Inhibition of Mammalian and Oncornavirus Nucleic Acid Polymerase Activities by Alkoxybenzophenanthridine Alkaloids

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SUMMARY

The alkoxybenzophenanthridine alkaloids (coralyne acetosulfate, fagaronine chloride, and nitidine chloride) have been reported to possess antileukemic activity in mice. These compounds were tested for inhibition of reverse transcriptase activity of an RNA tumor virus and DNA polymerase, RNA polymerase, and polyadenylic acid polymerase activities of NIH-Swiss mouse embryos. Reverse transcriptase and DNA polymerase activities were strongly inhibited by these antileukemic alkaloids, whereas RNA polymerase and polyadenylic acid polymerase activities were only moderately affected. Viral and cellular DNA polymerase activities were potently diminished by the alkaloids when poly(d(A-T)), poly(dA)-oligo(dT), and poly(rA)-oligo(dT) template primers were used in the reaction mixture; however, no inhibition of enzyme activity was obtained with poly(rC)-oligo(dG) as template primer. These results suggest that alkoxybenzophenanthridine alkaloids inhibit DNA polymerase activity by interaction with A:T base pairs of the template primer.

INTRODUCTION

Nucleocapsids of RNA tumor viruses contain RNA-directed DNA polymerase (reverse transcriptase) activity that catalyzes the formation of DNA from an RNA template (13, 18, 20). This enzyme is involved in viral DNA (provirus) formation (7, 13, 18, 20) which, on integration into the host genome, can lead to cell transformation (13, 18, 20). Besides viral reverse transcriptase, other host enzymes and factors may also be involved in provirus formation (13, 18, 20). Host DNA-directed RNA polymerase is involved in formation of viral genome (2, 12, 14). Viral RNA at its 3' end contains poly(A) sequences (4, 5, 8), and host poly(A) polymerase activity is probably responsible for the addition of these sequences. If RNA tumor viruses are involved in human neoplasia (3, 17), viral and host nucleic acid-polymerizing enzymes become important for oncogenesis. Specific inhibitors of these enzyme activities might, therefore, be useful prophylactic agents in cancer.

With a view to establish in vitro test systems for potential antitumor activity, we are examining synthetic compounds and natural products for the inhibition of virus transformation of normal cultured cells, viral and cellular replication, and transcription enzymes (11, 15).

A number of alkoxybenzophenanthridine alkaloids exhibit interesting biological and pharmacological activities. Among these, fagaronine chloride (NSC 157995), isolated from Fagara zanthoxyloides, has shown good activity against P388 leukemia in mice (19). Nitidine chloride (NSC 146397) isolated from Fagara macrophylla (23), is highly cytotoxic and has shown activity in L1210 and P388 leukemias and Lewis lung carcinomas (16, 23, 25). Coralyne acetosulfate (NSC 154890) has also displayed inhibitory activity against both L1210 and P388 leukemias in mice (25). This report describes the inhibition of mammalian and oncornavirus nucleic acid-polymerizing enzyme activities by these alkoxybenzophenanthridine alkaloids.

MATERIALS AND METHODS

Reagents and Templates

Tritium-labeled nucleotides were obtained from Schwarz/Mann, Orangeburg, N. Y. Unlabeled nucleotides were purchased from Worthington Biochemical Corp., Freehold, N. J., or from P-L Biochemicals, Milwaukee, Wis. Radioactive nucleotides were evaporated to dryness under vacuum to remove ethanol and dissolved in water. The specific activity of each nucleotide was adjusted to the desired level by the addition of unlabeled nucleotide. Phosphocreatine (disodium), creatine phosphokinase, and calf liver tRNA, type IV, were purchased from Sigma Chemical Co., St. Louis, Mo. Calf thymus DNA and DNase I were obtained from Worthington. Crystalline bovine serum albumin and poly(A) were from Miles Laboratories, Elkhart, Ind. Poly(dA)-oligo(dT), poly(rA)-oligo(dT), and poly(d(A-T)) were purchased from P-L Biochemicals. Poly(rC)-oligo(dG) was obtained from Schwarz/Mann. DEAE-cellulose filter discs (Whatman DE 81) were obtained from H. Reeve Angel & Co., Inc., Clifton, N. J. Polyethylene glycol 6000 was purchased from Schwarz/Mann. All other reagents were of analytical grade.

All templates were dissolved in 0.01 M Tris-HCl (pH 7.0), 0.1 mM EDTA, and 0.1 mM NaCl. Denatured calf thymus DNA...
was prepared by heating the DNA in a boiling water bath for 15 min followed by quick chilling in ice water. Activated calf thymus DNA was prepared by incubating 6 mg DNA in 10 ml buffer containing 0.05 M Tris-HCl (pH 7.0), 5 mM MgCl₂, and bovine serum albumin (500 μg/ml) with 0.01 μg crystalline pancreatic DNase for 15 min at 37°C. DNase was inactivated by incubating at 70°C for 5 min. All templates were stored at 4°C.

**Enzyme Assays**

**RNA Polymerase.** The assay mixture of 100 μl contained 5 μmoles Tris-HCl (pH 8.0); 25 nmoles each ATP, CTP, and GTP; 2.5 nmoles [³H]UTP (200 to 300 cpm/pmol); 5 μg phosphocreatine kinase; 0.4 μmole creatine phosphate; 25 μg bovine serum albumin; 0.2 pmole MnCl₂; 0.2 μmole M₃Cl₇; 0.1 μmole NaF; 0.1 μmole dithiothreitol; 10% (v/v) glycerol; 15 μg denatured calf thymus DNA; and the enzyme fraction. The reaction mixture was incubated at 37°C for 30 min.

**DNA Polymerase.** The assay mixture of 100 μl contained 100 μmoles sodium glycinate (pH 8.8); 10 nmoles each dATP, dCTP, and dGTP; 2 nmoles [³H]ATP (200 to 300 cpm/pmol); 1 μmole MgCl₂; 0.1 μmole NaF; 10% (v/v) glycerol; 15 μg activated thymus DNA; and enzyme. The reaction mixture was incubated for 30 min at 37°C.

**Poly(A) Polymerase.** The assay mixture of 100 μl contained 10 μmoles Tris-HCl (pH 8.0), 0.1 μmole MnCl₂, 0.4 μmole dithiothreitol, 0.5 μmole sodium fluoride, 0.4 μmole phosphocreatine, 5 μg phosphocreatine kinase, 25 nmoles [³H]ATP (20 to 40 cpm/pmol), 200 μg tRNA, 10% (v/v) glycerol, 25 μg bovine serum albumin, and enzyme fraction. The reaction mixture was incubated at 37°C for 60 min.

**Viral DNA Polymerase (Reverse Transcriptase).** The assay mixture of 100 μl contained 5 μmoles Tris-HCl (pH 7.3), 8 μmoles KCl, 0.1 μmole MnCl₂, 0.5 μmole dithiothreitol, 2 nmoles [³H]UTP (300 to 400 cpm/pmol), 2 μg poly(rA)-oligo(dT), 20 μg bovine serum albumin, 10% (v/v) glycerol, and enzyme fraction. The reaction mixture was incubated at 37°C for 30 min.

**Termination of Enzyme Reactions and Determination of Activity.**

Enzyme reactions were terminated by chilling in ice and by the addition of 25 μl 0.1 M EDTA. One hundred μl of each reaction mixture were spotted uniformly onto a 2.5-cm circular Whatman DE-81 filter, kept at room temperature for 15 min and washed batchwise by swirling 4 times in 10 ml of 5% Na₂HPO₄/7 H₂O per filter, followed by 2 washings each of water and 95% ethanol. Filters were dried, and radioactivity was counted in a toluene-based scintillation fluid.

**Determination of Protein and Nucleic Acid.**

Protein content was determined by the method of Lowry et al. (10) using crystalline bovine serum albumin as a standard. Protein in Tris-HCl buffer or in salt solution was precipitated with 5% trichloroacetic acid, centrifuged to a pellet, and dissolved in the alkali reagent for protein determination. Nucleic acid concentrations were determined spectrophotometrically.

**Purification of Enzyme Activities**

**Reverse Transcriptase from Oncornaviruses.** SSV-1, derived from tissue culture fluids of chronically SSV-1-infected NC-37 cells, was obtained from Pfizer, Inc., Maywood, N. J. Chicken plasma containing AMV was obtained from Life Sciences Research Laboratories, St. Petersburg, Fla., through the courtesy of Dr. J. Beard. Viral DNA polymerase was purified by a modification of the procedure of Abrell and Gallo (1). Five mg virus were sedimented by centrifugation, and the pellet was extracted with Buffer A (0.05 M Tris-HCl (pH 7.9), 0.05 M each NaCl and KCl, 0.02 M dithiothreitol, 1 mM EDTA, and 0.25% Triton X-100). The viral extract was applied to a DEAE-cellulose column and eluted with Buffer B (0.05 M Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 20% (v/v) glycerol, 0.025% Triton X-100, and 0.3 M KCl). The eluate was exhaustively dialyzed against Buffer B without KCl and applied to a phosphocellulose column that was previously saturated with bovine serum albumin. Enzyme activity was eluted from the column by a linear KCl gradient. The peak enzyme activity eluted at 0.25 to 0.3 M KCl. Active enzyme fractions were pooled and concentrated by dialysis against Buffer B without KCl, but with 30% polyethylene glycol 6000. The enzyme preparation was stored at −80°C. The amount of protein in the enzyme preparation, due to its very low content, could not be determined. The activities of SSV-1 and AMV DNA polymerases were 4.5 and 6 nmoles dTMP incorporated per 30 min per ml, respectively.

**Purification of Poly(A) Polymerase.** Poly(A) polymerase was purified from the cytoplasmic fraction of 12- to 16-day old NIH-Swiss mouse embryos according to a procedure established in this laboratory (6). In this study, poly(A) polymerase purified by phosphocellulose column chromatography was used. The specific activity of the enzyme was 60 nmoles AMP incorporated per hr per mg protein.

**Purification of RNA and DNA Polymerases.** RNA polymerases I and II from the nuclei of NIH-Swiss mouse embryos were purified by procedures established in our laboratory (14). The specific activity of RNA polymerases I and II purified by DEAE-cellulose column chromatography were 100 pmoles UMP incorporated per 30 min per mg protein and 3 nmoles UMP incorporated per 30 min per mg protein, respectively. DNA polymerases α and β from the cytoplasmic fraction of NIH-Swiss mouse embryo homogenate were purified according to a procedure developed in this laboratory. The specific activities of DNA polymerases α and β purified by phosphocellulose column chromatography were 2.5 and 1.7 nmoles dTMP incorporated per 30 min per mg protein, respectively.

**Testing of the Alkaloids for Inhibition of Enzyme Activities.** Two types of reaction mixture, one containing enzyme, buffer, and bovine serum albumin (Mix 1) and the other having template, substrates, metal ions, dithiothreitol, and glycerol (Mix 2), were made. The final concentrations of the ingredients of the reaction mixture were the same as described.

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3 V. S. Sethi and P. Okano, submitted for publication.
scribed above for enzyme assays. An appropriate concentration of the alkaloid was added to Mix 1 and placed in ice for 10 min before adding Mix 2. Control experiments contained an equivalent amount of dimethylsulfoxide:water (9:1) mixture in which alkaloids were dissolved. The reaction mixtures were incubated at 37°C for the desired time, after which the reactions were terminated and the enzyme activity was determined. Inhibition results were calculated and expressed as percentage of control activity.

The amounts of various enzymes used in each assay mixture were: reverse transcriptase from SSV-1 or AMV, 5 μl; DNA polymerase α, 11.5 μg; DNA polymerase β, 7.5 μg; RNA polymerase I, 80 μg; RNA polymerase II, 20 μg; and poly(A) polymerase, 10 μg.

RESULTS

Inhibition of SSV-1 Reverse Transcriptase, Mouse Embryo DNA Polymerase, RNA Polymerase, and Poly(A) Polymerase Activities by Coralyne, Nitidine, and Fagaronine. Benzophenanthridine alkaloids (Chart 1) were examined for their effects on the enzyme activities of SSV-1 DNA polymerase, mouse embryo cytoplasmic DNA polymerases α and β, mouse embryo mRNA polymerases I and II, and mouse embryo cytoplasmic poly(A) polymerase at increasing concentration of the alkaloids. Viral and cellular DNA polymerase activities were strongly inhibited by coralyne (Chart 2A). Similar results were obtained with nitidine chloride (data not given). The 50% enzyme-inhibitory doses of coralyne and nitidine for DNA polymerase activities were 20 to 25 and 30 to 45 μg/ml, respectively. RNA polymerase activity was inhibited by these alkaloids at higher dose levels, and poly(A) polymerase activity was only moderately
inhibited (Chart 2B). Results similar to those described above were obtained with fagaronine chloride and nitidine chloride (data not shown).

**Mechanism of DNA Polymerase Inhibition by Benzophenanthridine Alkaloids.** In order to elucidate the mechanism of enzyme inhibition, different template primers [poly(rA)-oligo(dT), poly(dA)-oligo(dT), and poly(rC)-oligo(dG)] were used for AMV reverse transcriptase inhibition studies. The enzyme activity with poly(rA)-oligo(dT) and poly(dA)-oligo(dT) was strongly inhibited by coralyne and nitidine, but with poly(rC)-oligo(dG) there was no inhibition (Table 1). These results suggest that inhibition was not due to interaction of the alkaloid with the enzyme protein. Moreover, the inhibition was also not due to interaction or competition with KCl or substrate ([3H]TTP or [3H]dGTP), since these were present in the reaction mixture in large excess relative to the alkaloid. It was also observed that enzyme activity remained unchanged by increasing MnCl₂ in the assay mixture (data not shown), indicating that the metal ions did not interact with the alkaloids. The data of Table 1 further suggested that the alkaloids had less affinity for (rC)ₙ or G:C pairs. The strong inhibition of enzyme activity with poly(rA)-oligo(dT) and poly(dA)-oligo(dT) as template primers suggested that inhibition was due to interaction of the alkaloids with (rA)ₙ, (dT)ₙ, or A:T pairs. Similar results have been reported for fagaronine chloride (15).

When the amount of AMV reverse transcriptase was increased from 5 to 30 µg in the reaction mixture inhibited by coralyne and nitidine, the level of enzyme inhibition remained unaffected (Chart 3). These data support the conclusion that the alkaloids did not interact with the enzyme protein (Table 1). However, when poly(rA)-oligo(dT) concentration was increased from 2 µg to 40 µg/assay mixture, inhibition of reverse transcriptase by coralyne and nitidine was completely overcome (Chart 4). Similar data have been reported for fagaronine chloride (15). Inhibition of the cellular DNA polymerase activity by coralyne and nitidine was also reversed by increase of the excess of poly(dA-T)] (Chart 5). These results further substantiate our previous findings that the benzophenanthridine alkaloids exhibit an affinity for A:T base pairs (15).

In the above-described experiments, the alkaloid was added to the reaction mixture before incubation at 37° and inhibitor effects were directed to free template primer and its complexes with enzyme, substrate, and metal ions. In another situation, where the enzyme molecules were elongating the polynucleotide chain, the effect of addition of coralyne acetosulfate was examined. Addition of coralyne acetosulfate during AMV DNA polymerase reaction at 5, 10, and 20 min stopped [3H]dTMP incorporation instantly, and

### Table 1

**Inhibition of AMV DNA polymerase activity by coralyne and nitidine with different template primers**

<table>
<thead>
<tr>
<th>Template primer</th>
<th>Coralyne acetosulfate</th>
<th>Enzyme activity (pmoles/assay)</th>
<th>Nitidine chloride</th>
<th>Enzyme activity (pmoles/assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(rA)ₙ-(dT)ₙ-x₁-x₂</td>
<td>28.3 (100)</td>
<td>28.3 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rA)ₙ-(dT)ₙ-x₁</td>
<td>0.60 (2.4)</td>
<td>0.90 (3.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(dA)ₙ-(dT)ₙ-x₁-x₂</td>
<td>4.5 (100)</td>
<td>4.5 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rC)ₙ-(dG)ₙ-x₁-x₂</td>
<td>0.25 (5.5)</td>
<td>0.16 (3.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rC)ₙ-(dG)ₙ-x₁</td>
<td>8.55 (100)</td>
<td>8.55 (100)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(rC)ₙ-(dG)ₙ-x₁</td>
<td>11.88 (139)</td>
<td>8.44 (98)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

 airing assay conditions as described in "Materials and Methods," except 5 µg of the template primer were used. Five µg of the alkald per assay were used.

1 Numbers in parentheses, percentage.

2 Standard assay mixture contained 5 µg of the template primer.

3 Five µg of the inhibitor per assay were used.

4 Standard assay mixture contained 5 µg of the template primer and 2.2 nmoles of [3H]dGTP (340 cpm/pmol). Five µg of the alkaloid per assay were used.
Poly d (A-T) (µg/assay)

Chart 5. Effect of increasing concentration of polyd(A-T) on the alkaloid inhibited mouse embryo DNA polymerase I reaction. The standard assay mixture of 0.10 ml contained 16.2 µg of the enzyme and no dCTP and dGTP. Five µg each of coralyne acetosulfate (O) or nitidine chloride (D) per assay were used. The control values at 5, 10, 20, and 30 µg poly[d(A-T)] per assay were 8.8, 7.5, 7.2, and 3.9 pmoles dTMP incorporated per assay, respectively.

TIME (MINUTES)

Chart 6. Addition of coralyne acetosulfate during AMV reverse transcriptase kinetic reaction. Two ml of standard assay mixture containing 50 µl of enzyme per ml were made and distributed into 0.6-ml (A), 0.5-ml (B), 0.5-ml (C), and 0.4-ml (D) aliquots. From A, 0.10-ml sample was withdrawn at 0 min. A to D were incubated at 37°. Samples (0.10 ml) from A were withdrawn at 5, 10, 20, and 30 min postincubation. Coralyne acetosulfate (50 µg/ml) was added to B, C, and D at 5, 10, and 20 min, respectively. One min after addition of the alkaloid, 0.10-ml samples were withdrawn from B, C, and D at 5-min intervals. The enzyme activity of each sample was determined. Control; O, alkaloid treated; □, Arrows, addition of drug at various times.

there was no decrease of the synthesized product on further incubation (Chart 6). These data clearly indicate that coralyne halted the elongation of nascent polynucleotide chains, probably by interacting with the template primer. Moreover, these results further indicate that degradation of the enzyme product did not take place in the presence of the alkaloid. Similar results were obtained with nitidine chloride (data not shown) and fagaronine chloride (15).

**DISCUSSION**

Cellular and oncornavirus nucleic acid-polymerizing enzymes (DNA-directed RNA polymerase, DNA polymerase, or reverse transcriptase), which require a double-stranded DNA as template or template primer, are more potently inhibited by alkoxybenzophenanthridine alkaloids (coralyne, fagaronine, or nitidine) than is poly(A) polymerase, which requires a single-stranded RNA primer with free 3' terminus. The alkaloid inhibition of viral or cellular DNA polymerase reaction is reversed by the addition of excess template primer. Increase of enzyme, metal ions, or substrate has no effect on the alkaloid inhibition. These results indicate that the alkaloids inhibit the enzyme reaction by interacting with the template primer.

Among the synthetic templates [poly[d(A-T)], poly(rA)-oligo(dT), poly(dA)-oligo(dT), and poly(rC)-oligo(dG)] used for examining inhibition of viral DNA polymerase by the alkaloids, the incorporation of [3H]dGMP by the enzyme with poly(rC)-oligo(dG) as template primer is not inhibited, whereas the incorporation of [3H]dTMP using other template primers is strongly inhibited. These results indicate a preferential interaction of these alkaloids with A:T pairs of the DNA or DNA-RNA hybrids. Indeed a change in the absorption spectrum of coralyne acetosulfate when added to a calf thymus DNA solution has been reported (24), suggesting a binding of the alkaloid to the DNA. Experiments conducted in this laboratory show that the absorption maxima of coralyne acetosulfate and nitidine chloride are shifted toward higher wavelengths on addition of synthetic polynucleotides (V. S. Sethi, unpublished results). These results confirm the previous report (24) and indicate an interaction of the polynucleotides with coralyne acetosulfate and nitidine chloride.

The preferential inhibition by the alkoxybenzophenanthridine alkaloids of DNA synthesis by viral reverse transcriptase or mammalian DNA polymerases from an A:T template primer may have some implication for the biology of RNA tumor viruses. The RNA of RNA tumor viruses, at its 3' end, contains poly(A) sequences similar to mammalian mRNA (4, 5, 8). The exact role of poly(A) sequences is still unknown, but one possibility is that these sequences may be the attachment site for an initiator polynucleotide. If RNA tumor viruses are involved in the etiology of human cancer, inhibitors that interfere with the initiation of viral RNA transcription could be effective agents in cancer chemotherapy. Other antitumor drugs, such as adriamycin, daunomycin, and acridinylaniside inhibit DNA polymerase reactions more strongly when template primers with A:T pairs, such as poly(dA)-oligo(dT), poly(rA)-oligo(dT), or poly[d(A-T)] are used.

These findings suggest a stronger or preferential binding of these drugs to A:T regions of the DNA, and thus may be related to the differential fluorescent staining of chromosomes by these agents (9). Furthermore, preferen-

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tial inhibition by drugs of DNA polymerase activity utilizing A:T template primers may prove to be a useful in vitro test for the screening of antitumor compounds.

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