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NUCLEOSIDES OF 2-AZA-PURINES—CYTOTOXICITIES AND ACTIVITIES AS SUBSTRATES FOR ENZYMES METABOLIZING PURINE NUCLEOSIDES

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Abstract—Nucleosides of 2-aza-adenine, 2-aza-hypoxanthine and 4-amino-7H-pyrazolo[3,4-*d*]-*v*-triazine were examined: (a) for cytotoxicity to cultured H. Ep. No. 2 cells and to sublines resistant to certain analogs of purines and purine nucleosides and deficient in certain enzymes of purine metabolism, and (b) as substrates for adenosine kinase, adenosine deaminase, and nucleoside-cleaving enzymes. 2-Aza-adenosine was much more toxic than 2-aza-adenine and was a good substrate for both the kinase and the deaminase. The responses of the resistant cell lines indicated that the cytotoxicity of 2-aza-adenosine was due both to its direct phosphorylation and to its conversion to 2-aza-hypoxanthine. 2-Aza-inosine, 2′-deoxy-2-aza-inosine and 2-aza-hypoxanthine had similar cytotoxicities, and the responses of the resistant cell lines showed that the cytotoxicities of both of the nucleosides resulted from their conversion to 2-aza-hypoxanthine. The ribonucleoside of 4-amino-7H-pyrazolo[3,4-*d*]-*v*-triazine was less toxic than 2-aza-adenosine, and was a good substrate for adenosine kinase but a poor substrate for adenosine deaminase. 2-Aza-inosine and 2′-deoxy-2-aza-inosine were cleaved to 2-aza-hypoxanthine by cell-free supernatants, but the conversions were poor relative to those of inosine and 2′-deoxyinosine. 3′-Deoxy-2-aza-adenosine and the α-arabinosyl, β-arabinosyl and β-xylosyl derivatives of 2-aza-adenine were not toxic. 3′-Deoxy-2-aza-adenosine and β-xylosyl-2-aza-adenine were moderately good substrates for the deaminase, but α-arabinosyl-2-aza-adenine was not deaminated and β-arabinosyl-2-aza-adenine was deaminated at a rate less than 5 per cent that of β-arabinosyladenine. These results indicate that some of these nucleosides, particularly 2-aza-adenosine, may merit further study as growth-inhibitory and potential antitumor agents.

Nucleosides of purines and purine analogs have been found to exhibit a broad spectrum of biological activity, which includes activity against viruses and experimental tumors [1]. Our observations on the antitumor activity of 8-aza-inosine [2] and on the metabolism of 8-aza-inosine and 8-aza-adenosine [3] have led to interest in a related series of compounds, the nucleosides of 2-aza-purines. The free bases, 2-aza-adenine and 2-aza-hypoxanthine, have long been known to inhibit growth of both microbial and mammalian cells [4–9], but the biological activity of 2-aza-purine nucleosides has not been examined. We report here observations on the cytotoxicities of 2-aza-purines and their nucleosides and on the activities of the nucleosides as substrates for enzymes acting on purine nucleosides.

MATERIALS AND METHODS

Compounds. The 2-aza-purines and nucleosides used in this study were synthesized in our laboratories by Dr. J. A. Montgomery and his associates. 2-Aza-adenine and 2-aza-hypoxanthine were synthesized by the method of Shaw and Woolley [4], and 2-aza-adenosine, 2-aza-APP ribonucleoside,* 9-β-D-arabinosyl-2-aza-adenine, 9-β-D-xylosyl-2-aza-adenine, and 2′-deoxy-2-aza-adenosine by procedures described by Montgomery and Thomas [10]. 2-Aza-inosine, previously prepared chemically by Kawana *et al.* [11], was prepared for our study by the enzymatic deamination of 2-aza-adenosine. 3′-Deoxy-2-aza-adenosine was prepared by a ring opening–reclosure sequence similar to that used for the synthesis of other nucleosides of 2-aza-adenine [10–12].

Cell cultures. The cultures used were the H. Ep. No. 2 line established initially by Moore *et al.* [13] and sublines selected in our laboratory for resistance to specific purine analogs and deficient in certain enzymes of purine metabolism [14]. These cell lines and their enzyme deficiencies are listed in Table 1. Compounds were assayed as inhibitors of these cell lines by determining their effects on colony formation as described in Table 2.

Enzyme assays. Nucleosides were assayed as substrates for partially purified preparations of adenosine kinase and adenosine deaminase and for nucleoside phosphorylase and hydrolase activities present in crude supernatants from H. Ep. No. 2 cells; details of the assays are given in Table 3. The adenosine kinase was a preparation 675-fold purified from H. Ep. No. 2 cells as previously described [15]. The adenosine deaminase preparation was from calf intestine (Sigma Chemical Co., St. Louis, Mo.). The rate of deamination was determined by changes in absorption at or near the absorption maxima for each nucleoside.

* Abbreviations: 2-aza-APP ribonucleoside, 4-amino-7-β-D-ribofuranosyl-7H-pyrazolo[3,4-*d*]-*v*-triazine; 6-MP, 6-mercaptopurine; 6-MeMPR, 6-methylthiopurine ribonucleoside; PRTase, phosphoribosyltransferase.

Table 1. Description of H. Ep. No. 2 cell lines

Cell line	Resistant to	Enzyme deficiency
H. Ep. No. 2 MP	6-MP	Hypoxanthine (guanine) PRTase
H. Ep. No. 2 FA	2-Fluoroadenine	Adenine PRTase
H. Ep. No. 2 MeMPR	6-MeMPR	Adenosine kinase
H. Ep. No. 2 MP FA	6-MP and 2-fluoroadenine	Hypoxanthine (guanine) PRTase and adenine PRTase
H. Ep. No. 2 MP MeMPR	6-MP and 6-MeMPR	Hypoxanthine (guanine) PRTase and adenosine kinase
H. Ep. No. 2 FA FAR	2-Fluoroadenine and 2-fluoroadenosine	Adenine PRTase and adenosine kinase

Table 2. Inhibition of H. Ep. No. 2 cell lines by some 2-aza-purines and nucleosides of 2-aza-purines*

Compound	50% Inhibitory concn for parent H. Ep. No. 2 cells (μM)	Ratio of 50% inhibitory concentrations: Resistant cell line Parent cell line					
		MP	FA	MeMPR	MP FA	FA FAR	MP MeMPR
2-Aza-hypoxanthine	0·4	>600	2	1	>600	2	>600
2-Aza-adenine	11	1	>27	2	>27	>27	3
2-Aza-inosine	0·9	>150	0·8	0·6	>150	0·4	>150
2-Aza-adenosine	0·2	0·7	1	2	0·9	3	>600
2′-Deoxy-2-aza-inosine	0·6	>250		1			
2′-Deoxy-2-aza-adenosine	7						
3′-Deoxy-2-aza-adenosine	>75						
9-β-D-Arabinosyl-2-aza-adenine	>75						
9-β-D-Xylosyl-2-aza-adenine	>75						
9-γ-D-Arabinosyl-2-aza-adenine	>75						
2-Aza-APP-R	15						
9-β-D-Arabinosyl-2-aza-hypoxanthine	37·75						

* Approximately 100 cells were placed in 4-oz. prescription bottles containing, for control cultures, 10 ml of SRI-14 medium [16] and for treated cultures, 10 ml of medium to which the inhibitor had been added. After the cultures had been incubated at 37° for 7–10 days, the medium was decanted and the cells adhering to the glass were washed with phosphate-buffered NaCl (0·85%), fixed with Bouin's fixative, and stained with Giemsa stain. The macroscopic colonies present were then counted. Cloning efficiency of control cultures was 40–70 per cent.

Prior to the assay these maxima and the molecular extinction coefficients were determined for each adenosine analog and its deamination product. The 2-aza-purine nucleosides have two absorption maxima at pH 7·0: for the nucleosides of 2-aza-adenine, at 255–258 nm and at 295–299 nm and for nucleosides of 2-aza-hypoxanthine at 248–253 nm and at 293–295 nm. The deamination of the 2-aza-adenine nucleosides was followed by changes in absorption at 260 nm; at this wave length the differences in molecular extinction coefficients (ΔE) of the adenosine analogs and their deamination products were all about the same (average, 4160; range 3900–4590). The absorption maximum for 2-aza-APP ribonucleoside was at 314 nm and that of its deamination product at 302 nm; the rate of deamination was determined at 320 nm, at which wavelength ΔE was 1500. The absorption maxima of β-arabinosyladenine and of β-arabinosylhypoxanthine were 260 and 247 nm; determination of the rate of deamination was at 265 nm, at which wavelength ΔE was 7100.

For preparation of cell-free supernatants, H. Ep. No. 2 cells, grown in suspension culture in SRI-14 medium [16] to a final concentration of about $1{\cdot}8 \times 10^5$ cells/ml, were collected by centrifugation and washed free of medium with 0·85% saline solution. The cells were suspended in 3 vol of water, homogenized in a glass-Teflon homogenizer, and centrifuged at 25,000 *g* for 45 min. The resulting supernatant, which contained 15–25 mg of protein ml, was used immediately. Conversion of the nucleosides of 2-aza-hypoxanthine to the free base was determined by the spectrophotometric method in which the purine base resulting from cleavage of the nucleoside is oxidized by xanthine oxidase [17]. The xanthine oxidase preparation used was from milk (Sigma Chemical Co.) and had a specific activity of 15 units/ml. 2-Aza-hypoxanthine is a known substrate for xanthine oxidase [5]. The oxidation product of 2-aza-hypoxanthine had an absorption maximum at pH 7 at 320 nm, and the ΔE value was 4800.

RESULTS AND DISCUSSION

Most purines and nucleosides and analogs thereof must by converted to nucleotides to be biologically active [18]. Since each of the resistant cell lines used in this study is deficient in one or two of the enzymes responsible for the conversion of purines or purine nucleosides to nucleotides, the responses of these cell lines give an indication of the pathways by which a cytotoxic purine, purine nucleoside or analogs of purines and nucleosides are converted to nucleotides.

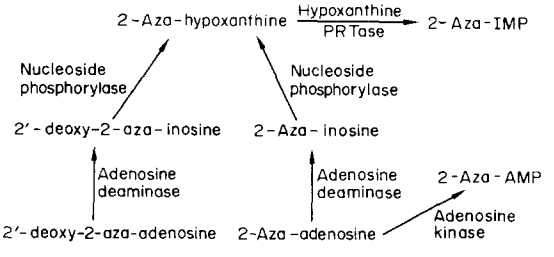

Fig. 1. Pathways for conversion of the ribonucleosides and deoxyribonucleosides of 2-aza-adenine and 2-aza-hypoxanthine to nucleotides.

Table 2 presents data on the responses of the parent and resistant cell lines to 2-aza-purines and 2-aza-purine nucleosides. 2-Aza-adenosine was the most cytotoxic compound of the series. Like many other adenosine analogs [19], it was much more toxic than its aglycone; this higher toxicity is presumably because the nucleosides are converted to the nucleotides more efficiently than are the free bases. It is noteworthy that resistance to 2-aza-adenosine did not result from loss of adenosine kinase (H. Ep. No. 2/MeMPR cells), adenine PRTase (H. Ep. No. 2/FA cells) or both of these enzymes (H. Ep No. 2/FA/FAR cells), but that H. Ep. No. 2/MP/MeMPR cells were highly resistant. From this pattern of response, it appears that 2-aza-adenosine may be converted to the nucleotide by two pathways: either by direct phosphorylation to yield 2-aza-AMP or by a three-step conversion to 2-aza-IMP involving the sequential action of adenosine deaminase, purine nucleoside phosphorylase, and hypoxanthine PRTase. Conversion to 2-aza-adenine is apparently not a factor in its cytotoxicity and would not be expected to be because of the relatively low cytotoxicity of 2-aza-adenine and the low activity of adenosine, and presumably of adenosine analogs, as substrates for mammalian nucleoside phosphorylase [20]. The ribonucleoside of 2-aza-APP has moderate cytotoxicity, whereas α-arabinosyl-2-aza-adenine, β-arabinosyl-2-aza-adenine, β-xylosyl-2-aza-adenine and 3′-deoxy-2-aza-adenosine (the 2-aza analog of the antibiotic, cordycepin) were not cytotoxic at concentrations 375-fold greater than the inhibitory concentration of 2-aza-adenosine.

The results with the nucleosides of 2-aza-hypoxanthine differed from those with the 2-aza-adenine nucleosides. The cytotoxicities of 2-aza-hypoxanthine, 2-aza-inosine and 2′-deoxy-2-aza-inosine were similar, and loss of hypoxanthine PRTase conferred resistance to all three compounds. This pattern of response demonstrates that these nucleosides are not phosphorylated to a biologically significant extent and that the principal pathway for their conversion to nucleotides is via conversion to 2-aza-hypoxanthine. These results with 2-aza-inosine are in contrast to those with the related analog, 8-aza-inosine, which showed that this compound could either be phosphorylated directly or converted to the nucleotide via the free base [2].

Shown in Fig. 1 are the pathways indicated by the responses of the cell cultures for the conversion of the ribonucleosides and deoxyribonucleosides of 2-aza-adenine and 2-aza-hypoxanthine to nucleotides. It was desirable to confirm some of these postulated conversions with isolated enzymes, and the results obtained are presented in Table 3. All of the expected conversions were found to occur. 2-Aza-adenosine was deaminated at about the same rate as adenosine, and 2′-deoxy-2-aza-adenosine was even more rapidly deaminated; the K_m values for these compounds were considerably greater than that for adenosine. 2-Aza-adenosine was also phosphorylated by partially purified adenosine kinase; both the K_m and the reaction velocity were greater than the values for adenosine. Relative to inosine and 2′-deoxyinosine, 2-aza-inosine and 2′-deoxy-2-aza-inosine were poorly cleaved to the free base by crude supernatants from H. Ep. No. 2

Table 3. Nucleosides of 2-aza-purines as substrates for certain enzymes*

Enzyme or conversion	Substrate	Relative activity	K_m (μM)
Adenosine kinase	Adenosine	100†	1·8
	2-Aza-adenosine	370	200
	2-Aza-APP-ribonucleoside	92	
Adenosine deaminase	Adenosine	100‡	29
	2-Aza-adenosine	102	670
	2′-Deoxy-2-aza-adenosine	230	1300
	β-Arabinosyladenine	22	120
	β-Arabinosyl-2-aza-adenine	0·8	260
	β-Xylosyl-2-aza-adenine	49	1250
	3′-Deoxy-2-aza-adenosine	14	500
	2-Aza-APP-ribonucleoside	0·2	440
	α-Arabinosyl-2-aza-adenine	ND§	
Nucleoside→base (crude supernatants)	Inosine	100	
	2′-Deoxyinosine	60	
	2-Aza-inosine	8	
	2′-Deoxy-2-aza-inosine	6	

* K_m values were determined by Lineweaver-Burk analysis at 15–18 different substrate concentrations for adenosine deaminase and at 5 different substrate concentrations for adenosine kinase. In both cases, concentrations higher and lower than the K_m value were used.

Assay for activity as substrates for adenosine kinase was carried out as described previously [15]. The incubation mixture contained, in a final volume of 0·2 ml, enzyme (675-fold purified from H. Ep. No. 2 cells); the substrate; $MgCl_2$ (0·25 mM); potassium phosphate buffer, pH 7·0 (50 mM); and ATP-γ-P^{32} (2·5 mM). After incubation at 37° for 45 min, the reactions were stopped by immersion in boiling water for 1 min, and the preparations were subjected to chromatography on Whatman paper in a solvent consisting of equal volumes of 93·8% *n*-butyl alcohol and 44% aqueous propionic acid. The paper strips were assayed for radioactivity on a Packard model 2701 scanner, and the amount of radioactivity present at the R_f for nucleoside monophosphates was taken as a measure of the amount of nucleoside that was phosphorylated.

For assay for substrate activity for adenosine deaminase the incubation mixture contained, in a final volume of 3·0 ml, the substrate; calf intestinal adenosine deaminase (220 units/mg of protein); and potassium phosphate buffer, pH 7·5 (50 mM). The extent of deamination was determined by the change in optical density at the absorption maximum for the nucleoside. See text for additional details.

For determination off the capacity of cell-free supernatants to convert nucleosides to the free bases, the reaction mixture contained: the substrate (0·125 mM); crude enzyme preparation (1·2 mg protein); potassium phosphate buffer (25 mM, pH 7·4); and xanthine oxidase (0·75 units), in a final volume of 4 ml. Incubation was at 25° for 30 min. The reaction was stopped by addition of 1 ml of 50% TCA and the extent of reaction was determined by the increase in optical density at 293 nm for inosine and 2′-deoxyinosine, and at 320 nm for the 2-aza-hypoxanthine nucleosides. See text for additional details.

† The V_{max} for adenosine was 69 nmoles/min/mg of protein.

‡ The V_{max} for adenosine was 435 μmoles/min/mg of protein.

§ ND = no detectable activity.

cells. This poor conversion appears at first sight to conflict with the conclusion that these nucleosides are converted to 2-aza-purine nucleotides via 2-aza-hypoxanthine. However, since mammalian cells have a high activity of purine nucleoside phosphorylase and since 2-aza-hypoxanthine is so highly cytotoxic, it is likely that this degree of activity as a substrate is sufficient for the conversion of 2-aza-inosine and 2′-deoxy-2-aza-inosine to cytotoxic concentrations of 2-aza-hypoxanthine. In addition to the pathways shown above, it is possible that other conversions take place, for example, conversion of 2-aza-IMP to 2-aza-AMP and 2-aza-GMP. The responses of the cell lines to inhibition by the bases and nucleosides cannot provide evidence for or against interconversions at the nucleotide level. Determination of whether these interconversions occur must await additional studies with labeled compounds.

Other noteworthy results in Table 3 are those with the arabinosyl derivatives of 2-aza-adenine and with 2-aza-APP ribonucleoside. The α-arabinosyl derivative had no detectable activity as a substrate for adenosine deaminase. The β-arabinosyl derivative had some activity, but it was less than 5 per cent that for β-arabinosyladenine. Similarly 2-aza-APP ribonucleoside was a much poorer substrate for the deaminase than was APP-ribonucleoside [21]. Thus, the substitution of N for C at position 2 of the purine ring reduces the activity of some, but not all, nucleosides as substrates for adenosine deaminase. With respect to substrate activity for adenosine kinase, 2-aza-adenosine was phosphorylated at a rate greater than that for adenosine, whereas the rate of phosphorylation of 2-aza-APP-ribonucleoside was about the same as that of adenosine but considerably less than that previously reported [15] for APP-ribonucleoside.

It would appear from these results that the nucleosides of 2-aza-purines may be worthy of further study and evaluation as growth-inhibitory and antitumor agents. This is particularly true of 2-aza-adenosine,

since it apparently has two routes for conversion to cytotoxic nucleotides and thus would be expected to differ in biological activity from 2-aza-adenine, 2-aza-hypoxanthine and 2-aza-inosine. Both of these pathways appear to operate efficiently, and each is capable, in the absence of the other, of providing a cytotoxic concentration of a metabolite, presumably, 2-aza-AMP, 2-aza-IMP or further metabolites thereof.

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