Dimethyladenosine Dialdehyde: Inhibition of Leukemia 1210 DNA Polymerase[1] (34893)

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The immunosuppressive and carcinostatic activities of the periodate oxidation products of purine and pyrimidine nucleosides have been demonstrated by Dvonch *et al.* (1), Bell *et al.* (2), and Kimball *et al.* (3). Periodate oxidation of β-D-ribosyl-6-methylthiopurine (methylthioinosine, MMPR) gave a product, (methylthioinosine periodate oxidation product, MMPR-OP) with a different mechanism of action than that reported for MMPR (4). The MMPR-OP was found to inhibit the thymidylate kinase and DNA polymerase of the Ehrlich ascites tumor (3). Methylthioinosine (MMPR) was demonstrated to have potent immunosuppressive activity (5) as was the MMPR-OP (2). In addition, the MMPR-OP appeared to have some specificity for the suppression of skin graft rejections. Recently, we began the use of the Leukemia 1210 in mice as an initial and stringent test system for the efficacy of potential immunosuppressive agents. The correlation between the carcinostatic and immunosuppressive activities for a given drug is excellent (6) although not absolute.

We report here several inhibitory properties of the periodate oxidation product of N^6-dimethyladenosine (DMAR-OP) on Leukemia 1210 (L1210).

Methods and Materials: *Preparation of the DMAR-OP.* The DMAR-OP was prepared from N^6-dimethyladenosine (DMAR, Nutritional Biochemicals) by the method of Dvonch *et al.* (1). The white crystalline product was shown to be free of starting material by paper chromatography in an ethanol-saturated borate–5 *M* ammonium acetate–0.5 *M* versene solvent system; 220:80:20:0.5, pH 9.5 (DMAR, $R_f = 0.67$; DMAR-OP, $R_f = 0.78$), and in a water-saturated *n*-butanol solvent system (DMAR, $R_f = 0.45$; DMAR-OP, $R_f = 0.84$).

Survival studies. The survival studies using female BDFI mice (18–20 g, Texas Inbred Mouse Co., Houston, Texas) bearing intraperitoneal implants of 5×10^4 L1210 ascites cells were carried out as described by Bell *et al.* (2). The DMAR-OP, 25 mg/kg, in saline which was 4% with respect to ethanol, was given by intraperitoneal injection every 3 hr for 24 hr on Days 1, 5, and 9. Control mice received saline (4% ethanol). Drinking water containing 50 mg/liter of streptomycin sulfate was given *ad libitum.*

In vitro studies: *Whole cell suspensions.* The *in vitro* incorporation of radiolabeled nucleic acid in protein precursors into whole-cell suspensions has been described (2, 3).

DNA polymerase. The assay for DNA polymerase in cell-free extracts has been described (3) except that dATP-8-^{14}C was used as the radioactive substrate. The protein concentration of the L1210 cell-free extracts as determined by the method of Lowry *et al.* (7) varied from 12 to 25 mg/ml.

Results. Survival studies. DMAR-OP increased the survival times of BDFI female mice bearing the L1210 ascites tumor (Table I). Treatment with DMAR-OP, 25 mg/kg every 3 hr on Days 1, 5, and 9 increased the mean survival times of the mice about 40% beyond the mean survival times of saline-treated control mice.

In vitro studies: *Whole cell suspensions.* Preincubation of L1210 cells with DMAR-OP, 10^{-3} *M*, inhibited the incorporation of thymidine-methyl-^{3}H (4×10^{-4} *M*, 5 μCi/μmole, Schwarz BioResearch, Inc.) into DNA about 50% (Fig. 1). This inhibition was

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TABLE I. L1210 Survival Study.[a]

Treatment	mg/kg q3H, days 1, 5, 9	MST[b]	T/C[c] (%)
Exp. I			
Saline	—	9.0 ± 0.1	—
DMAR-OP	25	13.0 ± 0.9	144
Exp. II			
Saline	—	9.0 ± 0.0	—
DMAR-OP	25	13.0 ± 0.3	144

[a] BDFI female mice (18–20 g) were each implanted with 5×10^4 L1210 ascites cells by the intraperitoneal route. Drug therapy was started 24 hr later every 3 hr for 24 hr. This regimen was repeated on Days 5 and 9. Each group contained seven mice.

[b] Survival times are reported as median survival time ± standard error.

[c] [Treated survival time/control survival time] × 100.

lacking when the DMAR-OP (10^{-3} *M*) was added 10 min after the incorporation of thymidine had begun (Fig. 1). Preincubation of

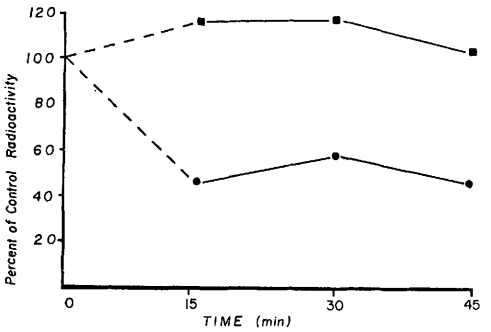

FIG. 1. Effect of DMAR-OP on the Incorporation of Thymidine into DNA. (●—●) Each reaction flask contained 8.6 ml Kreb's original Ringer buffer (no $CaCl_2$) at pH 7.4 to which had been added 55 μmoles glucose and 0.250 g cells. Ten μmoles of DMAR-OP in buffer or buffer (control) were added (1.0 ml) and cells were preincubated for 10 min at 37° before 4 μmoles TdR-^{3}H (5 μCi/μmole) in a volume of 0.4 ml were added. Two-milliliter portions were removed at the indicated intervals. (■—■) Each reaction flask contained 9.0 ml Kreb's original Ringer buffer, (no $CaCl_2$) pH 7.4, 55 μmoles glucose, 0.250 g cells, and 4 μmoles TdR-^{3}H. Cells were incubated for 10 min at 37° before 10 μmoles of DMAR-OP in buffer or buffer (control) were added (1.0 ml). Two-milliliter portions were removed at the indicated intervals for assay.

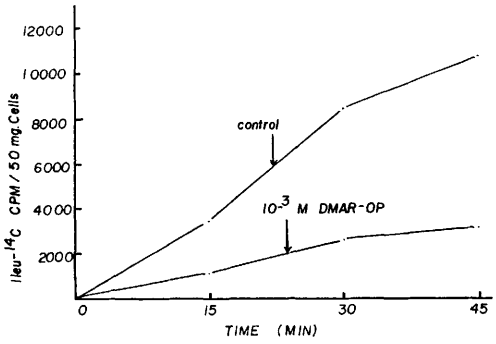

FIG. 2. Effect of DMAR-OP on the Incorporation of Isoleucine into Protein. Each reaction flask contained 8.6 ml Kreb's original Ringer buffer (no $CaCl_2$) at pH 7.4 to which had been added 55 μmoles glucose and 0.250 g cells. Ten μmoles of DMAR-OP in buffer or buffer were added (1.0 ml) and cells were preincubated for 10 min before 4 μmoles of ^{14}C-isoleucine (1 μCi/μmole) in a volume of 0.4 ml were added. Two-milliliter portions were removed for assay.

L1210 cells with DMAR-OP (10^{-3} *M*) also inhibited the *in vitro* incorporation of isoleucine-U-^{14}C (4×10^{-4} *M*, 1 μCi/μmole, Schwarz BioResearch, Inc.) into an acid-insoluble product (Fig. 2). This inhibition was somewhat greater (75%) than that for thymidine incorporation (50%). No reversal experiments were attempted with isoleucine in this study.

L1210 DNA polymerase. Table II shows

TABLE II. "DNA Polymerase" Activity in Enzyme Extracts of the L1210 Ascites Tumor.[a]

System	nmoles dATP-^{14}C incorporated	% Control
Complete	0.044	100
Minus dCTP, dGTP, and TTP	0.021	48
Minus ATP-regenerating system	0.008	18
Minus DNA template	0.000	0
Minus 1/2 enzyme	0.026	59

[a] The complete system contained 10 nmoles dATP-8-^{14}C (2.5 mμCi/nmole), 10 nmoles each of dCTP, dGTP, and TTP, 50 μmoles Tris buffer (pH 7.9–8.0), 5 μmoles creatine phosphate, 75 μg creatine phosphokinase, and 1 μmole ATP. The amount at extract added was 0.150 ml. Final volume was 0.450 ml. Incubations were carried out for 20 min at 37°.

several parameters affecting the L1210 DNA polymerase. The activity of DNA polymerase in the L1210 cell-free extract was somewhat low when compared with values reported for the polymerase from the Ehrlich ascites tumor (3). The incorporation of deoxyadenosine-5′-triphosphate-8-^{14}C (dATP-8-^{14}C, Schwarz BioResearch, Inc.) into an acid-insoluble product increased for 30 min and was linear for 15–20 min. The enzymic activity was dependent upon the addition of a DNA primer and an ATP-regenerating system consisting of creatine phosphate (5 μmoles), creatine phosphokinase (75 μg), and ATP (1 μmole). The additions of the triphosphates of deoxycytidine, deoxyguanosine, and thymidine increased the incorporation

TABLE III. Concentration Effect of DMAR-OP and DMAR on "DNA-Polymerase" Activity in Enzyme Extracts of the L1210 Ascites Tumor.[a]

System	nmoles of dATP-8-^{14}C incorporated	% Inhibition
Exp. I		
Control	0.207	
1.5×10^{-4} *M* DMAR-OP	0.180	13
3.0×10^{-4} *M* DMAR-OP	0.153	26
Exp. II		
Control	0.236	
6.0×10^{-4} *M* DMAR-OP	0.083	65
9.0×10^{-4} *M* DMAR-OP	0.052	78
Exp. III		
Control	0.152	
1.5×10^{-4} *M* DMAR	0.181	
3.0×10^{-4} *M* DMAR	0.196	
6.0×10^{-4} *M* DMAR	0.200	
9.0×10^{-4} *M* DMAR	0.214	

[a] The incubation tubes each contained 10 nmoles dATP-8-^{14}C (2.5 μCi/μmole), 10 nmoles each of dCTP, dGTP, and TTP, 50 μmoles Tris buffer (pH 7.9–8.0), 5 μmoles $MgCl_2$, 100 μg denatured DNA and an ATP regenerating system of 5 μmoles creatine phosphate, 75 μg creatine phosphokinase, and 1 μmole ATP. The amount of extract added was 0.200 ml; final volume was 0.500 ml. DMAR-OP and DMAR or H_2O was preincubated with the enzyme and all reagents except for dATP-8-^{14}C, dCTP, dGTP, and TTP for 10 min at 37°. After these were added at 0° incubation was allowed to continue for 30 min at 37°.

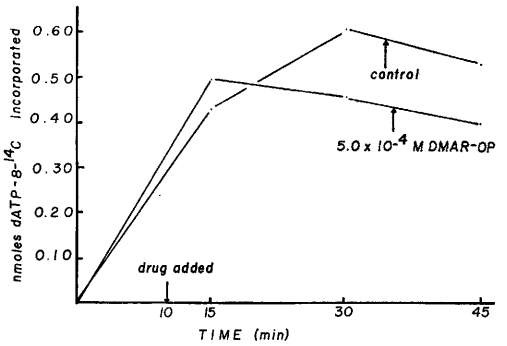

FIG. 3. Reversal of DMAR-OP Inhibition of "DNA Polymerase" in Cell-Free Enzyme Extract. Incubation tubes each contained 10 nmoles dATP-8-^{14}C (2.5 μCi/μmole), 10 nmoles each of dCTP, dGTP, and TTP, 50 μmoles Tris buffer (pH 7.9–8.0), 5 μmoles $MgCl_2$, 100 μg denatured DNA, and an ATP-regenerating system consisting of 5 μmoles creatine phosphate, 75 μg creatine phosphokinase, and 1 μmole ATP. The amount of extract added was 0.200 ml. Final volume was 0.500 ml. Incubations were carried out for the indicated intervals at 37°.

dATP-8-^{14}C only about 50%. This is probably due to the presence of very small pools of the deoxynucleoside triphosphates in the cell-free extracts. The DNA polymerase was inhibited by the DMAR-OP with the percentage of inhibition increasing with increasing molarities of drug (Table III). The 50% inhibitory molarity of DMAR-OP was determined to be about 5×10^{-4} *M*. No inhibition of DNA polymerase by the unoxidized N^6-dimethyladenosine (DMAR) were observed at the highest molarity used (9×10^{-4} *M*). Some inhibition of DNA polymerase by DMAR-OP (5×10^{-4} *M*) was found (Fig. 3) when the DMAR-OP was added 10 min after DNA synthesis had begun. This contrasts with the *in vitro* whole-cell experiments (Fig. 1) where no depression of thymidine incorporations was found when the drug was added at 10 min.

Discussion. The periodate oxidation product of N^6-dimethyladenosine (DMAR-OP) designed as a potential immunesuppressant was found to have antileukemic activity and resembles the activities of methylthioinosine (4) and its periodate oxidation product (2) in this respect. The L1210 subline used in

the survival studies reported here lacks IMP–GMP pyrophosphorylase and is resistant to 6-mercaptopurine (6 MP) as a consequence (4, 8). The DNA polymerase of this 6 MP-resistant L1210 subline was inhibited by DMAR-OP but not DMAR which suggests that the activity of DMAR-OP resides in the oxidized ribose moiety. Additional evidence for this possibility is that MMPR-OP also inhibits DNA polymerase (3) while methylthioinosine does not. However, further research will be required to ascertain if nonheterocyclic ring or aliphatic substituents modify this inhibitory activity for DNA polymerase. Preincubation of substrates for DNA synthesis before addition of DMAR-OP resulted in no inhibition by DMAR-OP in whole-cell suspensions but some inhibition in enzyme extracts. This suggests that DMAR-OP produced an effect or effects on whole-cell preparations that were not affected in enzyme preparations. In this connection, we conjecture that DMAR-OP may be acting on the cell membrane to inhibit the transport of thymidine into the cell. Future work is needed to confirm or rule out this possibility. If this proves to be the case, the nucleoside periodate oxidation products might also be useful compounds for the study of mechanisms of cellular transport.

Summary. The periodate oxidation product of N^6-dimethyladenosine, designed as a potential immunesuppressant drug, was found to have antileukemic activity. The DNA polymerase from the L1210 ascites tumor was inhibited by drug molarities at which molarities the N^6-dimethyladenosine was not inhibitory. The depression of DNA polymerase may be correlated with the increases in survival times of L1210-bearing mice although the possibilities of other mechanisms of action were not ruled out.

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