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Inhibition of Ribonucleic Acid-Directed Deoxyribonucleic Acid Polymerase of Murine Leukemia Virus by Polyribonucleotides and Their 2′-*O*-Methylated Derivatives

S. K. ARYA AND W. A. CARTER

Department of Medical Viral Oncology, Roswell Park Memorial Institute, Buffalo, New York 14203

J. L. ALDERFER AND P.O.P. Ts'o

Department of Biochemical and Biophysical Sciences, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205

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SUMMARY

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Poly(inosinic acid) [poly(I)], poly(2′-*O*-methylinosinic acid) [poly(Im)], poly(uridylic acid) [pol(U)], and poly(2′-*O*-methyluridylic acid) [poly(Um)] inhibit the RNA-directed DNA polymerase activity of Moloney murine leukemia virus. Poly(cytidylic acid) [pol(U)], and poly(2′-*O*-methyluridylic acid) [poly(Um)] inhibit the RNA-inhibition. The apparent inhibition kinetics for poly(I) and poly(Im) inhibition of the poly(A):oligo(dT)-directed reaction and that for poly(U) and poly(Um) inhibition of the poly(C):oligo(dG)-directed reaction are not consistent with a simple competitive inhibition with respect to the template. The following order of the potency of inhibition is observed: poly(I) > poly(Im) > poly(U) > poly(Um) >> poly(C) or poly(Cm).

INTRODUCTION

The activity of RNA-directed DNA polymerase associated with RNA tumor viruses seems to be required for the infection and transformation of cells by these viruses (1). A study of the selective inhibitors of this enzyme therefore may lead to the development of antiviral and antitumor agents. Additionally, such selective inhibitors may serve as diagnostic tools to distinguish viral DNA polymerase from cellular DNA polymerases, and thus contribute toward evaluating the role of viral functions in neoplastic transformation. Single-stranded polynucleotides have been shown to inhibit the viral RNA-directed DNA polymerase with little or no effect on cellular DNA polymerases (2–6). These polynucleotides reportedly inhibit viral functions in cell culture and animal systems as well (7–10). We have previously reported that polyadenylic acids inhibit the RNA-directed DNA polymerase activity of Moloney murine leukemia virus (5). They also

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inhibit Friend virus DNA polymerase.[1] To assess the effect of the base composition and 2'-*O*-alkylation of a polynucleotide on its inhibitory potency, we have now extended these studies to other polyribonucleotides. We present here the results of our studies with poly(inosinic acid) [poly(I)], poly(2'-*O*-methylinosinic acid) [poly(Im)], poly(cytidylic acid) [poly(C)], poly(2'-*O*-methylcytidylic acid) [poly(Cm)], poly(uridylic acid) [poly(U)], and poly(2'-*O*-methyluridylic acid) [poly(Um)].

MATERIALS AND METHODS

Poly[d(A-T)] (15.1 S), poly(A) (8.1 S), poly(I) (mol wt > 100,000), poly(C) (4.6 S), poly(U) (7.4 S), and oligo(dT)$_{12-18}$ were obtained from Miles Laboratories Poly(Um) (10.2 S) and oligo(dG)$_{12-18}$ were obtained from P-L Biochemicals. Poly(Im) (8 S) and poly(Cm) (5.5 S) were prepared by polymerization of the 2'-*O*-methylnucleoside diphosphates as described by Tazawa *et al.* (11). Tritium-labeled thymidine triphosphate (50 Ci/mmol) and deoxyguanosine triphosphate (6.5 Ci/mmole) were obtained from Schwartz/Mann. The concentration of polynucleotides in 0.01 M NaCl–0.01 M Tris-HCl, pH 7.2, was determined spectrophotometrically using the following millimolar nucleotide extinction coefficients: poly(d(A-T)], 6.8 mM^{-1} cm^{-1} at 260 nm; poly(A), 9.8 mM^{-1} cm^{-1} at 258 nm; poly(I), 9.6 mM^{-1} cm^{-1} at 248 nm; poly(Im), 9.9 mM^{-1} cm^{-1} at 248 nm; poly(C), 6.8 mM^{-1} cm^{-1} at 267 nm; poly(Cm), 6.2 mM^{-1} cm^{-1} at 268 nm; poly(U) and poly(Um), 9.8 mM^{-1} cm^{-1} at 260 nm.

Moloney murine leukemia virus, MLV (Moloney), was obtained from infected JLS-V9 cells in culture and purified by sucrose gradient centrifugation as described before (5). DNA polymerase assays were performed in a reaction mixture (50 or 100 μl) containing 0.05 M Tris-HCl (pH 7.9), 0.06 M NaCl, 0.001 M $MnCl_2$, 0.02 M dithiothreitol, 0.05% NP-40, 10 μg/ml of purified murine leukemia virus protein, and either 50 μM [^{3}H]thymidine triphosphate (2600 cpm/pmole) and 10–50:1–5 μM poly(A):oligo(dT) (molar ratio, 10:1) or 50 μM [^{3}H]deoxyguanosine triphosphate (2600 cpm/pmole) and 2–10:0.2–1 μM poly(C):oligo(dG) (molar ratio, 10:1). When poly[d(A-T)] was used as a template, the reaction mixture included 100 μM deoxyadenosine triphosphate. The template:primer complexes were prepared by mixing the appropriate polynucleotide and oligonucleotide in a molar ratio of 10:1, respectively, in 0.01 M Tris-HCl (pH 7.2) containing 0.1 M NaCl or 0.01 M NaCl and incubating them at 37° for 30 min followed by slow cooling.

The reaction mixture was incubated at 37°, and the radioactivity incorporated into acid-insoluble material was determined by withdrawing aliquots (10 or 20 μl) at specified times and placing them on Whatman No. 3MM filter paper discs. The discs were immersed in a cold 10% solution of trichloracetic acid containing 1% sodium pyrophosphate. They were then washed with five changes of a cold 5% solution of trichloracetic acid containing 0.5% sodium pyrophosphate, followed by washing with ethanol and acetone. After drying, the discs were immersed in a toluene-based scintillation fluid (Omnifluor, New England Nuclear) and radioactivity was measured in a Beckman scintillation counter. Under these assay conditions, the reaction mixture contains limiting concentration of enzyme and saturating concentrations of deoxyribonucleoside triphosphates, and there is negligible incorporation of precursors catalyzed by the endogenous murine leukemia virus template. The kinetic parameters were obtained by constructing Lineweaver-Burk and Dixon plots (12). The concentration of the template:primer was used as the substrate concentration, and the incorporation of precursor nucleoside triphosphate was taken as a measure of template:primer utilization.

RESULTS

The effect of polyribonucleotides on the kinetics of the poly[d(A-T)]-directed DNA polymerase activity of detergent-disrupted MLV (Moloney) is shown in Fig. 1. Poly(I) and poly(Im) strongly inhibit the polymerase activity, whereas poly(U) and poly(Um) show a lesser degree of inhibi-

[1] S. K. Arya, unpublished observations.

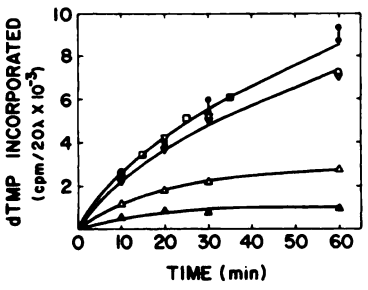

FIG. 1. *Effect of polyribonucleotides on kinetics of inhibition of poly[d(A-T)]-directed MLV (Moloney) DNA polymerase*

The reaction mixture contained 0.05 M Tris-HCl (pH 7.9), 0.06 M NaCl, 0.001 M $MnCl_2$, 0.02 M dithiothreitol, 0.05% NP-40, 100 μM dATP, 50 μM [^{3}H]dTTP (2600 cpm/pmole), 40 μM poly[d(A-T)], and 10 μg/ml of virus protein. Curves show poly[d(A-T)] in the absence (●——●) and presence of poly(I) (10 μM) (▲——▲), poly(Im) (10 μM) (Δ——Δ), poly(U) (10 μM) (▼——▼), poly(Um) (10 μM) (▽——▽), poly(C) (50 μM) (■——■), and poly(Cm) (50 μM) (□——□).

tion. Poly(C) and poly(Cm) fail to show significant inhibition even when the concentration of these two polyribonucleotides (50 μM) is 5 times that of the other polyribonucleotides (10 μM).

Figure 2 shows the degree of polymerase inhibition achieved with different concentrations of various polyribonucleotides. The template:primer for poly(I), poly(Im), poly(C), and poly(Cm) was poly(A):oligo(dT). Two concentrations of this template:primer were used: 10:1 μM (equal to ½ × K_m, ref. 5) and 20:2 μM (equal to K_m, ref. 5). With a poly(A):oligo(dT) concentration of 10:1 μM, the concentrations of poly(I) and poly(Im) required to achieve 50% inhibition of polymerase activity were about 0.6 μM and 15 μM, respectively (Fig. 2). When the concentration of poly(A):oligo(dT) was doubled, the concentrations of poly(I) and poly(Im) for 50% inhibition were also doubled (see Table 1). To assess the inhibitory potency of poly(U) and poly(Um), poly(A):oligo(dT) could not be used as a template:primer because of the base complementarity between the template and the inhibitor. Instead poly(C):oligo(dG) was used as a template:primer for these inhibitor polynucleotides. As shown in Fig. 2, the concentrations of poly(U) and poly(Um) required to obtain 50% inhibition of polymerase activity were 60 μM and 90 μM, when the poly(C):oligo(dG) concentration was 5:0.5 μM. [The K_m of poly(C):oligo(dG) for murine leukemia virus DNA polymerase is 4:04 μM (5)]. Reducing the concentration of poly(C):oligo(dG) in the reaction mixture resulted in a corresponding reduction in the concentrations of poly(U) and poly(Um) required for 50% inhibition (data not shown).

It is evident that, for a given concentration of the template:primer ($\leq K_m$) and lower fractional inhibition (<0.6), the degree of inhibition is linearly proportional to the polyribonucleotide (inhibitor) concentration. However, for higher values of fractional inhibition (>0.6), the degree of inhibition obtained is not a linear function of the polyribonucleotide concentration (Fig. 2). These results suggest that the polyribonucleotides may not be simple or classical competitive inhibitors of the template:primer. This notion is further substantiated by analyzing the kinetics of inhibition by Lineweaver-Burk ($1/v$ vs. $1/[S]$) and Dixon ($1/v$ vs. $[I]$) plots (12).

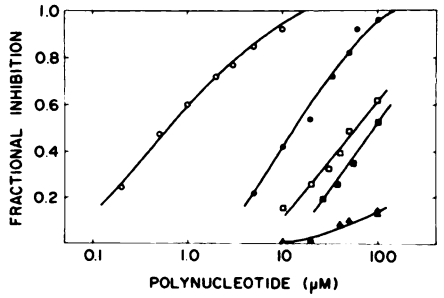

FIG. 2. *Effect of concentration of polyribonucleotides on degree of inhibition of MLV (Moloney) DNA polymerase*

The reaction mixture contained 0.05 M Tris-HCl (pH 7.9), 0.06 M NaCl, 0.001 M $MnCl_2$, 0.02 M dithiothreitol, 0.05% NP-40, 10 μg/ml of virus protein, and either 10:1.0 μM poly(A):oligo(dT) with 50 μM [^{3}H]dTTP (2600 cpm/pmole) for poly(I), poly(Im), poly(C), and poly(Cm) or 5:0.5 μM poly(C):oligo(dG) with 50 μM [^{3}H]dGTP (2500 cpm/pmole) for poly(U) and poly(Um). Fractional inhibition is based on the amount of [^{3}H]dTMP or [^{3}H]dGMP incorporated into acid-insoluble material in 10 min at 37°. O——O, poly(I); ●——●, poly(Im); □——□, poly(U); ■——■, poly(Um); Δ——Δ, poly(C); ▲——▲, poly(Cm).

Figure 3 shows the $1/v$ vs. $1/[S]$ plots for poly(I) inhibition of the poly(A):oligo(dT)-directed reaction. It is apparent that both the K_m and V_{max} of the reaction are affected by poly(I). The inhibition constant (K_i) for poly(I), estimated from the slopes of lines for inhibited and uninhibited reactions, is 0.53–0.72 μM. The $1/v$ vs [I] plots for poly(I) inhibition of the poly(A):oligo(dT)-directed reaction are shown in Fig. 4. It is evident that the plots deviate from linearity, especially at higher concentrations of the template:primer and inhibitor. These plots are not consistent with those

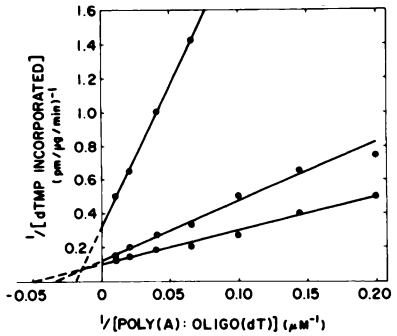

FIG. 3. *Kinetics of inhibition (1/v vs. 1/[S]) of poly(A): oligo(dT)directed MLV (Moloney) DNA polymerase by poly(I)*

The reaction conditions were the same as described in Fig. 2. Curves show poly(A):oligo(dT) in the absence (●——●) and presence of poly(I) (◐——◐, 1.0 μM; ⊖——⊖, 5 μM).

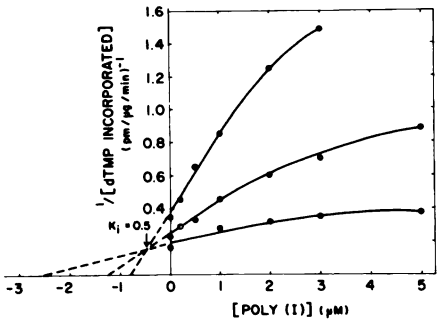

FIG. 4. *Kinetics of inhibition (1/v vs. [I]) of poly(A):oligo(dT)-directed MLV (Moloney) DNA polymerase by poly(I)*

The reaction conditions were the same as described in Fig. 2. ●—●, 10:1.0 μM poly(A):oligo(dT); ◐——◐, 20:2.0 μM poly(A):oligo(dT); ⊖——⊖, 50:5.0 μM poly(A):oligo(dT).

TABLE 1

Inhibition of MLV (Moloney) DNA polymerase by polyribonucleotides

Polyribonucleotide	I_{50}[a]	K_i[b]
	μM	μM
Poly(inosinic acid)	1.1	0.5–0.7
Poly(2'-*O*-methylinosinic acid)	32	12
Poly(cytidylic acid)	>100	
Poly(2'-*O*-methylcytidylic acid)	>100	
Poly(uridylic acid)	60	32
Poly(2'-*O*-methyluridylic acid)	90	45

[a] Concentration of polyribonucleotide yielding 50% inhibition of polymerase activity. The template:primer for poly(I), poly(Im), poly(C), and poly(Cm) was 20:2 μM poly(A):oligo(dT) (K_m = 20 μM), and that for poly(U) and poly(Um) was 5:0.5 μM poly(C):oligo(dG) (K_m = 4 μM).

[b] Estimated from $1/v$ vs. $[I]$ plots (see the text).

expected for apparent simple competitive inhibition, in which the inhibitor prevents binding of the substrate to the active site of the enzyme by direct competition. Similar plots were obtained for poly(Im) inhibition of the poly(A):oligo(dT)-directed reaction and poly(U) and poly(Um) inhibition of the poly(C):oligo(dG)-directed reaction (not shown). To obtain a measure of the relative inhibitory potency of various polynucleotides, we estimated the inhibition constants (K_i) from the intersection of the extrapolation of initial linear portions of $1/v$ vs. $[I]$ plots (Fig. 4). These are listed in Table 1. It is apparent that poly(I) is a more potent inhibitor than poly(Im) and that poly(U) is more potent than poly(Um). The inhibition constants for poly(I) and poly(U) may not be strictly comparable, since different template:primers were used. However, we have previously shown that in the case of polyadenylic acids the values of inhibition constants do not differ greatly (less than 50%) when either poly(A):oligo(dT) or poly(C):oligo(dG) is used as a template:primer (5) Therefore it is reasonable to assume that polyinosinic acids are more potent inhibitors than polyuridylic acids. This is supported by the effect of these polyribonucleotides on the poly[d(A-T)]-directed reaction of MLV (Moloney) DNA polymerase (Fig. 1).

DISCUSSION

Polyinosinic acids and polyuridylic acids inhibit MLV (Moloney) DNA polymerase, polyinosinic acids being more potent than polyuridylic acids. Polycytidylic acids show little or no inhibition. The potency of inhibition apparently depends on the base composition of the polynucleotide. A similar inference has been reported by Tuominen and Kenney (2) for MLV (Rauscher) DNA polymerase. The physicochemical basis of this composition dependence is unclear. In view of our previous study (5), it appears likely that purine polynucleotides may in general be more potent than pyrimidine polynucleotides as inhibitors of MLV (Moloney) DNA polymerase. On the other hand, for the poly[d(A-T)]-directed MLV (Rauscher) DNA polymerase, Tuominen and Kenney (2) reported that poly(U) is about 50 times more potent, and poly(C) about half as potent, as poly(A). Our collective results (this paper and ref. 5) with MLV (Moloney) DNA polymerase indicate that poly(U) is not significantly more potent than poly(A); in addition, poly(C) is less potent than poly(A) by several orders of magnitude. Tennant *et al.* (7) and Pitha *et al.* (8) have reported that poly(U) is less potent than poly(A) as an inhibitor of the replication of MLV (Moloney) in cultured cells. This is more in accord with our results with MLV (Moloney) DNA polymerase than with those of Tuominen *et al.* (2) with MLV (Rauscher) DNA polymerase. These observations raise the possibility that the two viral DNA polymerases respond differently to polyribonucleotide inhibitors.

The data suggesting variations in the response of DNA polymerases of RNA tumor virus across the species to poly(U) inhibition have already been reported. Whereas Tuominen and Kenney (2) observed a K_i of 0.2 μM for poly(U) inhibition of the poly[d(A-T)]-directed MLV (Rauscher) DNA polymerase, Abrell *et al.* (3) reported a K_i of 65 μM for poly(U) inhibition of the poly[d(A-T)]-directed Mason-Pfizer monkey tumor virus DNA polymerase. Additionally, Erickson *et al.* (4) reported the K_i of poly(U) inhibition of the poly(C):oligo(dG)-directed avian myeloblastosis virus DNA polymerase to be 0.3–2.5 μM, depending on the chain length of poly(U). We obtained a K_i of about 32 μM for the poly(C):oligo(dG)-directed MLV (Moloney) DNA polymerase. Recently Erickson and Grosch (13) reported apparent differences in the K_i values of poly(U) and its halogenated derivatives for detergent-disrupted virion polymerase activities of avian myeloblastosis virus, Rous sarcoma virus, and feline leukemia virus. Some of these variations may be related to differences in the assay conditions employed, such as the sizes of the templates and inhibitors, and to molecular species contaminating the polymerases in detergent-disrupted virions. The possibility remains, however, that the differences may be real. The cross-species differences are perhaps not unexpected. The DNA polymerases of RNA tumor viruses from different species are known to be different in such other properties as template-cation preference (14) and immunological cross-reactivity (15). The apparent differences now observed with DNA polymerases of the Moloney and Rauscher strains of MLV suggest that intraspecies differences in DNA polymerases may also exist. It may be pertinent that the template RNAs of Moloney and Rauscher strains of MLV reportedly show about 60% homology (16).

2′-*O*-Methylation of poly(I) and poly(U) decreases the inhibitory potency of these polyribonucleotides for MLV (Moloney) DNA polymerase. In contrast, 2′-*O*-methylation and 2′-*O*-ethylation of poly(A) increase its inhibitory potency (5). The reasons for these observations are not clear. It is possible that poly(Im) and poly(Um) undergo structural transitions under the assay conditions such that the effective single-stranded concentration is decreased. Poly(Im) is known to aggregate readily under conditions of high salt (17).[2]

Previously we reported that polyribonucleotide inhibitors, dissimilar in base composition to the template, yield kinetics of

[2] S. K. Arya, W. A. Carter, J. L. Alderfer, and P.O.P. Ts'o, unpublished observations.

inhibition which is inconsistent with that expected of simple competitive inhibition with respect to the template. Our current results further substantiate this observation. Given the complexity of the system, simple interpretation of the observed inhibition kinetics may ultimately prove to have been misleading. However, the results are not inconsistent with the suggestion that (a) there may exist polynucleotide-specific binding sites (or collection of subsites) on the polymerase, or (b) the various polynucleotides induce conformational changes in the enzyme which are dependent on the nature of the polynucleotide bound (5, 18). Additional explanations would include a polyribonucleotide (inhibitor) effect on chain initiation and propagation steps in some manner consistent with the observed inhibition kinetics.

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