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Evidence that the carbocyclic analog of adenosine has different mechanisms of cytotoxicity to cells with adenosine kinase activity and to cells lacking this enzyme

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The carbocyclic analog of adenosine (*C*-Ado*, (±)aristeromycin) is metabolized to triphosphates of *C*-Ado and of carbocyclic guanosine and has been observed to exert a number and variety of metabolic effects [1–3]. In our earlier studies with adenosine analogs [4, 5], *C*-ado was unique in that cells that lacked adenosine kinase (AK^- cells) were about as sensitive to inhibiton by *C*-Ado (as determined by inhibition of colony formation) as were cells with adenosine kinase activity (AK^+ cells). These results indicated several possibilities relative to inhibition by *C*-Ado: (a) phosphorylation of *C*-Ado was catalyzed by more than one kinase; (b) unphosphorylated *C*-Ado was inhibitory and was primarily responsible for the toxicity to both AK^+ and AK^- cells; (c) *C*-Ado inhibited by one mechanism in cells that could phosphorylate *C*-Ado and by another in cells that lacked kinase activity. We present here evidence in support of two independent mechanisms of cell kill by *C*-Ado. A preliminary report of some of these results has been presented [6].

Materials and methods

The effects of *C*-Ado were studied in cultured L1210 cells and a subline selected for resistance to 6-(methylmercapto)purine ribonucleoside (MeMPR) and devoid of activity of adenosine kinase [3]. The parent cell line henceforth is designated as L1210/0 and the MeMPR-resistant subline as L1210/MeMPR. These cells were maintained and grown in Fischer's medium [7]. Three criteria of cytotoxicity were used: (a) effects on the capacity to form colonies when cells were cloned in soft agar in the presence of the drug; (b) effects on the rate of proliferation of suspension cultures of cells in medium containing the drug; and (c) effects on the viability of cells, as determined by colony formation in soft agar, after various periods of exposure to the drug. The effects of *C*-Ado on macromolecular synthesis were determined, as described elsewhere [8], by measurement of effects on the utilization of [4,5-^{3}H]-L-leucine [0.06 Ci/mmole (New England Nuclear, Boston, MA)], [5-^{3}H]uridine [20 Ci/mmole (Moravek Biochemicals, Inc., Brea, CA)] and [*methyl*-^{3}H]thymidine [6,7 Ci/mmole (Moravek Biochemicals, Inc.)] For determination of the K_i of *C*-Ado for AdoHcy hydrolase from L1210 cells, we used the enzyme previously described [9], which was prepared by an adaptation of the method used by Hershfield [10] for the enzyme from WI-L2 human lymphoblasts; the assay involved determination of the [^{14}C]AdoHcy formed when [^{14}C]adenosine and homocysteine were incubated with the enzyme. Analysis of nucleotides by high pressure liquid chromatography (HPLC) was accomplished with a Series 4 liquid chromatograph (Perkin-Elmer, Norwalk, CT) and a Partisil-10 SAX anion exchange column (Whatman, Inc., Clifton, NJ) as described earlier [8].

Assays of effects on synthesis of *S*-adenosylhomocysteine (AdoHcy) in intact cells were performed essentially by the method of Zimmerman *et al.* [11, 12]. L1210 cells ($\sim 8 \times 10^8$ cells) were suspended in 20 ml of Dulbecco's phosphate-buffered saline containing 10% fetal calf serum. To this mixture was added 200 μCi of L-[2-^{3}H]methionine (sp. act. 3.9 Ci/mmole; Amersham Corp., Arlington Heights, IL). After 1 hr the cells were separated by centrifugation, washed with medium, and resuspended in 200 ml of medium. This suspension was divided into eight 25-ml samples, two of which served as controls. To the other six, *C*-Ado was added to various concentrations. After 2 hr the cells were separated by centrifugation, washed free of medium, and extracted with cold 0.5 N perchloric acid. The extracts were neutralized with $KHCO_3$, the $KClO_4$ was moved by centrifugation, and the supernatant solution was lyophilized to dryness. The residue was dissolved in water and subjected to HPLC on a μBondapak C_{18} column (Waters Associates, Milford, MA); elution was achieved with the gradient described by Zimmerman *et al.* [12].

Results and discussion

The concentrations of *C*-Ado required for 50% inhibition of colony formation by L1210/0 and L1210/MeMPR cells were the same (0.3 μM), as shown in Table 1, which contains, for comparison, previously published results [5] showing that AK^- HEp-2 cells also had, at best, only a low degree of resistance to *C*-Ado. In these determinations cells were exposed to drug for the entire incubation period (10 days), and it was therefore of interest to determine if any difference in sensitivity could be detected under other conditions of exposure. When the effects of *C*-Ado on the proliferation of cells were examined, AK^+ cells were much more sensitive to inhibition, as is shown in Fig. 1. The total number of cells present at 24 and 48 hr was reduced in both AK^+ and AK^- cultures, but the reduction was much less in the AK^- cultures. To investigate further this difference in response, the effects of *C*-Ado on the viability of AK^+ and AK^- cells were determined, with the results shown in Fig. 2. Incubation for 2 hr in the presence of *C*-Ado at concentrations up to 38 μM did not affect the viability of the AK^- cells, whereas a concentration of *C*-Ado of 20 μM reduced viability of AK^+ cells by more than 80%. Marked differences in response of the two cell lines also existed at 4 and 6 hr, but after 24-hr exposure even the lowest concentration of *C*-Ado studied (3.4 μM) reduced the viability of both cell lines almost to 0.

Table 1. Cytotoxicity of *C*-Ado to HEp-2 cells, L1210/0 cells, and sublines deficient in adenosine kinase

Cell line	IC_{50} (μM) for *C*-Ado
L1210/0	0.3
L1210/MeMPR	0.3
HEp-2	0.7
HEp-2/MeMPR	1.4

Cytotoxicities were determined by measurement of effects on the formation of colonies; see text. Values shown are averages of two or more experiments. The results with HEp-2 cells are taken from an earlier publication [5].

* Abbreviations: *C*-Ado, the carbocyclic analog of adenosine; MeMPR, 6-(methylmercapto)purine ribonucleoside; AdoHcy, *S*-adenosylhomocysteine; AK, adenosine kinase; and HPLC, high pressure liquid chromatography.

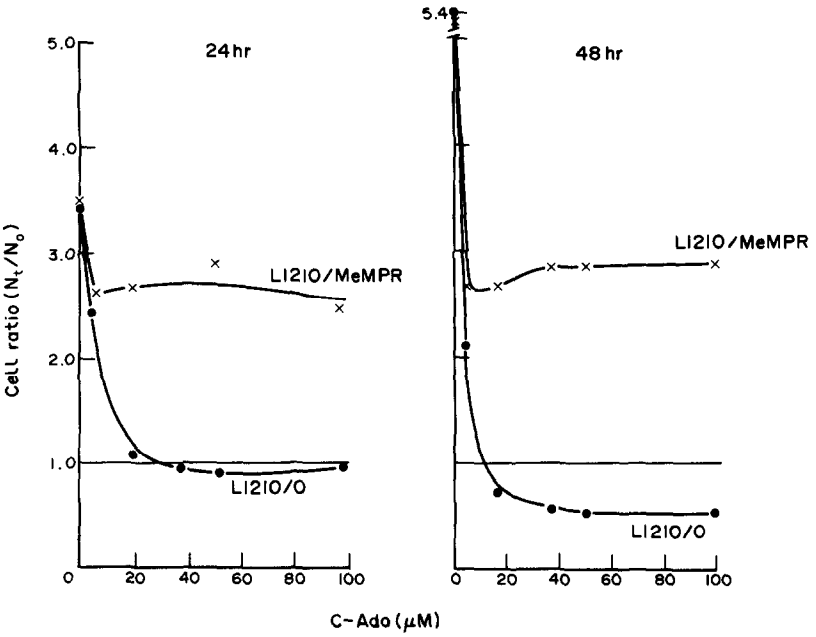

Fig. 1. Effects of *C*-Ado on proliferation of L1210/0 and L1210/MeMPR cells. To cells in suspension culture (~10^5 cells/ml) *C*-Ado was added at the indicated concentrations, and cells were counted 24 and 48 hr thereafter. Ordinates: Ratio of number of cells at indicated times to number present at initiation of the experiment. Points falling below a ratio of 1.0 (horizontal line) indicate a reduction of cell number below that of the initial inoculum.

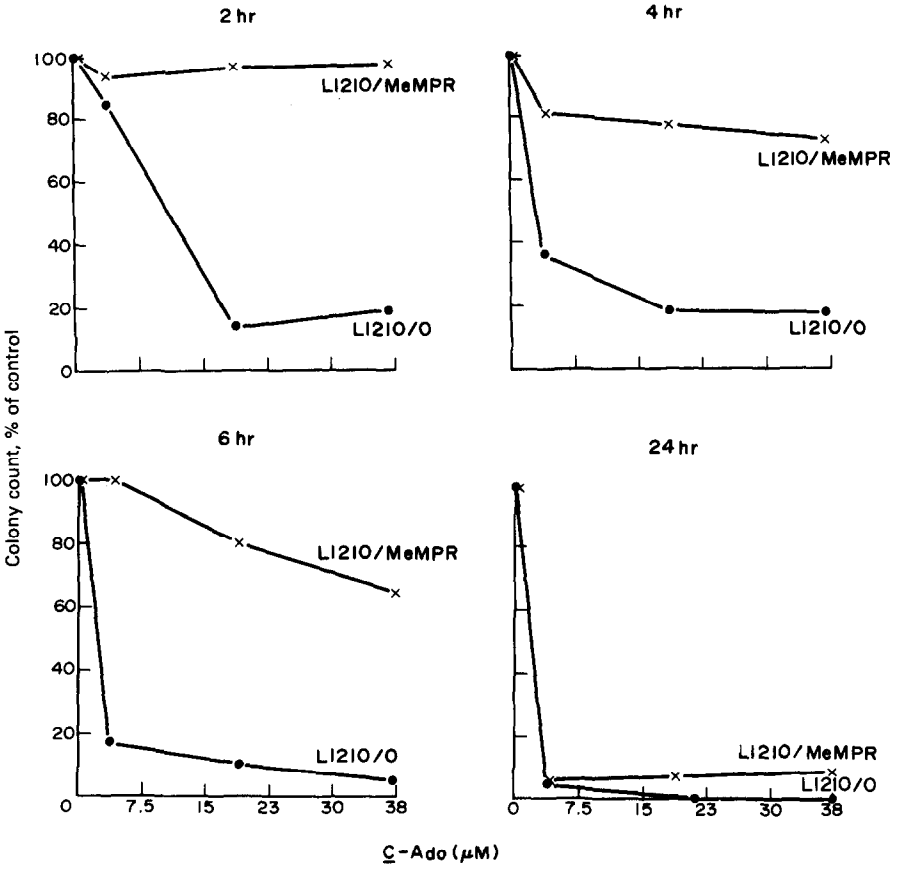

Fig. 2. Effects of *C*-Ado on the viability of L1210/0 and L1210/MeMPR cells. *C*-Ado was added at the indicated concentrations to suspension cultures (~10^5 cells/ml) in logarithmic growth. At 2, 4, 6, and 24 hr thereafter cells were harvested and washed free of drug-containing medium. Samples of 100 cells were then placed on soft agar, and the colonies formed were counted 10 days thereafter.

Further evidence of differences in action of C-Ado in the two cell lines is shown in the effects on synthesis of macromolecules (Fig. 3). In AK^- cells C-Ado at a concentration of 50 μM inhibited strongly, but without selectivity, the utilization of leucine, thymidine and uridine. Lesser degrees of inhibition of all three precursors, but still without selectivity, were observed with concentrations of C-Ado down to 10 μM (results not shown). In contrast, in AK^- cells C-Ado at a concentration of 100 μM had little or no effect on the utilization of any of the precursors (Fig. 3).

These results suggest that the mechanisms of cell kill are different in AK^- and AK^+ cells. However, a possible alternative explanation is that C-Ado may be a substrate for both AK and some other kinase, that its activity for the other kinase is poor, and that, as a result, a longer time is required for a toxic concentration of phosphates to accumulate in AK^- cells. We reported earlier that L1210/MeMPR cells do not form detectable amounts of C-Ado phosphates in a 4-hr incubation [3]. To examine this possibility more thoroughly, L1210/MeMPR cells were placed in medium containing C-Ado at a concentration of 25 μM, and HPLC analyses were performed at periods of 4, 8, 16, and 24 hr thereafter. No trace of C-Ado phosphates was detected. (These negative results, which are similar to those already published for the 4-hr study [3], are not shown.)

The nature of the lethal events in the two types of cells is a question of interest. C-Ado is known to inhibit multiple metabolic processes in AK^+ cells, and a primary site of action has not yet been identified [3]. In AK^- cells cytotoxicity must be due to an action of the unphosphorylated nucleoside. For this site of action, AdoHcy hydrolase appeared to be the most likely candidate because of its known high sensitivity to inhibition by C-Ado: the K_i for inhibition of AdoHcy hydrolase from beef liver was 5 nM [13]. The enzyme from L1210/0 cells was also found to be very sensitive to inhibition; the K_i, determined from Lineweaver–Burk plots (not shown), was 25 nM. Evidence that C-Ado inhibits AdoHcy hydrolase in intact L1210 cells is shown in Table 2. C-Ado produced an accumulation of AdoHcy in both AK^+ and AK^- cells, but the accumulation was greater in AK^- cells. In both cell lines a concentration of 20 μM produced an increase that was not much elevated as the concentration was increased to 150 μM. The greater effect in AK^- cells probably is due to the fact that the pool of free nucleoside should be more constant in these cells in contrast to AK^+ cells in which it is continuously decreasing as C-Ado is phosphorylated. The degree of accumulation

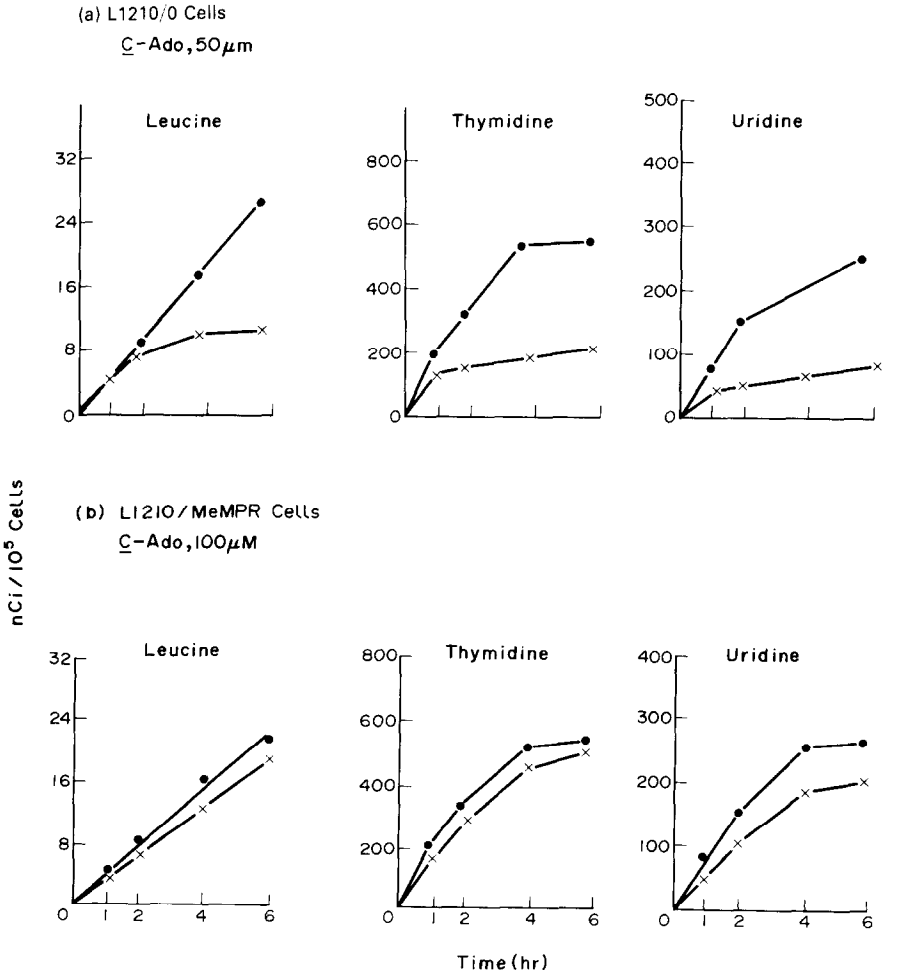

Fig. 3. Effects of C-Ado on the synthesis of macromolecules in L1210/0 and L1210/MeMPR cells. To cells (5×10^5 cells/ml) in logarithmic growth C-Ado was added followed 0.5 hr thereafter by [4,5-^{3}H]-L-leucine, [5-^{3}H]uridine, or [*methyl*-^{3}H]thymidine, each at a final concentration of 2 μCi/ml. Control cultures received only the radioactive substrate. At 1, 2, 4 and 6 hr after addition of the labeled substrate, cells were harvested and extracted with cold trichloroacetic acid. The acid-insoluble residues were assayed for radioactivity by liquid scintillation spectrometery. Key: (●—●) control cells; and (×—×) cells treated with C-Ado at the concentrations indicated in the chart.

Table 2. Effects of *C*-Ado on synthesis of *S*-adenosylhomocysteine in L1210/0 cells and L1210/MeMPR cells

C-Ado (μM)	*S*-Adenosylhomocysteine (concentration as percent of concentration in controls)			
	L1210/0 cells		L1210/MeMPR cells	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
20	180	335	780	690
50	240	371	820	653
150	311	430	708	641

The synthesis of [^{3}H]AdoHcy from L-[2-^{3}H]methionine was followed by HPLC as described in the text. Results of two representative experiments are given to show the degree of variation. In each of the experiments, L1210/0 and L1210/MeMPR cells were assayed in parallel.

of AdoHcy in L1210 cells is about the same as that found in 3T3-LI fibroblasts [13]. The fact that micromolar concentrations of *C*-Ado produced at best 6 to 8-fold accumulation of AdoHcy appears inconsistent with the low K_i of *C*-Ado for AdoHcy hydrolase; however, it has been noted by others [14] that some of the more potent inhibitors of the isolated enzyme produce only modest increases in AdoHcy in intact cells. For the purpose of the present study these results serve to demonstrate that AdoHcy hydrolase is inhibited by *C*-Ado in AK$^-$ cells; they do not, of course, prove that inhibition of this enzyme is the mechanism by which *C*-Ado kills AK$^-$ cells, but they are not inconsistent with it being so. Evidence of another kind that inhibition of AdoHcy hydrolase may be the lethal action of *C*-Ado in AK$^-$ cells is the recent report* that a line of AK$^-$ murine T lymphoma cells, selected for resistance to adenosine, had increased levels of *S*-adenosylmethionine and was cross-resistant to *C*-Ado.

In summary, these studies show that *C*-Ado was toxic at low concentrations to both AK$^+$ and AK$^-$ cells, but that the mechanisms of cell kill were different in the two types of cells. *C*-Ado apparently killed AK$^+$ cells by a relatively rapid process presumably mediated by phosphates of *C*-Ado, and killed AK$^-$ cells by a considerably slower process due to an action (possibly inhibition of AdoHcy hydrolase) of the unphosphorylated nucleoside analog.

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