INHIBITION OF RNA POLYMERASE AND FORMYLTETRAHYDROFOLATE SYNTHETASE ACTIVITY BY 6-CHLORO-8-AZA-9-CYCLOPENTYLPURINE. STRUCTURE-ACTIVITY RELATIONSHIPS

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The structural requirements for inhibition of bacterial RNA polymerase and rabbit liver formyltetrahydrofolate synthetase activity by a series of purine nucleoside analogs related to 6-chloro-8-aza-9-cyclopentylpurine (689) were investigated. To achieve an inhibitory effect, preincubation of the enzyme preparations with the purine analogs, prior to assay of enzyme activity, was required. The greatest inhibition was produced by analogs containing all three alterations of the purine nucleoside structure: the 6-halo, 8-aza, and 9-cyclopentyl groups. It is suggested that 689 inhibits the activity of enzymes involved in nucleic acid synthesis by a site-directed alkylation.

1. Introduction

The purine nucleoside analog 6-chloro-8-aza-9-cyclopentylpurine (689) has been shown to inhibit the synthesis of DNA, RNA, and protein independently in *Escherichia coli* [1, 2]. Further studies revealed that although thymine nucleotide formation *in vivo* was markedly inhibited, thymidylate synthetase activity measured *in vitro* was unaffected by 689 [2].

Enzyme inhibition by 689 is a rather selective action. As reported previously [2], only certain enzymes concerned with polynucleotide formation or metabolism appear to be affected by 689. Because of this selective effect, it was considered pertinent to determine the structural characteristics of the purine nucleoside analogs required to produce enzyme inhibition and whether the structure-activity relationships for inhibition of different drug-affected enzymes were similar. RNA polymerase, which was shown to be sensitive to inhibition by 689 [2], was studied along with formyltetrahydrofolate synthetase to explore further the structural requirements necessary for inhibition of enzyme activity. The inhibition of RNA polymerase activity takes on special significance because of the fact that there are few known inhibitors of this enzyme which act by interfering with the enzyme directly, rather than the template.

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2. Materials and methods

2.1. *Compounds*

The synthesis of 689 and 6-substituted-8-aza-9-cyclopentylpurine analogs has been reported [3]. The other analogs were generously contributed by either Drs. P.K.Chang and A.C.Sartorelli of the Department of Pharmacology, Yale University, New Haven, Conn.; Dr. J.A.Montgomery, Southern Research Institute, Birmingham, Alabama; Dr. R.K.Robins of the Department of Chemistry, University of Utah, Salt Lake, City, Utah; or Dr. H.J.Schaeffer, State University of New York, Buffalo. *dl*,L-tetrahydrofolic acid and 6-chloropurine riboside were purchased from the Sigma Chemical Co., adenine from Calbiochem, and ^{14}C-ATP (410 mCi/mmole) from New England Nuclear Corp.

2.2. *Enzymes*

RNA polymerase (nucleosidetriphosphate: RNA nucleotidyltransferase, E.C. 2.7.7.6), partially purified from *E. coli* or *Micrococcus lysodeikticus,* was obtained from Miles Laboratories. The enzyme preparations had a specific activity of 250–500 units/mg protein (a unit being that activity catalyzing the incorporation of 1 nmole of ATP into RNA in 10 min at 37° and pH 7.9 with calf thymus DNA as template). Formyltetrahydrofolate synthetase (formate: tetrahydrofolate ligase (ADP), E.C. 6.3.4.3) was prepared from rabbit liver by homogenizing the liver in a precooled blendor in 5 volumes of buffer (0.5 M tris-HCl–0.05 M EDTA disodium, pH 7.2). Solid ammonium sulfate was added to the homogenate slowly with stirring at 0° until 50% saturation was reached. The precipitate was discarded, and additional ammonium sulfate was added to the supernatant fraction to attain 70% saturation. The precipitate was collected by centrifugation and redissolved in tris-EDTA buffer (as above), divided into aliquots, and frozen. The enzyme fraction had a specific activity of 10 units/mg protein (1 unit is defined as a change in absorbance of 1 absorbance unit at 355 nm in 10 min) and was diluted with tris-EDTA prior to use. Formyltetrahydrofolate synthetase was also extracted and partially purified [5] from lyophilized *Clostridium cylindrosporum* obtained from Worthington Biochemical Corp. The final fraction had a specific activity of 5400 units/mg protein.

2.3. *Assay of RNA polymerase activity*

The enzyme preparation (25–50 μg protein) was preincubated at 37° for 60 min with gentle shaking, in a 0.26 ml reaction mixture containing 12.5% dimethylsulfoxide (necessary to dissolve the purine analogs), 20 mM tris-HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM KCl, 2.5 mM β-mercaptoethanol, and 25 μg of bovine serum albumin. The purine analogs were added in a concentration of 2 mM to the preincubation mixtures, and control samples were incubated under the same conditions but in the absence of drugs. RNA polymerase activity was assayed in a 0.4 ml reaction mixture containing 24 μmoles tris-HCl, pH 8.0; 4.5 μmoles $MgCl_2$; 0.85 μmole β-mercaptoethanol; 100 nmoles each of CTP, GTP, UTP; 10 nmoles ^{14}C-ATP (0.5 μCi); 30 μg calf thymus DNA (Worthington); and 10–25 units of preincubated polymerase. After incubation at 37° for 30 min, reactions were stopped by the addition of 3 ml of cold 5% trichloroacetic acid, filtered on Millipore filters (0.45 μm pore size), washed three times with cold 2.5% TCA, placed in counting vials, dried, and counted in 10 ml of toluene scintillator on a Packard Tri-Carb liquid scintillation spectrometer. All assays of enzyme activity were determined in duplicate samples.

2.4. *Assay of formyltetrahydrofolate synthetase activity*

The incubation mixture consisted of the following components: 50 mM tris-HCl (pH 7.5), 20 mM $MgCl_2.6\,H_2O$, 50 mM NH_4Cl, 5 mM ATP, 10 mM ascorbic acid, 1.2 mM tetrahydrofolic acid, 50 mM sodium formate, and enzyme in a total volume of 3 ml. A reaction mixture which included the potential inhibitor, but without sodium formate, was utilized as a blank for each compound tested. Unless otherwise specified, the enzymic extract was initially preincubated with the potential inhibitors in a total volume of 0.2 ml for 3 min at 37°, after which the remainder of the reaction components were added, and the entire mixture was incubated for 20 min. The reaction was terminated by the addition of 0.2 ml of 70% perchloric acid; the N^{10}-formyltetrahydrofolate formed during the reaction was thereby converted to N^5, N^{10}methenyltetrahydrofolate. The mixture was cooled, centrifuged, and the absorbancy of the supernatant due to the formation of N^5, N^{10}-methenyltetrahydrofolate was determined at 355 nm. All of the compounds tested were dissolved in dimethyl sulfoxide (DMSO) prior to use; comparable concentrations of DMSO were added to non-drug-treated extracts to serve as controls.

3. Results

Table 1 indicates the effect of 689 and various related purine nucleoside analogs on RNA polymerase activity. A concentration of 2 mM 689 produced virtually a complete loss of polymerase activity when the enzyme was preincubated for 60 min with drug (0.2 mM 689 produced a 25% decrease in activity), and the inhibition was essentially irreversible. No inhibition of RNA polymerase function was observed when purified calf thymus DNA was pretreated with 689 prior to utilizing the DNA as a template for the RNA

Table 1

Inhibition of RNA polymerase activity by 689 and related analogs. RNA polymerase from *E. coli* (Experiment 1) or *M. lysodeiktus* (Experiments 2 and 3) was preincubated for 60 min in buffer alone (control) or with 2 mM of the various purine analogs, and activity was assayed as described in **Materials and methods**. The lower incorporations of labeled precursor in Experiment 3 reflect a decrease in enzyme activity with storage of the enzyme preparation.

Agent	^{14}C-ATP incorporated/mg DNA (nmoles)	Decrease from control (%)
Experiment 1		
Control	23.6	
6-Chloro-8-aza-9-cyclopentylpurine (689)	2.2	91
6-Bromo-8-aza-9-cyclopentylpurine	1.2	95
6-Iodo-8-aza-9-cyclopentylpurine	4.2	82
6-Mercapto-8-aza-9-cyclopentylpurine	17.4	26
8-Aza-9-cyclopentylpurine	26.9	–
6-Chloro-8-azapurine	22.5	–
6-Chloropurine riboside	22.2	–
6-Chloropurine deoxyriboside	24.5	–
6-Chloropurine	26.4	–
Experiment 2		
Control	34.8	
689	22.3	36
6-Amino-2-aza-9-cyclopentylpurine	36.7	–
Experiment 3		
Control	7.8	
689	4.9	37
6-Chloro-9-cyclopentylpurine	8.2	–
6-Hydroxy-8-aza-9-cyclopentylpurine	8.1	–

synthesis reaction. Thus a concentration of 2 mM for each analog and a preincubation time of 60 min was used as a screening procedure to determine the relative activities of each agent as an RNA polymerase inhibitor.

It can be seen from table 1 that 689 produced 91% inhibition of *E. coli* RNA polymerase. The 6-bromo derivative was slightly more active, and the 6-iodo was somewhat less effective than 689. The 6-mercapto analog had only slight activity. If one of the three deviations of the purine nucleoside structure were absent, essentially no inhibitory activity was observed. The analogs lacking the 6-halo group or the cyclopentyl group were inactive, as were 6-chloropurine, its riboside, and deoxyriboside.

The data presented in Experiments 2 and 3 (table 1) were obtained using RNA polymerase from *M. lysodeikticus*. It is interesting that the enzyme from this source was much less sensitive to 689 than that derived from *E. coli*. The results of these experiments give further support to the concept that the basic alteration

Table 2
Effect of purine analogs upon formyltetrahydrofolate synthetase activity*

Compound	Concentration** (mM)	Decrease from control (%)
6-Chloro-8-aza-9-cyclopentylpurine (689)	0.3	17
	0.75	50
	1.5	80
	3.0	86
6-Choropurine	3.0	0
	30.0	10
6-Chloro-9-cyclopentylpurine	3.0	0
	30.0	25
6-Hydroxy-8-aza-9-cyclopentylpurine	3.0	0
	30.0	19
6-Mercapto-8-aza-9-cyclopentylpurine	3.0	13
	7.5	35
6-Amino-8-aza-9-cyclopentylpurine	7.5***	5
Adenine	3.0	0
9-Cyclopentyladenine	3.0	0

* Compounds were preincubated with the enzyme fraction at 37° for 3 min, after which the reaction components were added and the entire mixture was incubated for another 20 min.
** The concentration of inhibitor preincubated directly with the enzyme.
*** This compound was not soluble at the concentration indicated, and the reaction mixture was cloudy.

of the purine nucleus present in 689 are required for inhibition of polymerase activity. The agents lacking a halogen in position 6 or the 8-aza group were ineffective.

The results in table 2 indicate that the inhibition of formyltetrahydrofolate synthetase activity from rabbit liver was dependent upon the concentration of 689. The data in fig. 1 show that enzyme activity decreased with an increase in the duration of preincubation; thus, when a concentration of 0.68 mM 689 was preincubated with the enzymic fraction for 32 min, activity was reduced to 10% of the control value. Preincubation of the enzyme without 689 did not result in a loss of activity.

To determine the structural requirements for inhibition of formyltetrahydrofolate synthetase, several structurally related compounds were tested as inhibitors of this enzyme. The data in table 2 indicate that 689 inhibited formyltetrahydrofolate synthetase activity more effectively than did the other purine analogs examined. The other 6-chloro-containing analogs (6-chloropurine and 6-chloro-9-cyclopentylpurine) as well as several compounds possessing the 8-aza-9-cyclopentyl substitutions, such as 6-hydroxy-8-aza-9-cyclopentylpurine, 6-mercapto-8-aza-9-cyclopentylpurine, and 6-amino-8-aza-9-cyclopentylpurine, inhibited enzyme activity to a much lesser degree. The free base adenine and 9-cyclopentyladenine were not active at a concentration of 3.0 mM.

Experiments were carried out to test whether any of the three substrates, ATP, formate, or tetrahydrofolate, could protect the enzyme from inhibition by 689. Preincubation of either ATP, formate, or tetrahydrofolate with 689 or with enzyme prior to the addition of 689 did not protect the enzyme. In contrast, preincubation of the enzyme with magnesium ions

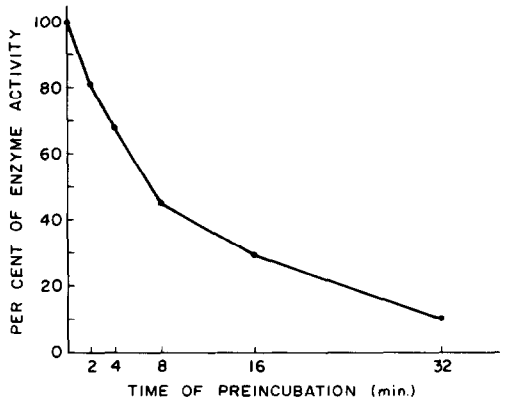

Fig. 1. The effect of the duration of preincubation with 0.68 mM 689 upon formyltetrahydrofolate synthetase from rabbit liver. After the desired time of preincubation, the reaction components (see **Materials and methods**) were added, and the total mixture was incubated for another 20 min.

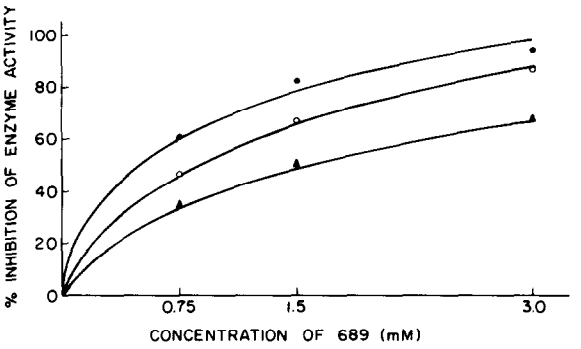

Fig. 2. The effect of preincubating Mg^{2+} with the enzyme fraction upon the subsequent inhibition by 689. The enzyme was incubated with Mg^{2+} for 15 min at 37°, after which 689 was added for an additional 5 min of incubation. The reaction mixture (see **Materials and methods**) was added and the entire mixture incubated for an additional 20 min. Concentration of Mg^{2+} preincubated with the enzyme: None, ●—●; 6.15 mM, ○—○; 12.3 mM ▲—▲.

($MgCl_2.6\ H_2O$, 12.3 mM) or with other cations (chloride salts of Mn^{2+}, Ni^{2+}, Co^{2+}, Ca^{2+}, Fe^{3+}, Sn^{2+}) prior to the addition of 689 resulted in less inhibition of enzyme activity. However, preincubation of the enzyme preparation with 689 prior to the addition of the cation resulted in an inhibition of activity similar to that observed in the absence of additional cation. The protective effect of the cations was studied using several concentrations of both inhibitor and magnesium chloride. The data in fig. 2 indicate that the protective effect of Mg^{2+} was dose dependent. Since it was possible that Mg^{2+} interacted with 689 and thereby prevented its effects upon the enzymes, 689 was incubated alone or with Mg^{2+} in tris EDTA buffer for 5 min at 37°, after which the spectrum between 240 nm and 300 nm of both mixtures was recorded. The peak of ultraviolet absorption (263.5 nm) was similar in both cases; it appeared, therefore, that Mg^{2+} did not directly interact with 689, at least in such a way that would lead to alteration of its absorbancy characteristics.

Preliminary experiments were also carried out with an enzyme fraction of higher specific activity obtained from *C. cylindrosporum*. Preincubation of the enzyme with 1.0 mM and 3.0 mM 689 for 3 min resulted in 45% and 70% inhibition, respectively.

4. Discussion

It was concluded from these experiments that the 6-halo, 8-aza, and 9-cyclopentyl components of 689 are all necessary to achieve full activity as an inhibitor of both RNA polymerase and formyltetrahydrofolate synthetase. It may be that the presence of the three groups in their respective positions in the purine ring is necessary to achieve a proper fit or attachment to a "receptor" site on the enzyme molecule and that the site of attack in both enzymes is similar. It has been suggested previously [2, 6] that the halogen atom in position 6 of the purine nucleus could make 689 an "alkylating agent" and that the drug inhibits enzyme activity by being directed into an active site of the enzyme as a result of its "nucleoside" structure and then irreversibly binding to it.

The data from our experiments suggest that 689-type analogs may have a differential sensitivity toward RNA polymerase from different organisms, a finding similar to that observed with the rifamycin group of antibiotics which also inhibit RNA polymerase activity by interaction with the enzyme rather than the DNA template [7,8,9]. It would seem that this series of analogs might prove to be very useful tools for examining the functions of the various RNA polymerase subunits which have recently been described [10].

The N^{10}-formyl tetrahydrofolate (FH_4) formed by formyltetrahydrofolate synthetase is subsequently converted to N^5, N^{10}-methenyl FH_4 and then to N^5, N^{10}-methylene FH_4. The latter coenzyme, also formed from the reaction between serine and tetrahydrofolic acid in the presence of L-serine hydroxymethyltransferase, is employed as the one-carbon donor in the synthesis of thymidylic acid [11]. It is conceivable that 689 inhibits the formation of thymidylate by inhibiting the formation of the necessary coenzymes. Since N^{10}-formyl FH_4 is only one of the possible coenzymes which can subsequently be used for thymidylate synthesis, the effect of 689 upon formation of N^5, N^{10}-methylene FH_4 in the L-serine hydroxymethyltransferase reaction should be explored.

The protective effect by Mg^{2+} upon enzyme activity may be due to binding of the cation to the enzyme and altering its conformation so that 689 may no longer attach to, or exert its effect upon, the enzyme. It is of interest that those divalent cations which cannot replace Mg^{2+} as the necessary metal for enzyme activity, namely Ni^{2+} or Co^{2+} [12], were also capable of protecting the enzyme from 689. An extension of these studies may give further insight into the nature of the binding site attacked by 689 and its relationship to enzymatic function.

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