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ACTIVITY OF ADENOSINE ANALOGS AGAINST A CELL CULTURE, LINE RESISTANT TO 2-FLUOROADENINE*

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Abstract—By serial transfer in the presence of 2-fluoroadenine, a subline of H.Ep. #2 cells in culture was selected for high resistance to 2-fluoroadenine. The resistance was stable when the cells were cultured in the absence of 2-fluoroadenine. The resistant cells were essentially devoid of AMP pyrophosphorylase activity, but retained IMP and GMP pyrophosphorylase and purine nucleoside kinase activities. Intact growing cells of the parent and resistant lines did not differ in capacity to incorporate into polynucleotides ^{14}C from adenosine-8-^{14}C and hypoxanthine-8-^{14}C, but, as compared to sensitive cells, the resistant cells incorporated ^{14}C from adenine-8-^{14}C very poorly. The resistant cells were cross-resistant to adenine analogs but did not differ from the parent cells in sensitivity to a series of adenosine analogs. Many nucleosides were much more toxic to the parent cell line than were the corresponding free bases. These results suggest that a purine nucleoside kinase, presumably adenosine kinase, is the critical enzyme in the activation of these cytotoxic analogs of adenosine.

AMONG the purine analogs of interest because of their tumor-inhibitory or other biological activity are certain analogs of adenine and adenosine, such as 2-fluoroadenine,[1, 2] 2-fluoroadenosine,[2–4] 4-aminopyrazolo(3,4-*d*)pyrimidine (for a review, see Brockman and Anderson[5]), tubercidin (7-deaza-adenosine),[6, 7] and others. Many types of study have shown that the active forms of the highly cytotoxic purine analogs are the nucleotides.[8] The nucleotides might be formed intracellularly by the following known pathways: (a) the action of a nucleotide pyrophosphorylase on the free base; (b) the action of a nucleoside kinase on the ribonucleoside; or (c) the sequential action of a nucleoside phosphorylase and a nucleotide pyrophosphorylase on the ribonucleoside. Less likely is the conversion of the free base to the nucleoside which might then be phosphorylated. The critical role of nucleotide pyrophosphorylases in the activation of certain purine analogs has been demonstrated repeatedly,[8] but until recently little attention has been given to the possible importance of nucleoside kinases, probably because for 6-mercaptopurine (6-MP), the purine analog with the most interesting antitumor activity, the pyrophosphorylase pathway is apparently the only route to the nucleotide, as shown by the fact that cells lacking IMP pyrophosphorylase were resistant to both 6-MP and 6-MP-ribonucleoside.[8] The failure of 6-MP-ribonucleoside to inhibit cells resistant to 6-MP might result from (a) the absence

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of the required kinase, presumably inosine kinase, which has not been reported in mammalian cells;[9] (b) the ready cleavage of 6-MP-ribonucleoside to 6-MP, which has been observed in mammalian cells;[10, 11] or (c) a combination of both these factors. Similar information is not available on the cytotoxic analogs of adenine and adenosine but, since adenosine kinase is known to occur in mammalian cells,[12, 13] the kinase pathway might be expected to be significant. To obtain information on the relative importance of these pathways in the activation of analogs of adenine and adenosine, it appeared desirable to have a cell line lacking a necessary enzyme for one of the pathways outlined above. This report is concerned with the isolation and metabolic characterization of a line of H.Ep. #2 cells highly resistant to 2-fluoroadenine and deficient in AMP pyrophosphorylase activity and with its response to a series of analogs of adenine, adenosine, and related compounds.

MATERIALS AND METHODS

Compounds. 8-Aza-adenosine was synthesized by Dr. Leon Goodman, Stanford Research Institute, and obtained from the Cancer Chemotherapy National Service Center through the courtesy of Dr. Harry Wood. 6-Methylthio-9-β-D-arabinofuranosylpurine, 6-methylthio-9-β-D-xylofuranosylpurine, 9-β-D-arabinofuranosyladenine, and 9-β-D-xylofuranosyladenine were also gifts from Dr. Leon Goodman. 4-Aminoimidazo(4,5-*d*)-pyridazine and its two isomeric ribonucleosides were gifts from Dr. John Carbon of Abbott Laboratories, and cordycepin (3′-deoxyadenosine)[14] was a gift from Dr. George Boxer of the Merck Institute for Therapeutic Research. Tubercidin was obtained from Dr. C. G. Smith of the Upjohn Co. and also from the CCNSC. 6-Mercaptopurine was obtained from Nutritional Biochemicals Corp.; 8-aza-adenine from Krishell Laboratories, Inc.; purine from Calbiochem; 6-methylpurine from Cyclo Chemicals; and 4-aminopyrazolo(3,4-D)pyrimidine and 2-azaadenine from the CCNSC. Other compounds used were synthesized in our laboratories; see Table 4 for references to methods of synthesis. Adenine-8-^{14}C, hypoxanthine-8-^{14}C, guanine-8-^{14}C, and adenosine-8-^{14}C, each with a specific activity of 1 mc/m-mole or greater, were obtained from various commercial suppliers.

Isolation of H.Ep. #2 *cells resistant to 2-fluoroadenine.* The cell line used in these studies was that designated H.Ep. #2, a human epidermoid cell line originally isolated by Moore *et al.*[15] and carried in our laboratories, either in stationary or suspension culture, by methods that have been described in detail elsewhere.[16] For selection of a resistant line, these cells were cultured, in serial transfer, in the presence of gradually increasing concentrations of 2-fluoroadenine, starting with a concentration of 0·05 μg/ml which inhibited growth of the initial culture almost completely. Resistance developed slowly but, after cells were obtained that grew well in the presence of 2–4 μg/ml, the concentration was raised rapidly to 40 μg/ml. The resistant cell line (designated H.Ep. #2/FA to distinguish it from the parent sensitive line, hereafter designated H.Ep. #2/S) was routinely carried in the presence of fluoroadenine at a concentration of 20 μg/ml. The resistant cells also grew well in the absence of fluoroadenine, and for particular experiments were cultured for long periods in the absence of the inhibitor.

Response of H.Ep. #2/S and H.Ep. #2/FA cell lines to inhibitors. Response of the parent and resistant cell lines to various inhibitors was determined by cloning procedures. For these experiments, 100 cells were placed in 4-oz prescription bottles

containing SRI-14[16] medium and varying amounts of the inhibitor. Six or seven days later the medium was decanted, after which the cells were washed with phosphate-buffered (pH 7·0), 0·85% NaCl solution, fixed with Bouin's fixative, and stained with Giemsa stain. The number of macroscopic colonies that had formed was then counted. Cloning efficiencies for both parent and resistant cell lines over a period of many months were in the range of 40–60 per cent.

Assays for nucleotide pyrophosphorylases. Assays for IMP, GMP, and AMP pyrophosphorylase activity were carried out under the conditions described in Table 1. These procedures are essentially those of Brockman *et al.*[17]

Assays for purine nucleoside kinase activity. Purine nucleoside kinase activity was assayed with 6-methylthiopurine-^{35}S ribonucleoside as substrate; it has been shown earlier that this nucleoside is phosphorylated by a phosphokinase present in H.Ep. #2 cells[18] and that the kinase involved is apparently adenosine kinase.[19] Adenosine could not be used as substrate in the crude cell-free system because of the high activity of adenosine deaminase. The conditions of the assay are given in Table 2. For comparison with the parent and fluoroadenine-resistant cell lines, kinase activity was also determined for a line of H.Ep. #2 cells (H.Ep. #2/MP) resistant to 6-mercaptopurine and devoid of pyrophosphorylase activity for IMP and GMP.[20]

Metabolism of purines and nucleosides. To suspension cultures of H.Ep. #2/S and H.Ep. #2/FA lines, containing at the beginning of the experiment about 3×10^8 cells in 800 ml SRI-14 medium, was added 5 μc adenine-8-^{14}C, hypoxanthine-8-^{14}C, or adenosine-8-^{14}C. Twenty-four hours thereafter the cells were harvested, and hot 80% ethanolic extracts were prepared. The water-soluble portions of the extract were subjected to two-dimensional paper chromatography in phenol–water and butanol–propionic acid, and from the resulting chromatograms, two-dimensional radioautograms were prepared.[21] From the residue remaining after the ethanol extraction, the sodium nucleates were isolated by extraction with hot 10% NaCl;[22] the amount of polynucleotides present was determined by measurement of ultraviolet absorption, and the ^{14}C present was assayed in a liquid scintillation spectrometer.

RESULTS

The high degree of resistance of the H.Ep. #2/FA cell line to 2-fluoroadenine is indicated by the fact that 2-fluoroadenine did not inhibit colony formation in the resistant cell line at a concentration 2000-fold greater than that producing marked inhibition of the sensitive line (Table 4). The cells of the resistant line, as compared to the sensitive line, were essentially devoid of AMP pyrophosphorylase activity, but retained activities of IMP and GMP pyrophosphorylases that did not differ significantly from the range of values obtained for the parent cell line (Table 1). Cells of the resistant line showed no increase in AMP pyrophosphorylase activity when cultured in the absence of drug in separate experiments for thirteen and for twenty generations. Both the H.Ep. #2/FA and H.Ep. #2/MP lines had about the same nucleoside kinase activity, which was about 70 per cent that of the lower extreme of the range found for the parent cell line (Table 2).

The metabolism of three precursors (adenine, hypoxanthine, and adenosine) by intact cells of H.Ep. #2/S and H.Ep. #2/FA lines was consistent with the specific loss of activity of AMP pyrophosphorylase observed in the experiments with cell-free extracts. Sensitive and resistant cells incorporated into polynucleotides about the same

Table 1. Nucleotide pyrophosphorylase activities of cell-free extracts of H.Ep. #2 cells and a subline resistant to 2-fluoroadenine

Cell line	Formation of nucleotide from corresponding free base (mμmoles nucleotide/20 min/mg protein)		
	AMP	GMP	IMP
Exp. 1			
H.Ep. #2/S	138	128	280
H.Ep. #2/FA*	1	298	145
H.Ep. #2/FA (normal medium 13 generations)	2	276	150
Exp. 2			
H.Ep. #2/S	128	289	172
H.Ep. #2/FA (normal medium 20 generations)	0	308	157

$$\text{Reaction: base} \xrightarrow[\text{enzyme}]{\text{PRPP, } Mg^{++}} \text{nucleotide}$$

The reaction mixture contained 5-phosphoribosyl-1-pyrophosphate (PRPP) (1 μmole); Tris (50 μmoles), the ^{14}C-labeled purine (0·25 μmole); the enzyme fraction (ca. 200 μg protein), and H_2O in a final volume of 0·5 ml, pH 7·6. The reaction was stopped by addition of EDTA and immersion in a boiling water bath. The nucleotides were separated by paper chromatography of the reaction mixture in isopropanol in an ammonia atmosphere and located by radioautography; the radioactive spots were cut out and assayed in a liquid scintillation spectrometer. The enzyme fraction used was the soluble portion of the acetone powder prepared directly from the harvested cells which had been washed free of medium with saline solution.

* These cells were grown in the presence of 2-fluoroadenine up to the time the cell-free preparation was made.

Table 2. Nucleoside kinase activities of cell-free supernatants from H.Ep. #2 cells and sublines resistant to 2-fluoroadenine or 6-mercaptopurine

Cell line	Nucleotide formation (mμmoles/min/mg protein) from 6-methylthiopurine-^{35}S ribonucleoside
H.Ep. #2/S	4–5
H.Ep. #2/FA	2·9
H.Ep. #2/MP	2·9

For preparation of the cell-free supernatant, cells suspended in 3 volumes of water were broken up in a Thomas glass–Teflon homogenizer and centrifuged at 25,000 *g*. The incubation mixture contained the following in a final volume of 1·0 ml: ATP, 2·5 μmoles; Mg^{++}, 0·25 μmole; potassium phosphate buffer, pH 7·0, 50 μmoles; cell-free supernatant, 5·0 mg protein; and 6-methylthiopurine-^{35}S ribonucleoside, 1 μmole. The reaction was stopped by immersion in a boiling water bath, protein was removed by centrifugation, and the supernatant was subjected to chromatography in a butanol–propionic acid solvent.[21] The nucleotides formed were located by radioautography and identified, after which the radioactive spots were cut out and assayed in a liquid scintillation spectrometer.

amount of ^{14}C from adenosine and hypoxanthine; but the incorporation of adenine by the resistant cells was very poor (Table 3). Two-dimensional chromatographic-radioautographic analysis of the soluble fractions of the cells from these same experiments failed to reveal any differences between the sensitive and resistant cells in the

TABLE 3. INCORPORATION OF PRECURSORS INTO POLYNUCLEOTIDES BY H.Ep. #2 CELLS AND A SUBLINE RESISTANT TO 2-FLUOROADENINE

Precursor	Specific activity ($\mu c \times 10^4$/mg) of polynucleotides*	
	H.Ep. #2/S	H.Ep. #2/FA
Adenine-8-^{14}C	140	5
Adenosine-8-^{14}C	100	98
Hypoxanthine-8-^{14}C	85	84

* RNA and DNA isolated by NaCl extraction after exposure of growing cells to the precursor for 24 hr. See text for details.

metabolism of adenosine and hypoxanthine; in the resistant cells, little radioactivity was present in the nucleotides when adenine-8-^{14}C was the precursor. Since these results are consistent with, and only tend to confirm, those for the polynucleotides, reproductions of these radioautograms have not been presented here.

A comparison of the response of the sensitive and resistant cell lines to various inhibitors (Table 4) indicates as a general pattern that the fluoroadenine-resistant cells were cross-resistant to adenine analogs but still sensitive to adenosine analogs. Thus, for example, the resistant cells were significantly cross-resistant to 4-amino-pyrazolo(3,4-*d*)pyrimidine, 8-aza-adenine, 2,6-diaminopurine, 4-aminoimidazo(4,5-*d*)-pyridazine, and 6-methylpurine, but were not different from the parent cells in sensitivity to, among others, 8-aza-adenosine, purine ribonucleoside, tubercidin, and 6-methylthiopurine ribonucleoside. The resistant cells were to some extent cross-resistant to fluoroadenosine, but the degree of resistance was small (20-fold) relative to the >2000-fold resistance to fluoroadenine. Some free bases (cf. 6-hydrazinopurine and 6-chloropurine) inhibited the parent line only at very high concentrations, and the fluoradenine-resistant cells were not cross-resistant to these compounds. As would be expected from the pyrophosphorylase data (Table 1), the parent and resistant cell lines did not differ in response to 6-MP and 6-MP-ribonucleoside. Toward the parent cell line many nucleosides were more toxic than the corresponding free bases; this is particularly striking for the nucleosides of 8-aza-adenine, purine,* 6-hydrazinopurine, 7-deaza-adenine, 6-methylpurine, and 6-chloropurine. In only one instance (i.e. 2,6-diaminopurine) was the free base more toxic than the nucleoside. Many nucleosides were relatively nontoxic to both parent and resistant cell lines: see for example 2-bromoadenosine, cordycepin, 2-dimethylaminoadenosine, and arabinosyladenine, which were not inhibitory at concentrations of 50 μM or greater.

The results with 4-aminoimidazopyridazine and its 1-ribosyl derivative (containing the ribosyl group in the same position as in adenine) differ from those with the other nucleosides and free bases in that, whereas 4-aminoimidazopyridazine had relatively

* The fact that purine ribonucleoside is much more toxic than purine in mammalian cells has been observed by others (see Gordon and Brown[23] for references).

Table 4. Response of H.Ep. #2/S and H.Ep. #2/FA cells to analogs of adenine and adenosine

Compound and reference	Inhibitory conc.* for H.Ep. #2 cells (μmoles/l.)	Ratio of inhibitory conc.: H.Ep. #2/FA / H.Ep. #2/S
A. Base-nucleoside pairs		
2-Fluoroadenine[1]	0·03	>2000
2-Fluoroadenosine[3]	0·02	20
7-Deaza-adenine	>20	(1)
7-Deaza-adenosine (tubercidin)	0·002	1
4-Aminopyrazolo(3,4-*d*)pyrimidine	3	>50
4-Aminopyrazolo(3,4-*d*)pyrimidine-1-R†[34]	0·2	1
8-Aza-adenine	20	>7
8-Aza-adenosine	0·8	1
6-Hydrazionpurine[35]	70	1 or >
6-Hydrazinopurine-R[36]	1·8	0·2–1·0
Purine	80	2 or >
Purine-R[37]	0·08	1
6-Chloropurine[38]	120	1
6-Chloropurine-R[37]	3	1
2-Chloroadenine[39]	10	>20
2-Chloroadenosine[40]	7	1–1·5
2, 6-Diaminopurine	20	7 or >
2, 6-Diaminopurine-R[41]	40	1
6-Methylpurine	4	>5
6-Methylpurine-R[42]	<0·02	1
4-Aminoimidazo(4,5-*d*)pyridazine	4	20 or >
4-Aminoimidazo(4,5-*d*)pyridazine-1-R	60	(1)
4-Aminoimidazo (4,5-*d*)pyridazine-3-R	20	>2
1-Methyladenine[43]	70	
1-Methyladenosine[44]	70	
B. Miscellaneous free bases		
2-Methyladenine[45]	>70	(1)
6-Mercaptopurine	0·6	1
C. Miscellaneous nucleosides		
6-Methylthiopurine ribonucleoside[46]	1	1
2-Bromoadenosine[47]	120	(1)
3′-Deoxyadenosine (cordycepin)	80	(1)
2-Dimethylaminoadenosine[40]	>30	
Isoguanosine (crotonoside)[48]	>70	
9-β-D-Arabinofuranosyladenine	>150	(1)
9-β-D-Xylofuranosyladenine	50	ca. 0·6
6-Methylthio-9-β-D-arabinofuranosyl-purine	>70	
6-Methylthio-9-β-D-xylofuranosylpurine	>60	
6-Mercaptopurine-R[49]	0·7	1

* Concentrations inhibiting the formation of colonies by 50 per cent or more; for details of assay, see text. The odd numbers are the result of the fact that compounds were assayed routinely at equal concentrations by weight (e.g. 1·0 μg APP/ml = 7 μM). The data given are a composite of many experiments. For many compounds, the highest concentration assayed (usually 10–20 μg/ml) failed to inhibit; hence the ">" designations. For compounds that failed to inhibit the parent cell line, a (1) designation in the ratio column indicates that the compounds also failed at the same concentration to inhibit the resistant cell line. A blank in the ratio column indicates that the compound was not assayed against the resistant line, and a "or >" designation following a ratio indicates that the compound at that concentration (i.e. conc. inhibiting sensitive line multiplied by the ratio) gave only borderline inhibition of the resistant line.

† R = β-D-ribofuranosyl (at the 9-position of the purine ring except where otherwise indicated).

high cytotoxicity, the nucleoside was inactive at the highest concentration studied. On the other hand, the 3-ribosyl derivative (ribosyl group at the position corresponding to the 7-position in adenine) was cytotoxic, but less so than the free base, and H.Ep. #2/FA cells were cross-resistant to it as well as to the free base.

Three sugar derivatives of 6-methylthiopurine were assayed. As has been reported earlier, 6-methylthiopurine ribonucleoside was highly cytotoxic to H.Ep. #2 cells,[18] and it is apparent in Table 4 that, like the other nucleosides, this compound was equally toxic to the resistant line. The arabinosyl and xylosyl derivatives were not toxic to the parent line even at much higher concentrations than the inhibitory concentration of the ribonucleoside; because of the small amounts of compounds available, the cytotoxic concentrations could not be determined.

DISCUSSION

The specific loss of AMP pyrophosphorylase activity by the H.Ep. #2/FA cell line represents another example of resistance apparently resulting from the loss of the pyrophosphorylase converting a purine analog to the nucleotide. Loss of IMP–GMP pyrophosphorylase has been observed in a variety of cells resistant to 6-MP, 6-thioguanine, and 8-azaguanine.[8] Loss of activity of AMP pyrophosphorylase has hitherto been reported as a mechanism of resistance to 8-aza-adenine in *Streptococcus faecalis*,[17] to 2,6-diaminopurine in Salmonella,[24] *Escherichia coli*,[25] and mammalian cells,[26, 27] and to 2-fluoroadenine in *E. coli*.[28] The present report appears to be the first one of a mammalian cell line selected for resistance to 2-fluoroadenine and devoid of AMP pyrophosphorylase activity.

The fact that the H.Ep. #2/FA cell line was resistant to the adenine analogs but still highly sensitive to the adenosine analogs is consistent with the observed loss of AMP pyrophosphorylase activity with retention of adenosine kinase activity. The response of the resistant cell line to the nucleosides suggests that the observed somewhat lower nucleoside kinase activity of the resistant cells as compared to the sensitive cells (Table 2) probably does not reflect a significant difference in the capacity of the intact resistant cells to phosphorylate nucleosides. The results of Table 4 suggest that, with the possible exception of fluoroadenosine, the nucleosides are phosphorylated directly and that the pathway—nucleoside → free base → nucleotide—probably does not represent a route of great significance in the conversion of these nucleosides to the nucleotides. Fluoroadenosine appears to be an exception because the fluoroadenine-resistant cell line was partially cross-resistant to fluoroadenosine; however, the relatively low degree of cross-resistance would indicate that the kinase pathway is probably the predominant route from fluoroadenosine to 2-fluoroadenylic acid. The conversion of some fluoroadenosine to fluoroadenine is consistent with the observation that fluoroadenosine was a substrate for a bacterial adenosine phosphorylase.[29] In indicating the activation of the nucleosides by a kinase, the results with the fluoroadenine-resistant cells complement those obtained with a line of H.Ep. #2 cells resistant to 6-methylthiopurine ribonucleoside, which had lost kinase activity but not AMP pyrophosphorylase activity, and which was resistant to adenosine analogs but still sensitive to adenine analogs.[19] With regard to the relative importance of nucleoside kinase and nucleotide pyrophosphorylase pathways, it is noteworthy that results with resistant lines of *E. coli* differ from those with H.Ep. #2

cells: lines of *E. coli* selected for resistance to either fluoroadenine or fluoroadenosine were each resistant to both compounds, and each had a marked loss of AMP pyrophosphorylase activity.[28] Evidence for the direct phosphorylation of some of the nucleosides used in this study, namely tubercidin,[30] cordycepin,[31] arabinosyladenine,[32] and 2-fluoroadenosine,[33] has been reported.

The results of studies of uptake of precursors by intact H.Ep. #2/FA cells (Table 3) are those to be expected of cells that had lost AMP pyrophosphorylase activity but had retained IMP and GMP pyrophosphorylases and adenosine kinase. However, it might be pointed out that although sensitive and resistant cells incorporated the same amounts of ^{14}C from adenosine-^{14}C, this is not in itself evidence for adenosine kinase activity, because these cells contain adenosine deaminase and inosine phosphorylase, and therefore the route adenosine → inosine → hypoxanthine → IMP → AMP represents a route from adenosine to AMP alternative to direct phosphorylation of adenosine.

The principal significance of these results lies in their indication that phosphorylation by a nucleoside kinase represents an important pathway for the activation of adenosine analogs, and that insofar as adenine and adenosine analogs are concerned, the kinase pathway appears to be operative for a greater variety of substrates than is the pyrophosphorylase pathway. Since many of these analogs have some anti-tumor activity, these results are of obvious potential significance for chemotherapy.

Several other points, not related closely to the above conclusions, appear worthy of some comment.

1. The relative cytotoxicities of adenine and adenosine analogs might be expected to correlate with their capacities to act as substrates for AMP pyrophosphorylase and adenosine kinase. The fact that most of the nucleosides were more toxic than the corresponding free bases suggests that the pyrophosphorylase is more fastidious than the kinase with respect to its sensitivity to structural variations in the natural substrate.

2. In the 2-haloadenosine series there was a decrease in cytotoxicity with increasing size of the halogen atom. It is yet to be determined whether this indicates that 2-chloroadenosine and 2-bromoadenosine are less readily phosphorylated than 2-fluoroadenosine or that 2-chloro-AMP and 2-bromo-AMP, if formed, are less toxic than 2-fluoro-AMP.

3. Among the nucleosides studied, the more toxic compounds were those derived from adenosine by modification of the purine ring or its substituents rather than by replacement of ribose by another sugar. Thus cordycepin, arabinosyladenine, and xylosyladenine were relatively nontoxic, and an even better illustration is the failure of the arabinosyl and xylosyl derivatives of 6-methylthiopurine to inhibit at concentrations more than 50-fold the inhibitory concentration of the ribonucleoside.

4. The ribonucleosides of 4-aminoimidazopyridazine had no advantage over the free base. The inactivity of the 1-ribosyl derivative in the sensitive line suggests that it is extensively neither phosphorylated nor cleaved to the free base. The activity of the 3-ribosyl derivative against the sensitive cells and its failure to inhibit the fluoroadenine-resistant cells suggest that it too is not phosphorylated and probably is inhibitory only after cleavage to the free base.

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