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EFFECT OF THE DIALDEHYDE DERIVATIVE OF 5′-DEOXYINOSINE ON PYRIMIDINE DEOXYRIBONUCLEOSIDE METABOLISM IN L1210 CELLS*

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Abstract—The effects of the dialdehyde derivatives of inosine (Inox) and 5′-deoxyinosine (5′-dInox) on L1210 cells were compared. The growth of L1210 cells was inhibited to a greater extent by 5′-dInox than by Inox. The increased inhibition of L1210 cell growth by 5′-dInox was also reflected by the increased inhibition of the incorporation of precursors into RNA, DNA and proteins. Even though 5′-dInox was a more potent inhibitor, Inox accumulated in the L1210 cells to levels 4- to 5-fold greater than 5′-dInox. The metabolism of [5-^{3}H]deoxycytidine and [5-^{3}H]deoxyuridine by L1210 cells in culture, in the presence of Inox or 5′-dInox, indicated that dCMP deaminase was an intracellular site of action for 5′-dInox. The dCMP deaminase activity in cell-free extracts prepared from 5′-dInox-treated cells was reduced markedly. This decrease in activity was not reversed by increased substrate concentrations nor was the activity subject to allosteric activation by dCTP. Deoxyuridine and deoxycytidine were able to reverse the effects of 5′-dInox on the inhibition of L1210 cell growth.

Dialdehyde derivatives of nucleosides have been shown to be effective antitumor agents in various animal models [1, 2]. Studies have shown that there are multiple metabolic sites at which these agents act as determined by effects of the compounds on specific enzymes [3–9]. The dialdehyde derivative of inosine (Inox)‡ has been tested in Phase I and II clinical trials [10–12]. Many of the properties of Inox were determined including the biochemical pharmacology [13, 14], the cell-cycle specificity [15, 16], chemical reactivity [17] and antitumor activity [16].

It had been shown that Inox was phosphorylated by intact tumor cells and it appeared that many properties of this compound were due to the phosphorylated derivatives [13, 18]. The dialdehyde derivative of cytidine was also shown to be phosphorylated by L1210 cells [2]. However, when the 5′-deoxy-analog of Inox, which could not be phosphorylated by the cells, was prepared and studied, it was found that 5′-dInox was, in fact, more active on a molar basis than Inox [19]. In this report we present data to show that 5′-dInox interferes with pyrimidine deoxyribonucleoside metabolism at the dCMP deaminase step.

METHODS AND MATERIALS

Growth of L1210 cells. L1210 cells were grown in suspension culture in RPMI 1640 medium which was supplemented with 10% horse serum and erythromycin (50 mg/l). Determinations of cell numbers were performed on a Biophysics Cytograf, model 6300A.

For growth studies, the L1210 cells were inoculated into 25 ml of fresh culture medium at a cell concentration of 1.0 to 1.5 × 10^5 cells/ml. Inox or 5′-dInox, at various final concentrations, was added to the culture medium before the addition of cells. At 24-hr intervals, duplicate aliquots (1.0 ml) were removed from each flask, and cell counts were made in triplicate.

[^{14}C]*Cytidine metabolism.* L1210 cells (approximately 1 × 10^5 cells/ml) were incubated in the presence of Inox or 5′-dInox for 90 min at 37°. [^{14}C] Cytidine (0.5 μCi, 485 mCi/mmole) was added to each flask, and the cultures were incubated for an additional 30 min. The cells were collected by centrifugation and subjected to the Schmidt–Thannhauser procedure [20] to separate the acid-soluble, RNA and DNA fractions. The acid-soluble fractions were neutralized and lyophilized. The acid-soluble samples were dissolved in a small volume of H_2O, and treated with snake venom to hydrolyze the nucleotides to nucleosides [8]. Deoxycytidine was separated from cytidine by chromatography on Dowex-1-borate [21].

[^{3}H]*Leucine metabolism.* L1210 cells were incubated as described above. The cells were labeled for 30 min with [^{3}H]leucine (1.0 μCi/flask, 140 Ci/mmole). The acid-insoluble fractions were extracted from cells pellets with 5% trichloroacetic acid (3 × 1.0 ml each). The pellet was solubilized in NaOH, and aliquots were taken for radioactivity and protein determinations.

Uptake studies. L1210 cells, in log phase, were incubated in RPMI 1640 medium. [^{3}H]Inox (0.4 μCi/

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‡ Abbreviations: Inox, the dialdehyde derivative of inosine (NSC 118994); 5′-dInox, the dialdehyde derivative of 5′-deoxyinosine; C-4′,C-5′-dehydroInox, the dialdehyde derivative of 9-(5-deoxy-β-D-erythro-pent-4-enofuranosyl) hypoxanthine; and PBS, phosphate-buffered saline consisting of 0.136 M NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 1.5 mM KH_2PO_4 and 8.1 mM Na_2HPO_4.

flask, 0.1 mM) or [^{3}H]5′-dInox (0.4 μCi/flask, 0.1 mM) was added to the flask. At various intervals, aliquots (750 μl) of the cell suspension were withdrawn, and the cells were pelleted rapidly in a Beckman microfuge. The pellets were washed twice with 1.0 ml of cold phosphate-buffered saline (PBS). The bottoms of the tubes were cut off and the cell pellets were analyzed for radioactivity [22].

To analyze for the distribution of radioactivity in the soluble extracts, the cell pellets from above were suspended in cold deionized water and homogenized for 1 min in a Potter–Elvehjem vessel. The homogenate was centrifuged for 45 min at 30,000 *g* at 4°. The supernatant fluid (1 ml aliquot) was layered on a Sephadex G-50 column (1.5 cm × 30 cm). The column flow rate was 0.8 ml/min, and fractions (2.0 ml) were collected and analyzed for radioactivity.

Metabolism of [5-^{3}H]*deoxycytidine via dCMP deaminase and thymidylate synthetase in intact L1210 cells.* L1210 cells were incubated in culture with various concentrations of Inox or 5′-dInox for 90 min. [5-^{3}H]Deoxycytidine (1.25 μCi/flask, 20 Ci/mmole) was added, and the incubation was continued for an additional 30 min. For controls, L1210 cells were incubated without drugs. The cells were collected by centrifugation, and the nucleotide fraction was prepared by extraction of the cell pellets with 6% perchloric acid (3 × 1 ml each). Aliquots (100 μl each) were taken for radioactivity measurements. Samples (2.4 ml) of the acid-soluble fractions were heated in a boiling H_2O bath for 30 min to hydrolyze the pyrimidine nucleoside di- and triphosphates to the mononucleotides. These samples were neutralized with 4.0 M KOH and 0.4 M KH_2PO_4. Aliquots (1.0 ml) were added to Dowex-50-H^+ columns (Pasteur pipet). The columns were washed with 50 mM HCl (3 ml) which was collected. Under these conditions, [^{3}H]dUMP and ^3H_2O were eluted from the column. The radioactivity in this column effluent served as a measure of dCMP deaminase activity. An additional aliquot (0.5 ml) was treated with Norit A charcoal (250 μl, 100 mg/ml) to absorb all the nucleotides. The charcoal was removed by centrifugation, and the supernatant fluid was analyzed for radioactivity. This sample contained any ^3H_2O generated via the thymidylate synthetase step.

Metabolism of [5-^{3}H]*deoxyuridine via thymidylate synthetase in intact L1210 cells.* L1210 cells were incubated in culture with various concentrations of Inox or 5′-dInox for 90 min. [5-^{3}H]Deoxyuridine (1.25 μCi/flask, 56 Ci/mmol) was added, and the incubation was continued for an additional 30 min. The acid-soluble fractions were prepared as above. Aliquots (0.5 ml) were treated with Norit A charcoal (250 μl, 100 mg/ml) to asborb the nucleotides. The charcoal was removed by centrifugation, and the supernatant fluid was analyzed for radioactivity. This sample contained any ^3H_2O generated via thymidylate synthetase.

[^{14}C]*Deoxycytidine metabolism.* L1210 cells were incubated in culture with various concentrations of Inox or 5′-dInox for 90 min. [^{14}C]Deoxycytidine (0.125 μCi/flask, 25.2 mCi/mmole) was added, and the incubation was continued for an additional 30 min. Acid-soluble extracts were prepared with 6% perchloric acid, neutralized and lyophilized. The samples were dissolved in 200 μl H_2O and analyzed by high pressure liquid chromatography on a Whatman Partisil SAX column. Separation was achieved via a linear gradient of ammonium phosphate from 0.01 M (pH 2.77) to 0.50 M (pH 4.82). Samples (1.0 ml) were collected directly into scintillation vials and were analyzed for radioactivity.

Enzyme activities in cell-free extracts. L1210 cells were removed from culture and collected by centrifugation. The cell pellets were washed with PBS, and resuspended in potassium phosphate (50 mM, pH 7.0) containing dithioerythritol (1 mM). The cells were homogenized in an Omni-Mixer for 2 min at full speed. The homogenate was centrifuged at 30,000 *g* for 45 min, and the supernatant fluids were used as the source of crude extracts for enzyme analyses.

The assay of dCMP deaminase was carried out by the method of Maley and Maley [23], using [^{3}H] dCMP as the substrate. The assay mixture normally contained in a final volume of 200 μl: [^{3}H]dCMP (0.2 mM, 2.5 μCi/ml), dCTP (0.11 mM), potassium phosphate buffer (50 mM, pH 7.0), and enzyme extract containing dithioerythritol (1 mM). The reactions were started with the addition of enzyme and were carried out for 15 min at 37°. The reactions were terminated by heating in a boiling H_2O bath for 4 min. H_2O (0.8 ml) was added to each tube. After mixing, the samples were applied to a Dowex-50-H^+ column (Pasteur pipet). The dUMP was eluted with 3.0 ml of HCl (50 mM). Aliquots of the effluent were taken for measurement of radioactivity. All assays were set up in triplicate. Blanks were run in which the enzyme was inactivated by heating prior to the incubation.

Thymidylate synthetase activity was assayed by the method of Roberts [24]. The assay mixture in a final volume of 115 μl was prepared as described by Roberts [24]. The reactions were started by the addition of enzyme and were carried out for 30 min at 37°. The reaction was stopped by the addition of trichloroacetic acid (15 μl, 33%) containing dUMP as carrier. Charcoal (200 μl, 100 mg/ml) was added to each tube, and the contents of the tubes were mixed and centrifuged. Aliquots of the supernatant fluids were taken for radioactivity measurements. For blanks, the enzyme was heated before the substrate was added. All assays were set up in triplicate.

Synthesis of 9-(5-deoxy-β-D-erythro-pent-4-enofuranosyl)hypoxanthine dialdehyde (C-4′,C-5′-dehydroInox). Heating of nucleoside dialdehydes causes an elimination reaction yielding a double bond between C-4′ and C-5′ [25]. Inox (100 mg) was dissolved in H_2O (7.0 ml) and refluxed for 90 min. An aliquot of the product was chromatographed on Whatman 3M in *n*-butanol–H_2O (86:14). No hypoxanthine was detected. A difference spectrum, utilizing Inox in the reference compartment, gave peaks at 277 nm and 240 nm. C-4′,C-5′-dehydroInox was more soluble in H_2O than Inox.

Miscellaneous. Protein, RNA and DNA determinations were carried out by the methods of Lowry *et al.* [26], Hurlbert *et al.* [27] and Burton [28] respectively.

Materials. Inox was obtained from the Drug and

Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, through the assistance of Dr. Leonard Kedda. 5′-dInox was prepared in this laboratory as previously described [19]. The biochemicals were purchased from the Sigma Chemical Co., St. Louis, MO. RPMI 1640 culture medium, horse serum and sodium bicarbonate were purchased from the Grand Island Biological Co., Grand Island, NY. The labeled nucleosides were purchased from Moravek Biochemical, City of Industry, CA. The L1210 cell line used in these studies was obtained from Dr. David Kessel, Wayne State University School of Medicine, Detroit, MI.

RESULTS

Effects of Inox and 5′-dInox on L1210 cells in culture. The effects of various concentrations of Inox and 5′-dInox on the growth of L1210 cells in culture were studied. As seen in Fig. 1, Inox at a concentration of 0.1 mM had very little effect on the growth of L1210 cells while complete inhibition of cell growth was obtained with 0.5 mM Inox. With 5′-dInox, L1210 cell growth was completely inhibited at 0.1 mM.

On a concentration basis, 5′-dInox was more inhibitory to the incorporation of [^{14}C]cytidine into RNA, to ribonucleotide reductase activity *in situ*, as measured by the formation of deoxycytidine nucleotides in the acid-soluble pool, and to the incorporation of the labeled precursor into DNA (Fig. 2). This effect was also observed on a time-dependent basis (data not shown).

In addition, a concentration-dependent decrease in [^{3}H]leucine incorporation into the protein was observed (Table 1) with both 5′-dInox and Inox. 5′-Deoxyinox caused greater inhibition at all concentrations studied. This effect was not on leucine uptake, since the total amount of radioactivity associated with the cells remained unchanged (data not shown).

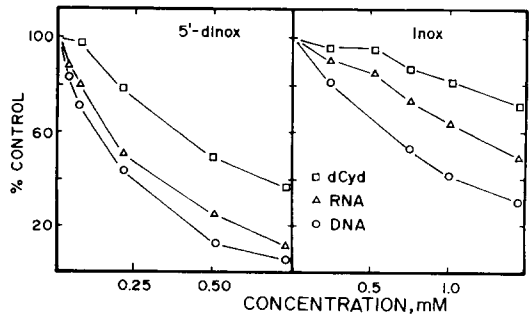

Fig. 2. Effects of Inox and 5′-dInox on [^{14}C]cytidine metabolism. L1210 cells were incubated for 1.5 hr in the presence of Inox or 5′-dInox as indicated. [^{14}C]Cytidine (0.04 μCi/ml, 485 mCi/mmole) was added to each culture, and the cells were incubated for an additional 30 min. The acid-soluble, RNA and DNA fractions were separated. The formation of deoxycytidine nucleotides (dCyd) is shown by the open squares (□), the incorporation into RNA by the open triangles (△) and the incorporation into DNA by the open circles (○).

Uptake of Inox and 5′-dInox by L1210 cells. Utilizing labeled Inox and 5′-dInox at equal concentrations and specific activities, the uptake of these compounds was studied in the L1210 cells. As seen in Fig. 3, the uptake of Inox was considerably greater than the uptake of 5′-dInox. High-speed supernatant fluids were prepared from the Inox- and 5′-dInox-treated cells. Analysis of these samples on Sephadex G-50 showed that labeled 5′-dInox or Inox was bound to macromolecules which eluted in the void volume. A second peak of radioactivity corresponding to free Inox or 5′-dInox was observed. No apparent difference in binding of 5′-dInox or Inox to the macromolecules was observed. When the peak

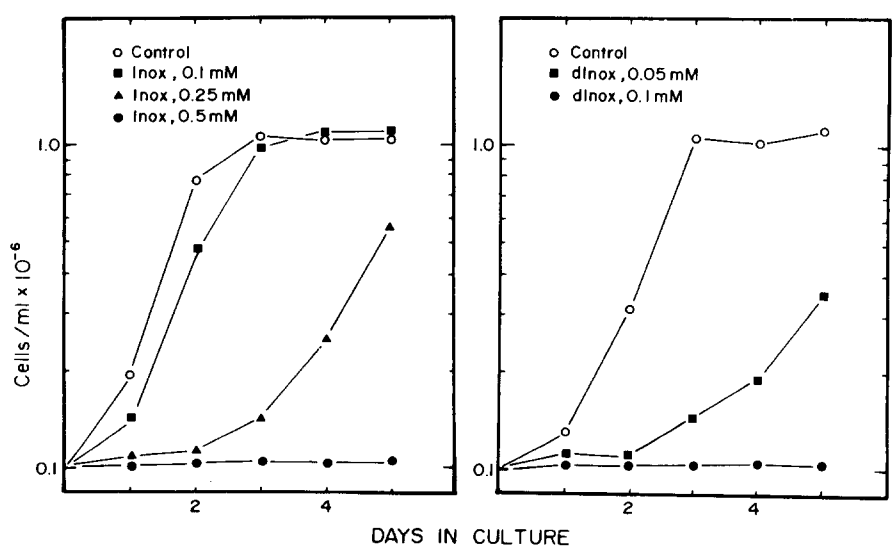

Fig. 1. Effects of Inox and 5′-dInox on the growth of L1210 cells in culture. The cultures were set up as described in Methods and Materials. Inox or 5′-dInox was added to the culture flask at day zero at the concentration indicated.

Table 1. Effects of 5′-dInox and Inox on leucine incorporation into protein*

	% Control
Control	100
0.1 mM 5′-dInox	50
0.5 mM 5′-dInox	8
0.1 mM Inox	84
0.5 mM Inox	40
1.0 mM Inox	10

* Cells were preincubated with the indicated concentration of dialdehyde for 90 min. [^{3}H]Leucine (1.0 μCi/flask, 140 Ci/mmole) was added, and the incubation was continued for an additional 30 min. The protein fraction was isolated by trichloroacetic acid precipitation and solubilized in NaOH.

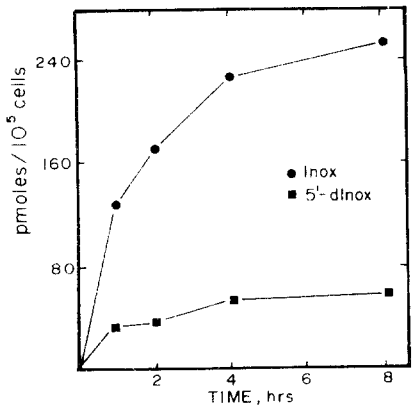

Fig. 3. Accumulation of [^{3}H]Inox and [^{3}H]5′-dInox in soluble extracts from L1210 cells. L1210 cells were incubated with [^{3}H]Inox (0.1 mM, 0.08 μCi/ml) or [^{3}H]5′-dInox (0.1 mM, 0.08 μCi/ml). Aliquots were removed at regularly timed intervals and treated to determine the amount of Inox or 5′-dInox taken up by the cells.

fractions from the void volume were rechromatographed on the Sephadex G-50 columns, all the radioactivity re-eluted with the void volume, indicating tight binding of Inox or 5′-dInox to the macromolecules. The efflux of either [^{3}H]Inox or [^{3}H]5′-dInox from the cells was negligible. In the L1210 cells treated with [^{3}H]Inox, approximately 90% of the Inox was found as a phosphorylated metabolite (90% of this was as the di- and triphosphate derivatives).

Effects of Inox and 5′-dInox on the metabolism of [5-^{3}H]deoxycytidine and [5-^{3}H]deoxyuridine in L1210 cells. The metabolism of [5-^{3}H]deoxycytidine in L1210 cells was studied to determine if Inox or 5′-dInox altered the metabolism of [5-^{3}H]dCyd through the dCMP deaminase and thymidylate synthetase steps. Using the approach described in Methods and Materials, the flux of deoxycytidine through these two steps could be estimated. As seen in Fig. 4, Inox had essentially no effect on the dCMP deaminase step and only a slight effect on thymidylate synthetase. With 5′-dInox, the effects on dCMP deaminase and thymidylate synthetase were more pronounced. The inhibition of the thymidylate synthetase step appeared to be greater than the inhibition of the dCMP deaminase step.

The decrease in flux of deoxycytidine through this pathway was not attributable to inhibition of deoxycytidine kinase. L1210 cells were preincubated with 5′-dInox (0.75 mM) or Inox (1.0 mM) for 90 min and were then labeled with [^{14}C]deoxycytidine (0.125 μCi/flask, 25.2 mCi/mmole) for 30 min. Acid-soluble extracts were prepared and analyzed by high pressure liquid chromatography (HPLC). The distribution of radioactivity in the nucleotides was similar in all conditions. Approximately 70–75% of the label existed as the triphosphates with approximately 20–25% as monophosphates.

To establish the specificity of the site which was altered by 5′-dInox, the L1210 cells were incubated with [5-^{3}H]deoxyuridine in the presence and absence of 5′-dInox. 5′-dInox had no significant effect on the metabolism of [5-^{3}H]deoxyuridine through the thymidylate synthetase step as measured by ^3H_2O release in intact cells.

The data would suggest that the effects observed on [5-^{3}H]deoxycytidine metabolism through the thymidylate synthetase step were secondary effects due to the inhibition at the dCMP deaminase step.

Thymidylate synthetase and dCMP deaminase activities in cell-free extracts from Inox and 5′-dInox-treated cells. L1210 cells were incubated in culture in the presence of Inox or 5′-dInox. Cell-free extracts were prepared from the control and drug-treated cells and used for the determination of enzyme activities. Thymidylate synthetase activity in the extracts from the drug-treated cells was the same as in the control cells. However, in the cell-free extracts prepared from 5′-dInox-treated cells, dCMP deaminase activity, when assayed under the optimal conditions of substrate and dCTP concentrations, was reduced to 40% of control. These data are shown in Table 2. When the enzyme activity was determined as a function of substrate (dCMP) concentration, it was observed that increasing the dCMP concentration had no effect on the dCMP deaminase activity. The

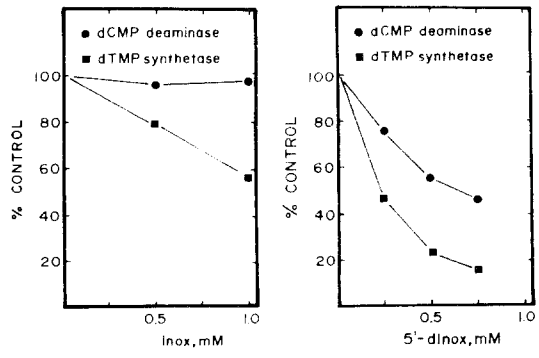

Fig. 4. Metabolism of [5-^{3}H]deoxycytidine in intact L1210 cells in the presence of Inox or 5′-dInox. L1210 cells were incubated for 1.5 hr in the presence of Inox or 5′-dInox at the concentration indicated. [5-^{3}H]Deoxycytidine (0.25 μCi/ml, 20 Ci/mmole) was added, and the incubation was continued for an additional 30 min. The cells were treated as described in Methods and Materials to determine the intracellular activity of dCMP deaminase and thymidylate synthetase.

Table 2. Thymidylate synthetase and deoxycytidylate deaminase activities in crude extracts from Inox and 5′-dInox-treated L1210 cells*

	Conc (mM)	Thymidylate synthetase (nmoles/min/mg)	dCMP deaminase (nmoles/min/mg)
Control		0.98 (100)	19.7 (100)
Inox	0.50	0.96 (98)	19.7 (100)
	1.00	1.11 (113)	
5′-dInox	0.10		13.8 (70)
	0.25	0.91 (93)	
	0.50	1.00 (102)	8.4 (43)
	0.75	1.11 (113)	

* L1210 cells were incubated in culture in the presence of Inox or 5′-dInox as indicated. After 2 hr, cell-free extracts were prepared from the control and drug-treated cells, and the enzyme activities were determined as described in the Methods and Materials. The values in parentheses are percent of control values.

apparent K_m values, with respect to dCMP, were the same (0.40 vs 0.48 mM), while the V_{max} of 5′-dInox-treated extracts was 44% of control (10.8 vs 18.7 nmoles/min/mg). This percent inhibition was observed at all concentrations of dCMP studied.

The activity of dCMP deaminase is known to be activated by dCTP [29]. The effect of dCTP concentration on the activity of dCMP deaminase in extracts from the control and 5′-dInox-treated cells was therefore studied. These data are shown in Fig. 5. In the absence of dCTP, the residual dCMP deaminase activity was slightly greater in the extract from the 5′-dInox-treated cells. When dCTP was added to the assay, the dCMP deaminase activity in the control extract increased as expected. However, the dCMP deaminase activity in the extract from the 5′-dInox-treated cells was not activated by dCTP. Chromatography of the extracts from the control and 5′-dInox-treated cells on Sephadex G-50 did not restore activity in the latter extract to that of the control.

Cell-free extracts were prepared from L1210 cells and were assayed for thymidylate synthetase and dCMP deaminase activities. The addition of exogenous 5′-dInox to the crude extract had no effect on the thymidylate synthetase activity. As seen in Fig. 6, the dCMP deaminase activity in the crude extracts was not stable over the 8-hr period studied. However, in the presence of 5′-dInox, the rate and extent of loss of dCMP deaminase activity were greater. The addition of dCTP to the crude extracts prevented the loss of deaminase activity in both the control and 5′-dInox-treated samples. These data, as well as the data shown in Fig. 5, indicate that 5′-dInox exerts its effect at the dCTP-binding site.

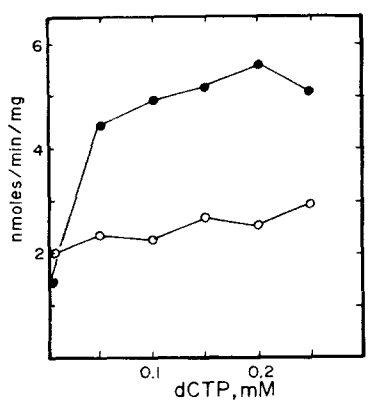

Fig. 5. Effect of dCTP on dCMP deaminase activity in extracts from 5′-dInox-treated cells. The dCMP deaminase activity was determined as a function of the concentration of dCTP. The dCMP deaminase activity in the extract from the control cells is shown by the closed circles (●) while the activity in the extract from the 5′-dInox-treated cells is shown by the open circles (○).

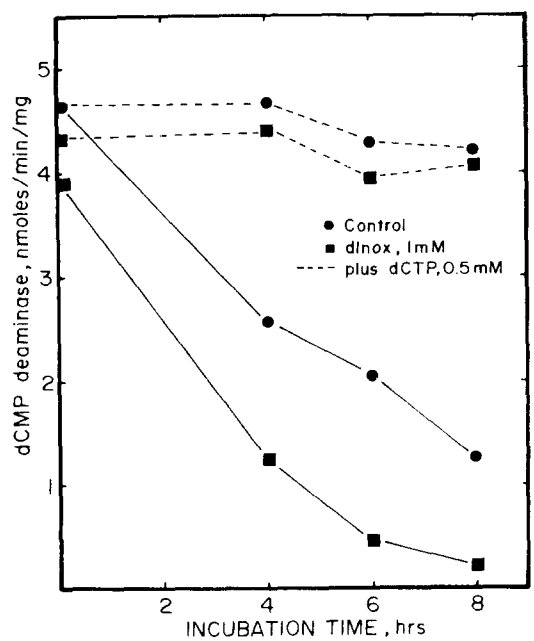

Fig. 6. Effect of 5′-dInox on dCMP deaminase activity in cell-free extracts. Crude cell-free extracts were prepared from L1210 cells. The cell-free extract was incubated on ice in the presence of 5′-dInox (1 mM) (■) or as the control (●). Aliquots were removed and assayed for dCMP deaminase activity. dCTP (0.5 mM) was added to the cell-free extracts at time zero in the presence of 5′-dInox (■—■) or to the control (●--●).

Table 3. Effect of C-4′,C-5′-dehydroInox on the incorporation of [^{14}C]cytidine into RNA and DNA*

Inhibitor (1 mM)	% Inhibition RNA	DNA
5′-dInox	99	99
Inox	40	75
C-4′,C-5′-DehydroInox	18	40

* L1210 cells were incubated in culture in the presence of drugs as indicated for 90 min. [^{14}C]Cytidine (0.20 μCi/flask, 485 mCi/mmole) was added, and the incubation was continued for an additional 30 min. The cells were collected and subjected to the Schmidt–Thannhauser procedure to separate the RNA and DNA fractions [20]. The cultures were set up in triplicate.

Effects of C-4′,C-5′-dehydroInox on the incorporation of [^{14}C]cytidine into RNA and DNA of L1210 cells. L1210 cells were incubated in the presence of C-4′,C-5′-dehydroInox at various concentrations. C-4′,C-5′-DehydroInox was much less active than either Inox or 5′-dInox. At equimolar concentrations (1.0 mM) of the drugs, the inhibition of incorporation of precursors into RNA and DNA was much less in the cells incubated with C-4′,C-5′-dehydroInox. These data are summarized in Table 3.

Effects of deoxyuridine and deoxycytidine on the growth of L1210 cells in the presence of 5′-dInox. Studies were carried out to determine if deoxyuridine could reverse the growth inhibition of L1210 cells in culture by 5′-dInox. These results are shown in Fig. 7. As previously shown (Fig. 1), 5′-dInox (0.1 mM) caused complete inhibition of L1210 cell growth. The presence of dUrd (5 mM), which itself had little effect on L1210 cell growth, was able to at least partially restore cell growth in the presence of 0.1 mM 5′-dInox. Deoxyuridine (5 mM) was not able to reverse or overcome the effects of 0.25 mM 5′-dInox on the growth of L1210 cells. Deoxycytidine, likewise, was able to partially reverse the inhibition of L1210 cell growth by 0.1 mM 5′-dInox (Fig. 8). The inhibition caused by a higher concentration of 5′-dInox (0.25 mM) was not reversed by deoxycytidine

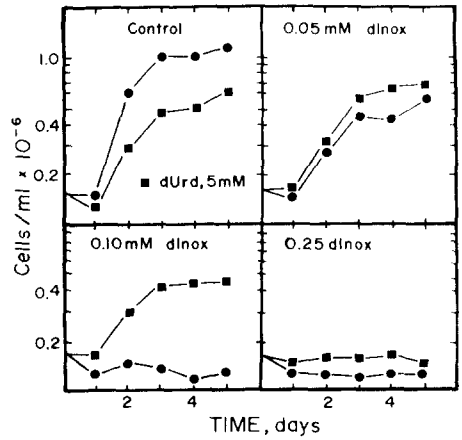

Fig. 7. Effect of deoxyuridine on the growth of L1210 cells in the presence of 5′-dInox. L1210 cells were set up in culture as described in Methods and Materials. Deoxyuridine (5 mM) (■) was added as indicated to the various culture flasks at the same time as 5′-dInox.

(5 mM). Deoxyadenosine, deoxyguanosine and thymidine were also added to the cultures in the presence of 5′-dInox. At the lower concentrations of these agents which did not inhibit cell growth, there were no effects of these deoxynucleosides on the 5′-dInox inhibition. Higher concentrations of deoxyandenosine, deoxyguanosine and thymidine alone inhibited L1210 cell growth.

DISCUSSION

The dialdehyde derivatives of nucleosides have been shown to interfere with tumor cell growth [1, 2] and to inhibit various enzymes [3–9] and certain metabolic processes [13]. It has been shown that the base moiety of the dialdehyde derivative influences the nature and degree of inhibition of enzymes in cellular functions. In a previous study, it was shown that the nature of the dialdehyde moiety also determined the activity of the dialdehyde derivatives of nucleosides. 5′-DeoxyInox was shown to be more inhibitory to ribonucleotide reductase and to inhibit RNA polymerase by a mechanism different from

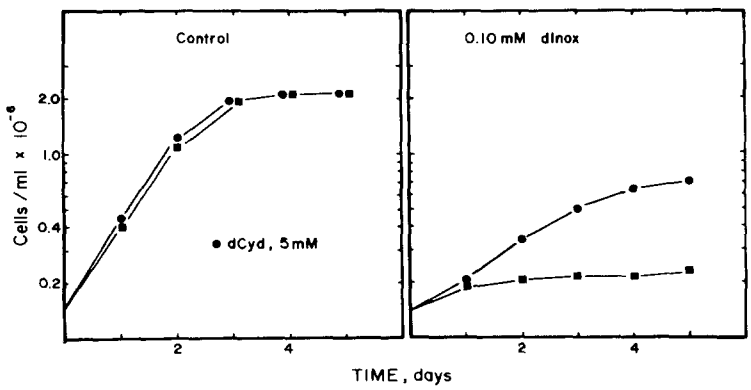

Fig. 8. Effect of deoxycytidine on the growth of L1210 cells in the presence of 5′-dInox. L1210 cells were set up in cultures as described in Methods and Materials. Deoxycytidine (5 mM) (●) was added as indicated to the culture flasks at the same time as the 5′-dInox.

Inox [19]. In the present study, the effects of 5′-dInox and Inox on L1210 cell growth and pyrimidine nucleotide metabolism were compared. 5′-Deoxy-Inox was at least five times more effective than Inox in inhibiting L1210 cell growth in culture. This was not due to increased uptake of 5′-dInox. The amount of Inox taken up by the cells was much greater than the amount of 5′-dInox. It had been shown previously that Inox is phosphorylated to mono-, di- and triphosphates [18]. Kinahan *et al.* [2] have shown that the dialdehyde derivative of cytidine is also phosphorylated by L1210 cells. It is possible that the phosphorylation of Inox resulted in the greater intracellular accumulation. However, with either Inox or 5′-dInox there was essentially no efflux of nucleoside dialdehyde from the L1210 cells. With either drug, a similar percentage of the intracellular drug was found bound to the macromolecules which were excluded from a Sephadex G-50 column. The bound dialdehyde did not dissociate from the macromolecules when the macromolecules in the void volume peak were rechromatographed on Sephadex G-50. Novikoff hepatoma cells are similarly more sensitive to 5′-dInox [19]. The increased inhibition of L1210 cell growth by lower concentrations of 5′-dInox was also reflected in the effects on the incorporation of [^{14}C]cytidine into RNA, conversion to deoxycytidine nucleotides via ribonucleotide reductase, and incorporation into DNA.

This effect could not be explained by a decrease in the cellular phosphorylation of the cytidine precursor via uridine/cytidine kinase and the nucleotide kinases. The nucleotide profiles and distribution of radioactivity in the acid-soluble extracts, as determined by HPLC, were the same in the control and drug-treated cells. The increased sensitivity to 5′-dInox was seen not only as a function of concentration but also as a function of time.

The comparison of the effects of 5′-dInox and Inox on [5-^{3}H]deoxycytidine metabolism in intact cells led to two observations. First, 5′-dInox had a much greater effect on [5-^{3}H]deoxycytidine metabolism than did Inox. Second, it appeared that 5′-dInox inhibited thymidylate synthetase activity in intact cells to a greater extent than dCMP deaminase activity. However, with the use of [5-^{3}H]deoxyuridine, the site of thymidylate synthetase as a metabolic step at which 5′-dInox exerted its effect was essentially ruled out. When the levels of dCMP deaminase and dTMP synthetase activities were determined in extracts prepared from drug-treated cells, it was observed that the levels of dTMP synthetase activity were the same in the extracts from the control and drug-treated cells, while the level of dCMP deaminase activity was reduced in the extract from drug-treated cells. This reduction could not be accounted for by a decrease in *de novo* synthesis of enzyme by protein synthesis inhibition. Concentrations of 5′-dInox and Inox which gave similar inhibition of protein synthesis had very different effects on dCMP deaminase activity. Moreover, cycloheximide, at a concentration which inhibited protein synthesis greater than 95%, had no effect on dCMP deaminase activity in Ehrlich ascites cells (C-A. Ohmstede and J. G. Cory, unpublished observation). In this cell type, 5′-dInox had the same inhibitory effect on dCMP deaminase as in the L1210 cells. The dCMP deaminase activity in the extract from 5′-dInox-treated cells was not brought back to control levels by increasing the effector (dCTP) concentrations in the assay mixture. These data would suggest that the loss of dCMP deaminase activity was due to the alteration of the effector site by 5′-dInox rather than the substrate site. This was supported by the data which showed that dCTP, which stabilized dCMP deaminase activity, also prevented the inhibition caused by 5′-dInox. These data and the data shown in Table 2 indicated that the apparent inhibition of dTMP synthetase activity in intact cells was due to a secondary effect caused by the decrease in flux of deoxycytidine through dCMP deaminase. While the data generated in the studies of [^{3}H]deoxycytidine metabolism in intact cells support the hypothesis that dCMP deaminase was a site of action, it must also be considered that deoxycytidine can be metabolized by two separate routes to dUMP. Deoxycytidine can be phosphorylated directly to dCMP followed by deamination, or dCyd could be deaminated to dUrd followed by phosphorylation to dUMP. The studies carried out in the intact cells would not distinguish between these pathways.

In previous studies, the role of the base moiety in influencing the biological properties of these nucleoside dialdehyde derivatives was shown [30]. As reported in this paper and previously [19], the importance of the nature of the dialdehyde moiety attached to the base was also demonstrated. Further, the relative lack of activity of the C-4′,C-5′-dehydroInox analog also provided evidence that the dialdehyde moiety plays an important role in the biological activity of these dialdehyde derivatives. It has been reported that the α,β-unsaturated dialdehydes are less hydrated in solution [25]. It may be that the extent to which these dialdehyde derivatives form hydrated species [31] determines their relative biological activity.

Leukemia L1210 cells did not grow in the presence of 0.1 mM 5′-dInox. High concentrations of deoxyuridine (5 mM) and deoxycytidine (5 mM) were able to restore partial growth to the 5′-dInox-treated cells. The effects of higher concentrations of 5′-dInox on cell growth could not be reversed by deoxyuridine or deoxycytidine. The partial protection of cell growth afforded by deoxyuridine was possibly the result of bypassing the dCMP deaminase step, or was due to the fact that deoxyuridine led to higher intracellular concentrations of dUMP or dTTP which, in turn, protected the dCMP deaminase from inactivation by 5′-dInox. The protection given by deoxycytidine (5 mM) was probably due to the increased intracellular concentration of dCTP which had been shown to protect dCMP deaminase from inhibition by 5′-dInox. Alternatively, deoxycytidine could have been deaminated to deoxyuridine followed by phosphorylation by thymidine kinase to dUMP, thereby bypassing the block at dCMP deaminase. Attempts to utilize deoxyadenosine, deoxyguanosine or thymidine to reverse the effects of 5′-dInox (0.1 mM) were not successful. Concentrations of deoxyadenosine, deoxyguanosine and thymidine which were not inhibitory to L1210 cell growth had no effect on the inhibition caused by 5′-dInox.

The data presented in this paper and the previous data reported indicate that there are multiple metabolic sites of action of these nucleoside dialdehydes. There does not appear to be a unique site which is responsible for the observed effects. Rather, it is more likely that the observed effect on cellular growth is the consequence of the partial inhibition of several key metabolic pathways. In the case of 5′-dInox, the increased biological effects may be related to effects on metabolic steps related to deoxyribopyrimidine nucleoside interconversions near or at the dCMP deaminase step.

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