Study of Ansamycin Inhibition of a Ribonucleic Acid-Directed Deoxyribonucleic Acid Polymerase by an Immobilized Template Assay

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A series of structurally related ansamycins have been analyzed, in a new immobilized template assay, to determine the mechanism by which they inhibit a ribonucleic acid-directed deoxyribonucleic acid (DNA) polymerase from Moloney murine leukemia virus. By this assay, we can better correlate specific structures of these drugs with inhibitory mechanisms. Using an immobilized template, we were also able to observe drug effects on the stability of complexes formed between the polymerase, a template (polyadenylic acid-agarose), and a primer, as well as to monitor the synthesis of DNA in the presence of drug. For each drug, we determined (i) the complex (intermediate in DNA synthesis) which was primarily affected and (ii) whether the effect was due to a destabilization process. Although the activity and specificity of the unsubstituted ansamycins (streptovaricins and rifamycin SV) were modulated by conformation of the molecule and electron density of the aromatic ring, the principal mode of inhibition is, apparently, drug binding to a polymerase-template complex; the drug binds in a manner which prevents subsequent formation of a polymerase-template-primer complex. However, some derivatives of rifamycin SV, when substituted at carbon-3 with bulky or hydrophobic side chains, displayed markedly different modes of action. For example, demethyl dimethyl rifampin prevented the formation of polymerase-template complexes, whereas rifazacyclo 16 acted by promoting the dissociation of polymerase-template-primer complexes.

Recently, we presented a new system utilizing polyadenylic acid [poly(A)]-agarose as an immobilized template to facilitate the analysis of various steps involved in deoxyribonucleic acid (DNA) polymerization (B. I. Milavetz and W. A. Carter, Pharmacol. Ther., in press; B. I. Milavetz, J. S. Horoszewicz, M. J. Evans, K. F. Manly, K. L. Rinehart, Jr., and W. A. Carter, Mol. Pharmacol., in press). The immobilized template also lends itself to an analysis of the molecular events whereby certain drugs inhibit DNA synthesis. Because of the possible value of an agent which can inhibit the ribonucleic acid (RNA)-directed DNA polymerase found in RNA tumor viruses (1, 26) (an enzyme necessary for cell transformation by this class of tumor viruses [13]), we used this approach to investigate the inhibitory action of ansamycins, a chemically well-characterized series of antibiotics. Streptovaricins and rifamycins (principal members) have established inhibitory activities for reverse transcriptase and viral expression (2, 4, 5, 9-12, 19, 21, 22, 24, 27-31).

From results of experiments on the mechanism of polymerization by Moloney murine leukemia virus (MoMuLV) DNA polymerase (Milavetz and Carter, in press; Milavetz et al., in press), we postulated that inhibitors of polymerization might either prevent formation of certain complexes (necessary for complete polymerization) or bind to these complexes (after their formation) in a manner that would alter their stability. Our results (Milavetz et al., in press) with the first four ansamycins tested (streptovar C, rifamycin SV, demethyl dimethyl benzyl rifampin, and rifazone 82) revealed that, in fact, both modes of action do occur. From the small number of compounds initially tested, it was not clear how the structural modifications might explain the various modes of action which were seen. By including streptovaricin C, streptovaricin C triacetate, streptovar C, and damavaricin C, and selected rifamycins (Fig. 1), we now report experiments designed to correlate certain structural modifications with the modulation of DNA synthetic activity; in so doing, we are able,
for the first time, to define some of the structural features necessary for inhibitors to produce specific lesions in overall viral DNA synthesis.

MATERIALS AND METHODS

Material. The following ansamycins were used: streptovaricin C, purified from streptovaricin complex (21), a gift of The Upjohn Co.; streptovaricin C triacetate (21), damavarcin C (20; B. I. Milavetz, Ph.D. thesis, University of Illinois, Urbana, 1975), and streptoval C (23) were prepared from streptovaricin C; rifamycin SV (29) and rifamipin (29) were purchased from Schwarz/Mann; rifamycin SV formyl N-octyl, oxime (29), and demethyl dimethyl benzyl rifamipin (29) were gifts of R. C. Gallo; and rifazacyclo 16 (27) and rifazine 8 (27) were gifts of M. Calvin. The poly(A)-agarose [1 mg of poly(A)/ml] was purchased from P-L Biochemicals, Inc. The oligodeoxythymidylic acid [oligo(dT)12-18] was purchased from Collaborative Research, Inc. Deoxynucleoside triphosphate (dTTP) was obtained from General Biochemicals, and [3H]dTTP was purchased from New England Nuclear Corp. in a solution of 1:1 ethanol-water which was dried under vacuum and reconstituted with distilled water before use. All chemicals used for the preparation of buffers and assays were reagent grade. The Moloney leukemia virus was isolated and purified by K. Manly (18).

Disruption of MoMuLV and purification of its polymerase. The MoMuLV was disrupted by adding 5 µl of virus (11 µg/ml) to 100 µl of 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.9), 1 mM dithiothreitol, 0.1 mM ethylenedia-
polymerase to template. A 100-μl amount of purified polymerase was diluted 1:3 with buffer B, and then demethyl dimethyl benzyl rifampin (previously dissolved in dimethyl sulfoxide) was added to give a final concentration of 100 μg/ml in 2% dimethyl sulfoxide. After 30 min of incubation at 4°C, the enzyme-drug mixture was applied to a poly(A)-agarose column (500 ml). The column was washed with 2 ml of buffer B, followed by elution with a 0 to 0.5 M KCl gradient (4 ml) in buffer containing 0.05% Triton X-100. Fractions of 10 drops were taken, and 25 μl of each fraction was assayed for polymerase activity.

RESULTS

By passing polymerase and primer through poly(A)-agarose columns, template-primer, polymerase-template, and polymerase-template-primer complexes were prepared. We then added drugs to each column and measured any displacement of polymerase, as well as the residual amount of poly(A)-agarose-bound enzyme. Table 1 shows the effects of several drugs, which generally were similar to levels of inhibition previously described with reverse transcription in solution (compare columns 2 and 3).

We also measured the effects of each drug on displacement of polymerase from a polymerase-template complex and a polymerase-template-primer complex. Additionally, we measured the amount of polymerase activity remaining after drug was added to a template-primer complex, a polymerase-template complex, or a polymerase-template-primer complex.

Streptovaricin C did not displace significant amounts of polymerase, its effect being primarily on a polymerase-template complex (18% of control value) and secondarily on template-primer and polymerase-template-primer complexes (56% and 58%, respectively). Streptovaricin C triacetate was much less active, its only effect being on a polymerase-template complex. In contrast, streptovaricin C was quite active and, indeed, specific for the polymerase-template complex (1% of control). Damavaricin C and rifamycin SV were similar to streptovaricin C, with a strong effect on the polymerase-template complex (32% and 38%, respectively, of control) and less effect on template-primer complexes (50% and 64%). These latter two compounds had little effect on polymerase-template-primer complexes (100% and 75% of control).

Of the substituted rifamycins, rifampin was clearly inactive. Demethyl dimethyl benzyl rifampin was active (column 2), but ineffective against any of the preformed complexes, since there was little polymerase displacement or effect on preformed polymerase-template complex (79% of control). To determine whether the inhibition was due to preventing the formation of a polymerase-template complex, we analyzed its ability to inhibit the initial attachment of polymerase to poly(A)-agarose. Figure 2 shows that this compound does, in fact, prevent a significant amount of polymerase (approximately 50%) from binding to this immobilized template.

Interestingly, rifamycin SV formyl N-octyl oxime affected all complexes (54%, 0%, and 3% for columns 6, 7, and 8, respectively); it also caused the displacement of significant amounts of polymerase from a polymerase-template complex (28,000 cpm) as well as from a polymerase-template-primer complex (68,000 cpm). Rifazone 82 affected these complexes similarly, but, in con-

<table>
<thead>
<tr>
<th>Drug, 100 μg/ml</th>
<th>Percent of control</th>
<th>Polymerase activity</th>
<th>Effect on complexes (% of control)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Immobilized template assay</td>
<td>From literature</td>
<td>Poly(A)-agarose</td>
</tr>
<tr>
<td>Streptovaricin C</td>
<td>40</td>
<td>50 (4)</td>
<td>1,600</td>
</tr>
<tr>
<td>Streptovaricin C triacetate</td>
<td>82</td>
<td>50 (4)</td>
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<tr>
<td>Streptovaroval C</td>
<td>18</td>
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<td>500</td>
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<tr>
<td>Damavaricin C</td>
<td>13</td>
<td>NA</td>
<td>200</td>
</tr>
<tr>
<td>Rifamycin SV</td>
<td>49</td>
<td>61 (19)</td>
<td>1,100</td>
</tr>
<tr>
<td>Rifampin</td>
<td>108</td>
<td>96 (19)</td>
<td>1,000</td>
</tr>
<tr>
<td>Demethyl dimethyl benzyl rifampin</td>
<td>20</td>
<td>1 (19)</td>
<td>1,500</td>
</tr>
<tr>
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<tr>
<td>Rifazone 82</td>
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<td>6,500</td>
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<tr>
<td>Rifazacyclo 16</td>
<td>0</td>
<td>NA</td>
<td>13,800</td>
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</tbody>
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* Complexes were formed and inhibitions were determined as described in Materials and Methods. Control reactions yielded 7.5 × 10⁴ cpm of trichloroacetic acid-precipitable radioactivity for a 50-μl sample of the reaction mix. NA, Data not available from literature.
trast, did not displace as much polymerase activity (6,500 cpm and 2,600 cpm, respectively). Finally, rifazacyclo 16 was similar to rifampin SV formyl N-octyl oxime in its effect, although it was more active than the other drugs on a template-primer complex (15% of control).

DISCUSSION

Recently, we have shown that poly(A)-agarose is useful as a template upon which complexes can be built which participate in polymerization of DNA (template-primer, polymerase-template, or polymerase-template-primer). We chose to study these particular complexes since they may occur during polymerization (e.g., the Kornberg model [15]), and other drugs, known to inhibit DNA synthesis, also affect them (e.g., actinomycin D and the template-primer complex [17]). In our assay, the effect of a drug can be observed on three features of the complex—its formation, stability, and activity—by drug addition at specific times and subsequent measurement of polymerase, either bound to poly(A)-agarose or present in the column eluent.

To determine the molecular targets of the ansamycins, we found it useful to assume that the Kornberg model of DNA polymerization (15) may also describe the mechanics of MoMuLV DNA polymerase. We have recently shown that reverse transcriptase does exhibit many binding interactions in common with the Escherichia coli polymerase I (Milavetz et al., in press), and thus this assumption is quite plausible. Additionally, our evidence suggests that reverse transcription is a distributive process, a conclusion consistent with the results of Chang (6) and McClure and Jovin (16). The drugs could (i) block the formation of certain complexes necessary for polymerization, (ii) promote the dissociation of preformed complexes, or (iii) increase the stability of a complex such that the dissociation step (ultimately necessary for distributive polymerization) would not occur. Since the ansamycins are chemically polyfunctional, they may demonstrate multiple modes of action. In fact, the unsubstituted ansamycins streptovaricin C and rifampicin SV appear to affect all complexes, although the greatest effect is on a polymerase-template complex. Our experiments do not yet identify the specific “sub”-target within each complex which is primarily affected, although several possibilities can be easily envisioned.

Some structure-function correlations: streptovaricins. Based upon nuclear magnetic resonance studies done recently with these compounds by members of our group (14, 20, 21; Milavetz, Ph.D. thesis, 1975), we can now compare our current drug inhibition results with certain structural properties which the nuclear magnetic resonance work delineated. In general terms, it appears that an active reverse tran-
script inhibitor requires specific features of size, conformation, and electron density to be active. Because of the complexity of the molecules, even relatively minor changes (such as acetylation) cause many long-range effects. For example, we obtained streptovaricin C triacetate by acetylation of the 7, 9, and 11 hydroxyl groups of streptovaricin C (21). The $^{13}$C nuclear magnetic resonance (14) indicated a marked change in the conformation of the ansa ring as well as a shift in the electron density of the quinone at C-21 and the phenol at C-27. It is now clear that the net effect of these changes is to abolish drug activity against both the template-primer complex and the polymerase-template-primer complex; similarly, a reduction in activity against the polymerase-template complex is noted.

This varying sensitivity of different complexes is illustrated also by streptoval C, a product obtained by periodate oxidation of the cis-glycol at C-13 and C-14 (23). As expected, the ansa ring is cleaved; a hemiacetal is formed, and there is again a shift in electron density within the nucleus (14). The product now demonstrates a near absolute specificity for the polymerase-template complex and no effect on other complexes. Damavaricin C is an oxidative hydrolysis product of streptovaricin C (20; Milavetz, Ph.D. thesis, 1975) with almost no change in the ansa ring, but profound differences in electron density of the napthoquinone ring (20; Milavetz, Ph.D. thesis, 1975). As might be expected, the compound is still active against the polymerase-template complex. The drug is slightly active against a template-primer complex and inactive against a polymerase-template-primer complex.

From this type of structure-function analysis, we propose several general hypotheses concerning modes of inhibition: (i) the binding site of the drug for each complex is apparently distinct; (ii) each drug can affect more than one complex; (iii) the drug binding site, within the template-primer complex, requires a certain conformation of the ansa ring; (iv) the polymerase-template binding site seems to possess the least specificity, in terms of requirement for conformation and electron density of the inhibitor; and (v) the polymerase-template binding site has a conformational and electron density requirement which may reflect hydrogen bonding between the drug template and/or polymerase.

Rifamycins. Since rifamycin SV has been modified at C-3 by addition of many different side chains, one can observe the effects of rather specific changes on enzyme inhibition. The additional side chain of rifampin, which confers its activity against bacterial RNA polymerases (and also increases the drug size), apparently prevents it from inhibiting reverse transcriptase. In fact, for all the rifamycin SV derivatives which we analyzed, the addition of bulky side chains suppresses the inhibitory activity seen with the parent compound. Our evidence suggests that demethyl dimethyl benzyl rifampin, known previously to attach to the polymerase (29), prevents the formation of a polymerase-template-primer complex; once this complex is formed, the drug becomes inactive. This indicates that the drug may bind to a template-primer binding site on the enzyme, thus preventing the initiation of DNA synthesis.

The other series of rifamycin SV derivatives in the present study are characterized by substitution of a hydrophobic side chain at position 3: these include rifamycin SV formyl N-octyl oxime (an eight-carbon side chain) (29), rifazone 82 (two eight-carbon side chains), and rifazacyclo 16 (a 16-membered ring) (27). All three derivatives are quite potent and appear to affect all the complexes; the principal mechanism of action for rifamycin SV formyl N-octyl oxime and rifazacyclo 16 seems to reside in their ability to cause dissociation of both polymerase-template and polymerase-template-primer complexes. This effect for rifamycin SV formyl N-octyl oxime has been previously noted (3). Among other possibilities, the hydrocarbon skeletons might intercalate with the polymerase molecule, resulting in protein denaturation and collapse of the respective complexes. This intercalation mechanism has been recently advanced with several novel hydrophobic probes (7, 25). Since rifazone 82 causes the displacement of significant amounts of polymerase activity, but much less than that of the two other derivatives, it may be acting by a somewhat different mechanism.

It is now possible to define the general mechanism of action for most of the compounds analyzed in the current study and to modulate these effects by specific structural modifications; however, it is not yet known which type of modification might ultimately yield a compound with specificity for the viral polymerase. By including other DNA polymerases in this type of immobilized template assay, it could ultimately be possible to determine which mechanism is more specific for reverse transcriptase, and thus which series of modifications would yield a chemotherapeutic agent.

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LITERATURE CITED


