DNA-Dependent RNA Polymerase: Inhibition of a Mammalian Enzyme by Methylthioinosine Dialdehyde[1] (35321)

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The periodate oxidation product of β-D-ribosyl-6-methylthiopurine (MMPR-OP) was found by Kimball *et al.* (1) to be a potent inhibitor of the utilization of thymidine for DNA synthesis in the Ehrlich ascites tumor. Enzyme studies demonstrated that this inhibition resulted from the sequential blockade of thymidine-5′-monophosphate kinase and DNA polymerase. Previously we also found that the β-D-ribosyl-6-methylthiopurine periodate oxidation product (MMPR-OP) was a potent inhibitor of protein biosynthesis (2) in the murine tumor, L1210. This inhibition of protein synthesis could have been at the level of DNA transcription into messenger RNA, the translation of messenger RNA into protein, or both. We now report that the MMPR-OP inhibits DNA-dependent RNA polymerase(s) in enzyme extracts of the Ehrlich ascites tumor.

Materials and Methods. In vitro *studies. Whole cell suspensions.* Methods for *in vitro* experiments with whole cell suspensions involving the incorporation of radioactive amino acids into proteins have been described (2).

DNA-dependent RNA polymerase. The preparation of enzyme extracts of the Ehrlich ascites tumor and the assay for DNA-dependent RNA polymerase are briefly described as follows: Ehrlich ascites tumor cells grown for 6 days in female Swiss mice (3) were harvested from the peritoneal cavity, pelleted at 1500*g* for 2 min and washed with physiological saline. The cell pellet was resuspended in 4 vol of ice-cold glass-distilled water and allowed to swell for 15 min at 3°. The cells were then homogenized by 20 strokes in a glass tissue homogenizer with a Teflon plunger. The homogenate was centrifuged at 100,000*g* in a Beckman Model L preparative ultracentrifuge for 70 min at 3°. The supernatant was decanted through loosely packed glass wool and immediately assayed (in duplicate) for DNA-dependent RNA polymerase(s) activity.

RNA polymerase assay. The assay mixtures (in duplicate) contained in 1.0 ml: enzyme extract, 6.6–8.0 mg of protein (4); 50 μmoles of Tris–HCl buffer (0.050 ml), pH 7.9; $MnCl_2$, 2.0 μmoles (0.025 ml); $MgCl_2$, 2.0 μmoles (0.025 ml); NaF, 20 μmoles (0.025 ml); 400 nmoles each of ATP, CTP, GTP (Sigma Chemical Company) (0.150 ml); 167 nmoles of ^{3}H-5-UTP (4250 cpm/nmole, Schwarz BioResearch) (0.100 ml); spermidine, 1.0 μmole (0.025 ml); and calf thymus native DNA, 100 μg (0.100 ml). The enzyme extracts (0.40 ml) were preincubated with MMPR-OP (0.100 ml) or water (0.100 ml), buffer, $MnCl_2$ and $MgCl_2$ for 15 minutes at 37°, cooled to 3° and the remainder of the assay mixture added. The complete reaction mixtures were then incubated at 37° for varying lengths of time. The reactions were terminated by the addition of 1.0 ml of ice-cold 10% trichloroacetic acid (TCA), and subsequent addition of 1.5 ml of 5% TCA afer standing at 3° for 10 min. The precipitates were pelleted at 1500*g* and washed three times with ice-cold 5% TCA. The washed precipitates were dissolved in 0.5 ml of a 0.2 *M* NaOH solution, decolorized with 0.05 ml of 30% H_2O_2 and added to 10 ml of a scintillation cocktail containing 5.0 g of PPO, 0.3 g of POPOP, 10 ml of saturated ascorbic acid, and 200 ml of BBS III

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TABLE I. Requirements for RNA Polymerase(s) Activity from the Ehrlich Ascites Tumor.[a]

Reaction mixture	^{3}H-5-UMP Incorp. (nmoles)	Inhibition (%)
Complete	1.28 ± 0.02	—
+15 μg RNase A	0.27 ± 0.03	79.2
—CTP, GTP, ATP	0.35 ± 0.01	72.8
—DNA	0.23 ± 0.01	82.3
+1 × 10^{-4} *M* MMPR-OP	1.16 ± 0.02	9.0
+5 × 10^{-4} *M* MMPR-OP	0.71 ± 0.01	44.5

[a] The reaction mixture (in duplicate) contained in 1.0 ml: enzyme extract, 8.0 mg of protein; 50 μmoles of Tris–HCl, pH 7.9; 400 nmoles each of ATP, GTP, and CTP; 2.0 μmoles of $MnCl_2$; 2.0 μmoles of $MgCl_2$; 100 μg of calf thymus DNA; 1.0 μmole of spermidine; 20 μmoles of NaF; and 167 nmoles of ^{3}H-5-UTP (4250 cpm/nmole). MMPR-OP or water was preincubated with enzyme extract in 50 μmoles of Tris–HCl buffer, 2.0 μmoles of $MnCl_2$, and 2.0 μmoles of $MgCl_2$ for 15 min at 37° before the addition of template and substrates and incubations were continued for 15 min. Values for ^{3}H-5-UMP incorporated (nmoles/8 mg of protein) are reported as average nanomoles ± average mean deviations.

(Beckman)/liter of toluene (5). Radioactivity was measured with a liquid scintillation spectrometer (Packard Model 3380) at an efficiency of 30%.

Preparation of DMAR-OP. The pure DM AR-OP was prepared as described earlier (6).

Results. RNA polymerase(s). The requirements for DNA-dependent RNA polymerase(s) in crude enzyme extracts of the Ehrlich ascites tumor are shown in Table I. The reaction was maximally dependent upon the addition of the proximal precursors for RNA synthesis and a DNA template. The addition of bovine pancreatic ribonuclease A destroyed the RNA product of the reaction. Preincubation of the enzyme(s) with MMPR-OP at 5 × 10^{-4} *M* inhibited the reaction. The lower molarity of drug employed (10^{-4} *M*) had no effect on the enzyme(s).

The kinetics of inhibition of DNA-dependent RNA polymerase(s) by MMPR-OP at 1 × 10^{-4} and 5 × 10^{-4} *M* are shown in Fig. 1. The enzyme(s) was inhibited about 50% at 5 × 10^{-4} *M* MMPR-OP throughout the 45-min incubation period in this study. Again there was little or no inhibition of the crude enzyme(s) by MMPR-OP at 1 × 10^{-4} *M*.

Whole cell suspensions. Inhibition of messenger RNA formation should be reflected in decreases in protein biosynthesis. Figure 2 shows the effect of MMPR-OP (1 × 10^{-3} *M*) on the incorporation of lysine-2-^{14}C into proteins of whole cell suspensions of the Ehrlich ascites tumor. The drug at 10^{-3} *M* completely halted the incorporation of lysine after 20 min of incubation. The same results were obtained when valine-1-^{14}C or isoleucine-2-^{14}C were used as precursors. This corroborates our previous findings for several other protein precursors (3).

Discussion. The periodate oxidation product of methylthioinosine (MMPR-OP) has been shown to inhibit DNA-dependent RNA polymerase(s) activity in cell-free extracts of the Ehrlich ascites tumor. We have previously reported inhibition of purified bacterial

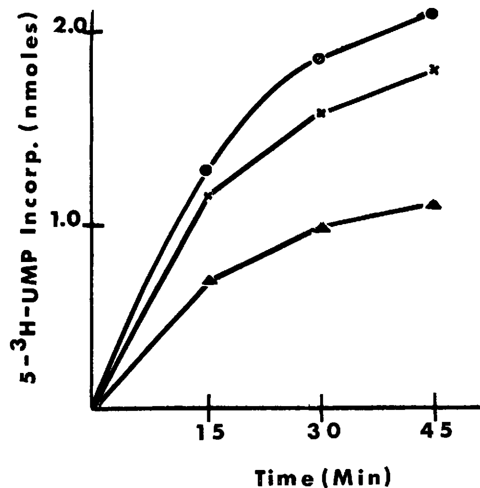

FIG. 1. Effect of 1 × 10^{-4} and 5 × 10^{-4} *M* MMPR-OP on the incorporation ^{3}H-5-UTP into an acid insoluble product. Refer to Table I, footnote *a*, for components and conditions. (○) control; 0 min, 0 μmoles; 15 min, 1.25 ± 0.02 μmoles; 30 min, 1.86 ± 0.01 μmoles; 45 min, 2.09 ± 0.05 μmoles. (X) MMPR-OP, 1 × 10^{-4} *M;* 0 min, 0 μmoles; 15 min, 1.14 ± 0.01 μmoles; 30 min, 1.57 ± 0.09 μmoles; 45 min, 1.81 ± 0.01 μmoles. (▲) MMPR-OP, 5 × 10^{-4} *M;* 0 min, 0 μmoles; 15 min, 0.71 ± 0.04 μmoles; 30 min, 0.99 ± 0.01 μmoles; 45 min, 1.13 ± 0.05 μmoles.

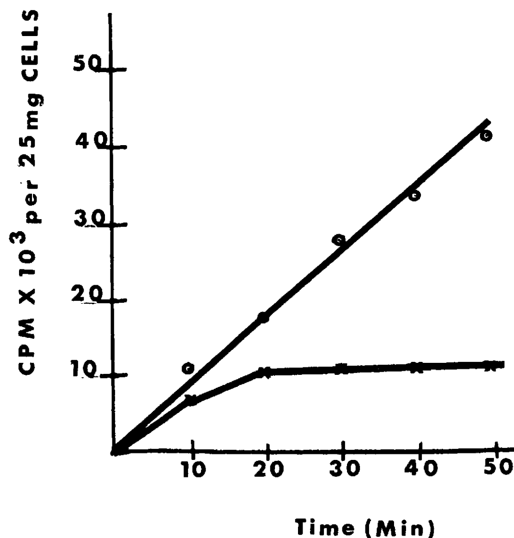

FIG. 2. Effect of 1×10^{-3} *M* MMPR-OP on the incorporation of lysine-2-^{14}C into proteins by Ehrlich ascites cell suspension: (O) control. (X) MMPR-OP, 1×10^{-3} *M*. The reactions which were carried out in Krebs original Ringer phosphate buffer (no $CaCl_2$), pH 7.4, in 10-ml volumes contained in 25-ml Erlenmeyer flasks the following: 10^{-3} *M* MMPR-OP or equivalent volume of buffer; glucose, 5.5×10^{-3} *M*; and 25 mg of wet weight of cells/ml of reaction mixture; and lysine-2-^{14}C, 1.25×10^{-4} *M*, 5 μCi/μmole. Cells in buffer were preincubated for 10 min at 37° with drug or buffer in a metabolic shaker before the addition of the lysine-2-^{14}C. At the designated times, 1.0-ml aliquots were removed for analysis.

RNA polymerase by MMPR-OP (7). Unlike α-amanitin which inhibits the yeast (8) and a form of the mammalian (9) but not bacterial DNA-dependent RNA polymerase (9); and rifamycin (10, 11) which inhibits the bacterial but not the mammalian enzyme, MMPR-OP inhibits both the mammalian and bacterial RNA polymerases. In addition, inhibition of the bacterial enzyme by increasing amounts of MMPR-OP is relatively linear (7). Discussion of the mechanism by which MMPR-OP inhibits the mammalian enzyme would be speculative at this time. However, the compound inhibited noncompetitively the incorporation of UTP into RNA and apparently binds to the 2′,3′-OH terminus site of the bacterial enzyme (7) active center at high substrate concentrations. Also, studies with bovine pancreatic ribonuclease A indicate that MMPR-OP inhibits this enzyme by forming a Schiff's base with the ϵ-amino groups in the enzyme (7). Studies of this nature on the Ehrlich ascites tumor enzyme(s) are underway in our laboratory.

MMPR-OP blocked the incorporation of lysine, valine, and isoleucine into proteins and this correlated with the inhibitory data on RNA polymerase(s). Although an effect at the translational level by MMPR-OP is not ruled out by these data, the observed inhibition of protein synthesis may be due, in part, to a blockade at the level of transcription.

Summary. DNA-dependent RNA polymerase(s) from the Ehrlich ascites tumor was inhibited by the periodate oxidation product of β-D-ribosyl-6-methylthiopurine. The inhibitions of RNA synthesis was correlated with inhibition of protein synthesis.

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