Inhibition of RNA-Dependent DNA Polymerase of Avian Myeloblastosis Virus by Pyran Copolymer

(RNA tumor viruses/polymerase inhibitors)

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ABSTRACT Pyran copolymer, a known immunostimulant, was found to be a potent inhibitor of purified DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase; EC 2.7.7.7) isolated from avian myeloblastosis virus. Unlike other inhibitors, pyran showed unique features of inhibition. It interacts with the polymerase at a region other than the template site. The inhibitory effect was overcome only by excess enzyme and not affected by excess template. The degree of inhibition was not template specific for the templates tested: 70S RNA from avian myeloblastosis virus, synthetic hybrid poly(rA)-oligo(dT),ë, synthetic copolymer poly(dA-dT), and activated calf-thymus DNA. The observed rate of inhibition by pyran was shown to vary with the different polymerases tested. Inhibition was shown with all oncarnoviral polymerases and, to a lesser extent, with mammalian polymerases. However, two of the three bacterial polymerases, by contrast, showed a marked activation.

The current investigations were initiated with the intention of obtaining a specific inhibitor of DNA polymerase (deoxynucleosidetriphosphate:DNA deoxynucleotidyltransferase; EC 2.7.7.7) of RNA tumor viruses. Such an inhibitor might be of major use in biological experiments. Based on the assumption that the synthesis of DNA with sequences that are potentially oncogenic may be catalyzed by this enzyme, specific inhibitors may also be of practical interest. A great variety of compounds have been tested, of which a large number have inhibitory activity (1–6). Although in many cases the mechanism of action is not known, most of the compounds interfere with the binding of the template to the enzyme (7, 8), either by direct interaction with the template or by competing with the template for the enzyme (6).

Single-stranded homopolymers of ribonucleotides are reported to be inhibitory to viral RNA-dependent DNA polymerase (6, 7). This observation suggests to us that it may be possible to design molecules that will possess some relationship to the template of the reaction.

The active compounds studied are pyran copolymers, the structure of which is shown in Scheme I. It is a copolymer of approximate molecular weight of 17,000. It is a divinyl ether of maleic anhydride which, in the presence of H2O, becomes hydrolyzed to the corresponding polycarboxylic acids. The compound is stable to chemical and enzymatic hydrolysis; it cannot serve as a template in the polymerase reaction. The highly negative charges mimic the negative charges of the nucleotide templates.

Abbreviations: AMV, avian myeloblastosis virus; RLV, Rauscher leukemia virus; PK15, pig kidney C-type particle.

In this report we describe the mechanism of action of pyran on the purified RNA-dependent DNA polymerase from avian myeloblastosis virus (AMV) obtained by the procedure of Kacian et al. (9).

MATERIALS AND METHODS

Reagents. Unlabeled triphosphates and deoxytriphosphates were purchased from P-L Biochemicals. Tritiated nucleoside triphosphates and deoxytriphosphates were obtained from Schwarz/Mann Bioresarch. Specific activities (Ci/mmol) were: [3H]dTTTP, 17.3; [3H]dATP, 20.9; [3H]dCTP, 30; [3H]dGTP, 20; [3H]UTP, 12. Whatman phosphocellulose, P-11, was from H. Reeve Angel, Inc., Clifton, N.J. Calf-thymus DNA and DNase I were from Worthington Biochemicals Corp., Freehold, N.J. Nonionic detergent, Nonidet P-40 (NP-40), was obtained from Shell Chemical Co.

Pyran Source. The pyran copolymers of various viscosities (indirect estimation of molecular weight) were kindly supplied by Dr. David S. Breslow, Hercules Research Center, Wilmington, Del.

Viruses. AMV was obtained from chicken plasma from infected chicks and provided by Dr. J. Beard through contract N01CP33291 within The Virus Cancer Program of the National Cancer Institute. Cell debris was removed by centrifugation at 600 X g followed by a 30-min centrifugation at 5000 X g. The virus was further concentrated and purified as described by Riman et al. (10).

Rauscher leukemia virus (RLV) was obtained from the office of Program Resources and Logistics of Virus Cancer Program, NCI.

Pig kidney C-type particle (PK15) was isolated from a chronically infected pig-kidney cell line as described by Woods.

Abbreviations: AMV, avian myeloblastosis virus; RLV, Rauscher leukemia virus; PK15, pig kidney C-type particle.

Scheme I.

FIG. 1. Effect of pyran on DNA synthesis directed by poly-(dA-dT). DNA polymerase assays were carried out in 0.1 ml of standard reaction mixture composed of 50 mM Tris-HCl buffer pH 8.3, 6 mM MgCl₂, 40 mM KCl, 6 mM dithiothreitol, 0.125 mM [³H]TTP (300 cpm/pmol), and 10 µg of purified AMV DNA polymerase.

et al. (11). Reptilian C-type particles were isolated from supernatant fluid of chronically infected viper cell line VSW (12).

Enzymes. RNA-dependent DNA polymerase from avian myeloblastosis virus was isolated and purified as described by Kacian et al. (9). The purified fraction shows two distinct bands on sodium dodecyl sulfate disk-gel electrophoresis, representing the two nonidentical subunits. Purified enzyme was used for subsequent studies. Assay conditions are those of Kacian et al. (9).

DNA-dependent RNA polymerase from Escherichia coli was purified by the procedure of Berg et al. (13) and was a gift from Dr. Robert Lazzarini. Assay conditions are those of Berg.

DNA-dependent DNA polymerase from E. coli was purchased from Biopolymers Laboratory, Dover, N.J. and was assayed as described by Richardson et al. (14).

DNA-dependent DNA polymerase from Micrococcus luteus was purchased from P-L Biochemicals and was assayed by procedure of Bollum (15).

RNA-dependent DNA polymerase from reptilian C-type particle was isolated and partially purified by chromatography on phosphocellulose (16). Material containing peak activity was used for experiments described below. Assay conditions used are the same as those of AMV DNA polymerase.

DNA polymerases I and II from normal human-blood lymphocytes were kindly donated by Drs. R. G. Smith and R. C. Gallo. Assay conditions are those of Smith (26).

Assay of RNA-Dependent DNA Polymerases from RLV and PK15 C-Type Particle. Conditions for assay are essentially the same as those for A-particle (17). The reaction mixture contained, in a final volume of 25 µl: 50 mM Tris-HCl (pH 8.3), 25% (v/v) glycerol, 370 µg/ml of bovine-serum albumin, 75 mM KCl, 0.5 mM MnCl₂, 1 mM dithiothreitol, 0.33% (v/v) Nonidet P-40, 0.5 mM ATP, 100 µg/ml of poly(dA-dT), enzyme as shown in the legend of Table 4, and 0.125 mM [³H]TTP (80–300 cpm/pmol). The reaction mixture was incubated at 37°C for 45 min; it was stopped by transfer of the tubes to a 0–4°C waterbath and addition of 100 µl of a solution containing 2 µg/ml of bovine-serum albumin, 2 µg/ml of yeast RNA, and 100 mM pyrophosphate followed by 1 ml of ice-cold 10% trichloroacetic acid. After 10 min at 0°C, acid-insoluble material was collected on a Whatman GF/C filter. The filters were washed with 10% trichloroacetic acid, and radioactivity was determined by scintillation counting.

Templates. Poly(rA)·(dT)₁₀, poly(dA-dT), oligo(dT)₁₀ were purchased from Collaborative Research, Inc. Native calf-thymus DNA was activated by a mild deoxyribonuclease treatment as described by Schlabach et al. (18). High-molecular-weight 70S RNA from AMV was extracted by the procedure of Stephenson et al. (19), and layered onto linear sucrose gradients of 5–30% (w/w) in STE buffer [0.1 M NaCl, 0.01 M Tris-HCl (pH 7.3), and 1 mM EDTA]. Centrifugation was performed for 3 hr at 41,000 rpm in a Spincero SW41 rotor. Fractions were collected from the bottom, and material was precipitated with ethanol. External markers used were 28S and 18S [³H]RNA from NC-37 cells (a generous gift of Dr. J. Schom, Columbia University, Institute of Cancer Research, New York).

RESULTS

Inhibition of AMV DNA Polymerase Activity by Pyran Copolymers of Various Viscosities. Pyran copolymers of various viscosities were tested for their ability to inhibit DNA synthesis of AMV DNA polymerase, in an effort to determine whether a correlation exists between viscosity and polymerase inhibitory activity. Although such correlation is not complete (Table 1), high-viscosity compounds showed greater inhibition. All subsequent studies were carried out with compounds that possess maximal inhibitory activity, the preparation that gave viscosity of 0.425 centipoises.

Effect of Pyran on DNA Synthesis Directed By Poly(dA-dT). Increasing concentration of pyran was followed by an increase in inhibition (Fig. 1). 50% inhibition was obtained at a concentration of 25 µg/ml.

Effect of Concentration of Synthetic Templates on the Inhibition of DNA Polymerase. In order to determine the nature of inhibition, template poly(dA-dT) was varied at two different concentrations of the inhibitor. The double-reciprocal plots were linear, constituting a family of converging lines intersecting on the horizontal axis (Fig. 2). This result indicates (a) that the inhibitor interacts with the enzyme but not with the template, and (b) the inhibitor does not affect the binding of the template to the enzyme.

TABLE 1. Inhibition of DNA polymerase activity from AMV by pyran copolymers of various viscosities

<table>
<thead>
<tr>
<th>Viscosity in centipoises</th>
<th>% Inhibition 50 µg/ml</th>
<th>% Inhibition 100 µg/ml</th>
</tr>
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<tbody>
<tr>
<td>0.080</td>
<td>19</td>
<td>38</td>
</tr>
<tr>
<td>0.120</td>
<td>32</td>
<td>35</td>
</tr>
<tr>
<td>0.210</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>0.276</td>
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<td>0.375</td>
<td>36</td>
<td>60</td>
</tr>
<tr>
<td>0.425</td>
<td>55</td>
<td>78</td>
</tr>
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</table>
Fig. 2. Effect of concentration of poly(dA-dT) template on the inhibition of polymerase. Lineweaver-Burk plot with poly(dA-dT) as the variable substrate. Reaction conditions are the same as in Fig. 1. (○) No inhibitor; (△) 30 μg/ml of pyran; (□) 100 μg/ml of pyran.

Effect of Excess AMV DNA Polymerase or Poly(dA-dT) on DNA Synthesis in the Presence of Pyran. To further verify that the inhibitor interacts with the enzyme rather than with the template, excess enzyme was added (10-fold increase in concentration). The inhibition was completely overcome (Table 2). In contrast, increasing the concentration of poly(dA-dT) template 10-fold failed to abolish the inhibition. Thus, the observed inhibition by pyran was due to interaction with the enzyme rather than with the template.

Effect of Pyran on DNA Synthesis with Different Templates. The inhibitory activity of pyran was tested with various natural and synthetic templates measuring DNA synthesis on RNA or DNA templates. (Table 3). The degree of inhibition was the same regardless of template used. This further strengthens the argument that the site of inhibition was the polymerase molecule and not the template.

**Table 2. Effect of excess AMV DNA polymerase or poly(dA-dT) on DNA synthesis in the presence of pyran**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>cpm</th>
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<tbody>
<tr>
<td>Complete</td>
<td>5300</td>
</tr>
<tr>
<td>+ pyran (100 μg/ml)</td>
<td>700</td>
</tr>
<tr>
<td>+ pyran (100 μg/ml) + 10×† AMV DNA polymerase</td>
<td>5610</td>
</tr>
<tr>
<td>+ pyran (100 μg/ml) + 10×† poly(dA-dT)</td>
<td>756</td>
</tr>
</tbody>
</table>

*[^H]TMP incorporation into acid-precipitable counts. Reaction mixture had the same composition described in Methods. Incubation time was 30 min with 6 μg/ml of AMV DNA polymerase and 0.4 A unit of poly(dA-dT).
†10× indicates a 10-fold increase in enzyme or substrate concentration.

Effect of Pyran on DNA Synthesis As a Function of Length of Incubation. The aforementioned studies indicated that template and pyran interact at different sites on the polymerase molecule. The question was now asked whether the enzyme can be protected from this inhibition by the substrates of the reaction. In these experiments, pyran was added either at 0 time or at 10 min after the initiation of the polymerization reaction. Results shown in Fig. 3 demonstrate that addition of pyran at 0 time or 10 min after the start of DNA synthesis resulted in an immediate inhibition of the polymerase. It is suggested from this data that pyran appears to interact at a site other than the catalytic site since the presence of the substrates did not protect the catalytic site. The classical noncompetitive nature of inhibition (Fig. 2) and the immediate cessation of the polymerization reaction (Fig. 3B) suggested that the inhibitor interfered with the initiation or elongation phase of the polymerization process rather than with initial binding of template to enzyme.

Effect of Pyran on DNA Polymerases. To determine whether pyran shows any specific inhibition with respect to other polymerases, we tested the effect of pyran on a variety of polymerases (Table 4). Four of these are RNA-dependent DNA polymerases from diverse origin: avian, murine, reptilian, and porcine; three are bacterial polymerases; and

**Table 3. Inhibition of AMV DNA polymerase by different templates**

<table>
<thead>
<tr>
<th>Template</th>
<th>% Inhibition</th>
</tr>
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<tbody>
<tr>
<td>AMV 70S RNA</td>
<td>86</td>
</tr>
<tr>
<td>AMV 70S RNA + oligo(dT)</td>
<td>80</td>
</tr>
<tr>
<td>Activated DNA</td>
<td>82</td>
</tr>
<tr>
<td>poly(dA-dT)</td>
<td>87</td>
</tr>
<tr>
<td>poly(α)-oligo(dT)</td>
<td>87</td>
</tr>
</tbody>
</table>

*Assays were performed as described in legend of Fig. 1, except that each assay (0.1 ml) contained 2 μg of the stated template. In all cases [H]TTP was used. With 70S RNA and activated DNA, the other three unlabeled deoxynucleoside triphosphates were added.
Pyran on RNA-dependent DNA polymerase is different from that of synthetic polynucleotides. Thus, pyran inhibits the reaction by not competing directly for the template site on the enzyme.

Pyran copolymer offers some similarities to nucleic acid templates by being polymeric and possessing a highly negative charge, with the added feature of not being able to serve as template for transcription or translation. Furthermore, it cannot be hydrolyzed by cellular degradative enzymes, as is the case of most polynucleotides used as inhibitors.

It has recently been reported (7) that analogs of polynucleotides can interfere effectively with virus replication in tissue culture and with the activity of viral RNA-directed DNA polymerase in vitro. Macromolecules such as these, as well as pyran, are taken into the cells through pinocytosis rather than by simple diffusion through the cell membrane. Since high pinocytotic activity appears to be characteristic of many tumor cells, macromolecules could be expected to be taken up preferentially by some type of neoplastic cells (24, 25).