On the mechanism of inhibitory effect of violamycin antibiotics on the transcription by bacteriophage T3-induced RNA polymerase

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ABSTRACT

The effect of three components of the anthracycline antibiotic violamycin on the transcription of bacteriophage T3 DNA by bacteriophage T3-induced RNA polymerase has been investigated in a cell-free system. The glycosides of violamycin BII possess the highest inhibitory activity, whereas those of violamycin BI and violamycin A show a reduced inhibitory effect. Concentrations of violamycin BII depressing the incorporation of (3H)UMP into RNA chains have only a slight effect on the binding of the T3 RNA polymerase to T3 DNA and on the incorporation of GTP as the first nucleotide. This shows that the primary target of the antibiotic is not the initiation of the RNA synthesis. The binding of violamycin BI to T3 DNA causes a strong reduction of the elongation rate of the RNA chains.

INTRODUCTION

Violamycin - an antibiotic with antimicrobial activity (1) - is a mixture of several glycosides of mainly six related aglycones (2). The antibiotic can be separated chromatographically into three biologically active components, named violamycin A (VA), violamycin BII (VBII), and violamycin BI (VBI), containing an increasing number of different sugar residues bound (2). The violamycin components bind to DNA. The strength of the interaction between the antibiotics and DNA increases in the order VA, VBII, VBI (3). This property of the antibiotics suggests that they may inhibit the RNA synthesis. To investigate this problem we have employed our model system of bacteriophage T3-specific RNA synthesis. The in vitro system consists of isolated T3 RNA polymerase and T3 DNA. It was found that from the three violamycin components studied VBI causes the highest inhibition of T3 RNA synthesis. This inhibi-
itory action of VBI is due to the retardation of T3 RNA chain elongation.

MATERIALS AND METHODS

Antibiotics

The violamycin components A, BII, and B I were isolated from fermentation cultures of Streptomyces violaceus IMET JA 6844 (1). VA (average molecular weight = 600) and VBII (average molecular weight = 800) were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the assay of the RNA synthesis was 5 % (v/v) after the addition of the antibiotics. VBI (average molecular weight = 1000) was dissolved in bidistilled water. The concentrations of the antibiotics given in the legends of the figures are final concentrations.

T3 RNA polymerase and T3 DNA

T3 RNA polymerase was prepared from the RNase-I-deficient strain Escherichia coli DG 156 according to Chakraborty et al. (4). The concentration of the T3 RNA polymerase used in the assays is given in units according to Chakraborty et al. (4).

Unlabelled and (32P)-labelled T3 DNA were isolated by the method described by Thomas and Abelson (5). The T3 phages were purified according to Stibenz et al. (6) or by banding in CsCl.

RNA synthesis assay

The reaction mixture (0.25 ml) for the RNA synthesis contained: 50 mmol/l Tris-HCl pH 7.8, 20 mmol/l MgCl2, 0.1 mmol/l dithioerythritol, 0.125 mg bovine serum albumin, 100 nmol each of GTP, ATP, (3H)UTP (specific activity 4 - 8 x 10^7 dpm/μmol), and CTP, 15 - 30 nmol (expressed as deoxyribonucleotide residues) of T3 DNA, 8 - 15 units of T3 RNA polymerase. The incubation time was 5 min unless otherwise indicated at 30°C and 35°C, respectively. The antibiotics were preincubated with T3 DNA and the reaction was started by the addition of T3 RNA polymerase and the nucleoside triphosphates. The reaction was terminated by the addition of 5 ml ice-cold 5 % CCl3COOH containing 10 mmol/l tetrasodium pyrophosphate and the amount of (3H)UMP incorporated into acid-insoluble material was deter-
The initiation of the RNA synthesis was assayed with \((\gamma-^{32}P)GTP\) (specific activity \(0.5 - 3 \times 10^5\) dpm/\(\mu\)mol). Before terminating the reaction with \(\text{CCl}_2\text{COOH}\) unlabelled GTP was added to give a final concentration of \(60\) mmol/\(l\). The washing of the acid precipitates was performed by the method described by Richardson (7).

**Gel electrophoresis**

The reaction mixture for the preparation of radioactive RNA species contained the same components described for the RNA synthesis assay. Except for labelling the RNA products \((^{3}H)UTP\) was replaced by \((\epsilon-^{32}P)\text{ATP}\) (specific activity \(0.2 - 2 \times 10^5\)dpm/\(\mu\)mol). The volume of the assay mixture amounted to 0.1 ml. The reaction was terminated by the addition of EDTA and sodium dodecyl sulfate to yield a final concentration of \(50\) mmol/\(l\) and \(0.5\) %, respectively. The gel electrophoresis was performed in glass tubes according to Golomb and Chamberlin (8). An aliquot of \(10 - 20\) \(\mu\)l of the reaction mixture was directly subjected to gel electrophoresis. After running the gels were sliced in 1.1-mm sections and the radioactivity content of the individual slices was measured. \((^{3}H)-\text{labelled 16 S and 23 S rRNA (E.coli)}\) and \((^{32}P)-\text{labelled T3 RNA (product of transcription of T3 DNA by T7 RNA polymerase, molecular weight } 2 \times 10^6 (8)\) were run in parallel gels as molecular weight markers.

**Nitrocellulose filter assay**

The binding of T3 RNA polymerase to T3 DNA was measured on nitrocellulose filters according to Chakraborty et al. (9). The reaction mixture contained in a total volume of 0.25 ml: 50 mmol/\(l\) Tris-HCl pH 7.8, 0.5 mmol/\(l\) \(\text{MgCl}_2\), 0.1 mg bovine serum albumin, 4 mmol/\(l\) dithioerythritol, 4 mmol of \((^{32}P)-\text{labelled T3 DNA (2 - 3 } \times 10^4 \text{ dpm/mmol)}\), 2.5 units of T3 RNA polymerase (assay 1). Moreover, the binding between the T3 RNA polymerase and T3 DNA was also determined under conditions described above for the assay of the RNA synthesis (assay 2). However, one of the nucleoside triphosphates (CTP) was omitted from the assay to prevent RNA chain elongation. After incubation for 5 min at 30°C or 35°C the reaction mixture was fil-
trated through membrane filters (Synpor, ČSSR, 0.4 μm pore size, 24 mm in diameter). Then the filters were washed with 2 x 1 ml cold buffer (50 mmol/1 Tris-HCl pH 7.8, 0.5 mmol/1 MgCl₂). The radioactivity retained on the filters was determined using a dioxane-based scintillator.

RESULTS

Comparison of the effect of the violamycin components A, BII, and BI

The effect of the three isolated components VA, VBII, and VBI on the transcription of T3 DNA by T3-induced RNA polymerase differs markedly (Fig. 1). The triglycosides of VBI causes a complete inhibition of the RNA synthesis above concentrations of 4 x 10⁻⁵ mol/l. In contrast to VBI the other two components VA (monoglycosides) and VBII (diglycosides) have a reduced inhibitory activity. VA reduces the RNA synthesis only slightly even at higher concentrations. These results demonstrate the influence of the number or kind of sugar residues on the inhibitory effect.

Effect of VBI on initiation and elongation

VBI shows a strong interaction with DNA (10). As expected,
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VBI inhibits the RNA synthesis by interfering with the template function of T3 DNA and not by interfering with the T3 RNA polymerase (unpublished results).

The influence of VBI on the RNA synthesis at the level of initiation and elongation was determined by examining its effect upon the incorporation of (\(\gamma^\text{-}3^2\text{P}\))GTP and \((^{\text{3}}\text{H})\)UMP into RNA. Since the RNA species synthesized in vitro by the T3 RNA polymerase on T3 DNA start exclusively with the sequence pppG(pG) (11) the incorporation of \((\gamma^\text{-}3^2\text{P})\)GTP indicates the frequency of initiation events. The incorporation of \((^{\text{3}}\text{H})\)UMP represents the elongation of the RNA chains. Fig. 2 shows the time course of the incorporation of the nucleoside triphosphates in the presence of \(6 \times 10^{-6} \text{ mol/1 VBI}\). In comparison to the control the incorporation of \((^{\text{3}}\text{H})\)UMP is strongly reduced beginning from the start of the reaction. The reaction inhibited by the antibiotic proceeds almost linearly during the first 16 min. On the other hand, the incorporation of \((\gamma^\text{-}3^2\text{P})\)GTP shows a decrease after the initial lag phase.

![Figure 2: Kinetics of the effect of VBI on the incorporation of \((\gamma^\text{-}3^2\text{P})\)GTP and \((^{\text{3}}\text{H})\)UMP into RNA chains. T3 DNA was preincubated with \(6 \times 10^{-6} \text{ mol/1 VBI}\) for 5 min at 35°C. The reaction was started by the addition of T3 RNA polymerase and the four nucleoside triphosphates. At the indicated times aliquots of 0.2 ml were withdrawn. \((\gamma^\text{-}3^2\text{P})\)GTP incorporation: control (\(\Delta-\Delta\)), incorporation in the presence of VBI (\(\triangle-\triangle\)). \((^{\text{3}}\text{H})\)UMP incorporation: control (\(\bigcirc-\bigcirc\)), incorporation in the presence of VBI (\(\bullet-\bullet\)).](image-url)
hand VBI has little influence on the incorporation of \((\gamma^{32P})GTP\) if measured 1 min after starting the reaction. Until the 16th min there is a slow, but linear increase of the amount of \((\gamma^{32P})GTP\) incorporated. The inhibition of the \((\gamma^{32P})GTP\) incorporation by VBI measured after 1 min and 10 min incubation depends on the concentration of the antibiotic (Table 1).

Actinomycin D tested as reference substance reduces the incorporation of the first nucleotide only to a little extent at concentrations which strongly depress the incorporation of \((^{3H})UMP\).

The results indicate that the initiation of the RNA chains is not or only slightly affected by VBI concentrations which inhibit the elongation of RNA chains. The increasing influence of VBI on the incorporation of \((\gamma^{32P})GTP\) during the time of incubation may be caused by a reduced reinitiation of the RNA synthesis in consequence of the reduced growth rate of the RNA chains in the presence of VBI. The strong inhibition of the in-

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Incorporation of ((\gamma^{32P})GTP) (% of the control)</th>
<th>Incorporation of ((^{3H})UMP) (% of the control)</th>
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<tbody>
<tr>
<td></td>
<td>1 min</td>
<td>10 min</td>
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<tr>
<td>Violamycin BI</td>
<td></td>
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</tr>
<tr>
<td>(3.3 \times 10^{-6})</td>
<td>96</td>
<td>67</td>
</tr>
<tr>
<td>(6 \times 10^{-6})</td>
<td>88</td>
<td>43</td>
</tr>
<tr>
<td>(20 \times 10^{-6})</td>
<td>31</td>
<td>14</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td></td>
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<tr>
<td>(0.8 \times 10^{-6})</td>
<td>117</td>
<td>78</td>
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The reaction mixtures containing the antibiotic concentrations indicated, T3 DNA and the nucleoside triphosphates were preincubated for 5 min at 35°C. The RNA synthesis was started by the addition of T3 RNA polymerase and terminated after 1 min and 10 min, respectively.
corporation of the first and the following nucleotides by high antibiotic concentrations can be accounted for by an additional influence on the binding reaction between the T3 RNA polymerase and T3 DNA before the start of the RNA synthesis.

**Molecular weights of the RNA transcripts**

The influence of VBI on the elongation rate of the RNA synthesis was investigated by separation of the transcription products by means of gel electrophoresis. Fig. 3 shows the growth of the maximum molecular weights of RNA formed during 15 min in the absence and in the presence of $3.3 \times 10^{-6}$ mol/l VBI. This antibiotic concentration reduced the incorporation of $({}^2H)UMP$ to 20% of the control measured after an incubation time of 5 min. In the control assay the RNA species with the highest molecular weight of $5.5 \times 10^6$ are synthesized within

![Graph showing molecular weights of RNA transcripts](image)

**Figure 3.** Kinetics of the growth rate of RNA chains in the presence of VBI. After preincubation of 29.6 nmol T3 DNA with $3.3 \times 10^{-6}$ mol/l VBI in an assay mixture of 0.5 ml for 3 min at $35^\circC$ GTP and T3 RNA polymerase were added. To synchronize the initiation of the RNA synthesis this reaction mixture was further incubated for 2 min. By addition of the missing nucleoside triphosphates the elongation of the RNA chains was started and at the indicated time points aliquots were withdrawn. The maximum molecular weight corresponds to the peak of the radioactivity on the gels. Control (O—O), RNA synthesis in the presence of VBI (●—●).
min, whereas in the presence of VBI the rate of chain growth is reduced. The maximum molecular weight of the RNA products is reached only after a prolonged incubation time. The analysis of the RNA products of the inhibited reactions in gels containing 5 - 10% acrylamide did not reveal any accumulation of species with very low molecular weights (data not shown). These results confirm the conclusion made in the above section that the main effect of VBI is the inhibition of the growth rate of the RNA chains.

Effect of VBI on the binding between T3 RNA polymerase and T3 DNA

The binding of T3 RNA polymerase to T3 DNA and the influence of the drug on this first step of the RNA synthesis was measured by the retention of the complex on nitrocellulose membrane filters. The stability of this complex is relatively weak (11). The binding reaction alone requires different ionic concentrations (Mg²⁺-concentration 0.5 mmol/l) than those optimal for the RNA synthesis. The effect of VBI on the complex formation was investigated by two kinds of assays. In assay 1 only the binding reaction between the enzyme and DNA was studied, whereas in assay 2 the complex formed after initiation of the RNA synthesis was investigated. By omitting one of the nucleoside triphosphates (CTP) the elongation of the RNA chains was prevented. The ratio of the T3 RNA polymerase to T3 DNA in the two kinds of assays was the same as that employed for the RNA synthesis reaction. At this concentration of the enzyme 15 - 20% of the (³²P)DNA added were retained in the absence of the nucleoside triphosphates and 35 - 40% in their presence. For 100% retention a tenfold higher concentration of T3 RNA polymerase is necessary but an unspecific binding of T3 RNA polymerase to T3 DNA cannot be ruled out at this high ratio of the enzyme to DNA.

In both methods of the assay the amount of (³²P)DNA retained on the filters was determined after incubation of increasing amounts of VBI with T3 DNA followed by the addition of the T3 RNA polymerase. In Fig. 4 can be seen that the binding reaction between T3 RNA polymerase and T3 DNA under both condi-
Violamycin BI (mol/l)

Figure 4. Effect of VBI on the binding between T3 RNA polymerase and T3 DNA. The preincubation mixture contained the indicated amounts of VBI and (32P)DNA. After incubation for 3 min at 30°C T3 RNA polymerase was added and the reaction mixture incubated further for 5 min at 30°C. The RNA synthesis was carried out in a parallel assay. Complex formation between T3 RNA polymerase and T3 DNA in the absence of nucleoside triphosphates (●—●), complex formation between T3 RNA polymerase and T3 DNA in the presence of the three nucleoside triphosphates GTP, ATP, and UTP (○—○), RNA synthesis (▲—▲).

The extent of the binding is not or only slightly affected by antibiotic concentrations which influence the rate of the RNA synthesis. In the presence of higher concentrations of VBI having a strong effect on the RNA synthesis the complex formation is progressively inhibited. Any retention of complexes between VBI and (32P)-labelled T3 DNA on nitrocellulose filters could not be observed as described by Neogy et al. (12) for anthracyclines and other DNA binding drugs.

In the presence of the nucleoside triphosphates the extent of the binding between the enzyme and DNA at a fixed antibiotic concentration is time-dependent. After prolonged incubation of the T3 RNA polymerase with T3 DNA which was preincubated with the antibiotic the amount of (32P)DNA retained is enhanced (Fig. 5). This was observed only under conditions optimal for the RNA synthesis and not under conditions optimal for the binding reaction in the absence of the nucleoside triphosphates. This can be explained by the fact that due to the attachment of
Figure 5. Dependence of the amount of T3 RNA polymerase bound to the T3 DNA-VBI complex on the time. The preincubation mixture contained T3 DNA and $5 \times 10^{-5}$ mol/l VBI. After incubation for 3 min at 30°C the amount of $^{32}$P-DNA retained was measured at the indicated time points after addition of T3 RNA polymerase. Triangles: binding of T3 RNA polymerase to T3 DNA in the absence of nucleoside triphosphates; amount of $^{32}$P-DNA retained in the control ($\Delta-\Delta$), binding in the presence of VBI in % of the control (A-A). Circles: binding of T3 RNA polymerase to T3 DNA in the presence of the nucleoside triphosphates GTP, ATP, and UTP; amount of $^{32}$P-DNA retained in the control (O-O), binding in the presence of VBI in % of the control (●-●).

The complex formation in the absence of the nucleoside triphosphates is relatively weak so that the binding of VBI to T3 DNA destroys the complex to a large extent. Fig. 6 shows the dissociation of the complex in dependence on the time after addition of $1.5 \times 10^{-6}$ mol/l VBI. After initiation of the RNA synthesis VBI has little influence observed over 20 min.

These data are in agreement with the results presented in Fig. 2 and demonstrate that the main target of VBI is not the initiation i.e. the binding of RNA polymerase to DNA and the binding of the first nucleotide GTP. Only in the presence of

the antibiotic to DNA the binding between the RNA polymerase and DNA is lowered. On the other hand the stability of the T3 RNA polymerase - T3 DNA complex is increased by the initiation of the RNA synthesis, thus promoting the binding reaction in the presence of the antibiotic.
Figure 6. Stability of the complex between T3 RNA polymerase and T3 DNA in the presence of VBI. Complex formation in the absence of the nucleoside triphosphates (●—●). After preincubation of T3 RNA polymerase and ($^{32}$P)DNA for 3 min at 30°C VBI (1.5 x $10^{-6}$ mol/l) was added. At the indicated time points aliquots of 0.2 ml were removed and the amount of ($^{32}$P)DNA retained was determined. Complex formation in the presence of GTP, ATP, and UTP (○—○). The preincubation mixture contained T3 RNA polymerase, ($^{32}$P)DNA, GTP, ATP, and UTP. After incubation for 3 min at 30°C VBI (1.6 x $10^{-6}$ mol/l) was added. 0.2 ml aliquots were removed at the indicated time points and the amount of ($^{32}$P)DNA retained was determined.

DISCUSSION

The violamycin component VBI has the strongest inhibitory effect on the transcription of T3 DNA by bacteriophage T3-induced RNA polymerase. The two other known components of the antibiotic - VBII and VA - inhibit the RNA synthesis to a considerably lower extent, even in the presence of high antibiotic concentrations tested. This can be due to their weaker interaction with DNA (3). For the biological action of anthracycline antibiotics and the binding to DNA the amino sugar residue plays a great role (13, 14, 15). We assume that also in the case of the violamycin components the number and (or) the structure of the respective sugar constituents contribute to the different binding affinity to DNA and, therefore, to their
gradual effect on the RNA synthesis.

The inhibitory effect of VBI on the RNA synthesis by binding to DNA corresponds to the behaviour of the other anthracycline antibiotics (16). Our more detailed investigations on the mode of action demonstrate that the main effect of VBI does not concern the initiation of the RNA synthesis (i.e. the binding of the T3 RNA polymerase to T3 DNA and the incorporation of the first nucleotide) as described for other intercalating substances such as ethidium bromide and proflavine, respectively (7, 17). The patterns of the gel electrophoresis of the RNA products formed in the presence of VBI show that the antibiotic mainly reduces the elongation rate of the RNA chains. The effect of VBI on the RNA synthesis can partly be compared with the mechanism of action of actinomycin D. Actinomycin D inhibits the rate of chain growth (18) by affecting the rate terms for both GTP and CTP incorporation (19). The two antibiotics differ in their influence on the incorporation of the first nucleotide. In contrast to VBI the presence of actinomycin D concentrations reducing strongly the RNA synthesis has no marked effect on the incorporation of \((\gamma^{32}\text{P})\text{GTP}\) as was shown by other authors, too (18, 19, 20). The difference between the mode of action of both DNA intercalating antibiotics actinomycin D and VBI may be caused by the binding of these two antibiotics to different sites on DNA. Löber et al. (21) have found that at saturating levels actinomycin D or netropsin do not significantly decrease the binding of VBI.

The reduced growth rate of RNA chains due to the attachment of the antibiotic VBI to T3 DNA causes an inhibition of the reinitiation of the RNA synthesis. For this reason the rate of inhibition for the incorporation of \((\gamma^{32}\text{P})\text{-labelled GTP}\) caused by VBI is greater if measured after 10 min than after 1 min of incubation. In the literature the incorporation of \((\gamma^{32}\text{P})\text{GTP}\) in the presence of various drugs determined 10 min after the start of the reaction is often taken as a measure for the effect of these substances on the initiation of the RNA synthesis. Our findings demonstrate that these experimental conditions do not allow a precise answer on the mode of action of the drugs. The reduction of \((\gamma^{32}\text{P})\text{GTP}\) incorporated during
10 min incubation can be the result either of an inhibited initiation right from the beginning of the RNA synthesis or of a reduced rate of reinitiation due to the decreased growth of RNA chains in the presence of the drugs.

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REFERENCES