Studies on the Biologic Activity and Mode of Action of 7-Deazainosine[1]

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SUMMARY

Deazainosine was prepared by deamination of the antibiotic deazaadenosine (tubercidin). Whereas tubercidin inhibited the growth of *Streptococcus faecalis*, deazainosine was inactive against this organism. In contrast, both nucleoside analogs inhibited the growth of Sarcoma 180 cells *in vitro* and Ehrlich ascites and leukemia P388 cells *in vivo*. The lack of activity against *S. faecalis* is accompanied by the inability of the organism to convert deazainosine to tubercidin derivatives. Since the sensitive mammalian cells do carry out the amination reaction, this metabolic conversion appears to be required for biologic activity. A comparative evaluation of the antitumor activity of the two compounds showed that approximately 0.25–0.5 mg/kg of tubercidin produced the same effect as 4–8 mg/kg of deazainosine. This difference in potency may reflect the rate at which the conversion of deazainosine to tubercidin nucleotides occurs in sensitive cells. The more limited spectrum of tissue toxicity of deazainosine as compared to tubercidin may result from differences in the capacity of the tissues to carry out the metabolic conversion.

INTRODUCTION

The antibiotic tubercidin (7-deazaadenosine) is a structural analog of adenosine in which a carbon atom replaces the nitrogen in position 7 of the heterocycle (Chart 1). This compound is a potent inhibitor of the growth of various microbial and mammalian cells *in vitro* and of a number of experimental tumors *in vivo*, (1, 4, 5, 15). It replaces adenosine in a variety of metabolic reactions and is incorporated into RNA and DNA and into a nicotinamide adenine dinucleotide analog (1, 2, 4, 17). Unlike adenosine, however, tubercidin is not subject to phosphorolysis or deamination (4). In the microbial system examined, the primary inhibitory effect of the antibiotic appears to be its interference with glycolysis (4).

The profound toxicity of tubercidin in animals suggested the desirability of examining related compounds with potentially greater selectivity towards tumors. Deazainosine (Chart 1), prepared by deamination of tubercidin, was found to be an effective growth inhibitor with more limited toxicity for certain tissues than has tubercidin (12). Studies on the biologic

and biochemical effects of this inosine analog were therefore undertaken and are reported in this paper. Part of this work was presented in preliminary communications (3, 14).
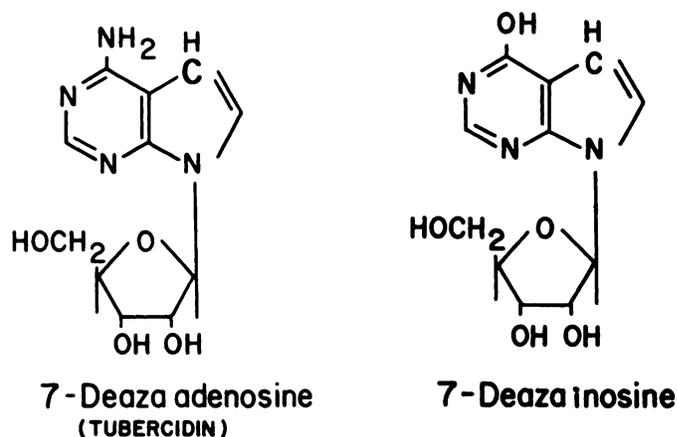

Chart 1. Structural formulas of 7-deazaadenosine and 7-deazainosine.

MATERIALS AND METHODS

Compounds. Tubercidin, deazaadenine, deazaadenine-2-^{14}C, tubercidin-5'-phosphate, and deazahypoxanthine were kindly furnished by the Upjohn Company through the courtesy of Dr. C. G. Smith and Dr. Wm. Schroeder. 7-Deazainosine-^{3}H was prepared by the Wilzbach technic by New England Nuclear Corporation. Its specific activity was 18.7 $\mu c/mg$. Its radiochemical purity, like those of the other labeled compounds used, was established by determining the correspondence of radioactivity with ultraviolet absorption following chromatography in the solvents listed in Table 1. Although deazainosine-^{3}H appeared to be stable in aqueous solution, the compound was rechromatographed at weekly intervals in solvents D and E respectively.

Preparation of 7-Deazainosine, 7-Deazahypoxanthine-2-^{14}C and 7-Deazainosine-5'-phosphate (deaza-IMP)

7-Deazainosine was prepared by dissolving 1 gm of tubercidin in 15 ml of 50% aqueous acetic acid. The solution was brought to ice-bath temperature, and 3 ml of an ice-cold aqueous solution containing 2 gm of sodium nitrite were added over a 15-min period with stirring. The mixture was allowed to warm to room temperature and was kept at this temperature for 16 hr. The solution was brought to dryness *in*

vacuo at 30°C, and the residue was purified by Celite column chromatography (10), using ethyl acetate:water:*n*-propanol (4:2:1) as the solvent system. A small quantity of unreacted starting material preceded deazainosine, and the latter compound was crystallized from hot methanol (m.p. 240°C, yield 79%). The elemental analysis agreed with the calculated values. Subsequent to this preparation the chemical synthesis of deazainosine was reported (13).

Deazahypoxanthine-2-^{14}C (0.9 mc/mmole) was prepared from deazaadenine-2-^{14}C in a similar manner, but separation of the products was carried out by descending chromatography on Whatman 3 MM paper with water, pH 10, (solvent D, Table 1).

Deaza-IMP was prepared chemically from deazainosine and enzymatically from synthetic tubercidin-5′-phosphate (TuMP). The chemical preparation proceeded as follows: 2′,3′-O-isopropylidene-7-deazainosine was prepared by the procedure of Hampton (11), except that triethylamine was used in place of tributylamine. Preparation of 2′,3′,-O-isopropylidene-7-deaza-IMP followed the procedure of Tener (16). However, the isopropylidene group was removed with 50% aqueous acetic acid at 100°C for 5 min only after precipitation of the nucleotide in the form of the barium salt. The free nucleotide was isolated from the reaction mixture by chromatography on N,N-diethylaminoethyl ether cellulose (Eastman) by gradient elution with triethylammonium carbonate buffer (0.01—0.1 M). The elemental analysis agreed with the calculated values.

For enzymatic deamination, 0.1 ml of 5′-adenylic acid deaminase (obtained from Sigma Chemical Company) was added to 2 mg of TuMP dissolved in 1.5 ml of 0.01 M citrate buffer, pH 6.9. Incubation proceeded for 2 hr at 37°C, after which the reaction mixture was separated in solvents B and C. The deaminated product, deaza-IMP, was identified by cochromatography with the chemically prepared deaza-IMP in the solvents B, C, and E, and by electrophoresis on Whatman 3 MM paper at 3000 volts for 90 min in 0.4 M citrate buffer, pH 2.9. Under these conditions, IMP moved 16.9 cm, deaza-IMP 16.3 cm, and TuMP 5.5 cm towards the positive pole.

Growth Inhibitory Activity of the Deaza Compounds *in Vitro*

The effect of the deaza compounds on the growth of *Streptococcus faecalis* (8043) and on AH/S cells (8), a subline of mouse Sarcoma 180, was determined by methods described previously (4, 9).

Metabolic Conversion of Deazainosine by Microbial and Mammalian Cells

Phosphorylation and Amination. The anabolic conversion of deazainosine was studied in intact cells and in cell-free extracts of *S. faecalis*, Sarcoma-180/AH/S, and of Ehrlich ascites. *S. faecalis* cells were grown overnight at 37°C in 500 ml of the medium of Flynn *et al.* (7), devoid of purines and pyrimidines and containing 2 mμg/ml of folate, harvested by centrifugation, and resuspended in 100 ml of fresh medium. The culture was incubated until resumption of growth, at which time 20 μc of deazainosine-^{3}H were added. Incubation proceeded for

Table 1

Compound	Solvent system				
	A	B	C	D	E
Deazahypoxanthine	0.69	0.84	0.50	0.53	
Hypoxanthine	0.67	0.58	0.54	0.65	
Deazainosine	0.70	0.63	0.65	0.74	0.69
Deazaadenine	0.66	0.97	0.30	0.33	0.79
Deazaadenosine	0.67	0.93	0.51	0.51	0.70
Inosine-5′-phosphate	0.48	0.37	0.80	0.95	0.44
Deazainosine-5′-phosphate	0.47	0.38	0.74	0.87	0.55
Deazaadenosine-5′-phosphate	0.45	0.63	0.62	0.86	0.48

R_F values of some 7-deaza purines. Determined on Whatman 3 MM paper at 20°C. Solvent systems (by volume): A, 1 M ammonium acetate (to pH 5, with HCl) and 95% ethanol (30:70); descending. B, isobutyric acid and 2.3 N NH_4OH (66:44); ascending. C, 5% aqueous sodium citrate and isoamyl alcohol (50:50); ascending. D, water, pH 10; descending. E, *n*-propanol and H_2O (60:40); descending.

90 min at 37°C, followed by centrifugation of the culture. The cells were extracted with three 2.5-ml portions of ice-cold 10% perchloric acid, and the extract was neutralized with KOH. The precipitated $KClO_4$ was removed by centrifugation and washed with two 2.5-ml portions of water, which were combined with the neutralized extract. The total extract was reduced to a small volume *in vacuo* at 30°C. Analysis of the extract for deazainosine or its derivatives was then carried out by paper chromatography as described below.

The anabolic conversion of deazainosine by S-180/AH/S and Ehrlich ascites cells was examined in a similar manner. S-180/AH/S cells grown in tissue culture or the ascites cells collected from the intraperitoneal cavities of two mice were suspended in Eagle's balanced salt medium (6) and were centrifuged for 10 min at 1000 rpm. Resuspension in 50 ml of Eagle's medium containing 20 μc of deazainosine-^{3}H was followed by incubation in a 5% CO_2 atmosphere or in air at 37°C for 90 min. The cells were centrifuged and extracted with cold 10% perchloric acid and the extract neutralized as described for the bacteria.

Portions of the extracts were applied to Whatman 3 MM paper and were analyzed by chromatography in solvents A, C, and D (Table 1). The identity of deaza-IMP and of the tubercidin nucleotides present in the extracts was confirmed by eluting the compounds from the chromatograms by shaking the appropriate portions cut from the paper in 5-ml aliquots of water. The paper was removed by centrifugation, and the volume of the solution was reduced *in vacuo* to 1 ml. One mg of snake venom in 1 ml of 0.1 M Tris buffer, pH 8.0, was added to each eluate together with 0.02 ml of 0.1 M $MgCl_2$. The mixtures were incubated for 2 hr at 37°C, and at the end of this period the tubes were placed into boiling water for 2 minutes. The volume of the reaction mixtures was then reduced *in vacuo* at 30°C, and aliquots were cochromatographed with tubercidin and deazainosine in solvents C and D. Portions of the various cell cultures incubated with deazainosine-^{3}H were also examined for incorporation of radioactivity into the nucleic acids by procedures outlined earlier (4).

Phosphorolysis. The possible cleavage of deazainosine was examined with partially purified *S. faecalis* extracts prepared at 2°C as outlined elsewhere for tubercidin (4) and with ex-

tracts from Ehrlich ascites prepared as described below. Deaza-inosine (0.5 μc) or 0.5 μc of inosine-8-^{14}C (32 mc/mmole, Schwarz BioResearch) were incubated for 1 hr at 37°C with 0.2 ml of 0.1 M phosphate buffer, pH 6.9, 0.02 ml of 0.1 M MgCl$_2$, and 0.5 ml of the respective cell extract in a total volume of 1 ml. As in all cases, the reactions were stopped by immersion of the tubes into boiling water for 2 minutes, and the reaction products were separated by chromatography in the solvents C and D. A strip measuring 1 cm in width was cut along the length of each chromatogram and was subdivided into 1-cm segments which were examined for radioactivity in a Packard Model 3000 Scintillation counter.

The interference of deazainosine with the phosphorolysis (or arsenolysis) of inosine was determined by two methods with the extract obtained from Ehrlich ascites cells dialyzed over-night against Tris buffer, pH 7.5. In one method, 0.1 ml of the extract (2.29 mg protein/ml) was added to 0.5 ml of 0.1 M Tris buffer, pH 7.5, 0.05 ml of 0.1 M MgCl$_2$, 0.1 ml of 0.1 M sodium phosphate (or sodium arsenate), and inosine and deazainosine at the concentrations indicated in Chart 3, in a total volume of 1 ml. After incubation for 15 min, the reaction mixtures were immersed in boiling water for 2 min and cleared by centrifugation. Following dilution of the super-natant with 9 volumes of 0.1 M Tris buffer, pH 7.5, 0.1 ml of xanthine oxidase (milk) was added to 1 ml of the dilution, and the resulting change in optical density, recorded at 248 mμ and 25°C, provided a measure of the amount of hypoxanthine formed from inosine. In the second method, inosine-8-^{14}C was added along with unlabeled inosine, and the amount of hypo-xanthine-8-^{14}C formed was determined in the scintillation counter, following chromatographic separation of the reaction products in solvent D.

Metabolic Inertness of 7-Deazahypoxanthine

The susceptibility of the base analog deazahypoxanthine to metabolic conversion to its nucleoside or nucleotide derivatives was examined in cell-free preparations of *S. faecalis* and Ehrlich ascites. Preparation of the bacterial extract was outlined elsewhere (4). The Ehrlich ascites extract was pre-pared by suspending Ehrlich ascites cells obtained from the peritoneal cavity of Ha/ICR Swiss mice in Eagle's balanced salt medium. Centrifugation for 10 min at 1000 rpm was followed by homogenization of the cells in 3 volumes of 0.1 M Tris buffer, pH 7.0, in a tissue grinder. The clear fluid obtained after cen-trifugation at 10,000 X g served as the crude enzyme prepara-tion. To determine their conversion to the nucleotide, hypo-xanthine-8-^{14}C (0.25 μc, 40 mc/mmole) or deazahypo-xanthine-2-^{14}C (0.25 μc, 0.9 mc/mmole) were incubated for 30 min at 37°C with 1 μmole of 5-phosphoribosyl-1-pyrophosphate, 0.02 ml of 0.1 M MgCl$_2$, 0.1 ml of 0.05 M phosphate buffer pH 7.0, and 0.5 ml of enzyme preparation in a total volume of 1 ml. To examine conversion to the nucleo-side, the two radioactive compounds were each incubated for 10 min at 37°C with 1 μmole of ribose-1-phosphate, 0.1 ml of 0.1 Tris buffer, pH 7.0, 0.02 ml of 0.1 M MgCl$_2$, and 0.5 ml of the respective enzyme preparation. Separation of the products of all experiments was carried out by chromatography in the solvents C and D.

Antitumor Activity

Sarcoma 180 solid (S-180) was implanted subcutaneously into female Ha/ICR Swiss mice by a standard trocar technic. Tumor growth was determined by caliper measurements per-formed through the skin according to well-established pro-cedures. Ehrlich carcinoma ascites and leukemias L1210, P288, P1534, L5178Y, and P388 were inoculated i.p. into Swiss Ha/ICR and DBA/2 Ha-DD respectively. Lymphoma AK 4 (AKR mice) was transplanted i.p. by the inoculation of spleen cell suspension. Tumor cells (1 X 10^6) were inoculated into each mouse. Treatments were given i.p. once daily, for the periods indicated, starting the day after tumor inoculation.

RESULTS

A comparison of the growth inhibitory properties of the 7-deaza analogs studied is presented in Table 2. Tubercidin decreased the growth of both the bacterial and tumor cells, but deazainosine interfered only with the growth of the tu-mor. Deazaadenine and deazahypoxanthine were not inhibi-tory for either system at the highest concentration tested. Neither adenosine nor inosine inhibit the growth of *S. faecalis* at concentrations up to 10^{-3} M. Growth of the S-180 cells is reduced by 50% at 1 X 10^{-4} M adenosine, but at this concen-tration inosine has no effect.

Table 2

Analog	Concentration (M) for 50% growth inhibition of	
	S. faecalis	S-180 cells[a]
Deazaadenosine	1.5 x 10^{-8}	2 x 10^{-7}
Deazainosine	>10^{-3}	5 x 10^{-6}
Deazaadenine	>10^{-3}	>10^{-4}
Deazahypoxanthine	>10^{-3}	>10^{-4}

Comparison of *in vitro* growth inhibition of *Streptococcus faecalis* and Sarcoma 180 cells by 7-deaza purines.

[a] The data shown in this column were kindly provided by Dr. M. T. Hakala of this department.

This differential inhibition is paralleled by the observation that deaza-IMP and tubercidin nucleotides were formed from deazainosine by the mammalian but not by the bacterial cells. Following incubation of S-180 cells for 1 hr, a typical distribu-tion of the total recovered radioactivity in the acid-soluble fraction was as follows: 38% corresponded to the nucleoside analog, 25% to deaza-IMP, and 37% was present as the tuber-cidin nucleotides. Similarly, tubercidin and deoxytubercidin formed from deazainosine were found in the respective nucleic acid fractions of the tumor, but not in those of the bacterial cells. The deaza analogs of hypoxanthine or adenine were not converted to the nucleoside or nucleotide stage (Chart 2) and cleavage of deazainosine to deazahypoxanthine was not seen. Although not a substrate, deazainosine interfered weakly but competitively with the phosphorolysis of inosine (Chart 3).

The effect of deazainosine and tubercidin on the growth of Ehrlich ascites is shown in Table 3. As measured by cell counts, a comparable decrease in the growth of Ehrlich ascites cells was produced by approximately 0.5—1.5 mg/kg/day of tubercidin and by 8—16 mg/kg/day of deazainosine, a ratio of
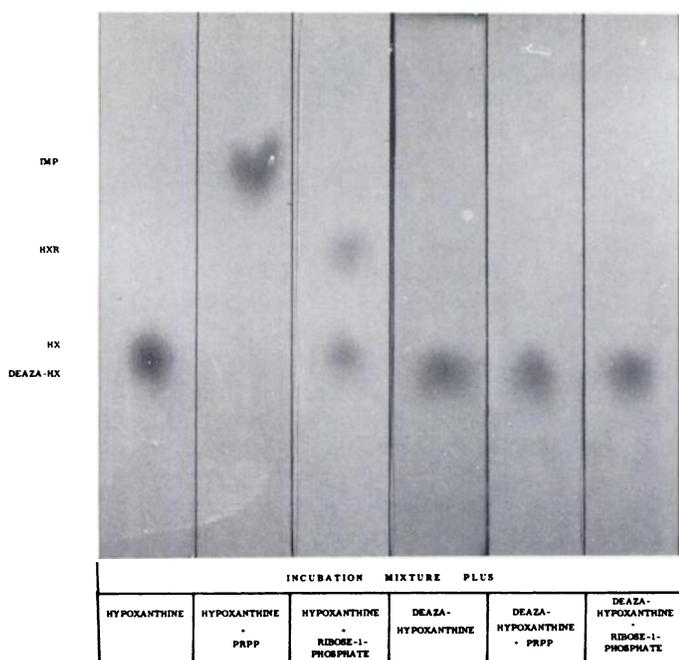
Chart 2. Comparison of the metabolic conversion of hypoxanthine (HX) vs deazahypoxanthine (deaza-HX). To determine their conversion to the nucleotide, hypoxanthine-8-^{14}C (0.25 μc, 40 mc/mmole) or deazahypoxanthine-2-^{14}C (0.25 μc, 0.9 mc/mmole) were incubated for 30 min at 37°C with 1 μmole of 5-phosphoribosyl-1-pyrophosphate (PRPP), 0.02 ml of 0.1 M MgCl$_2$, 0.1 ml of 0.05 M phosphate buffer pH 7.0, and 0.5 ml of S. faecalis or Ehrlich ascites extract, in a total volume of 1 ml. To examine conversion to the nucleoside, the two radioactive compounds were each incubated for 10 min at 37°C with 1 μmole of ribose-1-phosphate, 0.1 ml of 0.1 Tris buffer, pH 7.0, 0.02 ml of 0.1 M MgCl$_2$, and 0.5 ml of the respective enzyme preparation. Separation of the products of all experiments was carried out by chromatography and the radioactivity was located by radioautography. HXR, inosine; IMP, inosine-5'-phosphate.
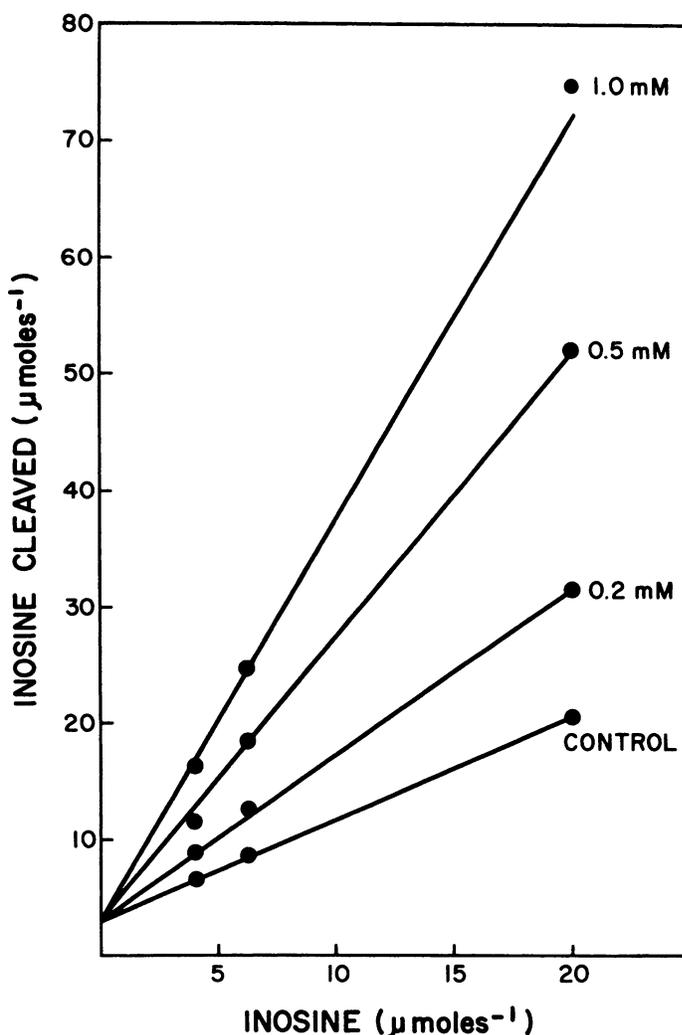

Chart 3. Interference of deazainosine with the phosphorolysis of inosine. The reaction mixture in a total volume of 1 ml contained 0.5 ml of 0.1 M Tris buffer, pH 7.5, 0.05 ml of 0.1 M MgCl$_2$, 0.1 ml of 0.1 M sodium phosphate, 0.1 ml of Ehrlich ascites extract, and inosine and deazainosine at concentrations indicated in the chart. The amount of hypoxanthine formed was determined from the change in optical density at 248 mμ following addition of xanthine oxidase.

1:16. At these doses the decrease in tumor growth was accompanied by a significant prolongation of average survival time. Approximately one-third of the animals survived for 50 days after treatment with 8 mg/kg/day of deazainosine. Comparable toxicity was seen after 1.5—2.5 mg/kg/day of tubercidin and 24 mg/kg/day of deazainosine. The similar relative potency of the two compounds was also shown by their slight effect on the growth of Sarcoma 180 solid (Table 4) and on leukemia P-388 (Table 5), where 0.5 and 8 mg/kg/day respectively (1:16) were equally effective. The compounds were inactive against the leukemias P-288, P-1534, L-5178Y, and L-1210 and against lymphoma AK4.

DISCUSSION

The extensive potency of tubercidin would be a desirable feature in chemotherapy if it were accompanied by greater tissue selectivity. Some change in toxicity for different tissues is achieved with deazainosine which can act as a metabolic precursor of tubercidin. Among the cells tested, tubercidin inhibited the bacterial, S-180, and Ehrlich ascites cells, where-

as deazainosine interfered only with the growth of the tumors. Similarly, whereas tubercidin toxicity extended to a wide variety of tissues, deazainosine toxicity was restricted to fewer tissues (12). The observation that the metabolic conversion of deazainosine to tubercidin derivatives occurs only in the deazainosine-sensitive and not in the refractory cells indicates strongly that this conversion is required for the observed inhibitory activity. The similarity in the antitumor activity of the two compounds supports this view. Those cells which are not inhibited by tubercidin are not affected by deazainosine either (Table 5). The difference in their relative potency, which in the mammalian systems tested was consistently 1:16, may be a reflection of the rate at which the conversion occurs. The observation that deazainosine, unlike tubercidin, inhibits the growth of mouse fibroblasts only after a lag period of 24

Table 3

Treatment[a] (mg/kg/day × 7)		8th day after inoculation		Average survival (days)	50-day survival (percent)
		(Average number × 10⁶/mouse)	Mortality (percent)		
Saline		614	0	19.7	0
Deazaadenosine	0.5	259	0	30.4	0
	1.0	41	0	27.0	0
	1.5	43	8	19.9	0
	2.0	36	10	10.0	0
	2.5	28	82	7.8	0
Deazainosine	8.0	103	0	34.3	28
	16.0	25	17	30.9	10
	24.0	1	90	6.9	0

Comparative effects of deazaadenosine and deazainosine on Ehrlich ascites carcinoma in mice.

[a]The drugs were inoculated intraperitoneally once daily from the day following that of tumor implantation.

Table 4

Treatment[a] (mg/kg/day)		No. of mice	8th day			15th day		
			Average change in body weight[b] (gm)	Average tumor diameter (mm ± S.D.)	Mortality (%)	Average change in body weight[b] (gm)	Average tumor diameter (mm ± S.D.)	Mortality (%)
Saline		48	+0.8	13.5 ± 2.8	0	−3.0	17.7 ± 3.0	15
Deazaadenosine	1.00	10	+1.5	12.9 ± 2.4	0	−1.2	16.9 ± 2.5	30
	2.00	25	−1.1	11.4 ± 2.4	0			100
Deazainosine	8.0	15	−1.8	13.8 ± 2.4	0	−1.9	17.0 ± 3.0	33
	16	20	−2.2	12.5 ± 2.7	5	−5.0	17.7 ± 1.3	95
	20	5	+0.6	15.0 ± 1.2	0	−1.4	17.2 ± 0.9	60
	24	10	−2.4	9.9 ± 2.6	40			100

Effects of 7-deazaadenosine and 7-deazainosine on Sarcoma 180 solid in mice.

[a]Treatment was given intraperitoneally once a day for 7 days starting the day following that of implantation.

[b]Average change of body weight from that on the day of tumor implantation.

hours (1) would support this assumption. A difference in response to deazainosine as compared to tubercidin might then be a consequence of the effectiveness with which a given tissue carries out the metabolic activation of deazainosine.

Since in mammalian systems inosine kinase activity has not, so far, been demonstrated, the presence of deaza-IMP in the acid-soluble extract requires examination of the sequence of reactions by which the conversion of deazainosine to the tubercidin nucleotide occurs. Deazainosine is not apparently cleaved to furnish the free base, and even if it were, deazahypoxanthine, unlike hypoxanthine, is not subject to phosphoribosyl transferase activity (Chart 2). Formation of the nucleotide must, therefore, occur either by direct phosphorylation of the nucleoside analog, under the influence of either a kinase or a transphosphorylase or by its amination to tubercidin prior to phosphorylation. Although there is no precedent for the latter reaction, if it were to occur, then the presence of deaza-IMP would imply the further intervention of a nucleotide deaminase. It is of interest to observe in this connection that, whereas tubercidin is not a substrate for adenosine deaminase (4), tubercidin-5'-phosphate is acted upon by a 5'-adenylic acid deaminase, demonstrating the difference in importance of the 7-position for determining substrate specificity for the two enzymes.

From the point of view of chemotherapy, the differential cell and tissue toxicity of deazainosine and tubercidin (12) may be of marked importance. If the extent of activation of

Table 5

Treatment (mg/kg/day × 6 i.p.)[a]	Leukemia					
	P 388	P 288	P 1534	L 5178Y	L 1210	AK 4
Deazaadenosine						
	16.8[b]	8.4	12.2	12.6	6.4	8.1
0.125		9.4		12.4		
0.25		9.0		12.0		
0.5	35.0	8.0	12.2	10.5	7.4	9.0
1.0	34.0		15.4	8.8	5.6	7.8
2.0	8.6		7.6	8.0		7.8
Deazainosine						
6		8.2	14.2	13.0		
8	44.0	8.6	12.8	12.5		8.0
12		7.8	10.6	10.8	6.8	
16	7.4		6.4	10.6	6.4	7.0

Activity of 7-deazaadenosine and of 7-deazainosine against mouse leukemias.

[a]Treatments the day after intraperitoneal tumor inoculation.

[b]Average survival in days.

deazainosine by a given cell type or tissue provides the compound with specificity for that cell or tissue, then this relationship would be well worth investigating with other precursors of chemotherapeutically active antimetabolites. If the difference in tissue specificity of a precursor analog is, indeed, related to the extent to which it is converted by a given tissue to the active form, then such biochemical differences existing between tissues could be similarly exploited for the preparation of precursors of other active analogs.

ADDENDUM

K. J. Pierre and G. A. LePage (Proc. Soc. Expt. Biol. Med. *127:* 432, 1968) have presented evidence that cell-free extracts from Ehrlich ascites tumor cells convert inosine to inosine 5'-monophosphate. The kinase catalyzing this conversion appeared to be distinct from adenosine kinase.

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