Inhibition of Vesicular Stomatitis Virus RNA Synthesis by 2',3'-Dideoxycytidine 5'-Triphosphate

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SUMMARY

The ability of the compound 2',3'-dideoxycytidine 5'-triphosphate (ddCTP) to serve as an inhibitor of viral RNA synthesis was examined using an in vitro system that supports vesicular stomatitis virus (VSV) protein synthesis, transcription and replication. Viral RNA synthesis was inhibited by 87 and 98% of control, respectively, in reactions containing 1 mM- and 10 mM-ddCTP in place of CTP. VSV RNA replication and transcription were inhibited equally by ddCTP. At a concentration of 1 mM-ddCTP, there was no inhibitory effect on viral protein synthesis; at 10 mM-ddCTP, total protein synthesis was inhibited by 30% as compared to control reactions. The presence of ddCTP had no effect on the size or relative molar amounts of each protein synthesized as analysed by electrophoresis on polyacrylamide gels. This is the first report describing a compound that will inhibit VSV RNA synthesis in vitro without compromising the concurrent synthesis and modification of proteins.

The nucleocapsid of vesicular stomatitis virus (VSV) consists of a single-stranded minus-sense RNA of approximately 12000 nucleotides complexed with the viral proteins N, the major nucleoprotein, NS, a phosphoprotein, and L, a polymerase component. In VSV virions, the nucleocapsid is surrounded by a lipoprotein membrane containing the viral matrix protein, M, and the glycoprotein, G. The viral polymerase of the nucleocapsid directs two distinct RNA synthetic processes: (i) the transcription of the five VSV mRNAs, a process that does not require concurrent viral protein synthesis, and (ii) the replication of the VSV genome, a process which in vivo requires the simultaneous production of viral proteins (Wertz & Levine, 1973). The products of VSV RNA replication, genome-length positive and negative strand RNAs, are found in the cell only in the form of nucleocapsids and as a result are resistant to degradation by ribonuclease (Soria et al., 1974).

Recently, we have established an in vitro system (Davis & Wertz, 1981, 1982) that carries out both transcription and replication of the VSV genome. This cell-free system consists of VSV intracellular nucleocapsids, a rabbit reticulocyte lysate and purified VSV mRNAs. As found in vivo, the replication of RNA in the system is dependent upon concurrent protein synthesis (Davis & Wertz, 1982). The in vitro replication products are predominantly resistant to degradation by ribonucleases (J. Patton, N. Davis and G. Wertz, unpublished observations). Toexploit this replication system, we required an inhibitor of VSV RNA synthesis that would satisfy the following criteria. The inhibitor must not (i) interfere with protein synthesis or modification, (ii) prevent the association of proteins with nucleocapsids, or (iii) affect the nucleocapsid structure.

Several compounds which interfere with the synthesis of mature VSV mRNAs or genome-length RNA have been described previously. These include analogues of ATP such as ara-ATP (Chanda & Banerjee, 1980) and toyocamycin (Moyer & Holmes, 1979), the pyrophosphate analogue phosphonoformate (Chanda & Banerjee, 1980), the trypsin inhibitor N-α-tosyl-1-lysyl-chloromethyl ketone (Witt & Summers, 1980), the amino acid analogue cycloleucine (Moyer, 1981) and also highly basic proteins such as protamine (Banerjee et al., 1981). Although the
### Table 1. Effect of ddCTP on VSV RNA synthesis, transcription and replication

#### (a) Effect on RNA synthesis*

<table>
<thead>
<tr>
<th>Concentration added (mM)</th>
<th>CTP</th>
<th>ddCTP</th>
<th>Counts/min/rxn†</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-6</td>
<td>0</td>
<td>2-2 × 10⁶</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>7-1 × 10⁶</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>2-9 × 10⁶</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>4-7 × 10⁴</td>
<td>98</td>
<td></td>
</tr>
</tbody>
</table>

#### (b) Effect on transcription and replication

<table>
<thead>
<tr>
<th>Concentration added (mM)</th>
<th>CTP</th>
<th>ddCTP</th>
<th>Counts/min/rxn‡</th>
<th>Percentage inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleocapsid RNA</td>
<td>0-6</td>
<td>0</td>
<td>1-6 × 10⁵</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2-0 × 10⁴</td>
<td>87</td>
</tr>
<tr>
<td>Messenger RNA</td>
<td>0-6</td>
<td>0</td>
<td>2-4 × 10⁶</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2-7 × 10⁵</td>
<td>89</td>
</tr>
</tbody>
</table>

* VSV (Indiana serotype) intracellular nucleocapsids were prepared for the replication system from baby hamster kidney cells (BHK-21/13) at 6 h post-infection as described earlier (Davis & Wertz, 1982). Components and conditions for the system are the same as those described in detail elsewhere (Davis & Wertz, 1982). Reactions in which purified virions were used in place of intracellular nucleocapsids contained 0-05% Triton N101. In reactions containing ddCTP, no exogenous CTP was supplied to the system. Furthermore, where 1 or 10 mM-ddCTP was present, 0-4 and 9-4 mM additional magnesium acetate, respectively, were added to the reactions. Reactions were performed in a final volume of 50 µl and consisted of 70% (v/v) micrococcal nuclease-treated rabbit reticulocyte lysate, 5 µg purified VSV mRNA, 12% (v/v) VSV intracellular nucleocapsids, 50 mM-HEPES pH 7-6, 10 mM-creatine phosphate, 1 mM-ATP, 0-6 mM each of CTP and GTP, 0-1 mM-UTP, 0-05 mM each of the 20 amino acids except that methionine was 12-5 µM, 2 mM-magnesium acetate, 2 mM-dithioerythritol, 66 mM-ammonium chloride, 14 mM-potassium acetate, and 2-5 µg rabbit liver tRNA. Viral RNA was labelled in vitro by adding 1 mCi/ml of [3H]UTP. Incorporation of isotope into product was assayed by counting radioactivity present in trichloroacetic acid precipitates of 2 µl samples taken from the reaction mixture.

† Represents total acid-precipitable counts per min incorporated by 90 min post-incubation minus the value at 0 min for each reaction.

‡ Represents the 3H-labelled RNA synthesized in the replication system that banded at the position of nucleocapsids (nucleocapsid RNA) or pelleted (messenger RNA) in a CsCl gradient (Davis & Wertz, 1982).

### Short communication

The compounds described above have been useful in characterizing certain aspects of VSV RNA synthesis, many of them may inhibit other macromolecular synthetic processes in addition to inhibiting VSV RNA synthesis. For example, some of these compounds are analogues of ATP and may interfere with viral protein synthesis, or are substances which affect the modification of viral proteins (e.g. phosphorylation). Additionally, many of these substances do not inhibit both VSV replication and transcription equally. In summary, no compound has been described that effectively inhibits VSV RNA synthesis without potentially interfering with other synthetic processes such as protein synthesis and maturation.

The compound 2',3'-dideoxycytidine 5'-triphosphate (ddCTP; P-L Biochemicals), a chain terminator of DNA synthesis, met, in theory, the criteria described above and, therefore, was tested in the in vitro replication system for its effects on viral transcription, replication and protein synthesis. The choice of this dCTP analogue was based on a report by Schubert & Lazzarini (1982) showing that the deoxynucleotides dCTP and dGTP can be incorporated into viral RNAs by the VSV polymerase. In this communication we demonstrate that ddCTP is an effective inhibitor of VSV RNA synthesis in vitro. At the concentrations of ddCTP examined, this inhibitor had little or no effect on the level of concurrent protein synthesis, nor did it affect the characteristics of the proteins synthesized as determined by polyacrylamide gel electrophoresis. This is the first report of a method for inhibiting VSV RNA synthesis in a coupled RNA–protein synthesis system that has little or no effect on the concurrent synthesis or modification of proteins.
Initially, we investigated the possibility that the synthesis of RNA in the replication system could be blocked simply by not adding the ribonucleotide, CTP, to the in vitro reactions. As shown in Table 1, the omission of added CTP from the replication system resulted in a level of RNA synthesis in vitro which was 30 to 35% of the control reaction (0.6 mM-CTP). Since RNA synthesis was not completely blocked by the omission of added CTP, this result indicates that a significant amount of endogenous CTP, presumably from the rabbit reticulocyte lysate, was present in the replication system. Thus, the omission of added CTP was not, by itself, sufficient to prevent the synthesis of RNA by this system.

The effect of ddCTP on the synthesis of 3H-labelled RNA in the VSV in vitro replication system was examined by the addition of the compound to a concentration of 1 or 10 mM in place of added CTP. The results of these experiments are presented in Table 1. In the presence of 1 mM-ddCTP the incorporation of [3H]UTP in vitro was decreased by 85 to 90% as compared to the control reaction (0.6 mM-CTP). At a concentration of 10 mM-ddCTP, incorporation of [3H]UTP by the in vitro system was essentially blocked (98% inhibition as compared to control). The data show that the level of inhibition achieved in the presence of 1 mM-ddCTP (and no added CTP) was significantly greater than that obtained by the omission of added CTP itself and that the substitution of added CTP by ddCTP effectively inhibited RNA synthesis in the replication system even in the presence of the endogenous CTP found in the system.

The 3H-labelled RNA products synthesized by intracellular nucleocapsids in the presence of 0, 1, and 10 mM-ddCTP were compared by electrophoresis in a 1.5% agarose gel containing 6 M-urea. Densitometric profiles of the fluorescent products from the dried gel are shown in Fig. 1. A scan of the 3H-labelled RNA products made in a reaction containing no ddCTP and 0.6 mM added CTP showed that genome-length RNA and the five viral mRNAs were synthesized in the replication system (upper panel). Consistent with the observation that 1 mM-ddCTP inhibited overall RNA synthesis by 85 to 90% (Table 1a), the quantity of each RNA species produced in the presence of 1 mM-ddCTP, as measured by densitometry (lower panel), was decreased by at
least 85%. (Although not detectable on the scan, fluorograms revealed that genome-length and L messenger RNAs were synthesized in reactions containing 1 mM-ddCTP.) An identical scan of 3H-labelled RNAs made in the presence of 10 mM-ddCTP produced no detectable peaks (data not shown). This is in agreement with results presented above (Table 1a) indicating that RNA synthesis was inhibited by 98% at this concentration of inhibitor. Fluorograms obtained after long exposure of gels of the 3H-labelled RNAs made in the presence of 10 mM-ddCTP showed that even at this level of inhibition, detectable amounts of full-length 3H-labelled genome and messenger RNAs were synthesized.

To determine whether or not the residual level of full-length 3H-labelled viral RNA synthesized in the presence of ddCTP represented the elongation of nascent transcripts associated with intracellular nucleocapsids used in the replication system, the nucleocapsids were replaced with purified VSV virions. Because virions do not have associated nascent transcripts, the synthesis of full-length VSV 3H-labelled mRNAs by virions in the presence of ddCTP would require the initiation and complete elongation of transcripts in vitro. When purified VSV virions were used in place of intracellular nucleocapsids in the replication system, analysis of the 3H-labelled RNAs produced in the control reaction (0-6 mM-CTP) by electrophoresis on agarose gels containing 6 M-urea showed that all five VSV mRNAs were synthesized. In reactions containing 1 mM-ddCTP and no added CTP, no messenger-sized RNA was synthesized (data not shown). This finding indicates that the appearance of 3H-labelled full-length viral mRNAs in reactions containing intracellular nucleocapsids and ddCTP probably represented the addition of a few 3'-terminal residues to nearly mature nascent transcripts.

We further investigated the possibility that ddCTP might inhibit either VSV replication or transcription preferentially. Because approximately 95% of total incorporation of [3H]UTP in the in vitro system is into transcription products with 5% into replicative products (Davis & Wertz, 1982), it is necessary to enhance the distinction between these two types of RNAs. We used three methods to distinguish between replicative and transcriptive products: (i) equilibrium sedimentation in CsCl gradients [replication products will band in CsCl gradients while mature mRNAs will pellet (Davis & Wertz, 1982)], (ii) RNase-treatment of replication products [negative-stranded RNAs made by the replication system are predominantly RNase-resistant while all transcriptive products are RNase-sensitive (J. Patton, N. Davis & G. Wertz, unpublished results)] and (iii) analysis of CsCl-banded RNA products by gel electrophoresis.

The effect of ddCTP on the amount of 3H-labelled RNA which pelleted in CsCl gradients showed that the amount of mature viral mRNA produced in the presence of 1 mM-ddCTP and no added CTP was decreased by 85 to 90% as compared to the control reaction (Table 1b). The amount of 3H-labelled RNA produced in the presence of 1 mM-ddCTP that banded at the position of nucleocapsids was also decreased by 85 to 90%. Analysis of the banded RNA products by gel electrophoresis confirmed that the production of nucleocapsid-associated 3H-labelled genome-length RNA was inhibited by at least 75% in the presence of 1 mM-ddCTP. However, while the CsCl-banded RNA includes full-length genome RNA, a significant amount of nascent replicative and transcriptive RNA is also associated with the templates. Therefore, to test directly whether replication and transcription were equally sensitive to inhibition by ddCTP, the nucleocapsid material obtained by centrifugation of reaction products in CsCl gradients was digested with 25 μg/ml pancreatic RNase for 30 min at 23 °C, thus leaving intact only replicative 3H-labelled RNA. In a typical reaction, 1 mM-ddCTP inhibited the amount of RNase-resistant RNA product by 82 to 85%. Thus, these data showed that replication of VSV RNA was inhibited to the same extent as mRNA synthesis in the presence of 1 mM-ddCTP.

The effects of ddCTP on protein synthesis in the replication system were examined by comparing the incorporation of [35S]methionine into acid-precipitable product in the presence of 0, 1, and 10 mM-ddCTP. The results are shown in Fig. 2. There was no significant difference between the levels of 35S-labelled proteins synthesized in the presence of 1 mM-ddCTP or absence of ddCTP. However, a 30% reduction was seen in the level of [35S]methionine incorporated in the presence of 10 mM-ddCTP as compared to the control. Since ddCTP was supplied to the replication system as a sodium salt, the reduction in protein synthesis seen at 10 mM-ddCTP may be due to the inhibition of translation by excess sodium ions. These results
show that, while high concentrations of ddCTP decreased [\( ^{35} \text{S} \)]methionine incorporation in the system by 30\%, low concentrations of ddCTP (1 mM) which were able to inhibit RNA synthesis by 85 to 90\% had no effect on concurrent protein synthesis.

To determine whether complete and properly modified viral proteins were synthesized in the replication system in the presence of ddCTP, \(^{35}\text{S}\)-labelled protein products were analysed by electrophoresis on 10% polyacrylamide gels containing 7 M-urea and 0.1% SDS (Kingsford & Emerson, 1980). In the presence of 1 or 10 mM-ddCTP, the viral proteins L, N, NS1, NS2, and M were synthesized and all co-migrated with the corresponding proteins made in infected BHK cells. The non-glycosylated precursor of the VSV G protein was also synthesized by the replication system. The relative molar amounts of viral proteins synthesized in vitro in the presence of 0, 1, and 10 mM-ddCTP were the same (data not shown). Hence, these results demonstrated that ddCTP had little or no effect on translation of the VSV mRNAs. The fact that the VSV phosphoproteins NS1 and NS2 synthesized by the replication system co-migrated with the corresponding proteins made in vivo also indicated that ddCTP does not interfere with protein phosphorylation.

This is the first report describing a compound that will inhibit VSV RNA synthesis without compromising the synthesis and modification of proteins in vitro. We demonstrate that the compound ddCTP effectively inhibits VSV RNA synthesis in an in vitro replication system and that it has little or no effect on viral protein synthesis. The compound ddCTP inhibits both VSV replication and transcription equally in vitro. This result indicates that both the VSV replicase and transcriptase are equally sensitive to inhibition by ddCTP which suggests that both processes are carried out by the same polymerase.

By inhibiting RNA synthesis in VSV replication systems with ddCTP, future studies can be undertaken to determine to what degree viral proteins synthesized by these systems can be associated with and perhaps alter the function of nucleocapsid templates. Also, the degree to which newly synthesized viral polymerase components (NS and L proteins) can exchange with the polymerase components of active and inactive nucleocapsids can be compared.

To what extent the synthesis of viral RNA by RNA viruses other than VSV may be inhibited by ddCTP remains to be tested. However, the results presented here suggest that ddCTP and perhaps other chain-terminating nucleotides may have wide utility for the study of RNA-synthetic events in vitro.
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REFERENCES


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