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Inhibition of Guanine Metabolism of Mammalian Tumor Cells by the Carbocyclic Analogue of Adenosine

DONALD L. HILL, SUZANNE STRAIGHT, PAULA W. ALLAN, AND L. LEE BENNETT, JR.

Kettering-Meyer Laboratory,[1] Southern Research Institute, Birmingham, Alabama 35205

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SUMMARY

To tumor cells in culture, the carbocyclic analogue of adenosine {9-[β-DL-2α,3α-dihydroxy-4-β-(hydroxymethyl)cyclopentyl] adenine, C-Ado} has a potent toxicity which is increased in the presence of guanine. C-Ado strongly inhibits guanine utilization by intact cells, causes small amounts of xanthosine to accumulate in the cells, and reduces the ratio of AMP to IMP. The effect on guanine metabolism is reflected by a decreased incorporation of guanine into nucleic acids and by a decreased accumulation of guanine metabolites in the alcohol-soluble fraction of cells. Assays of enzymes involved in guanine metabolism show that C-Ado monophosphate is a potent, competitive inhibitor of GMP kinase (ATP:GMP phosphotransferase, EC 2.7.4.8). The inhibition constant for C-Ado monophosphate is 12 μM, compared to a Michaelis constant for GMP of 41 μM. C-Ado monophosphate does not inhibit either hypoxanthine (guanine) phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) or GDP kinase (ATP:nucleoside diphosphate phosphotransferase, EC 2.7.4.6). Inhibition of GMP kinase may be responsible for the growth-inhibitory properties of C-Ado and may be involved in the increased toxicity of the analogue in the presence of guanine.

INTRODUCTION

The carbocyclic analogue of adenosine[2] has a methylene group replacing the oxygen atom of the ribofuranosyl ring. With this substitution, the labile glycosidic linkage is replaced by a stable C—N bond. A previous report (2) has shown that, of the carbocyclic analogues of adenosine, inosine, 6-mercaptopurine ribonucleoside, and 6-methylthiopurine ribonucleoside, only C-Ado[3] is highly cytotoxic to H.Ep. 2 cells and that sublines of these cells lacking adenosine kinase are resistant to this agent. In intact, sensitive cells, C-Ado is phosphorylated to the mono-, di-, and triphosphates and is deaminated to a small extent. It is not incorporated to a detectable degree into nucleic acids. In sensitive cells, C-Ado also inhibits an early step of purine biosynthesis *de novo*, but this may not be its primary site of action.

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[1] Affiliated with the Sloan-Kettering Institute for Cancer Research, New York.

[2] Aristeromycin is a naturally occurring, optically active form of the carbocyclic analogue of adenosine (1). Since the preparation available to us is a racemic mixture, we prefer to avoid the name aristeromycin.

[3] The abbreviations used are: C-Ado, carbocyclic analogue of adenosine, H.Ep. 2, human epidermoid carcinoma No. 2.

This report concerns inhibition by C-Ado of purine metabolism in intact mammalian tumor cells and studies of the enzymes involved.

MATERIALS AND METHODS

Dr. Y. F. Shealy and Mr. J. D. Clayton provided samples of 9-[β-DL-2α,3α-dihydroxy - 4 - β - (hydroxymethyl)cyclopentyl]-adenine, and also the carbocyclic analogues of inosine and 6-methylthiopurine ribonucleoside. The synthesis of these compounds has been described elsewhere (3, 4). They also supplied a sample of C-Ado monophosphate.[4] Formycin B [7-hydroxy-3-(β - D - ribofuranosyl)pyrazolo(4,3 - *d*)-pyrimidine] was obtained from Dr. H. Umezawa, Institute of Microbial Chemistry, Tokyo. Tritiation of a sample of C-Ado was performed by New England Nuclear Corporation by catalytic exchange between C-Ado and ^3H_2O. Purification of the labeled compound has been described (2). Other radioactive compounds were purchased from Schwarz BioResearch, Inc.

Adenocarcinoma 755 cells were grown in swirl cultures by the procedure of Kelley *et al.* (5), and cells were counted with a Coulter counter. For enzyme purifications, the cells were harvested by centrifugation and washed twice in 0.9% NaCl. Hypoxanthine (guanine) phosphoribosyltransferase (IMP:pyrophosphate phosphoribosyltransferase, EC 2.4.2.8) and GMP kinase (ATP:GMP phosphotransferase, EC 2.7.4.8) were partially purified by published procedures (6, 7). The assay for hypoxanthine (guanine) phosphoribosyltransferase was identical with that used previously (6). This enzyme was purified 120-fold, and the specific activity was 600 nmoles/min/mg. Standard reaction mixtures for GMP kinase consisted of the following in 0.25 ml: 100 mM Tris-chloride buffer (pH 7.5), 4 mM $MgCl_2$, 8 mM KCl, 0.16 mM ATP, 50 μM GMP-8-^{14}C, and enzyme (3.6–6.3 μg of protein). The reaction was started by adding the enzyme and was allowed to proceed for 15 min at 30°. It was stopped by adding 50 or 100 μl of 0.2 M EDTA. The GMP kinase was purified 80–100-fold and had a specific activity of 80–100 nmoles/min/mg. The enzyme preparation contained a nucleoside diphosphate kinase (ATP:nucleoside diphosphate phosphotransferase, EC 2.7.4.6) with a specific activity 10 times higher, so that the final product was GTP. The radioactive components of the reaction mixture, GMP and GTP, were separated by paper chromatography with either isobutyric acid–H_2O–NH_3 (57:39:4 by volume) or 1-butanol–acetic acid–H_2O (2:1:1 by volume) and were assayed with a Packard 7201 radiochromatogram scanner.

The possible phosphorylation of AMP by the enzyme preparation was tested by replacing GMP-8-^{14}C in the reaction mixture with AMP-8-^{14}C and assaying as usual. The possible phosphorylation of unlabeled C-Ado monophosphate was tested similarly, except that materials migrating in the nucleoside di- and triphosphate area of the chromatogram were eluted, hydrolyzed in 1 N HCl for 1 hr, and rechromatographed to check for the appearance of C-Ado.

Initial velocities (v) and substrate concentrations [S] from the kinetic experiments were fitted with the aid of an IBM 1132 computer to the equation $v = V[S]/(K_m + [S])$, where V = maximum velocity and K_m = Michaelis constant. The FORTRAN program developed by Cleland (8) was used. By this procedure, values for V, $1/V$, K_m, and K_m/V were obtained.

For incorporation experiments using intact cells, 100 ml of cells in swirl culture were used. The radioactive precursor (10 or 20 μCi) and potential inhibitor were added simultaneously. After 2 hr the cells were harvested by centrifugation, washed once with 0.9% NaCl, and poured into 40 ml of boiling ethanol. Soluble materials were extracted by boiling for 10 min. The residue was removed by centrifugation at 27,000 × *g* for 10 min, and the solution was evaporated under reduced pressure at 40° to about 5 ml. This material was lyophilized to dryness and dissolved in water (0.8 ml for each 10^8 cells present). A portion (60 μl) of the solution was spotted on paper for two-dimensional chromatography. The solvents were 70% phenol and 1-butanol–

[4] Y. F. Shealy and J. D. Clayton, unpublished results.

propionic acid–H_2O (9). Metabolites were located by radioautography and were cut from the chromatogram before counting in a liquid scintillation spectrometer or for elution and identification. The identification process involved several procedures. (a) All the radioactive metabolites were hydrolyzed to purine bases with HCl (1 N, 1 hr at 100°) and were then subjected to chromatography with water-saturated 1-butanol–NH_3 (100:1, v/v), together with the appropriate base standards. (b) Compounds migrating in the two-dimensional system as bases and ribonucleosides were rechromatographed, with standards, in the 1-butanol–propionic acid solvent. (c) Compounds migrating as nucleotides were rechromatographed in a solvent containing 1 liter of 0.1 M sodium phosphate (pH 6.8), 600 g of $(NH_4)_2SO_4$, and 20 ml of 1-propanol. (d) The ribonucleotides were also treated with crude snake venom, and the resulting ribonucleosides were characterized by chromatography in 1-butanol–acetic acid–water (5:2:3 by volume).

From the residue remaining after ethanol extraction, sodium nucleates were isolated by extraction with hot 10% NaCl, precipitated with ethanol, and assayed for radioactivity. The nucleates were treated overnight with 1 N KOH at room temperature, the solution was neutralized with $HClO_4$, and $KClO_4$ was removed by centrifugation. The soluble portion was acidified to pH 4 with acetic acid, and a few drops of 1 M $MgCl_2$ were added. Ethanol was added to precipitate the DNA, which was also assayed for radioactivity.

RESULTS

In a previous report, C-Ado was stated to have an inhibitory effect on the pathway of purine synthesis *de novo* in tumor cells in culture (2). In an attempt to reverse the toxic effect of C-Ado, we added purine bases to Adenocarcinoma 755 cultures treated with the analogue (Fig. 1). Neither adenine, 4-amino-5-imidazolecarboxamide, hypoxanthine, nor guanine reversed the toxic effect; but guanine, which was not toxic alone, potentiated the toxicity of C-Ado. The synergistic effect of guanine could not be reversed by adenine, although adenine prevented destruction of the cell inoculum. To cause the synergism, guanine could have exerted some effect on the metabolism of C-Ado, or the reverse could have been true. The first possibility was ruled out by results on the metabolism of ^{3}H-C-Ado in Adenocarcinoma 755 cultures to which varying amounts of guanine were added. There were no differences in the uptake of C-Ado or in its conversion to di- and triphosphates in cultures containing up to 37 μM guanine.

In an attempt to find the site of action for C-Ado on guanine metabolism, we tested this analogue as an inhibitor of guanine, adenine, and hypoxanthine metabolism of Adenocarcinoma 755 cells. We noted that the incorporation of both hypoxanthine and guanine into combined nucleic acids and into DNA was blocked to a greater extent than that of adenine (Table 1). Similar results were obtained with H.Ep. 2 cells. The inhibition of guanine and hypoxanthine metabolism was progressive with higher concentrations of C-Ado. Formycin B and the carbocyclic analogues of inosine and 6-methylthiopurine ribonucleoside had less effect on guanine and hypoxanthine metabolism, and 28 other purine and purine nucleoside analogues had little or no activity.

Analysis of the alcohol-soluble metabolites showed that the uptake of all three bases was reduced in the presence of C-Ado (Table 2). However, adenine uptake was reduced to a smaller extent than that of hypoxanthine and guanine. Another effect of C-Ado was to produce an accumulation of xanthosine in the cells when either hypoxanthine or adenine was added. Another compound (unidentified "A") also accumulated in cells exposed to adenine-8-^{14}C and C-Ado. C-Ado reduced the ratio of AMP to IMP in the cells when either adenine or hypoxanthine was added as substrate.

The inhibition of guanine metabolism led us to believe that one of the enzymes involved in converting guanine to GTP was inhibited by C-Ado or a metabolite of it. C-Ado at 100 μM was not an inhibitor of hypoxanthine (guanine) phosphoribosyl-

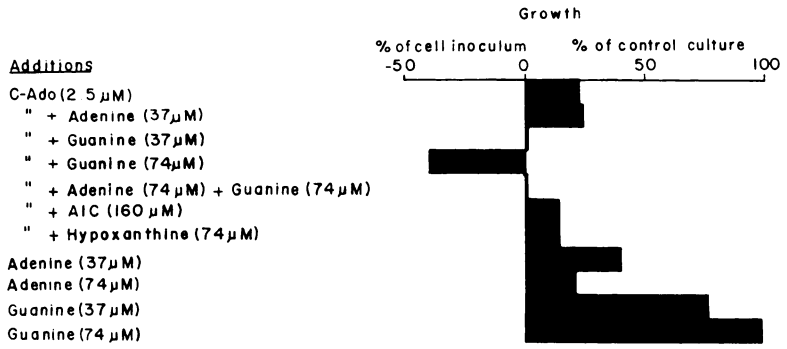

FIG. 1. *Attempted reversals of toxicity of C-Ado to Adenocarcinoma 755 cells in culture*

The positive values, on the right, denote the growth of cultures at 72 hr calculated as a percentage of the growth of control cultures, which increased in number about 20-fold. The negative values, on the left, represent destruction of the cell inoculum. The 100% control value represents 110,000 cells/ml. AIC, 4-amino-5-imidazolecarboxamide.

TABLE 1

Inhibition of incorporation of guanine-8-^{14}C, hypoxanthine-8-^{14}C, and adenine-8-^{14}C into nucleic acids of Adenocarcinoma 755 cells by ribonucleoside analogues

The amounts of purine base and radioactivity added to 100 ml of cells in culture were as follows: adenine-8-^{14}C, 20 μCi, 0.16 μmole; hypoxanthine-8-^{14}C, 10 μCi, 0.2 μmole; guanine-8-^{14}C, 10 μCi, 1 μmole. The incorporation of radioactivity into nucleic acids in the absence of inhibitor ranged from 200 to 400 pCi/mg.

Inhibitor	Concentration	Relative incorporation					
		Guanine		Hypoxanthine		Adenine	
		DNA + RNA	DNA	DNA + RNA	DNA	DNA + RNA	DNA
	mM	%	%	%	%	%	%
None		100	100	100	100	100	100
C-Ado	0.03	57	56	20	44	106	97
C-Ado	0.1	30	39	24	21	78	98
C-Ado	0.2	19	12				
Formycin B	0.1	74	62	80	73	97	76
Formycin B	0.2	50	50				
C-MeMPR[a]	0.1	54	56	80	80	113	117
C-Ino[a]	0.1	68	66	83	62	95	71

[a] C-MeMPR and C-Ino, the carbocyclic analogues of 6-methylthiopurine ribonucleoside and inosine, respectively.

transferase (6), and tests showed that C-Ado monophosphate was likewise not inhibitory.

GMP kinase was not affected by C-Ado, but it was strongly and competitively inhibited by C-Ado monophosphate (Fig. 2). The Michaelis constants for substrates of this enzyme were 15 μM for ATP (average of two determinations) and 41 ± 10 μM for GMP. The value for GMP (with standard deviation) was obtained from eight separate experiments. Substrate saturation curves for Mg^{++} did not give typical Michaelis-Menten plots. The optimum concentration of Mg^{++} for the standard reaction mixture was 4 mM. The inhibition constant for C-Ado monophosphate was 12 ± 1 μM, a value obtained from three separate experiments with varied amounts of in-

TABLE 2

Effects of C-Ado on metabolism of purine bases by Adenocarcinoma 755 cells

The experimental conditions were the same as those described for Table 1. The experiment was repeated twice, with essentially the same results.

Metabolite	Adenine-8-^{14}C		Hypoxanthine-8-^{14}C		Guanine-8-^{14}C	
	No C-Ado	0.1 mM C-Ado	No C-Ado	0.1 mM C-Ado	No C-Ado	0.2 mM C-Ado
	% total radioactivity		*% total radioactivity*		*% total radioactivity*	
Adenine	1.8	2.6	2.0	2.8		
Hypoxanthine			3.8	17.3		
Guanine			0.5	0.5	10	36.5
Adenosine	7.3	16.1				
Inosine	1.2	3.8	0.5	1.8		
Xanthosine	0.1	2.3	0.1	2.6		
Unidentified "A"	0.1	0.8				
NAD	2.9	3.8	3.0	3.6		
Unidentified "B"	0.3	0.4	0.3	0.4		
AMP	25.3	12.7	24.3	11.1	1.4	2.2
GMP			5.2	2.3	44.6	39.3
IMP	13.5	27.0	9.4	21.7		
Succinyl-AMP(?)	1.9	1.1	1.3	0.6		
ADP	32.2	21.0	30.9	23.3		
ATP	13.5	8.5	13.6	9.6		
GDP + GTP			4.8	2.1	44.0	21.9
Total radioactivity	405,400 dpm	169,500 dpm	206,600 dpm	55,800 dpm	20,300 dpm	4,800 dpm

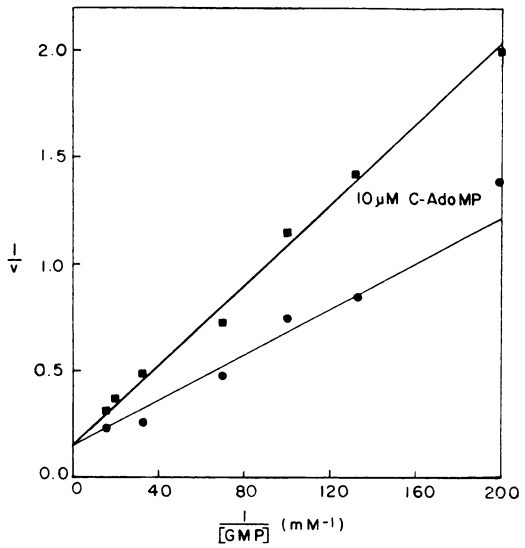

FIG. 2. *Inhibition of GMP kinase by C-Ado monophosphate*

Standard conditions of assay were used, except that the concentration of GMP was varied and C-Ado monophosphate (C-AdoMP) was added as indicated.

hibitor. Using these values, the K_i/K_m ratio is 0.31, an indication of potent inhibition. Moreover, if only one racemic form of C-Ado monophosphate accounted for the inhibition, the K_i would be 6 μM, and the K_i/K_m ratio would drop to 0.16. In contrast, the nucleotides ADP, dADP, and dGMP inhibited the reaction by 50% at about 300 μM. Even less inhibitory were AMP, 30 other ribonucleotides, and 55 ribonucleoside analogues. A combination of guanine and C-Ado monophosphate did not inhibit GMP kinase more than C-Ado monophosphate alone. C-Ado monophosphate inhibited GMP kinase without further phosphorylation; neither C-Ado monophosphate nor AMP in the reaction mixture could be converted to the di- and triphosphates. C-Ado monophosphate at a concentration of 100 μM did not inhibit the nucleoside diphosphate kinase of the enzyme preparation.

DISCUSSION

At present we are unable to offer a complete explanation for the synergistic effect

of C-Ado plus guanine on cultured tumor cells. Perhaps guanine or GMP is toxic if further metabolism is blocked. A similar potentiation by guanine of the cytotoxicity of 4-aminopyrazolo(3,4-*d*)pyrimidine observed for Ehrlich ascites cells in culture is unexplained (10, 11). Likewise, the reason for xanthosine accumulation by cells treated with C-Ado is not known with certainty. Bacteria blocked in the conversion of XMP to GMP by psicofuranine or 6-*N*-allyladenosine accumulate xanthosine in the medium (12, 13). End-product inhibition of this reaction by accumulated GMP could give rise to the xanthosine, but very little radioactivity from guanine is incorporated into GMP by cells treated with C-Ado. The reason for the lack of accumulation of GMP is probably that GMP is a strong end-product inhibitor of hypoxanthine (guanine) phosphoribosyltransferase (6). Inhibition of this enzyme by GMP can account for the reduced uptake of hypoxanthine by cells exposed to C-Ado.

A reasonable explanation for the effect on IMP:AMP ratios is that the reduced production of GTP prevents the conversion of IMP to succinyl-AMP, a precursor of AMP. GTP is required by the enzyme synthesizing succinyl-AMP (14). Furthermore, the reduced production of GTP, an inhibitor of AMP deaminase (15), could relieve inhibition by this nucleotide. Another possibility is that C-Ado triphosphate may be effective in activating the deaminase, which is stimulated by ATP (16).

Although we know that C-Ado is metabolized to the di- and triphosphates by intact cells (2), we do not have these compounds available for testing in the enzymatic systems. Our evidence indicates that C-Ado monophosphate inhibits GMP kinase without further phosphorylation, but it is possible that the di- and/or triphosphates could be even stronger inhibitors. The inhibition of GMP kinase by C-Ado monophosphate, an analogue of AMP, could hardly have been anticipated, for AMP is not an effective inhibitor of the enzyme, and ATP is a substrate.

The relatively high concentration of C-Ado required to block purine synthesis *de novo* (2) and the lack of reversal of C-Ado toxicity by either 4-amino-5-imidazolecarboxamide or hypoxanthine indicate that inhibition of this pathway is not a primary effect of the agent. The toxicity of 6-mercaptopurine (17) and 6-methylthiopurine ribonucleoside (18) can be reversed by 4-amino-5-imidazolecarboxamide or hypoxanthine. We do not know if blockade at GMP kinase is the event responsible for cell death in cultures treated with C-Ado, but this is a plausible site of action, for GMP kinase is essential in that it is the only enzyme catalyzing further phosphorylation of GMP.

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