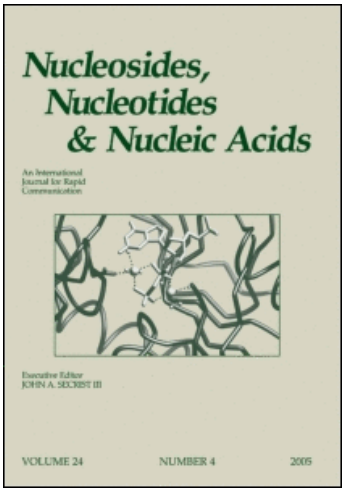

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Metabolism and Metabolic Effects of Halopurine Nucleosides in Tumor Cells in Culture

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METABOLISM AND METABOLIC EFFECTS OF HALOPURINE NUCLEOSIDES IN TUMOR CELLS IN CULTURE

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Summary. Within a series of halo derivatives of adenosine, deoxyadenosine and arabinosyladenine attempts have been made to correlate structure with cytotoxicity, substrate activity for adenosine deaminase and nucleoside kinases, and efficacy of the corresponding nucleotides against target enzymes.

INTRODUCTION

Although there exists a considerable literature on the biochemical properties and biological activities of purine nucleoside analogs, there is still a paucity of information that can be used to predict the effects of specific structural changes on activities of nucleoside analogs as substrates for enzymes that are involved in exertion of their biological activities. The pertinent enzymes are those that act directly on the nucleoside analog and also those that are the targets of the corresponding nucleotides, which are the biologically active forms of most nucleoside analogs. Knowledge of such structure-activity relationships is essential to the rational design of new biologically-active nucleoside analogs. We have begun such a study with an investigation of the effects of substitution at the 2- or 8-positions of adenosine (Ado), 2'-deoxyadenosine (dAdo), and 9-β-D-arabinofuranosyladenine (araA) on biochemical and biological properties. We had originally planned to include 8-fluoroadenosine in the series, but our synthetic route instead gave us 8-amino-6-fluoro-9-β-D-ribofuranosylpurine (8-NH_2-6-F-PuR). It has proven to be an interesting and biologically active compound, and we have included it with the 2- and 8-substituted purines reported upon herein. The halogenated nucleosides

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appear to be a series well-suited to such a study because (a) it is already known that some of them are biologically active; and (b) the several halogens offer a range of atomic sizes and electron-withdrawing capacities. Our study of these nucleosides is a three-part one. The first part is the determination of their cytotoxicities to HEp-2 cells in culture and their activities as substrates for adenosine deaminase (ADA), adenosine kinase (AdoK), and deoxycytidine kinase (dCydK). These three enzymes are those that act directly on analogs of Ado or dAdo. The action of ADA usually represents a detoxification because the resulting analogs of inosine (Ino) or 2'-deoxyinosine in most instances are nontoxic or less toxic than the Ado or dAdo analogs. AdoK is the kinase responsible for phosphorylation of analogs of Ado, and dCydK is the principal kinase acting on most analogs of dAdo. The second phase of the study is concerned with defining the sites of action of the analog and, finally, in the third phase, the phosphates of the analog are prepared and evaluated as inhibitors of the enzymes indicated as the primary sites of attack of the agents. When finished, this study should provide a complete picture of the influence of the halogen substituents on the activation and deactivation of the nucleoside analogs and on the activities of the phosphates on the isolated target enzymes. We report here the information obtained to date; this consists of results with fourteen nucleosides carried through the first part of the study and some data on the modes of action of two of the agents. The nucleosides studied are listed in Table 1; references to the syntheses are given in the table following the name of the compound.

CYTOTOXICITIES AND SUBSTRATE ACTIVITIES FOR ADA AND NUCLEOSIDE KINASES

2-Haloadenosines. The three members of this group showed a broad range of cytotoxicities. 2-F-Ado was the most cytotoxic. The cytotoxicity decreased as the size of the halogen increased; 2-Br-Ado was not toxic at the highest concentration assayed. All three compounds were very poor substrates for ADA; the K_m values were the same order of magnitude as that for Ado but the velocities were very low. Maguire and Sim[14] have also evaluated these derivatives with similar results. For AdoK the three halo derivatives had about the same K_m values but only for 2-F-Ado was the velocity high. Thus, in this series the toxicity correlates with substrate activity for AdoK in that the only compound with high cytotoxicity was the only compound that was well phosphorylated. The correlation is not quanti-

TABLE 1

Cytotoxicities and Substrate Activities of Halonucleosides

Nucleoside	Cytotoxicity[a] ID_{50} (μM)	Substrate Activity: ADA[b] K_m (μM)	ADA[b] V_{max}	AdoK[c] K_m (μM)	AdoK[c] V_{max}
ADO	--	29	100	1.7	100
2-F-Ado[1]	0.03	81	0.18	130	88
2-Cl-Ado[2]	3.5	110	0.01	140	7
2-Br-Ado[2]	>80	63	0.002	130	5
8-Cl-Ado[4]	~0.02	830	1.8	7	50
8-Br-Ado[d]	~3	250	0.009	9	67
8-NH_2-6-F-PuR[3]	~1.8	1,000	54	7	120

Nucleoside	dCydK[c] K_m (μM)	dCydK[c] V_{max}
dAdo	600	100
AraA	610	5
2-F-dAdo[5]	50	41
2-Cl-dAdo[5]	50	42
2-Br-dAdo[5]	43	42
8-Cl-dAdo[6]	ND[f]	--
8-Br-dAdo[7]	ND[f]	--
2-F-araA[8]	540	4
2-Cl-AraA[9]	ND[f]	--
2-Br-AraA[3]	ND[f]	--

Nucleoside	ADA[b] K_m (μM)	ADA[b] V_{max}
dAdo	28[e]	98[e]
AraA	120	22
2-F-dAdo[5]	71	0.4
2-Cl-dAdo[5]	47	0.01
2-Br-dAdo[5]	57	0.002
8-Cl-dAdo[6]	670	4
8-Br-dAdo[7]	500	0.03
2-F-araA[8]	220	0.02
2-Cl-AraA[9]	250	0.01
2-Br-AraA[3]	ND[f]	--

a. Cytotoxicities to HEp-2 cells were determined by colony counts[10]; ID_{50} = concentration required for 50% inhibition of colony formation. b. ADA from bovine intestine was assayed spectrophotometrically. V_{max} values are relative to that for Ado taken as 100. c. AdoK and dCydK from L1210 cells were assayed by use of [γ-^{32}P]ATP[11,12]. V_{max} values are relative to those for Ado or dAdo taken as 100. d. Commercial sample, Sigma Chemical Co., St. Louis, MO. e. Value taken from the literature[13]. f. ND = no detectable activity.

tative, however since 2-Cl-Ado and 2-Br-Ado had equal activities as substrates for AdoK but differed widely in toxicities. Thus other factors are involved, such as maintenance of nucleotide pools or differences in efficacy of nucleotides against target enzymes.

8-Haloadenosines. The cytotoxicities of these compounds were poorly reproducible, but both were toxic at low concentrations. The chloro derivative was the more toxic. As the size of the halogen increased, the substrate activity decreased sharply. For AdoK both compounds had about the same K_m values and velocities, and the latter were of the same order as that of Ado. Thus, the effects of halogen substitution on substrate activities for ADA and AdoK were quite different.

2-Halodeoxyadenosines. In this series all three compounds were cytotoxic, were poor substrates for ADA, and were moderately good substrates for dCydK. The chloro derivative was the most toxic. The relative toxicities obviously do not correlate with substrate activities for dCydK.

8-Halodeoxyadenosines. Only two compounds were available in this series. Both were resistant to ADA, although the chloro derivative was deaminated at a rate about 4% that of Ado. Neither was cytotoxic and neither had detectable substrate activity for dCydK. The failure of these compounds to be phosphorylated adequately explains their lack of toxicity.

2-Haloarabinosyladenines. The cytotoxicities of these compounds were all about the same, and all three were highly resistant to ADA. 2-F-araA, araA, and dAdo all had about the same K_m for dCydK, but the velocities for the arabinosyl compounds were only 4-5% that for dAdo. Neither 2-Cl-araA nor 2-Br-araA had detectable substrate activity for dCydK in the standard assay. This failure is possibly explained by the poor solubility of the compounds which prevented their study at concentrations as high as the K_m.

8-NH_2-6-F-PR. This compound was cytotoxic and was a good substrate for both ADA and AdoK. The action of ADA differs from that on the other compounds in Table 1 in that it represents a dehalogenation, a known action of ADA[15]. The identity of the product of the reaction as 8-NH_2-Ino was established by its comparison with an authentic sample of 8-NH_2-Ino.

Structure and Substrate Activity for ADA. As already noted, the presence of halogen at either the 2- or 8-position of Ado, dAdo, or araA markedly decreased substrate activity for ADA. The influence of the 2-halogen presumably is due to its electron-withdrawing capacity which decreases the basicity of the amino group and thereby decreases its capacity

to act as a leaving group. The influence of the 8-halo substituents is not so readily explained. The decrease in substrate activity from the 8-Cl to the 8-Br derivative suggests that bulk is the predominant factor. Whether this effect is due to bulk as such or is the result of effects on conformation of the nucleoside cannot be determined from the data at hand. It is known that 8-Br-Ado in solution is in a syn-conformation[16], and Ogilvie et al.[17] have shown that an adenosine analog locked in an anti configuration was a substrate for ADA. These results show that ADA can act on an anti conformation but do not prove that the reason for the lack of activity of 8-Br-Ado is because it is in a syn-conformation in solution. In fact, the energy of rotation around the glycosyl bond is sufficiently low that it is probable that 8-Br-Ado, in binding to the enzyme, can assume any conformation that Ado can unless steric restrictions in the region of the 8-carbon prevent it from doing so.

Structure and Substrate Activity for AdoK. The presence of a halogen atom at the 2-position decreased the activity of substrates, as shown by the fact that the K_m for 2-F-Ado was two orders of magnitude greater than that of Ado. But 2-F-Ado is to be regarded as a good substrate because its V_{max} was also high. AdoK apparently has little tolerance for bulk at the 2-position as shown by the poor activity of the Cl- and Br- derivatives. In contrast to ADA, AdoK has high tolerance for bulk at the 8-position.

Structure and Substrate Activity of dCydK. Since the 2-halo derivatives of dAdo were better substrates than dAdo (lower Michaelis constants and moderately high maximum velocities) it is apparent that not only is there tolerance for bulk at the 2-position but that the presence of a halogen at this position enhances activity for the kinase. In contrast, the 8-halo derivatives of dAdo did not have detectable substrate activity. Thus, dCydK is quite different from AdoK with respect to response to alterations at the 2- and 8-positions.

Implications for Design of New Nucleoside Analogs. These results have obvious application for the design of new nucleoside analogs. They show that it is possible to make structural changes in adenosine, deoxyadenosine, or araA that essentially preclude deamination but still permit phosphorylation. They indicate that it is possible to introduce large groups at the 8-position of Ado without unacceptable impairment of capacity of the nucleoside to be phosphorylated. In the opposite sense they show that biological activity is not to be expected from Ado derivatives with

bulky substituents at the 2-position or from derivatives of dAdo (and, by analogy, araA) with bulky substituents at the 8-position. In addition, it is obviously possible to make substitutions that enhance phosphorylation, as is illustrated by the activities of the 2-halo derivatives of dAdo. The results also provide further illustrations of the generalization that capacity to undergo phosphorylation is a requisite for most nucleoside analogs to exert biological activity.

MODES OF ACTION OF HALOGENATED NUCLEOSIDES

It would be expected that each member of a group of compounds, e.g., the 2-halodeoxyadenosines, would have the same mode of action, but that there might be considerable differences in effectiveness against the target enzymes. Since the phosphates, in most instances the triphosphates, are the active forms of the nucleosides, it is necessary to have the phosphates available for studies with the target enzymes, once these have been identified. To date we have initiated mechanism studies with two types of compounds: 8-NH_2-6-F-PuR and the 2-halo derivatives of dAdo and araA (grouped together because they appear to have similar modes of action). Studies with 2-F-araA have been completed and published[18,19]. This agent inhibits DNA synthesis and its triphosphate inhibits both DNA polymerase and ribonucleotide reductase. A comparison of its activities against these enzymes with those of araA revealed an interesting structure-activity relationship. The (μM) concentrations of 2-F-araATP and araATP, respectively, required for 50% inhibition of the reduction of ADP by reductase from three sources were: L1210 cells, 15 and 175; HEp-2 cells, 10 and 165; HeLa cells, 1 and 28. The (μM) concentrations of 2-F-araATP and araATP, respectively, required for inhibition of DNA polymerase-α were: L1210 cells, 11 and 11; HeLa cells 1.2 and 1.3. Thus, introduction of the F-atom was without effect on capacity to inhibit the polymerase but increased activity against the reductase by an order of magnitude. 2-F-dAdo, 2-Cl-dAdo and 2-Br-dAdo have also been found to selectively inhibit DNA synthesis: their triphosphates have been prepared and are currently being evaluated as inhibitors of DNA polymerase and ribonucleotide reductase. It is to be expected that these studies will reveal some interesting structure-activity relationships.

From the ribonucleosides, 8-NH_2-6-F-PuR was chosen for detailed study. To obtain quickly an indication of how this agent was metabolized, it was assayed as an inhibitor of lines of L1210 cells deficient in selected enzymes of purine metabolism (Table 2). The responses of these cell lines

TABLE 2

Response of L1210 Cells and Enzyme-Deficient Sublines
8-NH_2-6-F-PuR and 8-NH_2-Ino

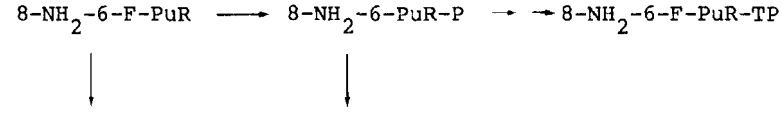
Cell Line	Enzyme Deficiency	Deoxycoformycin[a] (0.35 μM)	ID_{50} (μM)[b] 8-NH_2-6-F-PuR	ID_{50} (μM)[b] 8-NH_2-Ino
L1210/0	None	-	2	20
L1210/0	None	+	2	
L1210/MeMPR	AdoK	-	22	19
L1210/MeMPR	AdoK	+	>140	
L1210/MP	HPRT	-	2	22
L1210/MP/MeMPR	AdoK, HPRT	-	18	20
L1210/MP/MeMPR	AdoK, HPRT	+	>40	

a. +, deoxycoformycin present in medium at this concentration; -, no deoxycoformycin present. b. ID_{50} = concentration required for 50% inhibition of colony formation in soft agar.

showed the following: (a) 8-NH_2-6-F-PuR is most toxic to cells that have AdoK; (b) 8-NH_2-6-F-PuR is also toxic (but less so) to cells that do not have AdoK; (c) the presence of deoxycoformycin (dCF) does not affect toxicity to $AdoK^+$ cells but abolishes toxicity to $AdoK^-$ cells; (d) the toxicity of 8-NH_2-6-F-PuR is the same to $HPRT^+$ and to $HPRT^-$ cells; (e) cells that have lost both HPRT and AdoK are still sensitive to 8-NH_2-6-F-PuR. From these results the following conclusions can be drawn: (a) the most toxic metabolites of 8-NH_2-6-F-PuR are phosphates; (b) defluorination also leads to a toxic metabolite (8-NH_2-Ino); and (c) 8-NH_2-6-F-PuR is not itself toxic. Because of the evidence that 8-NH_2-Ino was itself toxic, its mechanism was also studied. 8-NH_2-Ino was found to be equally toxic to all of the cell lines identified in Table 2. In $AdoK^-$ cells its toxicity was the same as that of 8-NH_2-6-F-PuR. These results thus indicate that, in $AdoK^-$ cells, it is 8-NH_2-Ino that is the toxic metabolite of 8-NH_2-6-F-PuR.

HPLC analysis of cells grown in the presence of 8-NH_2-6-F-PuR revealed two metabolites. One had the retention time of a monophosphate. This metabolite was identified as 8-NH_2-IMP by (a) stop-flow scan of its uv spectrum, which was the same as that of 8-NH_2-Ino; and (b) elution, dephosphorylation, and rechromatography on a C_{18} reversed phase column where it had the same retention time and the same uv spectrum as a known sample of 8-NH_2-Ino. The second metabolite had a retention time about the same as ATP

and was detected by scanning the ascending portion of the ATP peak. The differences in the uv absorption spectra of Ado (λ_{max} = 262 nm) and 8-NH_2-6-F-PuR (λ_{max} = 271 nm) are of sufficient magnitude that 8-NH_2-6-F-PuR-TP may be detected in the presence of ATP. 8-NH_2-6-F-PuR-TP appeared to be present in smaller amounts than 8-NH_2-IMP. When the metabolism of 8-NH_2-6-F-PuR was studied in the presence of dCF, the amount of 8-NH_2-IMP formed was unchanged. This result indicates that 8-NH_2-IMP probably was formed by defluorination of the monophosphate of 8-NH_2-6-F-PuR, because the amount of dCF used was in excess of that required to inhibit ADA. To investigate the possibility that 8-NH_2-Ino, if formed, might itself be phosphorylated, we studied the metabolism of 8-NH_2-6-F-PuR in AdoK$^-$ cells in the absence of dCF. In the absence of AdoK, the only pathway open to 8-NH_2-6-F-PuR is conversion to 8-NH_2-Ino. Cells so treated contained no 8-NH_2-IMP. These results indicate that the metabolism of 8-NH_2-6-F-PuR is as shown below.



Thus, in cells treated with 8-NH_2-6-F-PuR there are three metabolites that potentially are responsible for its cytotoxicity: 8-NH_2-6-F-PuR-TP, 8-NH_2-IMP, and 8-NH_2-Ino. Clearly the phosphates (one or both) are more potent inhibitors than 8-NH_2-Ino, because the toxicity of 8-NH_2-6-F-PuR is less in cells that lack AdoK. Presently available results do not permit a decision as to whether 8-NH_2-IMP or 8-NH_2-6-F-PuR-TP is the more cytotoxic metabolite. Experiments underway, in which high levels of coformycin are being used to inhibit AMP deaminase and prevent formation of 8-NH_2-IMP from 8-NH_2-6-F-PuR-P, may answer this question. A remaining question is the mechanism by which 8-NH_2-6-F-PuR inhibits cell proliferation. HPLC analysis showed that the pools of adenine and guanine nucleotides were severely reduced in 8-NH_2-6-F-PuR-treated cells. In studies of effects on macromolecular synthesis, 8-NH_2-6-F-PuR (in the presence of dCF) did not inhibit protein synthesis but did inhibit incorporation of a number of precursors into RNA and DNA. The inhibition of synthesis of RNA and DNA may be the result of the depletion of pools of adenine and guanine nucleotides. Studies of macromolecular synthesis were also performed with 8-NH_2-6-F-PuR in AdoK$^-$ cells in the absence of dCF; under these conditions the metabolic effects observed

presumably are those of 8-NH_2-Ino. Concentrations up to 30 μM did not inhibit macromolecular syntheses; a concentration of 100 μM inhibited synthesis of protein and DNA but not RNA. The results of these preliminary experiments thus suggest that the primary effect of 8-NH_2-6-F-PuR is on polynucleotide synthesis and that of 8-NH_2-Ino is on protein synthesis.

Postscript. After this paper was presented, it was unexpectedly discovered that the product initially identified as 8-fluoroadenosine was actually 8-amino-6-fluoro-9-β-D-ribofuranosylpurine. The text has been changed to reflect this result.

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