Sulfhydryl Reactivity: Mechanism of Action of Several Antiviral Compounds—Selenocystine, 4-(2-Propinyloxy)-β-Nitro styrene, and Acetylaranotin

WILLIAM BILLARD AND EDWIN PEETS

Biological Research Division, Schering Corporation, Bloomfield, New Jersey

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The addition of 5 mM dithiothreitol to a cell-free assay system for influenza ribonucleic acid (RNA) polymerase activity reverses the inhibitory activity otherwise possessed by three established antiviral compounds: selenocystine, 4-(2-propinyloxy)-β-nitrostyrene, and acetylaranotin. Although 50% or greater enzyme inhibitory activity is repeatedly achieved for these compounds at a concentration of approximately 50 μg/ml (0.1 to 0.25 mM) in the absence of dithiothreitol, no inhibition is seen in its presence at inhibitor concentrations as high as 200 μg/ml. Against the deoxyribonucleic acid-directed RNA polymerases of Escherichia coli and chicken embryo cells, acetylaranotin and 4-(2-propinyloxy)-β-nitrostyrene caused very little inhibition. Only selenocystine significantly inhibited these two enzymes in the absence of reducing agent, but to an extent substantially less than that obtained against the viral enzyme. These results appear to suggest that influenza RNA polymerase is uniquely sensitive to a variety of structurally diverse antiviral compounds as a consequence of their sulfhydryl reactivity—a fact which might aid in the search for and development of more potent chemotherapeutic agents.

In the past several years, a number of reports have appeared in the literature describing compounds with inhibitory activity against a variety of ribonucleic acid (RNA) viruses. Included among these compounds are several which have been reported as specific inhibitors of the RNA-directed RNA polymerases of some of these viruses (2–7). Three such compounds are selenocystine (SC), 4-(2-propinyloxy)-β-nitrostyrene (4PβN), and acetylaranotin (AA), whose structures are shown in Fig. 1.

SC, an analogue of the amino acid l-cystine, has been reported to possess antiviral, antitumor, and antileukemia activity both in vitro and in vivo (6, 7, 23, 24). Ho et al. reported the compound to be specifically inhibitory to influenza PR8 RNA polymerase while having no effect on deoxyribonucleic acid (DNA)-dependent RNA polymerases (6, 7).

4PβN has been reported to be a specific inhibitor of Qβ replicase (2, 3) and RNA-dependent RNA polymerases isolated from Friend leukemia cells of mouse, polyivirus-infected HeLa cells, Ehrlich ascites tumor cells, and human acute myeloid leukemia cells (2, 3, 26). The specificity of 4PβN for RNA-dependent RNA polymerases was based on the failure of the compound to inhibit the DNA-dependent RNA polymerase from Escherichia coli.

AA is one of a series of epidithiapiperazinedi-one derivatives known for some time to be inhibitors of a variety of RNA viruses in tissue culture (13, 19, 20, 22) and animals (13, 22). Against poliovirus, AA is thought to exert its effect by blocking viral RNA synthesis, presumably through polymerase interaction (15, 21). Consistent with this, Ho and Walters reported this compound, along with others in this series, to be specific inhibitors of influenza RNA polymerase (5, 7).

In our cell-free screening procedure for inhibitors of virus-induced influenza A viruses 305 RNA polymerase, the above-mentioned compounds were tested and found to possess considerable antipolymerase activity. There were indications, however, that this activity was contingent upon the absence of reducing agent from the assay mixture. This seemed consistent with the chemical structure of each of the compounds: namely the presence of the reducible disulfide bond of AA, the diseleno bond of selenocystine, and the unsaturated nitrovinyl group of 4PβN—all of which possess the potential for sulfhydryl (SH) reactivity. The studies described here were designed to substantiate this possibility and to determine if SH reactivity could represent a probable common mechanism of antiviral action for these compounds.
Tris-hydrochloride, pH 8.2, in such a way that two units could be conveniently added to each assay mixture (0.5 ml) in a 10-μliter sample.

To avoid possible changes in the inhibitory properties of the compounds, which might have distorted an intercomparison of their effects on the three enzymes, all enzyme reactions were run for 30 min at pH 8.2 and 25°C. These were determined to be the optimal conditions for obtaining maximal influenza RNA polymerase activity. Such activity was linear through 40 to 50 min of reaction (Fig. 2). Under these same conditions, each of the other two enzymes displayed linear incorporating activity through at least 30 min. In both cases, pH 8.2 was very close to optimal, based on the rather broad pH optimum range for each of these enzymes. Only the reaction temperature was below the reported 37°C optimum. Despite this, very substantial *E. coli* and chicken embryo cell RNA polymerase activity was obtained by using the conditions described. In addition to the equalization of reaction conditions mentioned above, the concentra-

**MATERIALS AND METHODS**

**Source of materials.** DL-SC (molecular weight 334), A grade, was purchased from Calbiochem as were the unlabeled ribonucleoside triphosphates and calf thymus DNA; AA (molecular weight 504) was kindly supplied by M. Forbes of Lederle Laboratories; dithiothreitol (DTT) was purchased from Sigma Chemical Co.; and 4PβN (molecular weight 203) was synthesized in these laboratories by D. Blythin. Generally labeled [3H]guanosine 5'-triphosphate ([3H]-GTP, 14.5 Ci/mmole) was purchased from Schwarz/Mann.

**Enzyme systems.** The compounds were tested against three separate RNA polymerase enzymes: influenza A,Jap 305 RNA-dependent RNA polymerase, chicken embryo cell DNA-dependent RNA polymerase (Mn+++, (NH4)2SO4 activated), and *E. coli* K-12 DNA-dependent RNA polymerase (EC 2.7.7.6). The influenza RNA polymerase and its associated template were obtained by isolating the microsomal fraction of influenza-infected chicken fibroblasts grown in roller-bottle cultures (14). Maximal polymerase activity was obtained 10 h postinfection. The Mn+++,(NH4)2SO4-activated chicken embryo cell DNA-dependent RNA polymerase was assayed according to Widnell and Tata (25) by using whole nuclei. Nuclei were obtained by homogenization of the minced bodies of 11-day-old chicken embryos by using a Teflon-on-glass tissue grinder. Homogenization was performed in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4, containing 0.01 M KCl and 0.003 M ethylenediaminetetraacetic acid. The crude nuclear fraction obtained by centrifugation at 600 × g was suspended in 0.25 M sucrose-0.001 M MnCl2. *E. coli* K-12 RNA polymerase was purchased from Sigma at an activity of 500 units/0.44 ml (377 units/mg of protein). A unit of enzyme activity in this instance is defined as the amount of enzyme which catalyzes the incorporation of 1 nmol of guanosine 5'-monophosphate (GMP) per 15 min at 37°C with native *E. coli* DNA as a primer. The enzyme was diluted in 0.1 M Tris-hydrochloride, pH 8.2, in such a way that two units could be conveniently added to each assay mixture (0.5 ml) in a 10-μliter sample.

**FIG. 1. Compound structures.**

**FIG. 2. Time course of influenza RNA polymerase reaction at 35°C in the presence and absence of 50 μg of acetylaranotin, selenocystine, and 4-(2-propinyl)oxy)-β-nitrostyrene per ml.**
tions of the ribonucleoside triphosphates as well as the specific activity of labeled [\(^3H\)]GTP were the same for each of the enzyme assay solutions.

**Assay mixture compositions.** The assay mixture for influenza RNA polymerase contained in 0.5 ml: Tris-hydrochloride (pH 8.2), 50 \(\mu\)mol; \(MgCl_2\), 1 \(\mu\)mol; adenosine 5'-triphosphate (ATP), cytidine 5'-triphosphate (CTP), and uridine 5'-triphosphate (UTP), 0.2 \(\mu\)mol each; [\(^3H\)]GTP (adjusted to final specific activity of 2Ci/mmol), 0.0025 \(\mu\)mol; DTT, 2.5 \(\mu\)mol (present); and approximately 0.2 mg of enzyme preparation. The specific activity of the influenza RNA polymerase preparations used was between 100 to 150 pmol of GMP incorporated per mg of protein per 30 min at 25 \(C\). For every picomole incorporated, approximately 1,000 counts/min were registered. All compounds added to the assay mixture were first thoroughly suspended in a methylcellulose-H\(_2\)O vehicle by using an homogenizer and were added to the assay mixture in a 20-\(\mu\)litter sample to give the correct final concentrations.

The assay mixture for E. coli K-12 RNA polymerase contained in 0.5 ml: Tris-hydrochloride (pH 8.2), 20 \(\mu\)mol; \(MgCl_2\), 2.0 \(\mu\)mol; \(MnCl_2\), 0.5 \(\mu\)mol; KCl, 75 \(\mu\)mol; ATP, UTP, and CTP, 0.2 \(\mu\)mol each; [\(^3H\)]GTP (adjusted to a final specific activity of 2Ci/mmol), 0.0025 \(\mu\)mol; calf thymus DNA, 50 \(\mu\)g; E. coli RNA polymerase, 2 U.

Contained in the assay mixture for the chicken embryo cell Mn\(^++\), (NH\(_4\))\(_2\)SO\(_4\)-activated RNA polymerase were the following per 0.5 ml: Tris-hydrochloride (pH 8.2), 50 \(\mu\)mol; \(MnCl_2\), 1.5 \(\mu\)mol; (NH\(_4\))\(_2\)SO\(_4\), 200 \(\mu\)mol; ATP, UTP, and CTP, 0.2 \(\mu\)mol each, [\(^3H\)]GTP (adjusted to a final specific activity of 2Ci/mmol), 0.0025 \(\mu\)mol; and approximately 200 \(\mu\)g of nuclear material. The specific activity of incorporation was approximately 150 pmol of GTP per mg per 30 min at 25 \(C\), coincidentally close to that observed for the influenza polymerase. Again, approximately 1,000 counts/min were registered for every picomole of GTP incorporated.

For each of the enzyme systems, reaction was terminated by the addition of 0.5 ml of saturated sodium pyrophosphate followed by macromolecular precipitation with 5 ml of 10% trichloracetic acid. After 1 h at 4 \(C\), samples were either washed by repeated centrifugation and digested with Nuclear-Chicago Tissue Solubilizer prior to scintillation counting or were filtered onto 0.8 or 1.2 \(\mu\)m membrane filters (Millipore Corp.), washed with additional trichloracetic acid, dried, and monitored for radioactivity in toluene-based scintillation solution. Washing blanks and incubation blanks were routinely included in each experiment. These blanks represented from 1 to 3% of the total incorporation obtained in the typical assay.

**Preincubation study.** In this experiment, samples of the influenza polymerase were preincubated with SC, AA, and 4PBN for 10 min at 25 \(C\) at a concentration of 1 mg/ml. After this 10-min treatment, a portion of the mixture was removed, and DTT was added to it to make a concentration of 5 mM. This sample and the remainder of the original material not containing DTT were incubated for an additional 10 min at 25 \(C\), after which the enzyme activity of a standard amount of each sample was assayed for in the usual manner. This consisted of adding 25 \(\mu\)litters of the enzyme-drug preincubated mixture to a final volume of 0.5 ml of reaction mixture and incubation for 30 min at 25 \(C\).

**RESULTS**

Influenza RNA polymerase activity is essentially linear over a period of 40 to 50 min at 25 \(C\) (Fig. 2). Each time point represents the amount of [\(^3H\)]GMP incorporated per an equivalent 100-\(\mu\)litter sample of reaction mixture. The effect on this activity of 50 \(\mu\)g of SC, 4PBN, and AA per ml, as a function of time in the absence of DTT, is also shown. At time points beyond 10 min of incubation and through 50 min of incubation, the inhibition caused by each of these compounds as a percentage of control activity was fairly constant.

Figure 3 indicates the concentration-dependent nature of the inhibition caused by SC, 4PBN, and AA. All determinations were made after 30 min of reaction at 25 \(C\). On an equimolar basis the compounds were similar in terms of their inhibitory potency.

In repeated tests, in the absence of DTT, conducted at a fixed concentration of 50 \(\mu\)g/ml (0.1 to 0.25 mM depending on the compound), 50% or greater inhibition of the viral polymerase was achieved for each of the above compounds (Table 1). In the presence of 5 mM DTT, however, this concentration of each of the compounds failed to inhibit the enzyme. When the compounds were tested at 200 \(\mu\)g/ml, elimi-
nation of inhibitory activity was again achieved by 5 mM DTT.

Analogous results were obtained for the E. coli and chicken embryo cell polymerases in that observed inhibition of these enzymes could be prevented by DTT addition. In contrast to results obtained for the viral enzyme, however, AA and 4P9N caused very little inhibition of either the E. coli or chicken cell enzymes. Only SC significantly inhibited these two enzymes in the absence of a reducing agent. In both cases the level of inhibition was below that obtained for the viral enzyme.

Once the protective effect of DTT was established, experiments were performed in which drug and viral enzyme were allowed to interact prior to DTT addition. The objective was to determine if DTT could reverse the inhibitory effects of the compounds as distinguished from simply protecting the enzyme from interaction with them.

Such drug-enzyme preincubation was of little consequence in preventing the DTT effect (Table 2). Even after ample time was allotted for drug-enzyme interaction, DTT was still quite capable of effecting the elimination of inhibitory activity.

In contrast to the DTT-reversed inhibitory activity of the compounds described above, no such reversal could be achieved for several other known selective inhibitors of the enzymes under study (Table 1). The potent inhibitory effect of ethidium bromide upon influenza RNA polymerase activity, as well as its inhibition of the other two enzymes, could not be reversed by DTT. Also, the characteristic and expected inhibitory effects of actinomycin D, α-amanitin, and rifampin against one or several of the enzymes were all independent of the presence of DTT. HgCl₂, on the other hand, a known mercaptide forming reagent, displayed potent inhibition of both influenza RNA polymerase and E. coli RNA polymerase activities in the absence of DTT, and its inhibitory activity was readily prevented by excess DTT.

It should be noted that in these studies the removal of DTT from the assay mixtures had virtually no effect on the control activity (no compound present) of influenza and chicken embryo cell polymerases. E. coli RNA polymerase activity, however, was reduced from 25 to 50% whenever DTT was omitted from the assay mixture. For both the influenza and chicken cell polymerases, net total control incorporation of approximately 30 pmol of GMP per assay was obtained in these studies. For the E. coli polymerase, GMP incorporation was approximately 300 pmol/assay.

A distinction existed between the three compounds with respect to the concentration of

### Table 1. Effect of dithiothreitol on the inhibitory activity of various compounds against several polymerases

<table>
<thead>
<tr>
<th>Compound</th>
<th>Assay conc (μg/ml)</th>
<th>Inhibition of polymerase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fluorescent RNA polymerase</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-DTT</td>
</tr>
<tr>
<td>Selenocystine</td>
<td>50 (0.15 mM)</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>60</td>
</tr>
<tr>
<td>4-(2-Propinyloxy)-β-nitrostyrene</td>
<td>50 (0.25 mM)</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>74</td>
</tr>
<tr>
<td>Acetylaranotin</td>
<td>50 (0.1 mM)</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>57</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>5 (0.004 mM)</td>
<td>4</td>
</tr>
<tr>
<td>α-Amanitin</td>
<td>5 (0.005 mM)</td>
<td>0</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>10 (0.012 mM)</td>
<td>3</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>50 (0.13 mM)</td>
<td>99</td>
</tr>
<tr>
<td>Mercuro chloride</td>
<td>14 (0.05 mM)</td>
<td>99</td>
</tr>
</tbody>
</table>

### Table 2. Reversion of influenza RNA polymerase inhibition caused by sulphydryl-reactive compounds with dithiothreitol

<table>
<thead>
<tr>
<th>Compounds preincubated with enzyme</th>
<th>Inhibition of polymerase activity after preincubation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-DTT</td>
</tr>
<tr>
<td>Selenocystine</td>
<td>61</td>
</tr>
<tr>
<td>4-(2-Propinyloxy)-β-nitrostyrene</td>
<td>47</td>
</tr>
<tr>
<td>Acetylaranotin</td>
<td>46</td>
</tr>
</tbody>
</table>
DTT necessary to prevent inhibition of influenza RNA polymerase activity (Fig. 4). For AA and 4PβN, inhibitory activity began to diminish progressively at concentrations of DTT considerably below the molar concentration of the drug in the assay mixture. For SC, however, a greater than twofold molar excess of DTT was required to overcome inhibition. This may reflect the formation of 2 molar equivalents of SH-reactive product upon cleavage of the diseleno bond.

**DISCUSSION**

The results described here suggest that influenza RNA polymerase activity assayed for in a cell-free system is sensitive to several structurally diverse antiviral compounds as a consequence of their SH reactivity. Conceivably, such a mechanism might explain the ability of these compounds to inhibit the multiplication of a variety of RNA viruses in tissue culture and animal systems. An interpretation of this nature is tempting in light of recent reports. Trown and Billelo have shown, for example, that the inhibition of poliovirus RNA synthesis in infected HeLa cells by AA and structurally related compounds is either greatly decreased or abolished if DTT is simultaneously added to the tissue culture system (21).

With reference to 4PβN, Montagnier, et al., have demonstrated that the reducible nitrovinyl group is the substituent responsible for its in vitro and in vivo activity against ascitic tumor Krebs II (16). Additionally, the reported antileukemia and antitumor activity of both SC and 4PβN is consistent with recent findings concerning the potential usefulness of SH-blocking reagents in cancer chemotherapy and their inhibition of reverse transcriptase activity (9–12).

It is apparent from these results that the viral enzyme possesses greater sensitivity to the three compounds under consideration than was found for the other enzymes which were examined. For the chicken embryo cell polymerase this is not particularly unexpected, because HgCl₂, a known SH-reactive compound, also has considerably less inhibitory effect upon this enzyme than upon the viral enzyme (Table 1). *E. coli* RNA polymerase, on the other hand, is quite sensitive to HgCl₂ and possesses established sensitivity to certain other SH-reactive compounds (1, 8). Conceivably, the viral enzyme in association with its template may possess a much more vulnerable configuration than the *E. coli* RNA polymerase with respect to the chemical accessibility of various critical SH groups. This increased vulnerability and exposure of SH groups might render the viral enzyme more reactive to a much greater variety of compounds with such potential.

The exceptional sensitivity found for virus-induced influenza RNA polymerase to SH-reactive compounds may be a phenomenon applying to viral RNA polymerases in general. Certainly, the antiviral activity ascribed in the literature to the compounds mentioned here supports such speculation. Additional suggestions come from the recent findings of Ohki and Hori demonstrating the critical importance of SH groups to the catalytic function of Qβ replicase (17). In another, even more recent, report, Oxford (18) has convincingly demonstrated that selenocystamine (closely related to SC) inhibits the particle-associated RNA polymerase activity of a variety of influenza A and B viruses and, furthermore, that its inhibitory activity is reversed by β-mercaptoethanol. All of these results appear to indicate that the susceptibility of viral RNA polymerases to diverse thiol-reactive compounds may have considerable exploitative potential in terms of the development of new chemotherapeutic agents of increased potency.

**LITERATURE CITED**


