Antiviral Activities of Novel 5-Phosphono-Pent-2-en-1-yl Nucleosides and Their Alkoxalkyl Phosphonoesters

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Three acyclic nucleoside phosphonates are currently approved for clinical use against infections caused by cytomegalovirus (Vistide), hepatitis B virus (Hepsera), and human immunodeficiency virus type 1 (Viread). This important antiviral class inhibits viral polymerases after cellular uptake and conversion to their diphosphates, bypassing the first phosphorylation, which is required for conventional nucleoside antivirals. Small chemical alterations in the acyclic side chain lead to marked differences in antiviral activity and the spectrum of activity of acyclic nucleoside phosphonates against various classes of viral agents. We synthesized a new class of acyclic nucleoside phosphonates based on a 5-phosphono-pent-2-en-1-yl base motif in which the oxygen heteroatom usually present in acyclic nucleoside phosphonates has been replaced with a double bond. Since the intrinsic phosphate moiety leads to low oral bioavailability and impaired cellular penetration, we also prepared the hexadecyloxypropyl esters of the 5-phosphono-pent-2-en-1-yl nucleosides. Our earlier work showed that this markedly increases antiviral activity and oral bioavailability. Although the 5-phosphono-pent-2-en-1-yl nucleosides themselves were not active, the hexadecyloxypropyl esters were active against DNA viruses and hepatitis B virus, in vitro. Notably, the hexadecyloxypropyl ester of 9-(5-phosphono-pent-2-en-1-yl)-adenine was active against hepatitis B virus mutants resistant to lamivudine, emtricitabine, and adefovir.

The acyclic nucleoside phosphonates (ANPs) are an important class of antiviral drugs that are approved for treatment of viral infections, including infections with cytomegalovirus (cidofovir [Vistide]), hepatitis B virus (Hepsera), and human immunodeficiency virus type 1 (HIV-1; tenofovir disoproxil fumarate [Viread]). This important antiviral class inhibits viral polymerases after cellular uptake and conversion to their diphosphates, bypassing the first phosphorylation, which is required for conventional nucleoside antivirals. Small chemical alterations in the acyclic side chain lead to marked differences in antiviral activity and the spectrum of activity of acyclic nucleoside phosphonates against various classes of viral agents. We synthesized a new class of acyclic nucleoside phosphonates based on a 5-phosphono-pent-2-en-1-yl base motif in which the oxygen heteroatom usually present in acyclic nucleoside phosphonates has been replaced with a double bond. Since the intrinsic phosphate moiety leads to low oral bioavailability and impaired cellular penetration, we also prepared the hexadecyloxypropyl esters of the 5-phosphono-pent-2-en-1-yl nucleosides. Our earlier work showed that this markedly increases antiviral activity and oral bioavailability. Although the 5-phosphono-pent-2-en-1-yl nucleosides themselves were not active, the hexadecyloxypropyl esters were active against DNA viruses and hepatitis B virus, in vitro. Notably, the hexadecyloxypropyl ester of 9-(5-phosphono-pent-2-en-1-yl)-adenine was active against hepatitis B virus mutants resistant to lamivudine, emtricitabine, and adefovir.

The acyclic nucleoside phosphonates (ANPs) are an important class of antiviral drugs that are approved for treatment of viral infections, including infections with cytomegalovirus (cidofovir [Vistide]), hepatitis B virus (Hepsera), and human immunodeficiency virus type 1 (HIV-1; tenofovir disoproxil fumarate [Viread]), and human immunodeficiency virus type 1 (HIV-1; tenofovir disoproxil fumarate [Viread]) (11, 12). To show antiviral activity, ANPs must (i) undergo intracellular activation to the diphosphate, (ii) compete with endogenous deoxynucleotide triphosphates for binding to the viral polymerase, (iii) incorporate into the nascent DNA, and (iv) terminate viral replication. ANPs possess an enzymatically stable phosphonomethyl ether which, unlike conventional nucleoside antivirals, makes them independent of the first intracellular phosphorylation step.

In general, antiviral nucleosides and ANPs consist of a base moiety and a carbohydrate mimic. Structural features of the carbohydrate mimic influence the ability of the nucleoside analog to participate in the antiviral mechanism. For example, the 5′-triphosphates of stavudine (2′,3′-didehydro-3′-deoxymethylidine) and abacavir [Ziagen; (1S,4R)-cis-4-(2-amino-6-chloro-9H-purin-9-yl)-2-cyclopentene-1-methanol] are potent inhibitors of HIV replication, and the presence of a double bond in the sugar moiety appears to play a role in binding to the HIV reverse transcriptase (9). Likewise, potent antiviral activity is found in ANPs possessing a phosphonomethoxymethyl side chain; changes in the acyclic side chain or in the nucleoside base modulate activity and the antiviral spectrum against various virus classes (15).

We now describe the antiviral evaluation of a new series of acyclic nucleoside phosphonate analogs, the 5-phosphono-pent-2-en-yl (PPen) nucleosides, which incorporate a double bond into the phosphonomethyl ether side chain (Fig. 1) (9a). Since ANPs, as a class, are not readily taken up by cells because of their anionic character, we also prepared and evaluated hexadecyloxypropyl esters of each PPen nucleoside, because this strategy has been shown to increase the antiviral activities of cidofovir (4, 22, 23, 36), 9-(S)-(3-hydroxy-2-phosphonomethylpropyl)adenine [(S)-HPMPA] (5), and several other classes of ANPs (33, 35). The compounds and their hexadecyloxypropyl esters were evaluated for in vitro activity against cytomegalovirus (CMV), herpes simplex virus (HSV), vaccinia virus (VV), cowpox virus (CV), HIV-1, varicella-zoster virus (VZV), wild-type and drug-resistant hepatitis B virus (HBV), and Epstein-Barr virus (EBV).

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MATERIALS AND METHODS

Synthesis of PPen nucleosides. The PPen nucleosides and their hexadecyloxypropyl esters were synthesized as reported previously (9a). Proof of structure and purity (>98%) of all compounds was confirmed by 1H and 31P nuclear magnetic
FIG. 1. Structure of PPen nucleosides compared with stavudine. The 5-phosphono-pent-2-en-1-yl analogs of the bases (B) adenine, thymine, guanine, cytosine, and uracil and their hexadecyloxypropyl esters were synthesized and evaluated for antiviral activity.

resonance, electrospray ionization mass spectrometry, and thin-layer chromatography (silica gel plates with visualization by UV light Phosporyl [Supelco, Bellefonte, PA] and charring at 400°C).

Cells and viruses. Human foreskin fibroblast (HFF) cells were prepared as primary cultures and used in the CMV, VZV, VV, and CV assays. CMV strain AD-169 and VZV strain Ellen were propagated using standard virological techniques as reported previously (27, 38). VV strain Copenhagen and CV strain Brighton were kindly provided by John W. Huggins (Department of Viral Therapeutics, Virology Division, U.S. Army Medical Research Institute of Infectious Disease, Frederick, MD). Working stocks of these viruses were propagated in Vero cells obtained from the American Type Culture Collection (Manassas, VA). MRC-5 human lung fibroblast cells were propagated as described previously (4, 19).

Plaque reduction assays in HFF cells. The plaque reduction assays for CMV, VV, CV, and VZV were performed as described previously (5, 27, 37, 38), and standard methods were used to determine the compound concentration required to reduce plaque formation by 50% (EC50).

ELISA for EBV. EBV assays were done as described previously (38). An enzyme-linked immunosorbent assay (ELISA) was performed on cells fixed with 95% ethanol-acetic acid, rinsed with phosphate-buffered saline, and incubated with a monoclonal antibody to EBV viral capsid antigen (Chemicon, Temecula, CA) followed by an incubation with horseradish peroxidase-labeled goat antiamouse immunoglobulin G1 (Southern Biotechnology, Birmingham, AL), and the EC50 and 50% cytotoxic concentration (CC50) values were determined (38).

DNA reduction antiviral assays for activity against HSV-1 in vitro. The antiviral activities were determined against HSV type 1 (HSV-1) by DNA reduction with MRC-5 human lung fibroblast cells using HSV-1 DNA probes as described previously (19).

Neutral red uptake assay for cytotoxicity. HFF or MRC-5 cells were seeded into 96-well tissue culture plates. MRC-5 cells were plated sparsely and allowed to expand during drug treatment. With MRC-5 cells, the medium was replaced after 24 h with minimal essential medium containing 2% fetal bovine serum (FBS), and drug was added to the first row and then diluted serially fivefold from 100 μM to 0.03 μM. With HFF cells, the wells were allowed to become confluent prior to addition of drug-containing medium. The assays were done, and the CC50 values were determined as described previously (4, 5, 36–38).

HBV antiviral analysis. Confluent cultures of 2.2.15 cells were maintained on 96-well flat-bottom tissue culture plates in RPMI 1640 medium with 2% FBS as previously described (26). Cultures were treated with nine consecutive daily doses of the test compounds (six for each test concentration on two replicate plates). The culture medium was changed every day with medium containing the indicated concentrations of the test compounds. HBV DNA levels were assessed by quantitative blot hybridization 24 h after the last treatment. Cytotoxicity was assessed by neutral red dye and semiquantitative analysis of the absorbance of internalized dye at 510 nM (A492) 24 h following the last treatment (three cultures per test concentration) (26). Activity against lamivudine-resistant (2) and adefovir-resistant (3) HBV mutants was determined in a 5-day assay using a transient-transfection method in HuH7 cells, and cytotoxicity was determined as previously described (21).

HIV assays in MT-2 cells. MT-2 cells (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health) were maintained in RPMI 1640 supplemented with 10% FBS (JRH Biosciences, Lenza, Kans.) The antiviral activity of each compound was determined by inoculating MT-2 cells with HIV-1_92_L at a multiplicity of infection of 0.001 50% tissue culture infective dose/cell, followed by incubation in the presence of threefold serial drug dilutions (three wells per dilution) as previously described (13). The antiviral activity of each compound is expressed as the EC50, which is the concentration required to inhibit p24 antigen production by 50%.

MT-2 cytotoxicity. Cytotoxicity was assayed in rapidly dividing MT-2 cells inoculated with drug for 72 h and harvested. Flow count beads (Beckman Coulter, Miami, FL) were added to the cell suspension followed by propidium iodide staining and analysis using an Epics Elite flow cytometer (Beckman Coulter). The CC50 was calculated from the cell counts and viability (13).

RESULTS

The PPen nucleosides were tested against CMV, HSV, VV, CV, VZV, and EBV (Table 1). The unmodified PPen nucleosides were inactive, except for PPen-G, which showed low but measurable activity against CMV and VZV. However, when

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC50 (μM) in:</th>
<th>CC50 (μM) in:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HCMV</td>
<td>HSV-1</td>
</tr>
<tr>
<td>PPen-A</td>
<td>&gt;100</td>
<td>&gt;30</td>
</tr>
<tr>
<td>PPen-G</td>
<td>68.4</td>
<td>&gt;100</td>
</tr>
<tr>
<td>PPen-U</td>
<td>&gt;100</td>
<td>&gt;30</td>
</tr>
<tr>
<td>PPen-T</td>
<td>&gt;100</td>
<td>&gt;30</td>
</tr>
<tr>
<td>HDP-PPen-A</td>
<td>15.7 ± 6.2</td>
<td>18 ± 4.5</td>
</tr>
<tr>
<td>HDP-PPen-G</td>
<td>1.8 ± 1.5</td>
<td>5.8 ± 2.2</td>
</tr>
<tr>
<td>HDP-PPen-U</td>
<td>&gt;20</td>
<td>&gt;30</td>
</tr>
<tr>
<td>HDP-PPen-C</td>
<td>2.9 ± 0</td>
<td>4.7 ± 5.4</td>
</tr>
<tr>
<td>HDP-PPen-T</td>
<td>0.66 ± 0.03</td>
<td>0.90 ± 0.5</td>
</tr>
</tbody>
</table>

* Data are the average (± the standard deviation) of two or more determinations except for EBV, for which there was a single determination. Values indicated by a greater than sign were also single determinations. ND, not determined.

b The HCMV strain AD169 plaque reduction assay was performed in primary HFF cells.

c For HSV-1, a DNA reduction assay in MRC-5 cells was conducted.

d For VV Copenhagen, the plaque reduction assay was conducted in HFF cells.

e For CV Brighton, the plaque reduction assay was conducted in HFF cells.

f For VZV, the plaque reduction assay was conducted in HFF cells.

g The HCMV strain AD169 plaque reduction assay was performed in primary HFF cells.

h The CC50 was determined by neutral red staining as described in Materials and Methods. With stationary, confluent HFF cells, the assay measures cytotoxicity, while with sparsely plated MRC-5 cells, the assay measures both cell proliferation and cytotoxicity.
TABLE 2. Antiviral activities of PPen and HDP-PPen nucleosides against HBV

<table>
<thead>
<tr>
<th>Compound</th>
<th>HBV EC_{50} (µM)</th>
<th>Toxicity (CC_{50} [µM])</th>
<th>Selectivity</th>
</tr>
</thead>
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<tr>
<td>PPen-A</td>
<td>&gt;30</td>
<td>639 ± 8.6</td>
<td>–</td>
</tr>
<tr>
<td>PPen-G</td>
<td>&gt;30</td>
<td>610 ± 7.6</td>
<td>–</td>
</tr>
<tr>
<td>PPen-U</td>
<td>&gt;30</td>
<td>712 ± 19</td>
<td>–</td>
</tr>
<tr>
<td>PPen-C</td>
<td>&gt;30</td>
<td>600 ± 8.6</td>
<td>–</td>
</tr>
<tr>
<td>PPen-T</td>
<td>&gt;30</td>
<td>620 ± 9.6</td>
<td>–</td>
</tr>
<tr>
<td>HDP-PPen-A</td>
<td>1.5 ± 0.3</td>
<td>116 ± 7.8</td>
<td>77</td>
</tr>
<tr>
<td>HDP-PPen-G</td>
<td>2.4 ± 0.2</td>
<td>&gt;1,000</td>
<td>&gt;417</td>
</tr>
<tr>
<td>HDP-PPen-U</td>
<td>&gt;30</td>
<td>155 ± 8.2</td>
<td>–</td>
</tr>
<tr>
<td>HDP-PPen-C</td>
<td>3.9 ± 0.4</td>
<td>621 ± 8.9</td>
<td>159</td>
</tr>
<tr>
<td>HDP-PPen-T</td>
<td>2.0 ± 0.2</td>
<td>122 ± 10</td>
<td>61</td>
</tr>
</tbody>
</table>

*Antiviral results (EC_{50}s) were derived from virion DNA reduction assays in 2.2.15 cells as described in Materials and Methods. Values presented (±standard deviations) were calculated using data from two to four experiments. Cytotoxicity was determined by inhibition of the uptake of neutral red dye (26).*

The pyrimidine analogs HDP-PPen-C and HDP-PPen-T were resistant to L180M, M204V, M204I, and the LM/MV double mutant. All of the HDP-PPen nucleosides showed activity equal to wild type against an adefovir-resistant mutant (N236T); however, only HDP-PPen-A exhibited full activity against all five of the drug-resistant HBV mutants.

**DISCUSSION**

Nucleoside and nucleotide analogs are two important classes of drugs that selectively target viral polymerases and inhibit viral replication (11, 12). Nucleoside analogs require conversion to the 5’ mono-, di-, and finally the triphosphates before incorporation into viral DNA and include compounds such as acyclovir, ganciclovir, stavudine, abacavir, and lamivudine. The acyclic nucleoside phosphonates bypass the initial phosphorylation and are converted to their mono- and diphosphate derivatives by nucleoside kinases (12). Studies by Holy et al. of 9-[2-(phosphonomethoxy)ethyl], 9-(R)-[2-(phosphonomethoxy)propyl, and 5)-9-(3-hydroxy-2-phosphono-methoxy)propyl nucleotide analogs established the potent broad-spectrum antiviral activities available from ANPs and led to three FDA-approved phosphonates: cidofovir (Vistide), adefovir dipivoxil (Hepsera), and tenofovir disoproxil fumarate (Viread) (16). Other groups have focused on the design of phosphonate isosteres in which the 5’-oxygen of an antiviral nucleotide is either replaced or replaced by an enzymatically stable methylene group. This strategy led to phosphonate analogs of acyclovir (20), ganciclovir (8), stavudine (24), dideoxynucleosides (34), tetrahydrofuran derivatives (6), cyclopropane nucleosides (30, 39), and anti-HCV nucleosides (25). Acyclic phosphonates incorporating unsaturated acyclosugar side chains have also been evaluated (14, 32).

In our own efforts to identify selective virus inhibitors, we prepared a novel family of nucleotide analogs using a phosphopentenyl group as the acyclic sugar moiety. The general structure of the new compounds, 5-phosphono-pent-2-en-1-yl nucleosides, is shown in Fig. 1. The phosphopentenyl side chain was chosen so that the resulting analogs would resemble the corresponding portion of 2’,3’-didehydro-2’,3’-dideoxynucleosides, such as stavudine, 2’,3’-didehydro-2’,3’-dideoxyadenosine, and abacavir, nucleosides with potent anti-HIV activity. Choo et al. (9) found previously that the 2’,3’ double bond of these unsaturated nucleosides interacts with the aromatic moiety of Tyr115 of HIV-1 reverse transcriptase by hydrophobic π → π interaction. We hypothesized that the unsaturated acyclosugar side chain of the PPen structure might

**TABLE 3. Activities of HDP-PPen nucleosides against drug-resistant hepatitis B virus**

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC_{50} (µM)</th>
<th>CC_{50} (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild type</td>
<td>L180M</td>
</tr>
<tr>
<td>HDP-PPen A</td>
<td>9.2 ± 1.2</td>
<td>15.0 ± 1.8</td>
</tr>
<tr>
<td>HDP-PPen G</td>
<td>8.8 ± 0.9</td>
<td>&gt;100</td>
</tr>
<tr>
<td>HDP-PPen C</td>
<td>3.4 ± 0.5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>HDP-PPen T</td>
<td>3.0 ± 0.4</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

*Data are the EC_{50} values based on reduction of HBV replicative intermediates in Huh7 cells transiently infected with the indicated virus. Means ± standard deviations of two to four experiments are shown.
improve binding of the diphosphate to the target viral polymerases. The acyclic PPen side chain should also be more flexible than related cyclic analogs (24), which might increase binding within the polymerase complex and facilitate approach of the α-phosphorous to the 3′-OH of the replicating viral DNA.

The PPen nucleosides were evaluated in various antiviral assays, and the results are summarized in Table 1. No inhibitory activity was observed up to 100 μM for PPen-A, -C, -U, -T, or -G, and PPen-G was only weakly active against CMV (68.4 μM) and VZV (7.2 μM). The PPen nucleosides also were not antiproliferative or cytotoxic in MRC-5 or MT-2 cells up to 100 μM (Tables 1 and 2).

We have observed that ANPs frequently fail to exhibit biological activity in vitro because the double negative charge associated with the phosphate group impairs transport of drug through the cell membrane (1). In earlier work we prepared ANPs modified with lipophilic groups to create mimics of lysophosphatidylcholine, a dietary lipid which is absorbed intact from the gastrointestinal tract and readily metabolized in cells. We showed that cidofovir modified by esterification with a hexadecylxpropyl group (HDP-CDV) enters cells rapidly and is metabolized to give levels of CDV-diphosphate, the active metabolite, that are 100-fold higher relative to unmodified CDV (1). The in vitro activity of (S)-HPMPA was also enhanced using this strategy (5), and some weakly active ANPs displayed significant activity after lipid modification (33, 35). Esterification with alkoxyalkyl groups also makes ANPs orally bioavailable (10) and orally effective against lethal poxvirus infections (7, 31). This was the rationale for the synthesis of the hexadecylxpropyl esters of the PPen nucleosides. We evaluated them for antiviral effects against various viruses and found that several now showed significant activity (Table 1). The broadest antiviral activity was found in the guanine analog, HDP-PPen-G, which had EC50 below 12 μM for all viruses except HIV-1 and EBV. HDP-PPen-A was a slightly stronger inhibitor of HBV (EC50 1.5 μM) and showed substantial activity against EBV (EC50 0.1 μM). Against HIV-1, only the two pyrimidine analogs HDP-PPen-C and HDP-PPen-T showed significant activity but exhibited no antiviral selectivity. As expected, HDP-PPen-U was inactive in all the antiviral assays (VZV and EBV were not assayed).

Since four of the five HDP-PPen nucleoside compounds inhibited HBV replication and our previous work demonstrated that alkoxyalkyl esters of nucleoside phosphates and phosphonates are targeted to the liver (10, 17, 18), we evaluated the active analogs against a panel of drug-resistant HBV (Table 2). Hepatitis B virus is one of the 10 leading causes of mortality worldwide, and the emergence of strains resistant to the approved drugs, lamivudine and adefovir dipivoxil, is a major clinical concern (29). Resistance to lamivudine emerges rapidly during monotherapy and after 4 years affects approximately 70% of treated patients (28). Adefovir dipivoxil is effective in vitro and in vivo against lamivudine-resistant HBV (40). We found that like adefovir, HDP-PPen-A retains full activity against the lamivudine-resistant HBV polymerase mutants (L180M, M204V, M204I, and L180M/M204V) in transient infections in HuH7 cells. HDP-PPen-G was effective against M204V and M204I, but L180M and the double mutant, L180M/M204V, were resistant. All of the lamivudine-resistant strains exhibited resistance to the pyrimidine PPen analogs HDP-PPen-C and HDP-PPen-T.

Adefovir resistance has been associated with the selection of the mutant N236T, but its emergence is less frequent and delayed relative to lamivudine resistance (3). Interestingly, all of the HDP-PPen nucleosides retained activity equal to the wild type against the adefovir-resistant mutant (N236T). Among the compounds, only HDP-PPen-A retained full activity against all five drug-resistant HBV mutants, suggesting that this nucleotide analog should be evaluated as a salvage therapy in patients infected with drug-resistant HBV strains or used in combination with other drugs to prevent the emergence of resistance.

In summary, the PPen nucleosides are a new class of acyclic nucleoside phosphonates, possessing significant antiviral activity when esterified with the hexadecylxpropyl group. Further work will be required both to design analogs with improved antiviral activity and to investigate their mechanism of action.

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