INHIBITION OF ONCORNAVIRAL DNA POLYMERASES BY 5-MERCAPTO POLYCYTIDYLIC ACID: MODE OF ACTION

P. CHANDRA, U. EBENER and A. GÖTZ
Gustav-Embden-Zentrum der Biologischen Chemie, Abteilung für Molekularbiologie der J. W. Goethe-Universität, Frankfurt, West Germany

Received 25 February 1975

1. Introduction

Single stranded polyribonucleotides are known to act as efficient templates for the viral DNA polymerases in the presence of the complimentary oligodeoxyribonucleotide primer [1–5]. Chemical modification of such templates would be expected to alter the interaction between the template and the viral enzyme [6–10]. This appears to be a very useful approach for designing specific inhibitors of viral DNA polymerases, which might find application in the chemotherapy of cancer [11].

In a preliminary communication [6], we reported the inhibitory effect of a partially thiolated polycytidylic acid (MPC) on the DNA-polymerases from RNA tumor viruses. The initial results indicated that chemically modified polynucleotide template analogues may serve as effective and potentially selective inhibitors of DNA synthesis in the presence of various templates. It was suggested [8] that the inhibitory effects of modified polynucleotides may depend upon their structural similarities to the functional templates with which they presumably compete for their binding site(s) on polymerases. The present communication describes the mode of action of MPC in the DNA-polymerase reaction of Friend Leukemia virions (FLV).

2. Materials and methods

Labelled deoxynucleoside triphosphates were obtained from NEN-Chemicals GmbH, Germany, unlabelled deoxynucleoside triphosphates from Calbiochem, or Boehringer Mannheim, Tutzing. Polycytidylic acid (PC) was supplied by Miles Laboratories, Elkhart, Indiana, U.S.A., and oligo (dG) by Collaborative Research, Massachussetts.

Thiolated polycytidylic acid (MPC): The partially thiolated polycytidylic acid was prepared by the method described elsewhere [8]. The procedure consisted of the following two reaction steps: 1. Treatment of the polynucleotide with methyl hypobromite (MeOBr) in methanol (0°C, 1.5 hr, stirring), followed by evaporation of the reaction mixture in vacuo to dryness; and 2. subsequent reaction with sodium hydrosulfide (NaSH) in dimethyl acetamide (0°C, 1.5 hr, stirring under nitrogen atmosphere), followed by precipitation of the thiolated product with 3 M NaCl. Before treatment with these reagents, the polynucleotides were converted to their organic solvent soluble cetyl-trimethylammonium salts [12,13], and, after thiolation, they were reconverted to their corresponding sodium salts by application of the procedure of Jones [13]. The thiolated product was gel filtered through Sephadex G-25 and purified on agarose column (Bio-Gel A-1.5 m, purchased from Bio-Rad Laboratories, Munich, Germany). The MPC preparation employed in our present studies had 15% of the total cytosine bases converted to 5-SH derivative, measured spectrophotometrically [8].

Virion-associated DNA polymerases: Spleens from FLV-infected mice (AKR-strain, sacrificed on the 14th day after infection) were used to isolate virions. The detailed procedure for isolation and purification of virion-associated DNA polymerase has been described earlier [6]. The virion-associated DNA-polymerase activity was measured by [^{3}H] methyl-dTMP, or [^{3}H] dGMP incorporation into DNA.

North-Holland Publishing Company – Amsterdam

All reaction mixtures, regardless of the template, contained in 0.25 ml: 40 mM Tris–HCl (pH 7.8); 2 mM DTT; 0.1 mM each of unlabelled dATP, dGTP and dCTP; 1 μCi (0.02 mM) dTTP-[^{3}H] methyl; 2 mM manganese acetate, the indicated amounts of templates; and Nonidet P-40 (Shell Chemicals), at 0.2% final concentration. In experiments with oligo (dG) · poly (rC) and oligo (dG) · (MPC), [^{3}H] dGTP was used as the labelled precursor.

Product analysis of the DNA-polymerase reaction: The product analysis of DNA-polymerase reaction in the absence, or in the presence of MPC was carried out, as described earlier [15]. The reaction mixtures were dissolved with sodium-dodecyl sulfate (1%, w/w, final concentration), loaded on a hydroxylapatite column (1 g, Bio.Rad Lab., München), eluted with a sodium-phosphate gradient (0.05–0.4 M), collected into about 40 tubes (total vol. 100 ml) and the TCA insoluble radioactivity was collected on GF/C filters from Whatman, and counted in a liquid scintillation counter.

The protein content of enzyme preparations was determined by the method of Lowry et al. [16] with crystalline bovine serum albumin as standard.

3. Results and discussion

Viral DNA polymerases require a primer molecule, with a free 3-OH end for copying of single-stranded regions of templates to from the double-stranded product. The two synthetic template-primers that appear to be relatively specific for oncornaviral reverse transcriptases are oligo (dG) · poly (rC) and oligo (dT) · poly (rA), see review by Sarin and Gallo [17]. The template activities of oligo (dG) · poly (rC) and oligo (dG) · (MPC), i.e. 1:1 hybrid of oligo (dG) and mercapto-polycytidylic acid (MPC), for FLV DNA-polymerase system are shown in table 1.

As follows from table 1 the incorporation of [^{3}H] dGMP into DNA by the viral enzyme is stimulated 3 to 9-fold in the presence of oligo (dG) · poly (rC). However, under similar experimental conditions oligo (dG) · (MPC) fails to stimulate the [^{3}H] dGMP incorporation into DNA. The small response, observed at higher concentrations may be due to fact, that the thiolation of cytosine bases in the polymer is a random process, and some long stretches of unmodified cytosine bases may be used up by the enzyme. This interpretation is favored by the nearest frequency analysis of ^{35}S-labelled MPC (Dr Ho, unpublished results), and by our earlier results [6] where the inhibitory activity of MPC on viral DNA-polymerases was found to be directly related to the percent of thiolation.

The effect of oligo (dG) · (MPC) on the kinetic of the enzyme reaction at various concentrations of oligo (dG) · poly (rC) is shown in table 2. The addition of 1 to 4 μg of oligo (dG) · poly (rC) in the reaction mixture leads to a slight increase of [^{3}H] dGMP

Table 1
Template activities of oligo (dG) · poly (rC) and oligo (dG) · (MPC) in DNA-polymerase system from Friend Leukemia Virions (FLV)

Template used	Concentration (μg/reaction mixt.)	[^{3}H]dGMP incorporation into DNA c.p.m./reaction mixt.	% of Control
None (Endogenic)	–	856	100
Oligo (dG) · poly (rC)	1	3157	369
	2	5791	676
	4	7637	891
Oligo (dG) · (MPC)	1	953	111
	2	1228	143
	4	1363	159

Reactions were performed under standard conditions as detained in Methods. MPC is a partially thiolated polycytidylic acid containing 15% 5-mercaptocytidylate units.

Table 2
Oligo (dG) · poly (rC) - directed activity of DNA polymerase from FLV in the presence of oligo (dG) · (MPC)

Oligo (dG) · poly (rC) (μg/reaction mixt.)	Oligo (dG) · (MPC) (4 μg/reaction mixt.)	[^{3}H] dGMP incorporation into DNA c.p.m./reaction mixt.	% of Control
None (Endogenic)	–	903	100
None	+	1270	141
1	+	1310	146
2	+	1450	161
4	+	1670	185

Reactions were performed under standard reaction conditions as detailed in Methods. MPC is a partially thiolated polycytidylic acid containig 15% 5-mercaptocytidylic acid units.

incorporation, however the magnitude of stimulation exhibited by oligo (dG) · poly (rC) in the absence of oligo (dG) · (MPC), as shown in table 1, is not seen here. This indicates that the enzyme may have a higher binding affinity to the primer-template oligo (dG) · (MPC).

The data presented in table 3 show that the effect of oligo (dG) · (MPC) on the template functions of unmodified duplex, oligo (dG) · poly (rC), can be influenced at higher enzyme concentrations. Thus, at 56 μg protein enzyme, the incorporation of [^{3}H] dGMP into DNA is more than doubled, and at 84 μg protein enzyme it is more than 3 times to that obtained at 28 μg protein concentration of the enzyme. This would mean, that at these concentrations some of the free enzyme is available which is able to use up the functional primer-template.

The data presented in table 3 show that MPC acts by interacting directly with the enzyme. This has been confirmed by the ultracentrifugation studies in which the binding of ^{35}S-labelled MPC to a purified enzyme fraction was investigated (manuscript in

Table 3
Effect of oligo (dG) · (MPC) on the template activity of oligo (dG) · poly (rC) in the DNA-polymerase system from FLV at various enzyme concentrations

Template(s) used (4 μg/reaction mixt.)	Enzyme concentration μg viral protein per reaction mixt.	[^{3}H] dGMP incorporation into DNA c.p.m./reaction mixt.	% of Control
None (Endogenic)	14	489	100
Oligo (dG) · poly (rC)	14	1664	340
	28	3350	681
	56	6809	1390
	84	8820	1800
Oligo (dG) · poly (rC)	14	564	115
+	28	647	132.5
Oligo (dG) · (MPC)	56	1382	281
	84	2247	460

Reactions were performed under standard reaction conditions as detailed in Methods. MPC is a partially thiolated polycytidylic acid containing 15% 5-mercaptocytidylate units.

preparation). In the presence of enzyme fraction the peak of the radioactivity was partly shifted towards heavier fractions, showing that a part of MPC is bound to the enzyme.

Analysis of the endogenous products of the detergent disrupted virions exhibits 3 DNA species: single stranded DNA (ss-DNA), RNA–DNA hybrids (hy-DNA) and the double stranded DNA (ds-DNA). It has been shown earlier [15,18] that compounds which bind to hy-DNA selectively inhibit the formation of ds-DNA species in the viral polymerase system. The analysis of the endogenous products of DNA-polymerase reaction of FLV inhibited by MPC is shown in fig.1. These studies were carried out using [^{3}H] dTTP, since under endogenous conditions the dTMP incorporation into DNA is better.

As follows from fig.1, in the presence of MPC

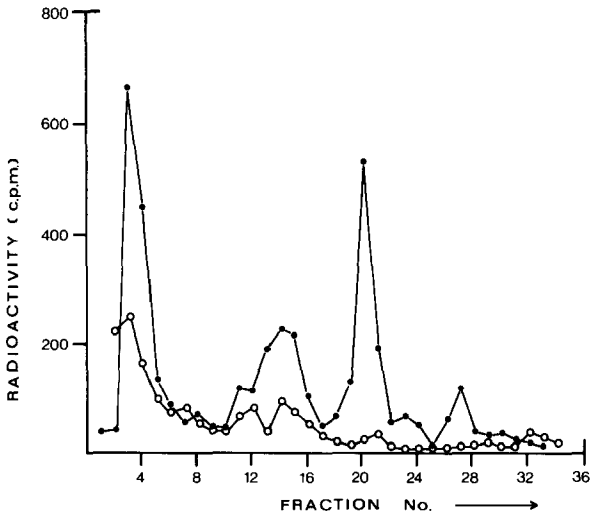

Fig.1. Analysis of the DNA species synthesized by FLV-DNA polymerase by elution from hydroxylapatite column. Each column was filled with 1 g of hydroxyapatite and carefully washed with 0.05 M sodium phosphate (approx. 50 ml.). The columns were loaded with the reaction products, as described under Materials and methods. The columns were washed with 0.05 M sodium phosphate buffer, pH 6.8, until equilibrium reached. Macromolecules were eluted from the column by a linear gradient of sodium phosphate (0.05–0.4 M). The first species to be eluted from the column contained ss-DNA, the second contained hy-DNA and finally, the ds-DNA, eluted in the last species. (●——●) DNA species synthesized in the absence of MPC. (o——o) DNA species synthesized in the presence of MPC. (20 μg/reaction mixture)

there is an over-all inhibition of [^{3}H] dTMP incorporation, indicating that the formation of all the 3 DNA species is blocked. This is to be expected since the inhibitor binds to the enzyme. However, this experiment does not rule out the possibility that some heteropolymeric regions of the functional endogenous template might undergo hydrogen-bonding with the added inhibitor. This would mean a bifunctional mode of MPC activity in the viral DNA-polymerase reaction.

In view of the fact that all the oncornalviral DNA polymerases examined so far do require a primer-template like double-stranded secondary structure for the initiation of DNA synthesis, it is no surprise that single-stranded synthetic polynucleotides (unprimed templates) can act as inhibitors of the polymerization reaction [19]. This, presumably, is due to hydrogen bonding of the base sequences between the added polymer and the functional template. Thus, the specificity of inhibition by such polymers is not limited to the viral enzyme system only. On the other hand, minor modifications in the chemical composition of synthetic polynucleotides might be useful to develop inhibitors, which interact directly with the DNA-polymerase. Of more practical value will be such polymers which though interact with the enzyme, but fail to be transcribed, i.e. they function as 'dead template' for the enzyme. The experimental data presented here indicate that the partially thiolated poly C (MPC) is functioning as a dead template in the DNA-polymerase system of FLV. It is interesting that though MPC acts as dead template in the DNA-polymerase system, its messenger coding properties in cell-free protein-synthesizing systems are similar to those of polycytidylic acid (Vazquez and Chandra, unpublished results). Synthesis of such template analogues may lead to the development of more selective inhibitors of reverse transcriptases of oncornaviruses.

Acknowledgement

One of us (P.C.) is grateful to Dr Robert C. Gallo and Dr P.S. Sarin both from the department of Tumor Cell Biology at National Cancer Institute, Bethesda for a very useful discussion in the initial phase of this study.

References

[1] Goodman, N. C. and Spiegelman, S. (1971) Proc. Natl. Acad. Sci., U.S. 68, 2203.

[2] Robert, M. S., Smith, R. G., Gallo, R. C., Sarin, P. S. and Abrell, J. W. (1972) Science 176, 798.

[3] Sarin, P. S. and Gallo, R. C. (1973) J. Int. Commun. Sys. 1, 52.

[4] Baltimore, D. and Smoler, D. (1971) Proc. Natl. Acad. Sci., U.S. 68, 1507.

[5] Wells, R. D., Fulgel, R. M., Larson, J. E., Schendel, P. F. and Sweet, R. W. (1972) Biochemistry 11, 621.

[6] Chandra, P. and Bardos, T. J. (1972) Res. Comm. Chem. Pathol. Pharm. 4, 615.

[7] Chandra, P. in: Biochemical Trends in Human Leukemia (R. Neth, ed.) J. F. Lehmanns Verlag, Munich, in press.

[8] Chandra, P., Barbos, T. J., Chakrabarti, P., Ebener, U. Ho. Y., Mikulski, A. J. and Zsindely, A. Cancer Res., submitted.

[9] Pitha, P. M., Teich, N. M., Lowry, D. R. and Pitha, J. (1973) in: DNA synthesis in vitro, (R. D. Wells and R. B. Inman, eds.) p. 369. MTP-Lancaster.

[10] Erickson, R. J. and Grosch, J. C. (1974) Biochemistry 13, 1987.

[11] Chandra, P. (1974) in: Current concepts in Chem. Res. 52, in press.

[12] Aubel-Sadron, G., Beck, G. and Ebel, J. P. (1961) Biochim. Biophys Acta 53, 111.

[13] Jones, A. S. (1963) Nature (Lond.) 199, 280.

[14] Wu, A. and Gallo, R. C. Biochim. Biophys Acta, in press.

[15] Chandra, P., Will, G., Gericke, D. and Götz, A. Biochem. Pharmacol., in press.

[16] Lowry, O. H., Rosebrough, N. I., Farr, A. L. and Randell, R. J. (1951) 193, 265.

[17] Sarin, P. S. and Gallo, R. C. in: Int. Rev. of Science, Vol. 6, Chapter 8, (K. Burton, ed.) Butterworth and MTP, Oxford, in press.

[18] Kotler, M. and Becker, Y. (1972) FEBS Lett. 22, 222.

[19] Tumominen, R. W. and Kenny, F. T. (1971) Proc. Natl. Acad. Sci., U.S. 68, 2198.