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Effects of 6-*N*-Allyladenosine on Bacterial and Mammalian Cells

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SUMMARY

The adenosine analogue 6-*N*-allyladenosine is toxic to *Escherichia coli* and to mammalian tumor cells in culture. In the presence of 6-*N*-allyladenosine, *E. coli* cells excrete xanthosine into the medium; the inhibition of growth of the bacteria caused by this agent is reversed by addition of guanine. Consistent with these observations is the potent inhibition of bacterial GMP synthetase [xanthosine 5′-phosphate:ammonia ligase (AMP), EC 6.3.4.1] by 6-*N*-allyladenosine.

The toxicity of 6-*N*-allyladenosine toward mammalian cells is different from that toward bacteria, for it is dependent upon phosphorylation of the analogue by adenosine kinase. Mutants which lack this enzyme are resistant to 6-*N*-allyladenosine. The mammalian GMP synthetase [xanthosine 5′-phosphate:L-glutamine amidoligase (AMP), EC 6.3.5.2] is not strongly inhibited by 6-*N*-allyladenosine, but in intact cells the analogue, presumably present as the nucleotide, is a potent inhibitor of an early step in the purine-biosynthetic pathway. However, inability to reverse the toxicity of 6-*N*-allyladenosine by 4-amino-5-imidazolecarboxamide or hypoxanthine implies that such an effect is not solely responsible for growth inhibition.

INTRODUCTION

Structural analogues of adenosine have a variety of biological effects. Some 6-*N*-alkyladenosines are characterized by their high cytokinin activity (1–3). Others, including 6-*N*-allyladenosine, stimulate, by an undefined mechanism, the growth of human leukemia cells (6410) in culture when the analogues are present at very low levels. At higher concentrations, however, they are toxic to cultured cells and also to bacteria (3, 4). For neither type of cell is the mechanism of toxicity understood.

Growth inhibition and inhibition of enzymes for both bacterial and mammalian cells were evaluated in an attempt to elucidate critical sites of action of 6-*N*-allyladenosine. In this report, it is shown that 6-*N*-allyladenosine inhibits the GMP synthetase [xanthosine 5′-phosphate:ammonia ligase (AMP), EC 6.3.4.1] of *Escherichia coli* B, an effect similar to that of psicofuranine (β-D-psicofuranosyladenine). However, GMP synthetase [xanthosine 5′-phosphate:L-glutamine amidoligase (AMP), EC 6.3.5.2] of mammalian cells is not powerfully inhibited by either psicofuranine or 6-*N*-allyladenosine.

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MATERIALS AND METHODS

A sample of psicofuranine was supplied by the Upjohn Company, Kalamazoo, Mich. Azaserine was obtained from Dr. John Dice of Parke, Davis and Company, Detroit, through the Cancer Chemotherapy National Service Center. 6-*N*-Allyladenosine and most of the other adenosine analogues were obtained from Dr. J. A. Montgomery

TABLE 1

Standard assays for GMP synthetases

The final volume in each case was 250 μl. The enzyme preparation was added to initiate the reaction, and 750 μl of 3.5% perchloric acid were added to terminate it. The increased absorption at 290 mμ was measured (11). In the perchloric acid solution, XMP has no appreciable absorption at this wavelength.

Source of GMP synthetase	Molar composition						Protein	Incubation at 37°
	Tris-Cl, pH 8.4	$MgCl_2$	ATP	XMP	$(NH_4)_2SO_4$	Glutamine		
	μmoles						*μg*	*min*
E. coli B	40	4.0	1.0	0.60	40		5.6	30
Adenocarcinoma 755	40	2.4	0.60	0.24		1.0	300–500	45
H.Ep. 2	40	1.6	0.40	0.18		1.0	300–500	45

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E. coli B cells were grown in the medium developed by Gray and Tatum (5). This medium contains glucose, L-asparagine, and inorganic salts. Purification of GMP synthetase from this organism was carried out by a published procedure (6).

Xanthosine was isolated from psicofuranine- and 6-*N*-allyladenosine-treated cultures of *E. coli* B by the method devised by Slechta (7). The procedure involved absorbing the xanthosine on charcoal, eluting, and passing the preparation through a Dowex 1 anion-exchange column. Xanthosine was identified by its ultraviolet spectrum and by two-dimensional paper chromatography (8).

Adenocarcinoma 755 and H.Ep. 2[1] cells were grown in swirl cultures as described by Kelley *et al.* (9). Growth inhibition studies and attempts to reverse growth inhibition of Adenocarcinoma 755 cells were made with the swirl cultures. Such studies with the H.Ep. 2 cells were accomplished by using a cloning technique, which involved placing 100 cells in 4-ounce prescription bottles containing the culture fluid and counting the number of macroscopic colonies present after 7 days (9). For enzyme studies, the mammalian cells were harvested by centrifugation, washed in 0.9% NaCl, and used for acetone powder preparations. The acetone powders were extracted with dilute Tris-chloride buffer just prior to use. Protein was measured by the method of Lowry *et al.* (10). The standard assays for the GMP synthetases are given in Table 1.

The selection of the H.Ep. 2 cell lines resistant to analogues of adenosine has been described previously (12, 13).

Tests for pseudo-feedback inhibition were performed by the azaserine block technique (13–15). In the presence of ^{14}C-formate, azaserine blocks the purine-biosynthetic pathway and causes the

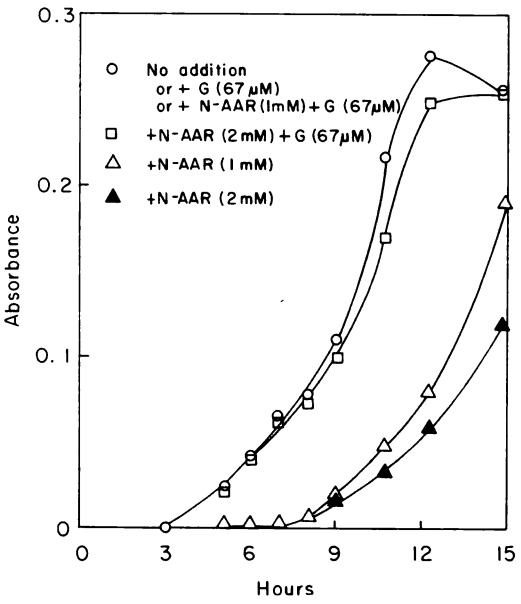

FIG. 1. *Guanine as a reversal agent of growth inhibition of E. coli B in the presence of 6-N-allyladenosine*

Growth conditions were the same as for Table 2. Abbreviations: G, guanine; N-AAR, *N*-allyladenosine.

[1] The abbreviation used is: H.Ep. 2, human epidermoid carcinoma No. 2.

accumulation of labeled 5′-phosphoribosyl-*N*-formylglycinamide. Agents which inhibit an earlier step of the pathway reduce this accumulation.

RESULTS AND DISCUSSION

6-*N*-Allyladenosine causes a moderate, transitory inhibition of growth of *E. coli* B (Fig. 1). The degree of inhibition is dependent on the amount of analogue present, and can be reduced or completely overcome by addition of guanine simultaneously with 6-*N*-allyladenosine. Hypoxanthine and 4-amino-5-imidazolecarboxamide are ineffective as reversal agents. The transitory inhibition of growth of *E. coli* B by another adenosine analogue, psicofuranine, is known to be reversed solely by guanine or guanine derivatives (7). Guanine also reverses the inhibition by psicofuranine of *Staphylococcus aureus* (16), *Streptococcus faecalis* (17), and *Bacillus subtilis* (18). Our observations confirm that growth inhibition of *E. coli* B by psicofuranine is reversed by guanine, but the inhibition by adenosine, 6-*N*-hydroxyadenosine, and 6-hydrazinopurine ribonucleoside is not reversed (Table 2). The latter analogues apparently have a mechanism of action different from that of psicofuranine and 6-*N*-allyladenosine. The extreme toxicity of adenosine to *E. coli* B has been noted previously (4).

The singular reversal by guanine indicates that an enzymatic reaction involved in the conversion of IMP to GMP is inhibited by the agent. The target for psicofuranine is the bacterial GMP synthetase, which is also inhibited, to a small extent, by adenosine (6, 11, 19). Reversal of growth inhibition is attained because the cell can

TABLE 2

Inhibition of growth of E. coli B and inhibition of GMP synthetases by adenosine and adenosine analogues

For studies on growth inhibition, additions were made to 5.0 ml of medium, so that the final volume was 6.0 ml. The tubes were inoculated with a loop from a 16-hr culture and allowed to grow at 37° for 630 min, at which time the growth was two-thirds maximal. The absorbance was measured at 600 mμ. Each assay was made in triplicate and was repeated at least once. Standard assays for enzymes were used, except that the designated inhibitor was added to the system at several different concentrations in order to determine the 50% inhibitory level. Each value was measured at least twice, and the figures given are averages. 6-*N*-Allyladenosine could be recovered without change after the period of incubation. Neither AMP nor 46 other compounds which could be considered analogues of adenosine were inhibitory to either the mammalian or the bacterial GMP synthetase.

Inhibitor	Amount required for 50% inhibition of growth of *E. coli* B	Reversal of inhibition of growth of *E. coli* B by guanine	Amount required for 50% inhibition of GMP synthetase		
			E. coli B	Adenocarcinoma 755	H.Ep. 2
	μM		μM	μM	μM
Psicofuranine	20	Yes	1.4	39	600
6-*N*-Allyladenosine	500	Yes	5.9	520	>1000
Adenosine	1.0	No	24	2100	>2000
6-*N*-Methyladenosine	>1000		25	2100	>2000
1-Methyladenosine	>1000		35	>3000	>2000
6-*N*-Hydroxyadenosine	26	No	85	>1000	>2000
6-Ethylpurine ribonucleoside	>1000		90	>3000	>2000
6-Hydrazinopurine ribonucleoside	40	No	135	>3000	>2000
1-Ethyladenosine	>1000		340	>3000	>2000
6-*N*-Dimethyladenosine	>1000		375	>1000	>1000
2′-Deoxyadenosine	>1000		700	>3000	>3000
C-Ado[a]	>1000		790	>3000	>3000
2-Methoxyadenosine			>2000	580	>1000
dATP			>2000	820	>1000

[a] The carbocyclic analogue of adenosine, 9-[β-DL-2α,3α-dihydroxy-4β-(hydroxymethyl)cyclopentyl]-adenine.

TABLE 3

Michaelis constants for GMP synthetases of E. coli B, Adenocarcinoma 755, and H.Ep. 2 cells

Except for varying the concentration of the appropriate substrate, standard assays were used. The constants were derived from Lineweaver-Burk plots, and the numbers in parentheses represent the number of individual determinations. Where more than one determination was made, the number given is the average.

Substrate	Michaelis constant		
	E. coli B	Adenocarcinoma 755	H.Ep. 2
	mM	*mM*	*mM*
$MgCl_2$	20 (1)	3.7 (1)	3.0 (1)
ATP	0.93 (2)	1.1 (2)	2.0 (1)
XMP	0.37 (5)	0.15 (2)	0.38 (3)
$(NH_4)_2SO_4$	34 (2)		
Glutamine		1.3 (1)	1.1 (1)

bypass the blockade at GMP synthetase by making guanine nucleotides directly from guanine.

A preparation of GMP synthetase from *E. coli* B purified 15-fold to a specific activity of 210 nmoles/min/mg of protein gives the kinetic values listed in Table 3. The K_m for XMP as a substrate for this enzyme is about 10 times higher than a value previously published, but the other values for substrates are similar (11). This enzyme is inhibited by 6-*N*-allyladenosine to almost the same extent as by psicofuranine (Table 2). Adenosine, 6-*N*-methyladenosine, and 1-methyladenosine are moderately effective, but a number of other adenosine analogues have little or no effect on the reaction. Reports that inhibition of *E. coli* B GMP synthetase by psicofuranine increases with time and that psicofuranine and inorganic pyrophosphate are synergistic (6, 11) have been confirmed.

The presence of psicofuranine in an *E. coli* system causes the cells to excrete xanthosine into the medium (7, 20), and this is also the case for 6-*N*-allyladenosine. Xanthosine can be isolated and identified from *E. coli* B cultures treated with either analogue. The material accumulating in the medium has the same spectral properties

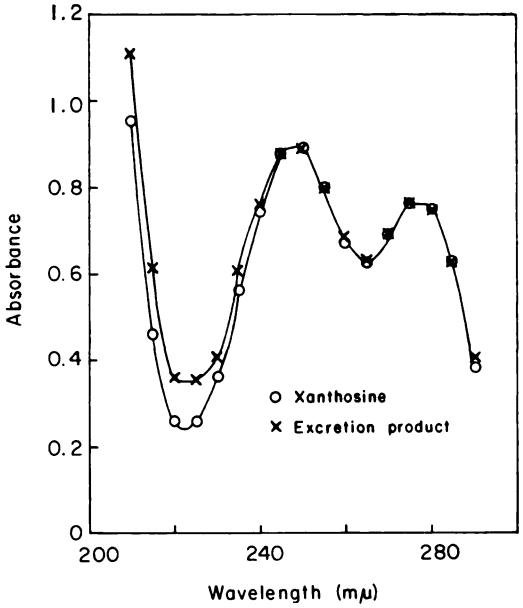

FIG. 2. *Ultraviolet absorbance spectra of xanthosine and the excretion product found in cultures of E. coli B grown in the presence of 6-N-allyladenosine (80 μM)*

(Fig. 2) and the same migration in a two-dimensional paper chromatographic system as an authentic sample of xanthosine.

Growth of cultured mammalian Adenocarcinoma 755 and H.Ep. 2 cells in a medium containing no added guanine is not retarded by psicofuranine at concentrations up to 170 μM, but in the same system 6-*N*-allyladenosine inhibits growth by 50% at 5 μM. The inhibition is not reversed by either guanine, hypoxanthine, or 4-amino-5-imidazolecarboxamide. 6-*N*-Allyladenosine is a substrate for the purified adenosine kinase from H.Ep. 2 cells (21), but psicofuranine is not.[2] The toxicity of 6-*N*-allyladenosine toward H.Ep. 2 cells is dependent on phosphorylation of the analogue, for mutant cell lines selected for resistance to 2-fluoroadenosine and to 6-methylthiopurine ribonucleoside and lacking adenosine kinase tolerate 6-*N*-allyladenosine at 130 μM.

The K_m values for the substrates of the GMP synthetases from Adenocarcinoma 755 and H.Ep. 2 cells are given in Table 3.

[2] D. L. Hill, unpublished observations.

TABLE 4

Inhibition of 5'-phosphoribosyl-N-formylglycinamide accumulation in azaserine-treated cells by 6-N-allyladenosine

To flasks containing 4 × 10^7 H.Ep. 2 cells in 100 ml of medium were added azaserine (10 μg/ml), 6-*N*-allyladenosine 30 min thereafter, and sodium ^{14}C-formate (25 μCi) 60 min thereafter. After 2 hr of incubation, the cells were harvested and extracted with hot 80% ethanol. The extracts were subjected to two-dimensional paper chromatography, and radioactive spots were located by radioautography and counted in a liquid scintillation counter.

Concentration of 6-*N*-allyl-adenosine	Accumulated 5'-phosphoribosyl-*N*-formylglycinamide	Percentage of control
μM	*cpm*	
0	72,000	100
1.0	59,000	82
1.6	20,500	28
3.3	12,600	17
6.5	5,000	7.0
19.0	570	0.8
38.0	690	1.0

These values are for enzyme preparations consisting of acetone powder extracts of the cells. Appreciable purification of the mammalian enzymes has not been achieved. The GMP synthetases of the mammalian cells are not very sensitive to inhibition by adenosine or adenosine analogues (Table 2). Most effective of all is psicofuranine, which, at a concentration of 39 μM inhibits the Adenocarcinoma 755 enzyme by 50%. The lack of potent inhibition of mammalian GMP synthetase by 6-*N*-allyladenosine tends to rule out this enzyme as a strong site of action in these cells.

Another possible mechanism of action of 6-*N*-allyladenosine in mammalian cells is pseudo-feedback inhibition of the purine-biosynthetic pathway. This inhibition presumably occurs after phosphorylation of the analogue by adenosine kinase. In the experiments involving accumulation of 5'-phosphoribosyl-*N*-formylglycinamide following the administration of azaserine, 6-*N*-allyladenosine at 1.6 μM reduced the accumulation to 28% of the control value (Table 4). Such inhibition implies that the analogue is a potent inhibitor of an early step of the pathway for purine biosynthesis. However, if this site of action of 6-*N*-allyladenosine is responsible for growth inhibition, the toxicity of the analogue should be reversed by 4-amino-5-imidazolecarboxamide or hypoxanthine, as is the case for 6-mercaptopurine (22) and 6-methylthiopurine ribonucleoside (23). No reversal of inhibition of Adenocarcinoma 755 cells or H.Ep. 2 cells by either 4-amino-5-imidazolecarboxamide at 200 μM or hypoxanthine at 150 μM was observed. This probably means that although an enzyme participating in an early step of the mammalian purine-biosynthetic pathway is strongly inhibited by a metabolite of 6-*N*-allyladenosine, this effect is not solely responsible for growth inhibition. The most critical site of action by this analogue on mammalian cells remains unknown.

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