Visna Virus as an In Vitro Model for Human Immunodeficiency Virus and Inhibition by Ribavirin, Phosphonoformate, and 2',3'-Dideoxynucleosides

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Inhibition of visna virus replication in vitro by several compounds previously reported to inhibit replication of human immunodeficiency virus (HIV) was examined. Ribavirin concentrations as high as 1 mM reduced virus production by <50% relative to controls. The concentration of phosphonoformate reducing virus replication by 50% was 80 μM. 2',3'-Dideoxynucleosides were potent inhibitors of visna virus replication. The 50% inhibitory concentrations for dideoxyguanosine, dideoxyadenosine, and dideoxythymidine were 0.1, 0.2, and 0.3 μM, respectively. In contrast, weak inhibition was produced by 100 μM dideoxythymidine. These results are consistent with the reported susceptibility of HIV replication to inhibition by these compounds in vitro. The interaction of visna virus reverse transcriptase with several inhibitors was also examined. Reverse transcriptase was inhibited by phosphonoformate, ribavirin 5'-triphosphate, ddATP, ddCTP, ddGTP, and ddTTP. The last four compounds inhibited incorporation of homologous 2'-deoxynucleoside 5'-triphosphates into polynucleotides by a competitive mechanism. In view of the biological similarities between visna virus and HIV and the similar in vitro susceptibility of visna virus replication to known inhibitors of HIV, visna virus may provide a good model for studying the inhibition of HIV replication in vitro. Because visna virus is not pathogenic to humans, this model may facilitate the identification of compounds for further investigation into the treatment of HIV-induced disease.

Many laboratories are interested in screening new compounds for activity against human immunodeficiency virus (HIV). Because antiviral testing on a large scale involves increased risk of accidental human exposure, it would be desirable to identify a virus related to HIV which is nonpathogenic for humans yet shows similar in vitro drug susceptibilities. Recently, several laboratories have reported similarities in gene sequences, genomic organization, and possible mechanisms of pathogenicity between visna virus and HIV (4, 13, 15, 32). HIV is now classified in the retrovirus subfamily Lentivirinae, along with several animal viruses including visna virus, caprine arthritis encephalitis virus, and equine infectious anemia virus.

In view of the importance of HIV and its relatedness to visna virus, it is important to investigate whether visna virus shares antiviral compound sensitivities with HIV. This study reports inhibition of visna virus replication in vitro by several compounds known to inhibit replication of HIV in cell culture. In addition, we demonstrate the kinetic interaction of visna virus reverse transcriptase with 5'-triphosphates of several 2',3'-dideoxynucleosides. These results suggest that visna virus provides a model for HIV replication in vitro which may be useful to laboratories interested in identifying new chemotherapeutic agents for use against HIV.

MATERIALS AND METHODS

Chemicals. All chemicals used were reagent grade or better. 2',3'-Dideoxynucleosides, their 5'-triphosphate derivatives, synthetic homopolymer, and oligo(dT)-cellulose were obtained from Pharmacia, Inc., Piscataway, N.J. Unlabeled and 3H-labeled deoxynucleoside 5'-triphosphates (dNTPs) were from ICN Biochemicals, Cleveland, Ohio.

and ICN Radiochemicals, Irvine, Calif. Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide) and its 5'-triphosphate were from Viratek, Inc., Costa Mesa, Calif. Phosphonoformate (PFA) was purchased from Sigma Chemical Co., St. Louis, Mo. Poly(A)-tailed mRNA was isolated from rabbit reticulocytes (Pel-Freez Biologicals, Rogers, Ark.) by magnesium precipitation followed by oligo(dT)-cellulose affinity chromatography (1, 17, 23).

Cell culture. All cell culture reagents were obtained from GIBCO Laboratories, Grand Island, N.Y., unless otherwise specified. A line of sheep choroid plexus (SCP) cells was established from a newborn lamb essentially as described by Sundquist and Larner (33) and maintained as monolayer cultures in antibiotic-free Eagle minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (Flow Laboratories, Inc., McLean, Va.). Cells were free of mycoplasma contamination as determined by broth/agar culturing under anaerobic conditions and by use of a [3H]DNA probe directed against homologous mycoplasma rRNA (Genprobe, San Diego, Calif.). Senescent SCP cultures were renewed every few months from cells which had been stored in liquid nitrogen at low passage number. SCP cells may also be obtained from the American Type Culture Collection, Rockville, Md.

To investigate cytotoxic effects, logarithmically growing SCP cells were trypsinized and adjusted to 2 × 10⁴ cells per ml in EMEM supplemented with 10% fetal bovine serum. Aliquots of 0.1 ml were used to seed 96-well plates which were then incubated for 24 h at 37°C in a humidified 5% CO₂ atmosphere. Serial dilutions of test compound were added to produce a final volume of 200 μl. Plates were incubated for 120 h at 37°C and then rinsed with Earle balanced salt solution, trypsinized to single cell suspensions, and counted with a model ZM Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.).

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**Virus propagation.** Visna virus, strain 1514, was a gift from Opendra Narayan of Johns Hopkins University. Slightly subconfluent SCP cell monolayers were rinsed with phosphate-buffered saline, and then virus was adsorbed for 1 h at 37°C in a small volume of serum-free EMEM at a multiplicity of infection of 0.05 PFU per cell. After adsorption, EMEM supplemented with 1% heat-inactivated lamb serum (56°C for 30 min) and gentamicin (25 μg/ml) was added. After 2 days at 37°C, medium was changed and incubation was continued until virus-induced cell pathology was extensive (days 4 to 6). Cells and culture supernatant were then pooled and stored at −70°C.

Virus was titered by plaque assay in six-well SCP cell cultures. Cells were rinsed with phosphate-buffered saline and then infected with 200-μl volumes of virus which had been serially diluted in unsupplemented EMEM. After adsorption for 1 h at 37°C, inoculum was aspirated and infected cell monolayers were overlaid with EMEM containing 1% heat-inactivated lamb serum, 25 μg of gentamicin per ml, and 0.5% low-gelling-temperature agarose (SeaPlaque; FMC Corp., Marine Colloids Div., Rockland, Maine). After 6 days of further incubation at 37°C, monolayers were stained with 1% crystal violet in 20% ethanol.

**Antiviral assays.** Plaque reduction assays were performed in six-well SCP plates, following a published procedure (28). Virus sufficient to produce 100 to 200 plaques was exposed to test compound for 10 days, and then plaques were counted. Cytotoxic effect (CPE) inhibition assays were performed in 96-well SCP microtiter plates (30). Cells were infected at a multiplicity of 0.01 PFU per cell prior to addition of test compound. After 5 days of incubation, CPE in untreated cultures was considered to have reached 100% and treated cultures were compared to these. Yield reduction assays were performed by infecting 24-well SCP plates with virus at 0.05 PFU per cell prior to addition of test compounds. After 4 days of incubation, cultures were frozen at −70°C. Upon thawing, culture fluid was titered by plaque assay. Alternatively, culture fluid from yield reduction assays were assayed for reverse transcriptase activity.

**Reverse transcriptase assay.** Frozen, virus-infected cultures were thawed and cellular debris was pelleted at 350 × g for 10 min at 4°C. Reverse transcriptase was extracted and assayed essentially as described by Vrang and Oberg (35). Briefly, supernatant was transferred to ultracentrifuge tubes and virus was pelleted at 100,000 × g for 2 h in a 70.1 Ti rotor. Supernatant was aspirated and virus pellet was allowed to soften on ice in the presence of 500 μl of extraction buffer (33 mM Tris chloride, pH 8.0; 500 mM KCl, 2.5 mM dithiothreitol, 0.33% Triton X-100). After 1 h of extraction, pellet was homogenized by rapid pipetting with a Pipetman micropipetter and aliquots of 10 μl were assayed for reverse transcriptase activity. Unless otherwise indicated, reaction mixtures contained 50 mM Tris chloride (pH 8.0), 150 mM KCl, 6 mM MgCl2, 4 mM dithiothreitol, 100 μg of heated bovine serum albumin per ml, 50 μg of poly(rA)·oligo(dT)12-18 per ml, and 5 μM 3H[dGTP (2 × 104 cpm/μmol) in a volume of 100 μl. After incubation for 20 to 60 min at 37°C, aliquots of 50 μl were spotted onto GF/A filter circles (Whatman, Inc., Clifton, N.J.) and washed with ice-cold 5% trichloroacetic acid–1 mM sodium pyrophosphate. One enzyme unit is defined as the activity catalyzing incorporation of 1 nmol of TTP per h into polynucleotide under these conditions. Reaction progress was linear for at least 60 min at 37°C.

**RESULTS**

The replication of visna virus was resistant to inhibition by ribavirin in vitro (Fig. 1A). Inhibition of reverse transcriptase production by 1 mM ribavirin was <50%. Visna virus replication was more susceptible to inhibition by PFA (Fig. 1B), with a 50% inhibitory concentration (ED50) of 80 μM.

The interaction of PFA with visna virus reverse transcriptase is shown in Fig. 2A. Data are presented as a Dixon plot. Since PFA has been shown to inhibit reverse transcriptases noncompetitively with respect to dNTPs (34), a K_I of 0.6 μM can be determined from this plot.

Ribavirin 5'-triphosphate inhibited reverse transcriptase noncompetitively with respect to dGTP (Fig. 2B), with a concentration of approximately 250 μM producing 50% inhibition. However, inhibition of reverse transcriptase did not increase linearly with increasing concentrations of

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**FIG. 1.** Replication of visna virus. Confluent SCP cell monolayers in 24-well plates were infected with visna virus and then treated with either (A) ribavirin or (B) PFA at the concentrations indicated. All conditions were performed in quadruplicate. After 4 days of incubation, virus was pelleted from supernatants; then reverse transcriptase was extracted and assayed as described in Materials and Methods.

**FIG. 2.** Inhibition of visna virus reverse transcriptase by PFA and ribavirin 5'-triphosphate. (A) Reaction conditions were identical to those described in Materials and Methods except that PFA was added to produce the concentrations indicated. (B) Reaction conditions were identical to those described in Materials and Methods except that poly(rC)·oligo(dG)12-18 was used as template/primer and 3H[dGTP (2 × 104 cpm/μmol) was used as labeled substrate. Ribavirin 5'-triphosphate was added to produce the concentrations of 0 (○), 200 (■), 400 (●) and 800 (▲) μM. Reaction velocity is expressed as picomoles per hour.
ribavirin 5'-triphosphate, indicating that reaction velocity cannot be driven to zero by infinitely high concentrations of ribavirin 5'-triphosphate.

The effect of 2',3'-dideoxynucleosides upon visna virus replication in vitro is shown in Fig. 3. 2',3'-Dideoxycytidine (ddC) and 2',3'-Dideoxythymidine (ddT) were also potent inhibitors, with ED₅₀ values of 0.2 and 0.3 μM, respectively. In contrast, inhibition by 2',3'-dideoxythymidine (ddT) was weak at concentrations as high as 100 μM.

The toxicity of these compounds for actively dividing SCP cells was determined and compared with antiviral effects (Fig. 3). ddA, ddC, and ddG each produced only slight inhibition of cell growth at 100 μM, demonstrating the antiviral selectivity of these compounds in this culture system. ddT was not significantly toxic at the highest concentration used (100 μM).

To validate reverse transcriptase recovery as an accurate indicator of virus replication, the antiviral effects of 2',3'-dideoxynucleosides were examined by several methods. Results are summarized in Table 1. ED₅₀ values obtained by CPE inhibition, virus yield reduction, and reverse transcriptase recovery were in excellent agreement with each other. However, virus appeared three to five times more sensitive to inhibition by these nucleosides when assayed by the plaque reduction method.

![Graph showing antiviral and cytotoxic activities of 2',3'-dideoxynucleosides](image)

**TABLE 1.** Inhibition of visna virus replication in vitro by 2',3'-dideoxynucleosides compared by four methods

<table>
<thead>
<tr>
<th>Compound</th>
<th>ED₅₀ (μM)</th>
<th>CPE inhibition</th>
<th>Plaque reduction</th>
<th>Yield reduction</th>
<th>Reverse transcriptase induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddA</td>
<td>0.3</td>
<td>0.08</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>ddC</td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>ddG</td>
<td>0.1</td>
<td>0.02</td>
<td>0.1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>ddT</td>
<td>&gt;1,000</td>
<td>100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td></td>
</tr>
</tbody>
</table>

**DISCUSSION**

Visna virus is a nononcogenic retrovirus causing several chronic diseases of sheep including pneumonia, slow neuro-

**TABLE 2.** Kₐ and Kᵣ values of nucleoside 5'-triphosphates for visna virus reverse transcriptase

<table>
<thead>
<tr>
<th>Nucleoside 5'-triphosphate</th>
<th>Kₐ (nM)</th>
<th>ddNTP</th>
<th>Kᵣ (nM)</th>
<th>Kₐ/Kᵣ</th>
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<tr>
<td>dATP</td>
<td>1,330</td>
<td>ddATP</td>
<td>37</td>
<td>36</td>
</tr>
<tr>
<td>dCTP</td>
<td>550</td>
<td>ddCTP</td>
<td>140</td>
<td>4</td>
</tr>
<tr>
<td>dGTP</td>
<td>500</td>
<td>ddGTP</td>
<td>13</td>
<td>38</td>
</tr>
<tr>
<td>TTP</td>
<td>650</td>
<td>ddTTP</td>
<td>40</td>
<td>16</td>
</tr>
</tbody>
</table>

* Inhibition was competitive in all cases.
logical disease, and wasting (10, 14). The target of visna virus in vivo appears to be sheecl cells of the monocyte/macrophage lineage in which it causes a slow or latent infection (11, 12, 14, 21). However, rapid production of virus follows infection of SCP cells in vitro, facilitating growth of this virus in the laboratory. In view of the relatedness of visna virus to HIV (4, 13, 15, 32), we wanted to determine whether or not visna virus and HIV shared similar drug susceptibilities. This would allow visna virus to serve as an in vitro model for HIV infection, eliminating the risk of exposure to HIV for persons performing antiviral screening on a daily basis.

Several previous studies investigated the inhibition of retrovirus replication and reverse transcriptase by compounds used in this study. Ribavirin inhibits replication by Rou5 sarcoma and murine leukemia viruses in vitro (16, 26) and inhibits replication of both Rauscher murine leukemia and Friend leukemia viruses in mice (26, 27). PFA inhibits reverse transcriptase from a broad spectrum of retroviruses including avian myeloblastosis virus, Moloney murine leukemia virus, Rauscher murine leukemia virus, bovine leukemia virus, baboon endogenous virus, and simian sarcoma virus (18, 34). Reverse transcriptase from avian and murine retroviruses is inhibited by ddTTP (6, 9, 31), and 2',3'-dideoxyxinoses inhibit replication of several retroviruses in vitro (5, 9, 36). Therefore, reverse transcriptase appears to be a good target for antiviral chemotherapy.

A previous study by McCormick et al. (19) reported suppression of HIV replication in vitro by ribavirin concentrations of ≥200 μM. In the present study, however, the highest concentration (1 mM) of ribavirin used produced <50% inhibition of visna virus replication. Concentrations of ribavirin of >50 μM were observed to inhibit replication of SCP cells, but did not kill contact-inhibited cell monolayers (P. McKernan, unpublished observations). This is similar to a study by Balzarini et al. (2) in which ATH8 cells were not protected from the CPE of HIV by 400 μM ribavirin. Furthermore, these authors reported toxicity to ATH8 cells of ribavirin concentrations as low as 40 μM. Therefore, replication of visna virus in vitro is no more susceptible to inhibition by ribavirin than was replication of HIV in the study by Balzarini et al. (2).

Ribavirin 5'-triphosphate inhibited visna virus reverse transcriptase at high concentrations, with 50% inhibition occurring at 250 μM. This concentration can be achieved intracellularly following a 6-h exposure of SCP cells to 100 μM ribavirin (D. Smees, unpublished observations). However, the data in Fig. 1 show that 100 μM is not significantly antiviral, indicating that 50% inhibition of visna virus reverse transcriptase is not sufficient to inhibit viral replication. Higher concentrations of ribavirin will result in higher intracellular concentrations of ribavirin 5'-triphosphate, since triphosphate formation has been shown to be proportional to the extracellular concentration of nucleoside analogs (30). However, the partially noncompetitive inhibition pattern observed in Fig. 2 suggests that binding of ribavirin 5'-triphosphate to reverse transcriptase lowers the catalytic activity by as much as two-thirds, but does not block activity entirely. Therefore, infinitely high concentrations of extracellular ribavirin will not eliminate reverse transcriptase activity.

Ribavirin has shown significant activity in vivo against Rauscher and Friend leukemia viruses (26, 27) and is currently being tested clinically for use against HIV. It is possible that selective antiretroviral effects are produced by ribavirin only in vivo and in certain in vitro models and that antiviral effects in these systems are due to targets other than reverse transcriptase.

Visna virus replication was significantly inhibited by PFA. The ED$_{50}$ of 80 μM calculated in the present study is in good agreement with the values of 20 to 80 μM reported for visna virus by Sundquist and Larner (33). Previous in vitro studies with HIV reported 95 to 98% reduction of virus replication by 132 to 300 μM PFA (24, 25), indicating susceptibility similar to visna virus. In addition, PFA was a potent inhibitor of visna virus reverse transcriptase. The $K_I$ of 0.6 μM determined in the present study is similar to the 0.5 μM ED$_{50}$ previously reported for HIV reverse transcriptase (35). However, Sundquist and Larner (33) reported only 90% inhibition of visna virus reverse transcriptase by 100 μM PFA. Other laboratories have reported $K_I$s of <2 μM for HIV reverse transcriptase and complete inhibition by 5 μM PFA (24).

Recently, Mitsuya and Broder (20) reported potent and selective inhibition of HIV replication in vitro by several 2',3'-dideoxynucleosides. Although ED$_{50}$s were not estimated, ddC, ddA, and 2',3'-dideoxyinosine completely protected ATH8 cells from killing by HIV at the nontoxic concentrations of 1, 50, and 50 μM, respectively. ddT did not completely protect cells and was toxic at 300 μM. In the present study, ddG, ddA, and ddC produced 50% inhibition of visna virus replication at 0.1, 0.2, and 0.3 μM, whereas ddT produced slight inhibition at 100 μM. All four compounds inhibited cell growth by <50% at 100 μM. These results are consistent with those obtained by Mitsuya and Broder (20) with HIV, but differ in the relative antiviral potency of ddC.

To demonstrate the accuracy of reverse transcriptase detection as an indicator of virus titer, the antiviral effects of 2',3'-dideoxynucleosides were also investigated by using CPE inhibition, plaque reduction, and yield reduction. In all cases, ddG was more potent than ddA and ddC, whereas the antiviral effects of ddT were minimal. Therefore, the different relative potency of ddC in our studies may reflect differences in the metabolism of nucleosides by SCP cells and human T cells.

To demonstrate the mechanisms by which viral DNA synthesis is inhibited by ddNTPs, kinetic experiments were performed with reverse transcriptase extracted from visna virus. Preliminary studies had indicated a $K_m$ of 6 to 7 μM for TTP during copying of poly(rA) · oligo(dT)$_{12-18}$ (data not shown), which is consistent with results obtained by others with HIV reverse transcriptase and this template/primer (3, 8, 22). However, it is more appropriate to examine the interaction of nucleoside 5'-triphosphates with reverse transcriptase during replication of templates such as primed mRNA which support incorporation of all four naturally occurring dNTPs. This allows use of an identical template/primer in all studies and simulates an early event in visna virus replication, the reverse transcription of viral genome.

The virus enzyme had high affinity ($K_m$, 500 to 1,330 nM) for dNTPs during replication of primed mRNA. Similar high substrate affinities have been observed previously with herpesvirus DNA polymerase (7) and may confer upon the virus an advantage as it competes with host replicases for available nucleoside 5'-triphosphates. Each ddNTP competitively inhibited incorporation of the homologous naturally occurring dNTP into polynucleotide. Presumably, this leads to termination of nascent polynucleotide chains.

Visna virus reverse transcriptase had higher affinity for ddNTPs ($K_I$, 13 to 140 nM) than for dNTPs. The $K_I$/$K_m$ ratios suggest a mechanism for the selective inhibition of
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activity.

ddTTP for visna virus replication was equal to that of 3'-azidodeoxythymidine 5'-triphosphate for HIV reverse transcriptase previously reported by Furman et al. (8). The present studies reveal a good correlation between inhibition of visna virus replication in vitro and the reported activities of several compounds as inhibitors of HIV replication. This suggests that visna virus may be useful to laboratories interested in screening new compounds for possible efficacy against HIV. Our laboratory screens approximately 3,000 compounds per year for possible in vitro activity against visna virus. The rationale for this practice is as follows: (i) the vast majority of compounds entering antiviral screens have little or no efficacy; and (ii) unlike HIV, visna virus is not pathogenic to humans. Therefore, visna virus lends itself to routine large-scale use in primary screening of compounds for antiviral activity. The smaller number of compounds showing inhibition of visna virus replication may then be further evaluated with HIV-infected cultures and, ultimately, in humans.

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LITERATURE CITED


