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THE INFLUENCE OF 2-CHLORO-2′-DEOXYADENOSINE ON METABOLISM OF DEOXYADENOSINE IN HUMAN PRIMARY CNS LYMPHOMA

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Abstract—The effects of 2-chloro-2′-deoxyadenosine (2CdA) on the activity of enzymes important for the metabolism of deoxyadenosine were studied in lysates prepared from human primary central nervous system (CNS) lymphomas and normal human lymphocytes. Strong inhibition (approximately 100%) of the phosphorylation of deoxyadenosine to its deoxynucleotide phosphate derivatives was produced in both systems in the presence of 2CdA, which was phosphorylated concomitantly to 2-chloro-2′-deoxyAMP. Interestingly, 2CdA was also found to be an inhibitor of the deamination of both deoxyadenosine (over 50%) and AMP (70%). These findings add to our understanding of the mechanisms of toxicity of this drug, especially considering that 2CdA is resistant to deamination by adenosine deaminase. These results challenge the existing theories of 2CdA toxicity, which have been limited to the formation of phosphate derivatives of 2CdA. The present *in vitro* studies have demonstrated that 2CdA also inhibits both phosphorylation and deamination of deoxyadenosine (dAdo), suggesting that its mechanism of toxicity includes a block in dAdo metabolic pathways. This has important implications for the perturbation of cell methylation, a functionality associated with, for example, apoptosis.

Key words: cladribine; 2-chloro-2′-deoxyadenosine; deoxyadenosine; CNS lymphoma cells

2CdA§ (cladribine) is an analogue of deoxyadenosine, which is used in the treatment of lymphoid malignancies [1, 2]. According to published data, this drug induces cell apoptosis [3], and the mechanism of 2CdA cytotoxicity occurs via phosphorylated derivatives causing inhibition of DNA synthesis, which involves ribonucleotide reductase, DNA polymerases, and DNA repair [2, 4, 5]. The precise mechanism of action of cladribine in either dividing or nondividing cells is still unknown, primarily because knowledge of the metabolism of 2CdA is limited. Previously, we reported that 2CdA, in addition to phosphorylation to the mono-, di-, and triphosphate chlorodeoxyadenosine, can be inefficiently cleavaged to 2-chloroadenine and deoxyribose-1-phosphate by methylthioadenosine phosphorylase [6]. Moreover, we reported a dramatic decrease in activity of both ADA and SAH hydrolase (approx. 70% and 60%, respectively) in erythrocyte lysates of patients with advanced CLL plus hemolytic anemia after 7 days of treatment with 2CdA [7].

In the present study, the effect of 2CdA on the metabolism of deoxyadenosine in lysates of human primary CNS lymphoma was investigated. We found that 2CdA had several unreported effects, causing a complete block of deoxyadenosine phosphorylation and significant inhibition of deamination of both dAdo and AMP. We suggest that both the depletion of the dATP level and the imbalance of the pool of nucleotides, observed by others [8], result from the inhibitory effect of 2CdA on enzymes involved in the metabolism of deoxyadenosine. Moreover, based on our previous results of studies *ex vivo* of the inhibitory effect of 2CdA on SAH hydrolase activity, we propose that the mechanism of 2CdA toxicity, which originates in a blocking of dAdo metabolism with inactivation of SAH hydrolase occurring as a secondary mechanism due to inhibition by the accumulating dAdo, synergically leads to induction of cell apoptosis.

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§ Abbreviations: SAH, S-adenosylhomocysteine; MTA, 5′-deoxy,5′-methyl-thioadenosine; dAdo, 2′-deoxyadenosine; 2CdA, 2-chloro-2′-deoxyadenosine; dCF, deoxycoformycin; TBA, tetrabutylammonium hydrogen sulphate; PRPP, 5-phosphorylribose-1-pyrophosphate; CNS, central nervous system; ADA, adenosine deaminase; APRT, adenine phosphoribosyltransferase; AMP, adenosine monophosphate; and IMP, inosine monophosphate.

MATERIALS AND METHODS

Lymphoma cells

Human primary CNS lymphoma was obtained postoperatively, and excess tissue cut down and frozen at −70°C until use. A small portion of tissue was minced and homogenized in 50 mM Tris/HCl buffer, pH 7.4, containing 1 mM EDTA. The homogenated lysate of CNS lymphoma was used as a source of the following enzymes: ADA, AMP deaminase, adenine phosphoribosyltransferase (APRT), SAH hydrolase, methylthioadenosine (MTA) phosphorylase, and purine 5′-nucleotidase. For deoxyadenosine phosphorylating activity assays, the lysate was centrifuged at 7000 g for 15 min, and supernatant was used.

Lymphocytes

Heparinized blood was obtained immediately before experiments from healthy laboratory personnel, and the lymphocytes were isolated by Histopaque-1077 density gradient centrifugation at 360 g for 30 min. The cells were washed twice with isotonic Tris/HCl buffer, pH 7.4, containing 1 mM EDTA and lysed by sonication.

The lysed and sonicated lymphocytes were centrifuged at 7000 g for 15 min. After centrifugation, the supernatant was used for deoxyadenosine phosphorylating activity assays, and the sonicated lymphocytes were used as a source of the remaining enzymes.

Enzyme assays

ADA, SAH hydrolase, and MTA phosphorylase activity in lysates of both CNS lymphoma and lymphocytes were assayed as previously reported [9, 10, 11, respectively]. Reaction products (inosine plus hypoxanthine, SAH, and adenine, respectively) were determined by HPLC using a 5 μ Spherisorb ODS-2 column (12.5 × 0.45 cm), and separated from substrate and inhibitor by isocratic elution with ammonium acetate buffer (40 mM), adjusted to pH 4.2 with HCl, containing 9% v/v methanol.

Purine 5′-nucleotidase activity was assayed according to the method of Van Laarhoven *et al.* [12]. The product (adenosine) was determined by HPLC using the same column and separation conditions as for the above enzymes. APRT activity and AMP deaminase activity were assayed utilizing the HPLC method of Fairbanks *et al.* [9]. Formation of AMP and IMP, respectively, was determined using a 5 μ Spherisorb ODS-2 column (12.5 × 0.45 cm) and isocratic elution with buffer: TBA (40 mM) and ammonium acetate (5 mM), adjusted to pH 2.70 with HCl. For 2CdA inhibitory studies, 2CdA was tested at double the concentration of substrate in the incubation mixture of all the above mentioned enzymes. Deoxyadenosine phosphorylating activity was assayed as follows. The standard incubation mixture in a final volume of 105 μL contained 50 mM Tris/HCl buffer (pH 7.4), 10 mM ATP, 5 mM $MgCl_2$, 0.015 mM deoxycoformycin (dCF), and approximately 0.1 mg of protein per sample. The substrate, [8-^{14}C] dAdo, was used at 0.05 mM final concentration, with or without 0.1 mM 2CdA. Formation of radioactive dAMP, dADP, and dATP, as well as incorporation of the dAdo into ATP, was determined by HPLC using a 5 μ Pheno menex amino column (250 × 3.2 mm) and the phosphate buffer system described previously [13], with an in-line radiodetector (Reeve Analytical, Glasgow). Some assays included 1.0 mM phosphoribosylpyrophosphate (PRPP) to activate APRT.

Protein assay

Protein was measured by Sedmak's method with Coomassie brilliant blue reagent [14].

Chemicals

D,L-homocysteine, MTA, AMP, adenosine, adenine, deoxyadenosine, [8-^{14}C]-labelled deoxyadenosine, and Histopaque-1077 were obtained from Sigma Chemical Co (U.K.). 2-Chloro- 2′-deoxyadenosine was kindly donated by Prof. Z. Kazimierczuk (Dept. Biophys., Inst. Experimental Physics, Univ. of Warsaw, Poland) and Ortho-Biotech (U.S.A.).

RESULTS

Deoxyadenosine phosphorylating activity

The possible influence of 2CdA on the phosphorylation of deoxyadenosine in the presence of dCF, an inhibitor of ADA, was evaluated in lysates of normal human lymphocytes and of CNS lymphomas. The effect of 2CdA on the phosphorylation reaction is shown in Fig. 1. The HPLC chromatograms of radioactivity showed that 2CdA strongly inhibited formation of mono-, di-, and triphosphate derivatives of dAdo in lysates of both human lymphocytes (Fig. 1a) and CNS lymphoma cells (Fig. 1b), and that this inhibition of dAdo phosphorylation was competitive. Simultaneous UV-detection showed that the phosphorylation of 2CdA to 2-chlorodeoxy-AMP was evident (chromatograms are not shown). On chromatograms showing radiolabelled products, peaks corresponding to ATP were evident when PRPP was present in the incubation mixture (which makes reaction with ARPT possible) (Fig. 1c). Incorporation of dAdo to ATP was not inhibited by 2CdA, even when phosphorylation of dAdo was completely blocked.

ADA and AMP deaminase activity

The effects of 2CdA, which is resistant to the action of adenosine deaminase, on ADA and AMP deaminase were assayed in lysates of both normal human lymphocyte and CNS lymphoma cells. Results of experiments are shown in Table 1. 2CdA showed significant inhibitory activity, causing a decrease in ADA activity (competitive inhibition) and AMP deaminase of 65% in both lymphocytes. For lysates of CNS lymphoma cells, ADA was inhibited by 55% and AMP deaminase by 75%.

SAH hydrolase, 5′-nucleotidase, MTA phosphorylase and APRT activity

In the lysate of CNS lymphoma, the inhibitory effects of 2CdA on the activity of SAH hydrolase and 5′-nucleotidase fluctuated slightly in individual experiments around 20% (Table 1). For the lymphocyte lysate, the activity of SAH hydrolase was reduced by only 10%, while the inhibition of 5′-nucleotidase activity reached 25%. 2CdA had no effect on MTA phosphorylase activity, measured as adenine released from MTA, in lysates of CNS lymphomas. Results did not show any inhibition of APRT activity by 2CdA (Table 1).

DISCUSSION

In the present study we have demonstrated that 2CdA, an analoque of dAdo that is resistant to deamination by ADA, significantly influenced dAdo metabolism. To investigate this effect, we used both human primary CNS lymphoma (frequently associated with the symptoms of immunosuppression) and normal human lymphocytes. The selection of these two cell systems results from the following premise: The common feature of both systems is that the lymphoma are lymphocytic cells but are characterized by neoplastic growth. At the same time, the latter feature differentiates lymphoma cells from normal human lymphocytes. It is commonly thought that the normal function of immune system cells depends on undisturbed dAdo degradation [15, 16]. It seems advisable to undertake a study encompassing the interaction of 2CdA and the metabolism of dAdo in human lymphocytes; up to the present, this research system has not been the object of a study concerning 2CdA toxicity.

Until now, the mechanism of cytotoxicity of the new antileukemic drug 2CdA has been attributed to 2-chlorodeoxy-ATP formation and its inhibitory effects on DNA synthesis. Our studies show that in the presence of 2CdA the conversion of dAdo to its other metabolites is greatly inhibited. We observed that the dAdo phosphor-

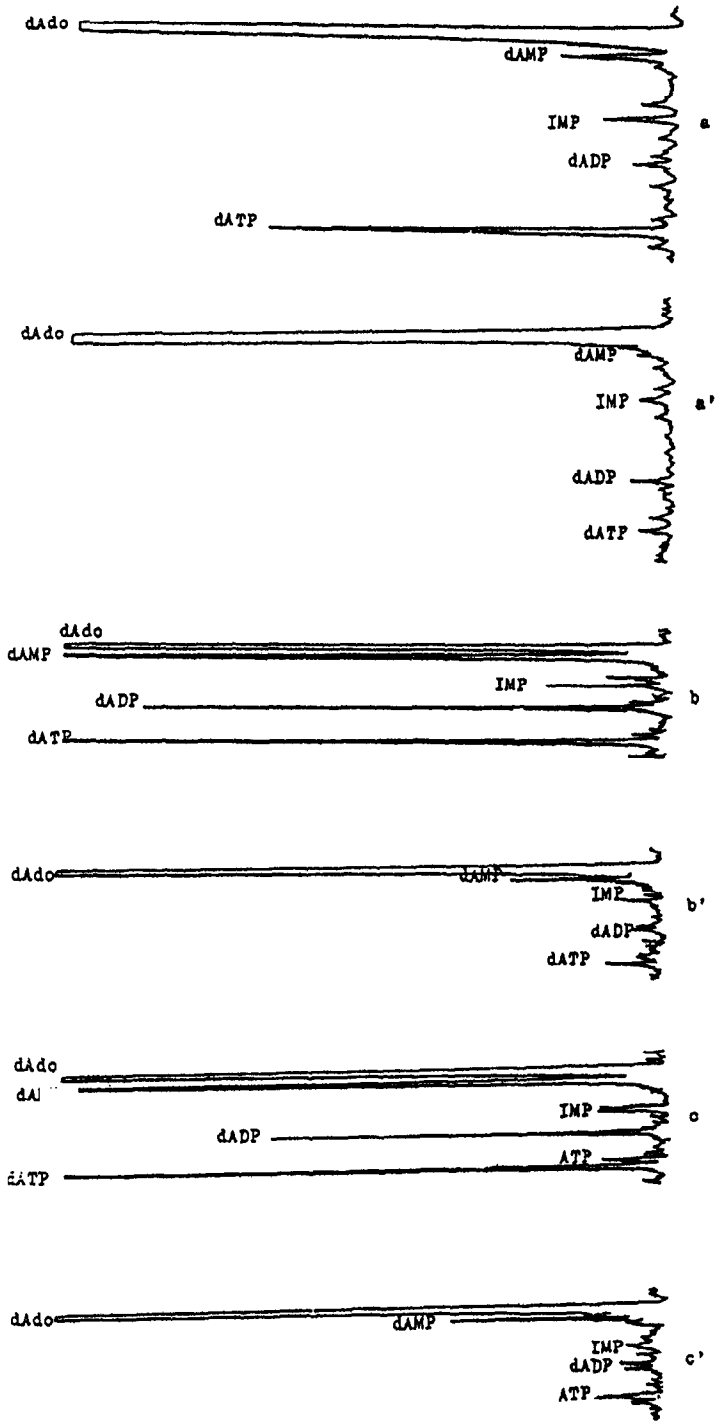

Fig. 1. Effect of 2-chloro-2′-deoxyadenosine on deoxyadenosine phosphorylating activity. Elution profile of labelled metabolites present in mixture after incubation (1 hr) with (a) extracts of human lymphocytes, (b) extracts of CNS lymphoma, and (c) extracts of CNS lymphoma and PRPP (1 mM). The concentration of substrate, [8-^{14}C]dAdo, was 0.05 mM and 0.23 μCi per sample. The concentration of inhibitor, 2CdA (chromatograms: a′, b′, c′, respectively), was 0.1 mM. Specific phosphorylating activity for dAdo: in lymphocytes 1.44 nmol/mg protein/hr and in CNS lymphoma, 8.6 nmol/mg protein/hr.

ylation is inhibited by 2CdA by almost 100%. It is necessary to stress that the product (i.e., dAMP calculated together with dADP and dATP) cannot be converted to other products. To eliminate non-enzymatic and enzymatic degradation of dAMP, the results were first estimated both with and without 2CdA in incubation mixture under the same reaction conditions. Secondly, to prevent dAMP from acting on purine 5′-nucleotidase, a supernatant of lyzed cells (i.e., without membrane fraction) of both systems was used. Moreover, since dAMP

Table 1. Effect of 2-chloro-2′-deoxyadenosine on the activity of some enzymes of the adenine salvage pathway in lysates of human lymphocytes and CNS lymphoma. Enzyme activities were determined as described in Materials and Methods. Each value of activity (nmol/mg protein/hr) represents the mean ± SEM of four experiments. Results of % inhibition are the average of at least four experiments, using double concentration of inhibitor compared to substrate. The differences between separate experiments with inhibitor did not exceed 10%.

Enzyme	Human lymphocytes		CNS lymphoma	
	Activity nmol/mg protein/hr	Inhibition by 2Cda %	Activity nmol/mg protein/hr	Inhibition by 2Cda %
ADA	2017.0 ± 140.0	65.0	5649.0 ± 390.0	55.0
AMP deaminase	47.0 ± 4.0	65.0	52.3 ± 4.0	75.0
SAH-hydrolase	51.0 ± 4.0	10.0	67.5 ± 5.0	18.0
5′-nucleotidase	221.0 ± 15	25.0	566.0 ± 40.0	17.0
Phosphorylating activity for dAdo	1.44 ± 0.2	99.0	8.6 ± 0.6	98.0
APRT	298 ± 8.0	5.0	96.0 ± 7.0	0.0
MTA phosphorylase	—	—	15.2 ± 1.0	0.0

is not a substrate of AMP deaminase [17], conversion of dAMP to dIMP was not possible. Thus, it is clear that the depletion of dATP observed by Hirota in mouse mammary tumor cells after treatment with 2CdA is not only due to inhibition of ribonucleotide reductase [4, 8], but is also the consequence of the inhibition of dAdo phosphorylation. Because deoxycytidine kinase is the enzyme mainly responsible for dAdo phosphorylation in human lymphocyte lysate [18] and because the deoxyadenosine phosphorylating activity in lysate erythrocytes was not inhibited by 2CdA (results not shown), we suggest that 2CdA inhibited only deoxycytidine kinase and not adenosine kinase in lymphocytes and CNS lymphoma. From Seto's studies [19], it is known that deoxycytidine is a competitive inhibitor of the phosphorylation of dAdo and 2CdA in non-dividing lymphocytes. In our study, the analysis of linear plots according to Dixon's method [20] also reveals that 2CdA is a competitive inhibitor of dAdo phosphorylation. Comparison of the kinase activities for dAdo and 2CdA in human lymphocytes supports this statement [21, 22].

In the present study, 2CdA is also shown to be an inhibitor of ADA in both lymphocytes and CNS lymphoma lysates. As the character of inhibition of ADA activity by 2CdA is competitive, and 2CdA is resistant to the enzyme just as other 2-halogenated adenosine analogues [4] that prevent the beginning of enzymatic deamination [23], it seems that 2CdA effectively replaces the natural substrate in the active centre of ADA. Inhibition of ADA activity has also been demonstrated in our previous studies *in vivo* where we observed a rapid decrease in ADA activity (by 70%) in erythrocyte lysate of patients after 7 days' treatment with 2CdA [7] (*in vivo* experiments estimating enzyme activities resulting from metabolic conversion of dAdo within human organism after drug, i.e., 2CdA, application). Moreover, our present studies show that AMP deaminase activity is also inhibited by 2CdA, and recently reported data have attributed the inhibition of deoxycytidine deaminase activity in human lymphocytes to 2CdA [24]. Thus, it seems that the inhibition of deamination reactions by 2CdA might also result in the imbalance of the pool of nucleotides.

Regarding the experiments showing incorporation of dAdo to ATP pool during the determination of phosphorylating activity in lysates of CNS lymphoma, it is evident that although 2CdA significantly blocks phosphorylation and deamination of dAdo, it neither inhibits the release of adenine from dAdo nor does it change the percent of incorporation of dAdo into the pool of ATP. The incorporation of dAdo into ATP, with low efficacy, was previously observed in erythrocytes, and the release of adenine was attributed to SAH hydrolase [13]. It is worth pointing out that in lysate of CNS lymphoma, only about 1.5% of total radioactivity was found as ATP, 10-fold less than for both human lymphocytes and also intact erythrocytes [13].

In the study that examined the effect of 2CdA on SAH hydrolase activity, we noted only slight inactivation of this enzyme. Previously, we noted a decrease in SAH hydrolase (of 60%) in lysates of erythrocytes of patients after 7 days' treatment with 2CdA [7]. Diminution of this same enzyme activity in lymphocytes of patients treated with 2CdA was also noted (results not shown). Thus, if 2CdA does not influence SAH hydrolase activity as a direct mechanism of toxicity (experiments *in vitro*), it seems that for *in vivo* conditions where the metabolism of dAdo is rapidly reduced by 2CdA, SAH hydrolase is inactivated by dAdo. Additionally, the inhibition of adenosine deaminase results in an increase not only of deoxyadenosine, but also of adenosine, a strong inhibitor of SAH hydrolase [13]. The significance of the inactivation of SAH hydrolase by dAdo was stressed by Henderson several years ago [25], and we would also like to stress the importance of the inhibitory effect of dAdo on SAH hydrolase activity created by 2CdA, involving perturbation of SAM-dependent methylation reactions. This has been confirmed by more recent data: Laird supports an exceptional role of perturbation of DNA methylation on gene expression and generating mutations of cytosine residues [26]; Roberts indicates that a close relationship exists between methylation reactions in which S-adenosylmethionine is the donor of methyl groups and the action of DNA repair systems [27]; and Endresen describes that adenosine analogues (i.e., 3-deazaadenosine) may contribute to the toxic mechanism that induces cell apoptosis [28]. Although we did not estimate the integrity of DNA of each cell system after exposition of 2CdA, it has been previously documented that 2CdA induced programmed cell death in lymphocytes from patients with chronic lymphocytic leukemia (CLL), and that the drug-induced DNA fragmentation was both time- and dose-dependent [3].

Moreover, it is known that the inactivation of SAH

hydrolase by dAdo is associated with the irreversible binding of NAD [29]. Thus, the observation that depletion of NAD levels is the result of 2CdA therapy can be attributed not only to consumption of NAD for poly-(ADP-ribosyl) synthesis [30], but also to inactivation of SAH hydrolase by non-metabolized dAdo.

In summary, it is conceivable that the imbalance of the nucleotide pool, the depletion of NAD levels, and the impairment in DNA repair or synthesis may be attributed to the toxic effect of 2CdA in significantly reducing dAdo metabolism and thus methylation reactions. According to our above hypothesis, the cytotoxic mechanism of 2CdA that leads to induction of apoptosis may also originate in inhibition of both deamination and phosphorylation of dAdo with subsequent inactivation of SAH hydrolase as a natural consequence secondary to dAdo accumulation. This may explain why the action of both deoxynucleotides—deoxyadenosine in the presence of dCF and 2-chlorodeoxyadenosine—are similar [19, 31].

Further investigations relating to 2CdA cytotoxicity are currently being carried out in our laboratories in an attempt to verify our hypothesis.

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