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Effect of 6-Mercaptopurine Ribonucleotides on DNA-Dependent RNA Polymerase Activity

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SUMMARY

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6-Mercaptopurine ribonucleoside 5′-triphosphate was found to be an inhibitor of DNA-dependent RNA polymerases from *Escherichia coli* and chicken myeloblastosis cells. The inhibitor appears to compete with GTP for its binding to RNA polymerase, resulting in a reduction of RNA chain initiations. Neither 6-mercaptopurine ribonucleoside nor its monophosphate derivative caused the inhibition. These studies suggest that the antineoplastic action of 6-mercaptopurine may in part be owing to the inhibition of the activity of DNA-dependent RNA polymerase, resulting in an inhibition of cellular RNA synthesis.

INTRODUCTION

6-Mercaptopurine (6-MP),[1] an analogue of hypoxanthine, has been widely used as an antileukemic agent. Despite its extensive use and relatively lengthy history of clinical applications, the mechanism by which 6-MP brings about cell death has not yet been fully established. Many metabolic effects have been reported for 6-MP and a number of 6-MP metabolites, including the methylated derivatives, have been identified in blood samples of patients who receive 6-MP therapy (1–5). It is generally considered that 6-MP is biologically active only after conversion to its 5′-monophosphate nucleotide, thioinosinate (6-thio IMP), which inhibits both *de novo* synthesis of IMP and interconversion of purine ribonucleotides (1). However, studies of the effect of 6-MP on mouse lymphoma L5178 cells revealed that a delay existed between exposure of the mouse tumor cells to 6-MP and physical manifestations of toxicity by the cells (6). The delayed cytotoxic activity of 6-MP was found not related to the reduction of cellular purine nucleotide pools (7), suggesting that inhibition of purine nucleotide synthesis by 6-MP was alone insufficient to account for its cytotoxic effects. Nelson *et al.* (8) later reported that long-term incubations of red cells with 6-thiopurine analogues and the technique of high-performance liquid chromatography could reveal the presence of nucleoside triphosphates not previously identified. 6-MP ribonucleoside 5′-triphosphate (6-thio ITP) was thus found to accumulate in erythrocytes incubated with 6-MP at a linear rate over the 24-h incubation period (8). The formation of analogue triphosphates may result in altered nucleic acid biosynthesis which could be related to the cell-killing effects of these analogues. The present studies report the inhibitory effect of 6-thio ITP on *in vitro* DNA-dependent RNA polymerase activities and its possible biochemical mechanism.

MATERIALS AND METHODS

6-Thioinosine, 6-thio IMP (barium salt), 6-thio ITP (tetrasodium salt), unlabeled nucleoside triphosphates, and dithiothreitol (DTT) were obtained from P-L Biochemicals (Milwaukee, Wis.). [^{3}H]UTP was obtained from New England Nuclear (Boston, Mass.). 6-MP and *Escherichia coli* RNA polymerase (Type II) were purchased from Sigma Chemical Co. (Saint Louis, Mo.). Denatured calf thymus DNA was prepared by heating the native DNA at 100°C for 6 min and quick-cooling in an acetone–dry ice bath.

Preparation of chicken myeloblastosis RNA polymerase II. Chicken myeloblastosis (leukemic) cells were prepared and isolated from peripheral blood by a procedure of Beard (9) and frozen at −70°C before grinding. The frozen cells were ground with dry ice using a prechilled mortar and pestle and stored at −70°C until use. DNA-dependent RNA polymerase II was isolated from

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[1] The abbreviations used are: 6-MP, 6-mercaptopurine; 6-thioinosine, 6-mercaptopurine ribonucleoside; 6-thio IMP and 6-thio ITP, the 5′-mono- and triphosphates of 6-mercaptopurine ribonucleoside; DTT, dithiothreitol; ara-CTP, cytosine arabinoside triphosphate.

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the nuclei of the leukemic cells and purified through DEAE-Sephadex column chromatography and 10–40% glycerol gradient centrifugation by a procedure previously described (10). RNA polymerase IIa, which represents the major species of RNA polymerase II in chicken myeloblastosis cells (10), was used for the present studies.

Assay for RNA polymerase activity. The reaction mixture (0.1 ml) for the assay of chicken myeloblastosis RNA polymerase II contained 50 mM (pH 7.9) Tris–HCl, 2 μg pyruvate kinase, 4 mM phosphoenolpyruvate, 1 mM $MnCl_2$, 10 mM KCl, 1 mM DTT, 0.2 mM each ATP, GTP, and CTP, 0.04 mM UTP and [^{3}H]UTP (450–750 cpm/pmol), 10 μg denatured calf thymus DNA, and 2 μg RNA polymerase. The reaction mixture for *Escherichia coli* RNA polymerase was similar to that described above except that it contained *E. coli* RNA polymerase (0.1 unit), 4 mM $MgCl_2$, 5 mM DTT, and native calf thymus DNA. After incubation for 45 min at 37°C, the reactions were stopped by adding 0.1 ml of cold 0.1 M sodium pyrophosphate (pH adjusted to 7.0) containing RNA, 2 mg/ml, bovine serum albumin, 2 mg/ml, 5 mM UTP, and 30% trichloroacetic acid, 0.5 ml. Acid-precipitable radioactivity was collected on Whatman GF/C filters and washed more than 10 times with 5% trichloroacetic acid. Filters were then dried and counted in a scintillation counter.

Formaldehyde/sucrose gradient centrifugation. The size of RNA synthesized in an RNA polymerase reaction was analyzed by the formaldehyde/sucrose gradient centrifugation procedure as we previously described (11). Samples (0.2 ml) containing RNA were added to 20 μl of 5% sodium dodecyl sulfate. After incubation for 3 min at 37°C, the samples were made up to 0.3 ml in a solution containing 16 mM sodium phosphate buffer (pH 7.7) and 1.1 M formaldehyde and incubated for 15 min at 65°C. The chilled, formaldehyde-treated sample was layered directly on a 5-ml 10–30% linear sucrose gradient containing 0.1 M sodium phosphate buffer (pH 7.7) and 1.1 M formaldehyde. The sucrose was pretreated with diethyl pyrocarbonate (12) to inactivate any nuclease present. After centrifugation, the gradients were fractionated by dripping the solution from the bottom of the tube. Fractions were collected in tubes containing 0.2 mg of bovine serum albumin and 0.2 mg of yeast RNA and precipitated by adding 0.5 ml of 30% trichloroacetic acid. The precipitates were collected and washed with 5% trichloroacetic acid, and their radioactivity was determined. Marker RNAs were processed like the samples and centrifuged in a parallel gradient. Absorbance at 260 nm of the fractions collected from the marker gradients was read in a spectrophotometer. Marker RNAs were prepared from *Escherichia coli* or *Bacillus subtilis* by a procedure described previously (13).

RESULTS AND DISCUSSION

The effects of 6-thioinosine, 6-thio IMP, and 6-thio ITP on purified DNA-dependent RNA polymerases from *Escherichia coli* and chicken myeloblastosis cells are shown in Fig. 1. Since 6-thio IMP was supplied as barium salt and 6-thio ITP sodium salt, we have tested the salt effects of Ba^{2+} and Na^+ on the activities of RNA polymerases in the absence of the thio nucleotides. It was found that Na^+, up to a concentration of 200 mM, had no effect on either *Escherichia coli* or chicken myeloblastosis RNA polymerases. On the other hand, Ba^{2+}, which showed no effect on *Escherichia coli* RNA polymerase activity, inhibited significantly the activity of chicken myeloblastosis enzyme (the inhibition curve of barium superimposed that of 6-thio IMP-Ba^{2+}). The data shown in Fig. 1 were results corrected from salt effects and clearly show that both RNA polymerases were highly sensitive to 6-thio ITP inhibition. The results also indicated that 6-thioinosine had no effect on the RNA polymerases, while 6-thio IMP caused a slight decrease in *Escherichia coli* RNA polymerase activity. These studies also suggested that caution should be taken in interpreting results where compounds studied are commercially available in various salt forms.

Further studies showed that the inhibition by 6-thio ITP on RNA polymerase activities could be reversed by increasing GTP concentrations of the assay system. GTP was the only nucleotide that could cause the reversal of inhibition. A double reciprocal plot of the RNA synthesis versus GTP concentrations revealed that 6-thio ITP was a competitive inhibitor for the binding of GTP to either *Escherichia coli* RNA polymerase (Fig. 2) or chicken myeloblastosis RNA polymerase II (Fig. 3): K_m was increased in the presence of 6-thio ITP without a change in V_{max}. The apparent K_m value for GTP and the apparent K_i value for 6-thio ITP inhibition were estimated to be

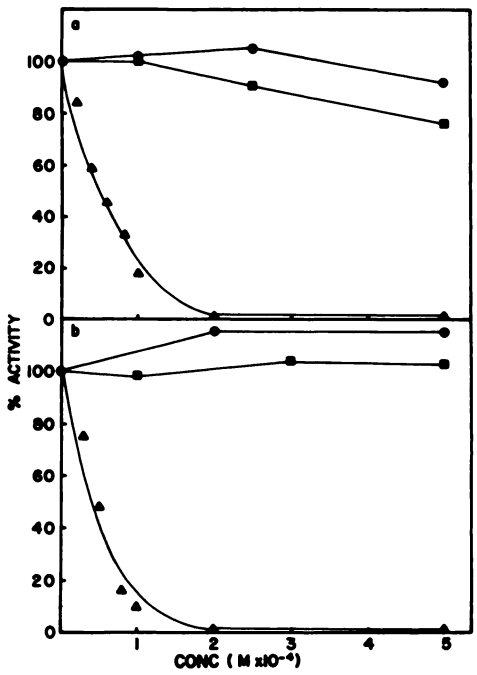

FIG. 1. *Dose–response of 6-thioinosine, 6-thio IMP, and 6-thio ITP on in vitro RNA polymerase activity*

Escherichia coli RNA polymerase (a) or RNA polymerase II isolated from chicken myeloblastosis nuclei (b) was assayed for activity in the presence of various concentrations of 6-thioinosine (●), 6-thio IMP (■), or 6-thio ITP (▲) as indicated. The reaction mixture and assay conditions were as described under Materials and Methods. The data presented here have been corrected from the salt effect (see text). Control (100%) activities were 96.0 and 8.6 pmol, respectively, for *Escherichia coli* and chicken myeloblastosis enzymes.

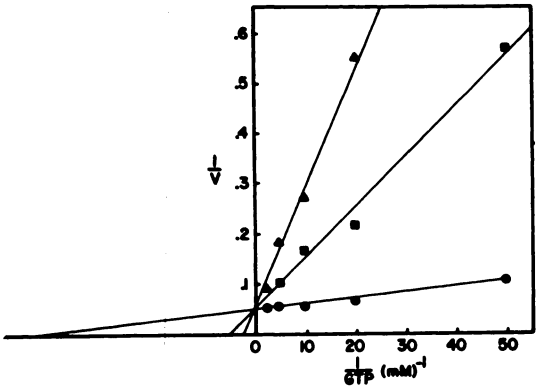

FIG. 2. *Effect of 6-thio ITP on the kinetics of RNA synthesis by Escherichia coli RNA polymerase (Lineweaver–Burk plot)*

The reaction mixture and assay conditions were similar to those described under Materials and Methods, except that GTP concentrations were varied as indicated, and the incubation was at 37°C for 15 min. 6-Thio ITP was included in the reaction mixture as follows: (●) control; (■) 10 μM 6-thio ITP; (▲) 33 μM 6-thio ITP. 1/*v* shown on the ordinate represents the reciprocal of picomoles UMP incorporated into RNA per 15 min.

21 and 1.2 μM, respectively, for *Escherichia coli* RNA polymerase and 46.5 and 13 μM, respectively, for chicken myeloblastosis RNA polymerase II.

To study the effect of 6-thio ITP on the chain length of RNA synthesized *in vitro*, the reaction mixture containing *Escherichia coli* RNA polymerase (Fig. 4) or chicken myeloblastosis RNA polymerase II (Fig. 5) after 45 min of incubation was treated with sodium dodecyl sulfate and formaldehyde, and the RNA, synthesized in the presence and absence of 6-thio ITP, was analyzed by sedimentation in 10–30% sucrose gradient containing formaldehyde. The results showed that in both prokaryotic and eukaryotic RNA-synthesizing systems, 6-thio ITP reduced the amount of RNA synthesized in a dose-related manner. Similar to the effect of adriamycin inhibition of RNA synthesis (14), 6-thio ITP, at various concentrations, did not change the sedimentation profile of the product RNA, suggesting the possibility that it reduces RNA chain initiation without affecting RNA chain length.

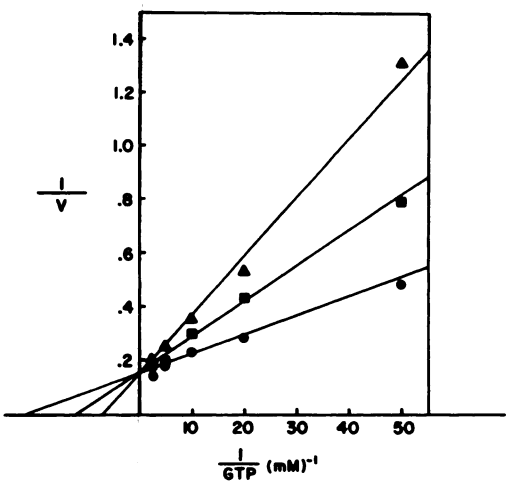

FIG. 3. *Effect of 6-thio ITP on the kinetics of RNA synthesis by chicken myeloblastosis RNA polymerase II (Lineweaver–Burk plot)*

The reaction mixture and assay conditions were similar to those described under Materials and Methods, except that GTP concentrations were varied as indicated, the incubation was at 37°C for 15 min, and the specific activity of [^{3}H]UTP was 1000 cpm/pmol. 6-Thio ITP was included in the reaction mixtures as follows: (●) control; (■) 15 μM 6-thio ITP; (▲) 25 μM 6-thio ITP. 1/*v* shown on the ordinate represents the reciprocal of picomoles UMP incorporated into RNA per 15 min.

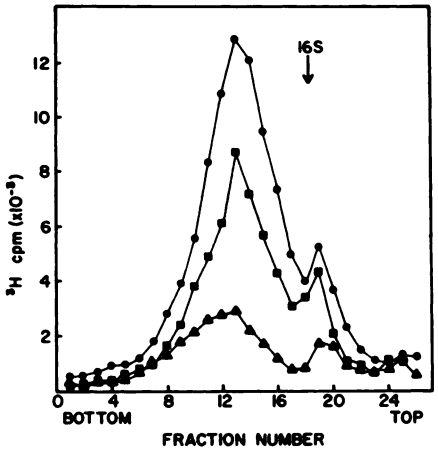

FIG. 4. *Effect of 6-thio ITP on the size of RNA synthesized in vitro by Escherichia coli RNA polymerase*

The reaction mixture was as described in Fig. 1, except that the assay volume was scaled up to 0.2 ml and the specific activity of [^{3}H]UTP was raised to 2000 cpm/pmol. After 45 min of incubation, the size of RNA synthesized by *Escherichia coli* RNA polymerase was analyzed by formaldehyde/sucrose gradient centrifugation procedure as described under Materials and Methods. The centrifugation was done at 50,000 rpm in an SW50.1 rotor at 16°C for 3 h, 15 min. The main peak of RNA synthesized was greater than 16 S in size (●) Control (■) 20 μM 6-thio ITP; (▲) 70 μM 6-thio ITP.

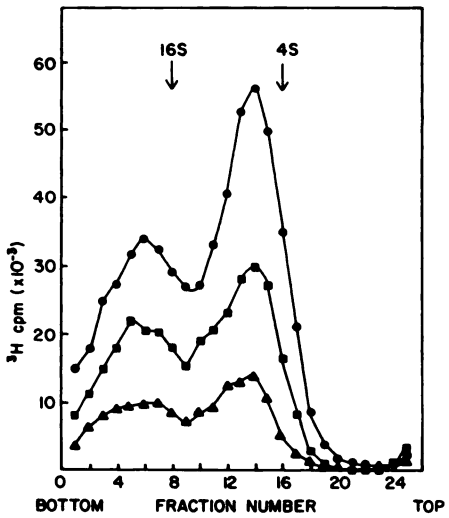

FIG. 5. *Effect of 6-thio ITP on the size of RNA synthesized in vitro by chicken myeloblastosis RNA polymerase II*

The reaction mixture was as described in Materials and Methods and the size of RNA synthesized by RNA polymerase II in the presence and absence of 6-thio ITP was analyzed as in Fig. 4, except that the specific activity of [^{3}H]UTP used was 6494 cpm/pmol and the centrifugation was done at 35,000 rpm in an SW50.1 rotor at 16°C for 18 h. The main peak of RNA synthesized was between 4 S and 16 S. (●) Control; (■) 40 μM 6-thio ITP; (▲) 80 μM 6-thio ITP.

We have previously reported that cytosine arabinoside inhibited RNA polymerase activity (15, 16). The inhibition involved ara-CTP being a competitive inhibitor for the binding of CTP to RNA polymerase (15), which resulted in a premature termination of RNA chains (16). The present studies present a similar kinetics of competitive inhibition and yet a completely different mode of action with regard to the product RNA synthesized in the presence of the drug. Although the present studies do not permit us to define a precise mechanism by which 6-thio ITP affects RNA chain initiations, the competitive nature of the inhibition with regard to GTP (Figs. 2 and 3) suggests to us that 6-thio ITP may impair the transcriptive initiation by competing with GTP at the initiation site (17) on DNA for RNA synthesis. This would be consistent with the previous findings of the relative inability of other purine nucleoside triphosphates, such as ITP (17) or 8-azaGTP (18), to replace GTP for initiation. However, this does not preclude the possibility that 6-thio ITP may incorporate into RNA as guanine nucleotide during RNA synthesis, and the incorporation alters the ability of the RNA–enzyme complex to dissociate upon reaching the termination signal and subsequently prevents RNA chain reinitiation. Further studies on this aspect are in progress.

The above studies demonstrated that *in vitro* RNA synthesis by DNA-dependent RNA polymerase was inhibited by 6-thio ITP. 6-Thio IMP or 6-thioinosine caused little or no inhibition of the enzyme. Whether the inhibition of RNA polymerase activity by 6-thio ITP is the primary mechanism by which 6-MP reduces cellular RNA synthesis remains to be determined. It would appear that the degree of inhibition of RNA synthesis at the level of RNA polymerase, and its relationship to the cell-killing effect of 6-MP, will depend largely upon the physiological (local) concentration of GTP, the concentration of 6-MP used, and the K_m/K_i value of the enzymic reaction. The formation of 6-thio ITP as 6-MP metabolite after prolonged incubation (8), the delayed cytotoxic effect of 6-MP (6, 7), and the high affinity (low K_i value) between the inhibitor and the enzyme (Figs. 2 and 3) suggest that inhibition of RNA polymerase should be considered, at least in cell culture systems, as one of the mechanisms of the cytotoxic action of 6-mercaptopurine.

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