Université Catholique de Louvain, Louvain-la-Neuve, Belgium. [8-^{3}H]CdA (24.2 or 4.7 mCi/mmol) (ini-

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§ *Abbreviations:* CdA, 2-chloro-2′-deoxyadenosine; CAde, 2-chloroadenine; CAdo, 2-chloroadenosine; CdAMP, 2-chlorodeoxyAMP; CdADP, 2-chlorodeoxyADP; CdATP, 2-chlorodeoxyATP; B-CLL, B cell chronic lymphocytic leukemia; FBS, fetal bovine serum; IC_{50}, concentration required to reduce viability by 50%; and MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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tial purity varying from 97.4 to 99.8%) was purchased from Moravek Biochemicals. CAde was prepared from CdA by acid hydrolysis for 120 min at 37° in the presence of 0.1 N $HClO_4$; was used after neutralization with 3 M K_2CO_3. It was verified that addition to cells of neutralized blank extract was without effect. MTT was purchased from Sigma-Aldrich. FBS and penicillin/streptomycin were purchased from BioWhittaker Europe, RPMI-1640 was from Life Technologies, and all tissue culture reagents were from GIBCO. Ficoll-Paque PLUS (density: 1.077 ± 0.001 g/mL) was from Pharmacia Biotech. All other chemicals, materials, and reagents were of the highest quality available.

Cell Preparation and Incubation

The continuous cell line EHEB, derived from a patient with B-CLL [10], was cultured in RPMI-1640 medium with Glutamax, supplemented with 10% heat-inactivated FBS, at 37° in an atmosphere of 5% CO_2 in air. B-CLL cells were isolated by fractionation by Ficoll-Paque sedimentation from freshly obtained peripheral blood from B-CLL patients. Mononuclear cells were washed with cold PBS and resuspended in RPMI-1640 supplemented with 10% FBS and 1% penicillin–streptomycin. Cells were counted, diluted to the indicated concentrations in RPMI, and incubated at 37° in 5% CO_2.

Cytotoxicity Analysis

EHEB cells (resuspended at a concentration of 0.2×10^6 cells/mL) and B-CLL cells (resuspended at a concentration of 1.5×10^6 cells/mL) were incubated in the presence of various concentrations of CdA or CAde in 96-well plates. After 96 hr, cell viability was measured using the MTT assay, according to Mosmann [11] with the modifications of Morabito *et al.* [12]. Controls and drug concentrations were set up in triplicate. The O.D. of each well was measured at 540 nm with a multiwell scanning spectrophotometer. Cell viability was calculated using the equation: (mean O.D. of treated wells/mean O.D. of control wells) × 100%. The IC_{50} (concentration required to reduce viability by 50%) was determined graphically.

Measurement of Intracellular Nucleotides

To measure intracellular nucleotides, EHEB cells were incubated at a concentration of 1×10^6 cells/mL and B-CLL lymphocytes at a concentration of 10×10^6 cells/mL. At the indicated time points, 5 mL (for EHEB cell suspensions) or 3 mL (for B-CLL lymphocyte suspensions) was washed twice in 5 mL of cold PBS before addition of 350 μL of 1 M $HClO_4$ to the washed pellet. The supernatant obtained after centrifugation was neutralized with 3 M K_2CO_3. Nucleotides were separated by HPLC on a 110 × 4.7-mm Partisphere 5 SAX column by the method of Hartwick and Brown [13], modified by Vincent *et al.* [14]. UV detection of nucleotides was performed at 254 nm. Fractions corresponding to 1 min of elution were collected and their radioactivity determined by scintillation counting. The amounts of nucleotides synthetized were calculated from the specific radioactivity of the precursor. Statistical significance was analyzed by the two-tailed Student's *t*-test.

Analysis of CdA and CAde

CdA and CAde were analyzed by HPLC on a 250 × 4.6-mm Alltima C18 column eluted at a flow rate of 1 mL/min. Separation was performed with a gradient consisting of two buffers. Buffer A was KH_2PO_4 50 mM, pH 5, and buffer B consisted of a mixture of H_2O/acetonitrile/methanol (75/45/90 by vol.). The chromatographic conditions were: 20 min at 100% of buffer A, 1 min at up to 50% of buffer B, and holding for 4 min. The gradient was then returned over 1 min to 100% of buffer A, thus restoring the initial conditions.* CAde and CdA were eluted at 23.3 and 25.9 min, respectively. When indicated, CdA was purified on the same column by isocratic elution with 30% buffer B, buffer A being replaced by H_2O. Under these conditions, CAde eluted after 8.7 min, whereas CdA eluted after 16.7 min.

Other Analytical Methods

High molecular weight DNA fragmentation was analyzed by pulsed-field gel electrophoresis as described by Huang *et al.* [15]. Apopain activity was determined with the Fluoro-Ace Apopain Assay from Bio-Rad and expressed as mg of protein measured in the supernatant obtained after centrifugation of the lysed cell extract. Protein was measured by the method of Bradford [16], using bovine γ-globulin as the standard.

RESULTS

Synthesis of Nucleotides in EHEB Cells Incubated with [8-^{3}H]CdA

Incubation of various lymphoblastoid cell lines [1, 3] and B-CLL cells [17] with CdA, as well as administration of CdA to patients [18], results in the intracellular synthesis of CdAMP, CdADP, and CdATP. CdAMP and CdATP are the major nucleotides measured, while CdADP is present at a much lower concentration. When EHEB cells were incubated in the presence of 0.5 μM [8-^{3}H]CdA, we observed that two major peaks were detected by HPLC in the triphosphate region (Fig. 1), where only CdATP was expected. In order to verify that all the nucleotides synthesized from CdA corresponded to deoxyribonucleotides, samples were treated with $NaIO_4$, which specifically destroys ribonucleotides as described by Garrett and Santi [19]. As also shown in Fig. 1, this treatment decreased the height of the peak eluted in the monophosphate region, nearly completely suppressed the peak eluted in the diphos-

* Vincent MF, personal communication.

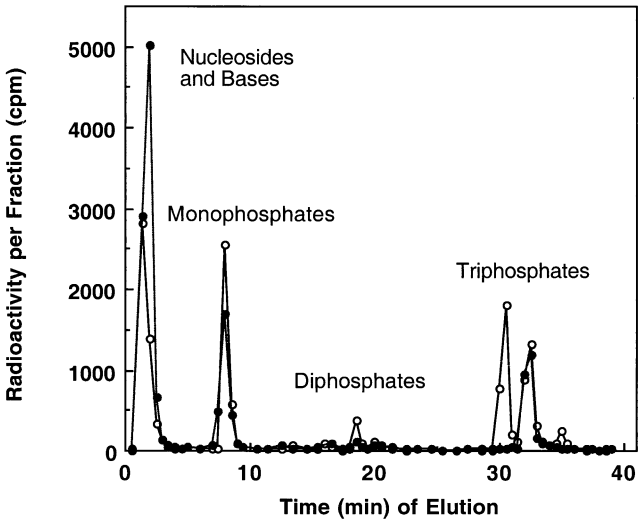

FIG. 1. Nucleotide synthesis from labeled CdA in EHEB cells. Elution profile of labeled metabolites present in neutralized perchloric extracts of EHEB cells incubated for 5 hr with 0.5 μM [8-^{3}H]CdA (2.5 μCi/mL). The samples were treated (●) or not (○) with $NaIO_4$ before analysis by HPLC.

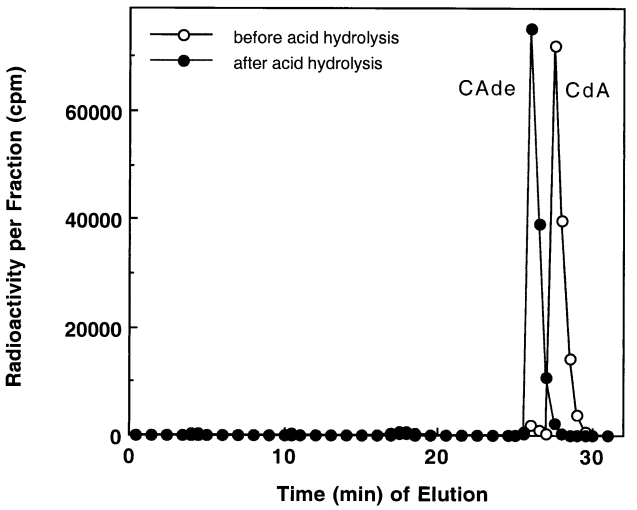

FIG. 2. Analysis of purity of labeled CdA. Elution profile of labeled CdA and CAde. An amount of [8-^{3}H]CdA corresponding to 0.1 μCi was applied on the column. The sample was incubated (●) or not (○) for 2 hr at pH 1 before analysis by HPLC.

phate region, and completely suppressed the first peak eluted in the triphosphate region. A minor, unidentified third peak also disappeared. This result indicates that ribonucleotides, in addition to deoxyribonucleotides, accumulated in the presence of labeled CdA. Synthesis of these ribonucleotides was not affected by addition of 50 μM deoxycytidine which, as expected, completely suppressed the synthesis of CdA nucleotides by competitive inhibition of deoxycytidine kinase (results not shown).

Purity of the labeled CdA used for the experiments described in Fig. 1 was analyzed by HPLC and found to be 97%. The major contaminant (2.3% of total radioactivity) was eluted approx. 2 min before CdA (Fig. 2, open circles), with the same retention time as CAde produced by a 2-hr incubation of [8-^{3}H]CdA at pH 1 and 37° (Fig. 2, closed circles). It was therefore postulated that the major contaminant of [8-^{3}H]CdA corresponded to CAde. CAde was not detected in unlabeled CdA.

When EHEB cells were incubated for 5 hr with labeled CdA purified just before use as described in Materials and Methods, ribonucleotides did not accumulate (results not shown). This indicates that EHEB cells can utilize CAde present in unpurified labeled CdA, but that they do not produce it, at least at detectable rates.

Metabolism of [8-^{3}H]CAde

IN EHEB CELLS. As illustrated in Fig. 3, incubation of EHEB cells with labeled CAde resulted in the synthesis of chloro-AMP, -ADP, and -ATP. These were eluted at the same time points as the ribonucleotides produced during incubation of EHEB cells with unpurified labeled CdA. The triphosphate was by far the major nucleotide produced from CAde, contrary to what is observed with CdA. As expected, all nucleotides produced from CAde could be completely destroyed by $NaIO_4$ treatment (results not shown). Synthesis of nucleotides from CAde and of the ribonucleotides produced during incubation with unpurified labeled CdA was inhibited by addition of adenine (results not shown), suggesting that CAde is metabolized, like adenine, by adenine phosphoribosyltransferase.

The influence of increasing concentrations of CAde on the synthesis of chloro-ATP over 5 hr is shown in Fig. 4A. Chloro-ATP increased proportionally to the concentration of CAde, at least up to 50 μM CAde. At concentrations above 200 pmol/10^6 cells, chloro-ATP was UV-detectable: it was verified that it had the same spectrum as CAde and CdA. The same triphosphate was detected when EHEB cells were incubated with CAdo (results not shown). High

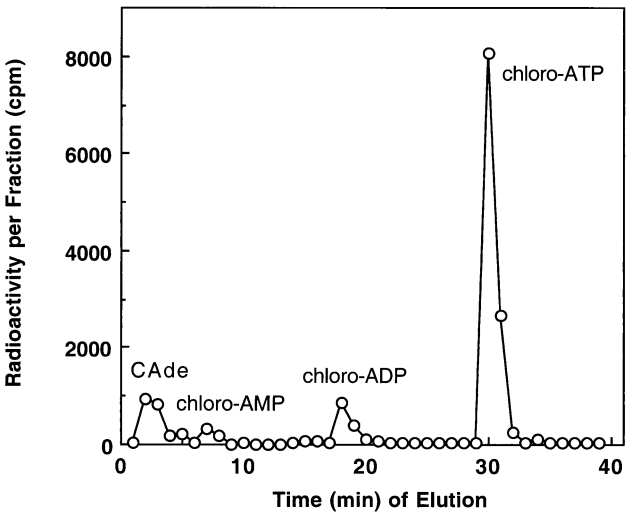

FIG. 3. Synthesis of nucleotides from 2-chloroadenine in EHEB cells. Elution profile of labeled metabolites present in neutralized perchloric extracts of EHEB cells incubated for 5 hr with 0.04 μM [8-^{3}H]CAde (0.25 μCi/mL).

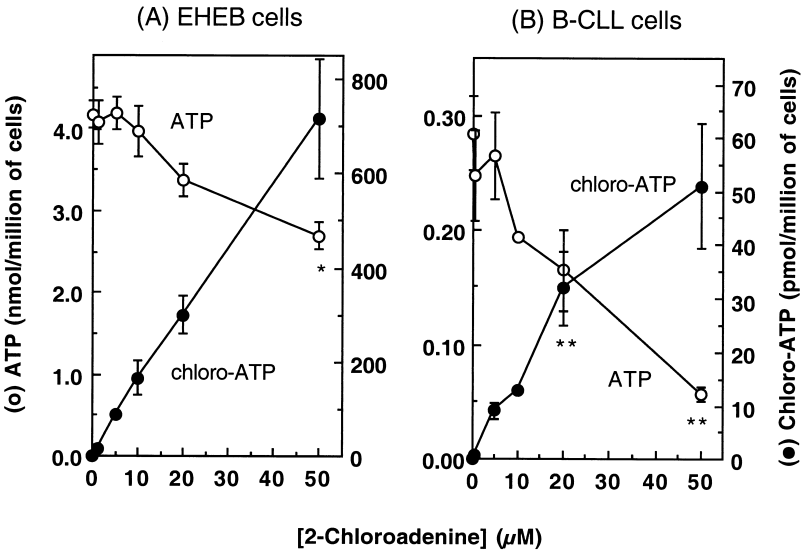

FIG. 4. Dose–effect of 2-chloroadenine on chloro-ATP and ATP concentrations in EHEB (A) and B-CLL cells (B). EHEB and B-CLL cells were incubated for 5 hr in the presence of increasing concentrations of [8-^{3}H]CAde (0.13 μCi/mL). Concentrations of chloro-ATP were calculated from the specific radioactivity of CAde. Results are means ± SEM of 3 separate experiments. *$P < 0.02$; **$P \leq 0.01$, when compared with ATP in the absence of CAde.

accumulation of chloro-ATP was accompanied by a decrease in ATP. This decrease was even more pronounced after 24 hr of incubation, reaching 40% of the initial value at 20 μM CAde and 70% at 50 μM CAde (results not shown). To determine if chloro-ATP could be incorporated into nucleic acids, radioactivity was measured in the acid-insoluble fraction of the cellular extract: it was found to represent only 1.1 to 1.3% of the radioactivity measured in CAde mononucleotides, at all the concentrations of CAde investigated.

IN B-CLL CELLS. Since CAde is the major metabolite of CdA measured in the plasma of patients treated with CdA [9], we investigated whether CAde was metabolized by lymphocytes isolated from patients with B-CLL (Fig. 4B). CAde was converted into nucleotides by B-CLL lymphocytes, although to a lesser extent than in EHEB cells. As in EHEB cells, chloro-ATP was also the major nucleotide produced from CAde in B-CLL cells, and its accumulation was accompanied by a decrease in ATP, which was much more pronounced than in EHEB cells.

In Vitro *Toxicity of 2-Chloroadenine*

The potential cytotoxicity of CAde was evaluated by MTT assay after 4 days of exposure of the cells to the drug (Fig. 5). A concentration-dependent decrease in cell survival was observed, with an IC_{50} of 16 μM in EHEB cells. In B-CLL lymphocytes, cytotoxicity occurred at lower concentrations, with an IC_{50} of approx. 5 μM. This IC_{50} is distinctly higher than that observed for CdA in B-CLL cells, which reaches 0.36 μM [17]. It is noteworthy that the IC_{50} recorded for CdA in B-CLL cells was not significantly modified when determined in the presence of 100 nM CAde, the maximum concentration prevailing in plasma after treatment with CdA [9] (results not shown).

Cytotoxicity can occur through necrosis or through apoptosis. Activation of apopain/caspase-3, a member of the ICE (interleukin-1β converting enzyme) protease family, is a crucial event in programmed cell death [20, 21]. As shown in Fig. 6, CAde induced a dose-dependent increase in apopain activity in EHEB cells incubated for 24 hr in the presence of CAde. It is interesting to note that the activation of apopain measured after 24 hr of incubation in the presence of 50 μM CAde was similar to that induced by 50 μM CAdo. Activation of apopain by CAde was also observed in B-CLL cells; however, it occurred at lower concentrations of CAde than in EHEB cells, in agreement with the outcome of the cytotoxicity assay shown in Fig. 5. These results indicate that cells incubated in the presence of CAde undergo apoptosis or programmed cell death. This is confirmed by the fact that high molecular weight DNA fragments, characteristic of the apoptotic process, were

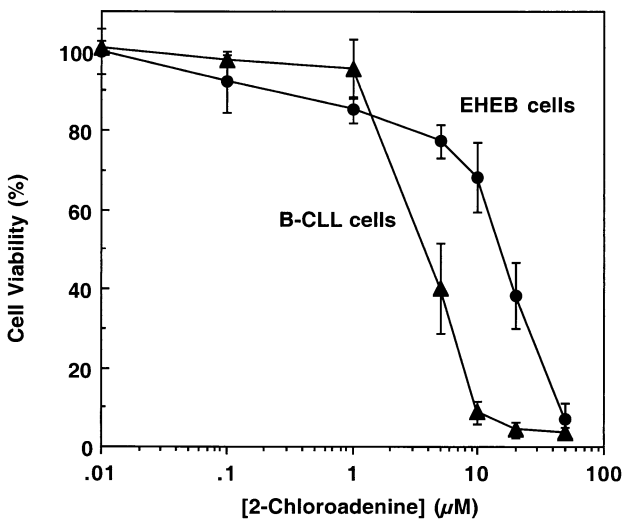

FIG. 5. Effect of CAde on viability of EHEB and B-CLL cells. Cell viability was measured by the MTT assay after 4 days of incubation in the presence of various concentrations of CAde. Results are means ± SEM of 3 separate experiments.

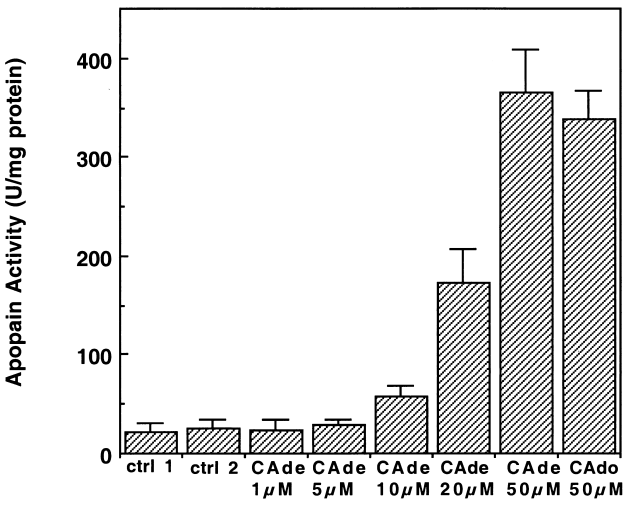

FIG. 6. Effect of CAde on apopain activity in EHEB cells. EHEB cells were incubated for 24 hr in the presence of various concentrations of CAde or in the presence of 50 μM CAdo before determination of apopain activity. Ctrl 1 corresponds to cells incubated without addition; ctrl 2 corresponds to cells incubated with an amount of neutralized blank extract equivalent to that introduced when 50 μM CAde was added to cells. Results are means ± SEM of 3 separate experiments.

detected at concentrations of CAde which induce a loss of cellular viability (results not shown).

DISCUSSION

The results reported in this paper show that CAde, the major catabolite found in plasma of patients after administration of CdA [9, 22], can be actively metabolized. We have indeed observed that CAde is converted into the corresponding nucleoside mono-, di-, and triphosphate in EHEB cells, a lymphoblastic cell line (Figs. 3 and 4A), as well as in lymphocytes isolated from patients with B-CLL (Fig. 4B). In both cell types, chloro-ATP was the major metabolite synthesized from CAde, whereas CdATP never exceeded CdAMP in cells incubated with CdA [3, 17, 23]. The enzyme responsible for CAde metabolism is most probably adenine phosphoribosyltransferase, since CAde conversion into nucleotides is inhibited in the presence of adenine. The rate of metabolization of CAde is markedly higher than that of CdA, particularly in EHEB cells, as indicated by the fact that almost equivalent amounts of CAde and CdA nucleotides were produced during incubation with CdA contaminated by only 2% of CAde (Fig. 1). In B-CLL lymphocytes, the rate of metabolization of CAde is lower than in EHEB cells. Nevertheless, the concentration of chloro-ATP reached after 5 hr of incubation in the presence of 0.5 μM CAde (0.83 ± 0.12 pmol/10^6 in Fig. 4B) was almost 3-fold higher than the concentration of CdATP reached after the same time of incubation in the presence of 0.5 μM CdA [17].

Despite a high rate of metabolism, CAde is less toxic for cells than CdA. CdA is indeed known to be cytotoxic in the nanomolar range in proliferating [23] and B-CLL cells [17], whereas cytotoxicity of CAde in the latter cells was detected only at micromolar concentrations (Fig. 5), which are not reached therapeutically *in vivo*. On the other hand, we have observed that CAde at 100 nM, a concentration prevailing in the plasma after the administration of CdA to patients, does not modify the sensitivity of B-CLL cells to CdA. These results indicate that CAde, although produced *in vivo* from CdA and thereafter metabolized by lymphocytes, probably does not play a role in the therapeutic effect of CdA.

Cytotoxicity of CAde, like that of CdA, results from induction of apoptosis. This is shown by the fact that CAde, similarly to CdA, induces DNA fragmentation. Furthermore, CAde provokes activation of apopain/caspase-3 (Fig. 6), which plays a pivotal role in programmed cell death [20, 24]. CdA-induced cell death results from the accumulation of intracellular CdATP, leading to initiation of programmed cell death [2]. The mechanism by which CAde triggers apoptosis remains to be investigated. It could be mediated by chloro-ATP, which similarly accumulates after incubation in the presence of CAdo and has also been reported to induce apoptosis in human thymocytes [25, 26] and peripheral blood mononuclear cells [27]. Although CAdo is often considered a metabolically stable adenosine analogue, it has been shown to be phosphorylated not only in EHEB cells (this paper), but also in the lymphoblastoid cell line WI-L2 [28, 29] and in isolated hepatocytes [30]. Whereas Szondy [25, 26] concluded from his studies that CAdo induces apoptosis through a membrane receptor, Barbieri *et al.* [27] have shown that the apoptotic effect of CAdo was strongly decreased by 5-iodotubercidin, an inhibitor of adenosine kinase, indicating that chloro-ATP could play a role in the induction of apoptosis.

The pathway of production of CAde from CdA *in vivo* is still speculative. The fact that CAde concentration in plasma is higher after oral than intravenous administration of CdA [9] suggests acid hydrolysis in the stomach or enzymatic degradation by gut bacterial phosphorylases. However, CAde could also be produced in the liver during first pass, since most of the drug was recovered as CAde following perfusion of rat liver with CdA [31]. Moreover, Fabianowska-Majewska *et al.* [32] have shown that CdA can be catabolized by phosphorolytic cleavage to CAde by human liver methylthioadenosine phosphorylase (5′-deoxy-5′-methylthioadenosine:orthophosphate methylthioribosyltransferase, EC 2.4.2.28). Since this enzyme is also found in other human organs, including prostate, placenta, and muscle, as well as in peripheral blood cells [33, and references therein], all these tissues could contribute to the overall production of CAde. In contrast, the capacity of EHEB cells and B-CLL lymphocytes to cleave CdA seems poor, as indicated by the fact that neither CAde nor CAde nucleotides were detected after incubation with purified CdA in our experiments, at least during the incubation time investigated. This observation accords with the fact

that methylthioadenosine phosphorylase is absent in a number of transformed cell types [34].

Taken together, our results show that EHEB cells are capable of metabolizing CAde at a much higher rate than its parent compound, CdA. However, CAde, at concentrations recorded in the plasma of patients treated with CdA, neither displayed cytotoxicity nor influenced the killing efficiency of CdA. In addition, our data stress the fact that results obtained with labeled precursors have to be interpreted with caution, since, as reported here, contaminants are sometimes used more extensively than the precursor compound itself.

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