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DIALDEHYDE DERIVATIVE OF 5′-DEOXYINOSINE AS A MORE POTENT ANALOG OF THE DIALDEHYDE DERIVATIVE OF INOSINE (NSC 118994)*

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Abstract—The dialdehyde derivative of 5′-deoxyinosine (5′-deoxyinox) was prepared from 5′-deoxyadenosine by HNO_2 deamination and periodate oxidation. 5′-Deoxyinox was shown to inhibit ribonucleotide reductase activity in cell-free extracts and to inhibit RNA and DNA syntheses in intact cells. Further, 5′-deoxyinox inhibited the conversion of cytidine nucleotides to deoxycytidine nucleotides in intact cells. In comparative studies with the dialdehyde derivative of inosine (Inox), 5′-deoxyinox was shown to be more active on a molar basis in inhibiting RNA or DNA synthesis in intact cells. In addition, 5′-deoxyinox was more inhibitory to the growth of Novikoff hepatoma cells in culture than was Inox. 5′-Deoxyinox, in addition to being more active than Inox, also differed from Inox in its biochemical properties. Inox did not inhibit RNA polymerase activity when added to isolated nuclei. On the other hand, 5′-deoxyinox showed a marked inhibition of the RNA polymerase activity when added to the isolated nuclei. Further, inhibition of the RNA polymerase activity in the nuclei from Inox-treated cells was reversed completely by the addition of exogenous polydeoxyadenylate-deoxythymidylate as template, whereas the inhibition caused by 5′-deoxyinox was not reversed by this treatment. These studies show that, in addition to the variation in activity caused by altering the purine component, the nature of the dialdehyde moiety also plays a role in the mode of action of this class of compounds.

Previous studies from this laboratory have shown that the dialdehyde derivative of inosine (Inox, NSC 118994) is metabolized by Ehrlich ascites tumor cells with the formation of the nucleotide derivative and the incorporation of the compound into RNA [1]. The inhibition of RNA synthesis caused by the incubation of the tumor cells in the presence of Inox was shown to be due to impairment of template function, rather than inhibition of the RNA polymerase [2]. In order to determine the role of the phosphorylation of Inox in its mode of action, the dialdehyde derivative of 5′-deoxyinosine (5′-deoxyinox), a compound which cannot be phosphorylated, was prepared and studied.

In this report we present data which show that 5′-deoxyinox is not only quantitatively more active than Inox but, in addition, has a different biological effect with respect to the inhibition of RNA synthesis.

METHODS AND MATERIALS

Preparation of 5′-deoxyinox. 5′-Deoxyinox was prepared from 5′-deoxyadenosine by nitrous acid deamination [3] to produce 5′-deoxyinosine followed by periodate oxidation [4] to form the dialdehyde derivative of 5′-deoxyinosine. 5′-Deoxyadenosine was a gift from Dr. Ray A. Olsson and was custom synthesized by Terra Marine Bioresearch, Inc., LaJolla, CA. The product was isolated and characterized as described previously for Inox [5].

Ehrlich tumor cells. The Ehrlich tumor cells were grown in female ICR mice (Laboratory Supply Co. Indianapolis, IN) by weekly inoculation of recipient mice with 0.2 ml of tumor cells taken from the donor mice. The cell culture experiments were carried out under sterile conditions as described previously [6].

Ehrlich tumor cells were incubated in the presence and absence of Inox or 5′-deoxyinox at 37° for varying periods of time. [^{14}C]cytidine (374 mCi/m-mole; 0.2 μCi/flask) was added to the cultures and the incubations were carried out for an additional 0.5 hr. The cells were collected by centrifugation, and the cell pellet was subjected to the Schmidt–Thannhauser procedure to separate the acid-soluble, RNA and DNA fractions [7]. The acid-soluble fractions were treated further to determine the conversion of cytidine to deoxycytidine nucleotides as a measure of ribonucleotide reductase activity in the intact cells [6]. To determine the distribution of [^{14}C]cytidine nucleotides in the acid-soluble fraction, the trichloroacetic acid was removed by ether extraction and the samples were lyophilized. The lyophilized material was dissolved in 200 μl H_2O. The samples were analyzed by high pressure liquid chromatography (h.p.l.c.) using a Whatman Partisil SAX column (25 × 0.46 cm) on a Varian 4200 gradient system. A linear gradient with (0.01 M $NH_4H_2PO_4$(pH 2.77) to 0.5 M $NH_4H_2PO_4$(pH 4.82) was run with a gradient change of 8 per cent/min and a flow rate of 2 ml/min. The effluent from the u.v. monitor was collected directly into scintillation vials (one vial/min). Under these conditions, the CMP, CDP and CTP standards eluted at 1.9, 8.9 and 14.5 min respectively.

RNA polymerase activity in isolated nuclei. The method of Muramatsu *et al.* [8] was used to isolate nuclei from the tumor cells. The reaction mixture for the polymerase assay [9] contained in a final volume of 0.25 ml: 40 nmoles each of ATP, GTP and CTP and 10 nmoles [^{3}H]UTP (0.2 μCi/nmole); 25 μmoles Tris–HCl(pH 8.1); 18.7 μmoles KCl; 2.5 μmoles

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mercaptoethanol; 1.25 μmoles magnesium acetate; 125 μmoles ammonium sulfate; 1.25 μmoles manganese chloride; and nuclei (200–300 μg DNA). The reactions were carried out for 10, 20 and 30 min at 37° and were stopped by the addition of 10% trichloroacetic acid (0.25 ml). The precipitate was collected by centrifugation and the pellet was resuspended and washed with 5% trichloroacetic acid (0.5 ml). The pellet was dissolved in 0.2 N NaOH (0.5 ml) and reprecipitated with 10% trichloroacetic acid (0.5 ml). The final pellet was dissolved in 0.2 N NaOH (0.1 ml), and H_2O (0.9 ml) was added. The sample was transferred to a scintillation vial. All RNA polymerase assays were carried out in triplicate. Heated controls served as blanks. The data are presented as [^{3}H]UMP incorporated into acid-insoluble material/mg of DNA in the nuclei.

In some experiments, the cells were incubated with Inox or 5′-deoxyinox and then the nuclei were isolated and the RNA polymerase assays carried out. In other experiments, the nuclei were isolated and then incubated with Inox or 5′-deoxyinox.

Ribonucleotide reductase assays. Partially purified ribonucleotide reductase was prepared from Ehrlich tumor cells through the ammonium sulfate step [10]. CDP and ADP reductase assays were determined by the methods of Steeper and Steuart [11] and Cory *et al.* [12] respectively. [^{3}H]CDP and [^{3}H]ADP were used as the labeled substrates. The assays were carried out in triplicate.

Ribonucleotide reductase activity in cell-free extracts prepared from 5′-deoxyinox-treated cells. Ehrlich tumor cells were incubated for 2 hr in culture in the presence and absence of 5′-deoxyinox (0.5 mM). The cells were collected by centrifugation and homogenized in 0.02 M Tris–HCl buffer (pH 7.0), containing dithioerythritol (1 mM). The homogenates were centrifuged at 27,000 *g* for 1 hr. The supernatant fluids were then passed over Sephadex G-25 columns (27 × 1 cm) and the protein was eluted with Tris buffer. The proteins in the void volume peaks were concentrated by ultrafiltration on Amicon PM-10 membrane. The concentrated fractions were used for the reductase assays.

Growth of Novikoff hepatoma cells. The Novikoff hepatoma cell line, the wild type, N1-S1 was originally obtained from Dr. Van R. Potter. The Novikoff cells were grown in suspension culture at 37° in Medium S-69 containing 10% calf serum [13]. Cell density measurements were made using a Bio-Physics Cytograph model 6300A particle counter [13].

Miscellaneous. Protein, RNA and DNA determinations were made by the methods of Lowry *et al.* [14], Hurlbert *et al.* [15] and Burton [16] respectively.

RESULTS

Effect of 5′-deoxyinox on [^{14}C]cytidine incorporation into RNA and DNA. The tumor cells were incubated in culture for 1.5 hr in the presence and absence of 5′-deoxyinox as indicated. [^{14}C]cytidine was added and the cells were incubated for an additional 30 min. The results of this experiment are shown in Table 1. 5′-Deoxyinox at a concentration as low as 0.1 mM showed some inhibition of nucleic acid synthesis (30–40 per cent) whereas at 0.5 and 1 mM there was essentially complete inhibition of both RNA and DNA syntheses. In addition, the conversion of [^{14}C]cytidine to deoxycytidine nucleotides, which is a measure of ribonucleotide reductase activity in the intact cells, was also inhibited by 5′-deoxyinox in a concentration-dependent manner. The total uptake of [^{14}C]cytidine was decreased only 30 per cent at 1 mM 5′-deoxyinox. The incorporation of [^{14}C]thymidine into DNA was also inhibited by 5′-deoxyinox. The inhibition of [^{14}C]thymidine incorporation into DNA by 0.5 and 1.0 mM 5′-deoxyinox was 87 and 98 per cent respectively.

5′-Deoxyinox had no effect on the conversion of [^{14}C]cytidine to CMP, CDP or CTP in the acid-soluble fraction of the cells. In both the control and 5′-deoxyinox-treated cells (0.5 or 1.0 mM), the distribution of radioactivity was: CMP, 30 per cent; CDP, 8 per cent; and CTP, 62 per cent. In addition, these concentrations of 5′-deoxyinox had no effect on the profiles of the nucleotides in the acid-soluble fractions as determined by h.p.l.c.

Effect of 5′-deoxyinox on partially purified ribonucleotide reductase. The addition of 5′-deoxyinox to a partially purified ribonucleotide reductase preparation from Ehrlich tumor cells caused a concentration-dependent inhibition of the CDP and ADP reductase activities. These data are shown in Table 2. CDP reductase activity was inhibited to a greater extent than ADP reductase activity. Altering the substrate concentration (either CDP or ADP) had no effect on the degree of inhibition, indicating that the inhibition was not competitive with respect to substrate.

Ribonucleotide reductase levels in 5′-deoxyinox-treated cells. The Ehrlich tumor cells were incubated in the presence and absence of 5′-deoxyinox (0.5 mM) in culture for 2 hr. The cells were collected by centrifugation and washed with buffer. Cell-free extracts were prepared from the control and 5′-deoxyinox-treated cells. The cell-free extracts were passed over Sephadex G-25 columns, and the proteins in the void volume peaks were concentrated by ultrafiltration. Under these conditions, the ribonucleotide reductase activity in the

Table 1. Inhibition of nucleic acid synthesis in Ehrlich tumor cells by 5′-deoxyinox

5′-deoxyinox (mM)	Total uptake (cpm × 10^{-3})	Acid-soluble (cpm/μg DNA)	RNA (cpm/μg RNA)	DNA (cpm/μg DNA)	Cyt $\underset{(\%)}{\rightarrow}$ dcyt
0	270.2	114.6	93.0	7.17	3.01
0.1	283.7 (105)*	166.2 (145)*	68.6 (74)*	4.51 (63)*	2.35 (78)*
0.5	239.5 (90)	193.9 (169)	11.1 (12)	0.49 (7)	0.87 (29)
1.0	187.3 (70)	162.0 (141)	2.6 (3)	0.17 (2)	0.48 (16)

* Per cent of control values.

Table 2. Effect of 5′-deoxyinox on partially purified ribonucleotide reductase

5′-Deoxyinox (mM)	Reductase activity CDP (nmoles/30 min/mg protein)	ADP (nmoles/30 min/mg protein)
0	0.64	0.37
0.1	0.60 (93)*	0.36 (97)*
0.5	0.31 (48)	0.32 (86)
1.0	0.25 (39)	0.29 (78)
2.0	0.13 (20)	0.21 (56)

* Per cent of control values; there was no preincubation of 5′-deoxyinox with the enzyme prior to assay.

cell-free extract from the 5′-deoxyinox-treated cells was much lower than the reductase activity in the extract from the control cells. These data are shown in Table 3. Both the CDP and ADP reductase activities were decreased in the cell-free extracts prepared from the 5′-deoxyinox-treated cells. This experiment did not distinguish whether the 5′-deoxyinox was still bound to the enzyme after Sephadex G-25 chromatography and ultrafiltration, resulting in inhibition, or whether the enzyme had been irreversibly inactivated.

RNA polymerase activity in nuclei from 5′-deoxyinox-treated cells. Ehrlich tumor cells were incubated in culture for 1 hr in the presence of 5′-deoxyinox. The nuclei were isolated from these cells and the RNA polymerase activity was determined. The results are shown in Fig. 1. The inhibition of the RNA polymerase activity in the nuclei from the 5′-deoxyinox-treated cells was concentration dependent and also time dependent (data not shown).

Comparison of the effect of inox and 5′-deoxyinox on [^{14}C]cytidine incorporation into RNA and DNA. The effects of Inox and 5′-deoxyinox on [^{14}C]cytidine incorporation into the RNA, DNA and acid-soluble fractions were compared. The tumor cells were incubated in the presence and absence of the dialdehyde compound for 1.5 hr before the addition of [^{14}C]cytidine. As seen in Table 4, 5′-deoxyinox was more inhibitory than Inox to the incorporation of cytidine into RNA and DNA and conversion of cytidine to deoxycytidine nucleotides in the intact cells in culture.

Effect of Inox and 5′-deoxyinox on RNA polymerase activity in isolated nuclei. As reported previously [2], the addition of Inox to isolated nuclei did not result in the inhibition of the RNA polymerase activity. However, the addition of 5′-deoxyinox to the isolated

Table 3. Ribonucleotide reductase activity in 5′-deoxyinox-treated cells

Extracts*	Reductase activity CDP (nmoles/30 min/mg protein)	ADP (nmoles/30 min/mg protein)
Control	0.71	0.16
5′-Deoxyinox-treated	0.19 (27)+	0.04 (25)+

* Cell-free extracts were prepared from tumor cells which had been incubated in the presence and absence of 5′-deoxyinox (0.5 mM) for 2 hr and passed through Sephadex G-25 columns and finally concentrated by ultrafiltration.

+ Per cent of control values.

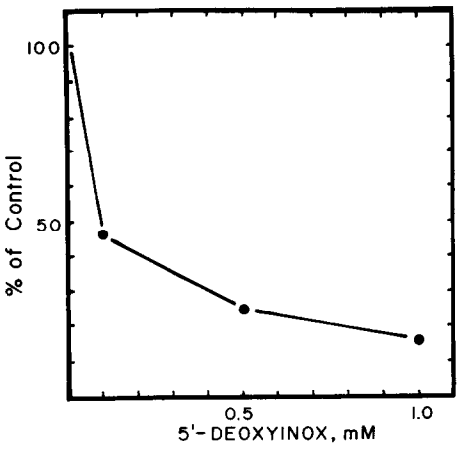

Fig. 1. RNA polymerase activity in nuclei from 5′-deoxyinox-treated cells. The Ehrlich tumor cells were incubated in the presence and absence of 5′-deoxyinox for 1 hr. The nuclei were isolated and the RNA polymerase activity was then determined. All assays were carried out in triplicate. The nuclei from control cells incorporated 166 cpm/μg of DNA/30 min.

nuclei resulted in the inhibition of the RNA polymerase activity. These data are shown in Fig. 2. The point to be made here is that, even with incubation, Inox did not inhibit the polymerase activity with respect to the control while 5′-deoxyinox was an effective inhibitor.

RNA polymerase activity in nuclei from Inox and 5′-deoxyinox-treated cells. Ehrlich tumor cells were

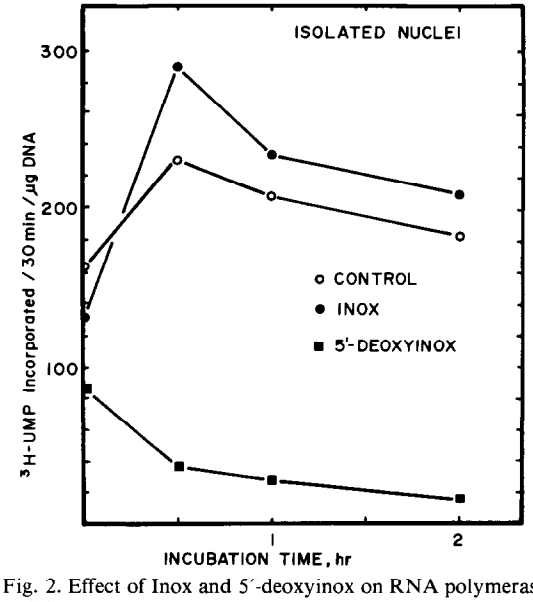

Fig. 2. Effect of Inox and 5′-deoxyinox on RNA polymerase activity in isolated nuclei. Nuclei were isolated from Ehrlich tumor cells and resuspended in 0.25 M sucrose containing 10 mM $MgCl_2$ and 0.1% Triton X-100. The nuclei were incubated in the presence and absence of Inox and 5′-deoxyinox (4 mM). Aliquots of the nuclei mixtures were removed at various time intervals and assayed for RNA polymerase activity at 37°. The final concentration of Inox or 5′-deoxyinox in the assay was 1.6 mM. The assays were carried out in triplicate. The reactions were carried out for 30 min.

Table 4. Comparison of Inox and 5′-deoxyinox on RNA and DNA syntheses and on ribonucleotide reduction in intact cells

	Total uptake (cpm × 10^{-3})	Acid-soluble (cpm/μg DNA)	RNA (cpm/μg RNA)	DNA (cpm/μg DNA)	Cyt → dcyt (%)
Control	281	76.8	323.8	6.5	3.54
Inox-treated*	283 (101)†	103.5 (135)†	255.2 (79)†	4.1 (62)†	2.57 (73)†
5′-Deoxyinox-treated*	276 (98)	184.2 (240)	57.1 (18)	0.6 (9)	1.22 (35)

* Cells were treated in culture with Inox (0.5 mM) or 5′-deoxyinox (0.5 mM).
† Per cent of control value.

incubated in the presence and absence of Inox (0.5 mM) and 5′-deoxyinox (0.5 mM) for 1.5 hr. The RNA polymerase activity in the isolated nuclei was then determined. The nuclei from the 5′-deoxyinox-treated cells showed a much lower level of RNA polymerase activity than did the nuclei from Inox-treated cells. These data are shown for Experiment 1 in Table 5. Further experiments were carried out to determine the level of RNA polymerase activity in the presence of actinomycin D and with poly(dA-dT) as the exogenous template [17]. Tumor cells were incubated in the presence and absence of Inox (0.5 mM) or 5′-deoxinox (0.5 mM) for 1.5 hr. The nuclei were isolated and then assayed in the presence and absence of actinomycin D and poly(dA-dT). Actinomycin D (20 μg/ml) was added to block RNA synthesis on the endogenous template of the nuclei. Poly (dA-dT) was added as the exogenous template since it does not bind actinomycin D. Consequently, the level of polymerase activity independent of endogenous template can be measured under these conditions. The results of this experiment are also shown in Table 5. In the case of the nuclei from the Inox-treated cells, in the presence of actinomycin D and poly(dA-dT), the level of RNA polymerase activity was essentially that of the nuclei from the untreated cells. However, in the case of the nuclei from the 5′-deoxyinox-treated cells, the addition of poly(dA-dT) as exogenous template did not reverse the inhibition of the RNA polymerase activity.

Comparison of effects of Inox and 5′-deoxyinox on growth of Novikoff hepatoma cells in culture. A comparison of the effects of Inox and 5′-deoxyinox on the growth of Novikoff hepatoma cells was made. These growth curves are shown in Fig. 3. In the Nl-Sl cell line, 5′-deoxyinox was more inhibitory to the growth of the cells than was Inox. From the comparison of the growth curves in the presence of 0.5 mM Inox and 0.1 mM deoxyinox (Fig. 3), it can be seen that 5′-deoxyinox was at least five times more active on a molar basis than was Inox in inhibiting the growth of the Nl-Sl cells. Similar observations were made in a fluorodeoxyridine-resistant Novikoff hepatoma cell line (data not shown).

DISCUSSION

The dialdehyde derivatives of nucleosides have been prepared and studied as potential antitumor agents [18–20]. It had been thought that the presence of the aldehyde functional groups on these compounds would react without specificity to the amino groups in the cell. However, several different studies have shown that the specific base moiety does, in fact, greatly influence the activity of the nucleoside dialdehyde derivatives, indicating that there was some specificity to the reaction of the aldehyde groups with amino groups on or in the cell [18, 21]. In experiments with partially purified enzymes, it has been shown that the nature of the purine moiety greatly influenced the degree of inhibition of the nucleotide dialdehyde [22]. Since we had shown that in Ehrlich tumor cells Inox was phosphorylated and incorporated into RNA as determined by several criteria [1], it was important to determine if an analog of Inox which could not be phosphorylated would still function as an inhibition of tumor cell

Table 5. RNA synthesis in nuclei from Inox- and 5′-deoxyinox-treated cells

		[^{3}H]UMP incorporated (cpm/μg DNA)		
		Addition to nuclei		
Experiment	Source of nuclei*	None	Act D	Act D + poly(dA-dT)
1	Control	266		
	Inox-treated	117 (44)†		
	5′-deoxyinox-treated	56 (21)		
2	Control	105.2	8.1	128.1
	Inox-treated	47.1 (45)	5.9	123.2 (96)†
3	Control	74.6	7.8	60.2
	5′-Deoxyinox-treated	9.4 (13)	1.6	20.8 (35)

* Nuclei were isolated from tumor cells which had been incubated in the presence and absence of Inox (0.5 mM) or 5′-deoxyinox (0.5 mM) for 1.5 hr.
† Per cent of control value.

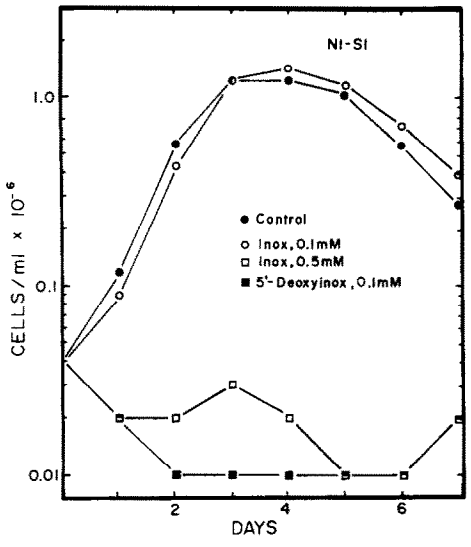

Fig. 3. Effect of Inox and 5′-deoxyinox on the growth of Novikoff hepatoma cells. The growth of N1-S1 cells was determined in the presence and absence of either Inox or 5′-deoxyinox as indicated.

growth and perhaps be able to distinguish those sites of inhibition which were dependent on the phosphorylation from those sites of inhibition which did not require the phosphorylated derivative. Plagemann *et al.* [23] reported that their attempts to determine whether Inox was phosphorylated by Novikoff hepatoma cells yielded results which were somewhat equivocal. It is entirely possible that the differences observed [1, 2] are dependent on the cell types used. It had been shown that certain cell types show differing sensitivities to Inox [23]. Further, Bhuyan and Fraser [24] reported that Inox inhibited DON cells at late G_1 and early S, while Plagemann *et al.* [23] reported that P388, L1210 and Novikoff hepatoma cells became arrested in $G_2 + M$. These apparent contradictions in results could, therefore, be due to the differences in cell types use by the various groups.

The results reported in this study show that 5′-deoxyinox inhibited the incorporation of [^{14}C]cytidine into RNA and DNA. In addition, ribonucleotide reductase activity was inhibited by 5′-deoxyinox as measured by the conversion of cytidine to deoxycytidine nucleotides in intact cells and in cell-free extracts. These results are similar to the results observed with Inox. However, in comparative studies, 5′-deoxyinox was much more inhibitory to these processes than was Inox. Further, qualitative differences were observed in addition to the quantitative differences. Incubation of nuclei with 5′-deoxyinox resulted in the inhibition of RNA synthesis. This inhibition apparently was the result of the direct inhibition of the RNA polymerase(s) since the addition of exogenous template (poly dA-dT) could not reverse the inhibition of RNA synthesis. On the other hand, the inhibition by Inox was reversed completely by the addition of exogenous template. The parent compounds 5′-deoxyadenosine and 5′-deoxyinosine did not inhibit the processes inhibited by the 5′-deoxyinox. However, they did inhibit the total uptake of [^{14}C]cytidine (J. G. Cory, unpublished data).

In cell-free experiments, low concentrations of 5′-deoxyinox (0.1 mM) were only slightly inhibitory to the enzyme activities mentioned. However, this concentration of 5′-deoxyinox completely inhibited the growth of Novikoff hepatoma cells. A possible explanation is that, in inhibiting multiple sites only partially, the net effect on the overall growth of intact cells is much greater. In addition, our earlier results indicated that complete inhibition of an enzyme was not required to completely shut off DNA synthesis [21].

These current studies have shown that at least two sites (ribonucleotide reductase and RNA polymerase), and probably more, are blocked by 5′-deoxyinox.

These results and the earlier results [18, 21] suggest that the activity of the nucleoside dialdehyde derivatives as antitumor agents depends not only on the nature of the base moiety but also on the nature of the moiety which contains the aldehyde group. Whether the increased inhibitory activity of 5′-deoxyinox compared to Inox is related to a difference in the transport of the compounds into the cell or to the difference in the effect on RNA sythesis is not known.

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