Susceptibilities of Zidovudine-Resistant Variants of Human Immunodeficiency Virus Type 1 to Inhibition by Acyclic Nucleoside Phosphonates

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The acyclic purine nucleoside phosphonates, a newly described class of broad-spectrum antiviral agents, effectively inhibit human immunodeficiency virus type 1 (HIV-1) replication in vitro and in animal AIDS models. 9-(2-Phosphonylmethoxyethyl)adenine (PMEA) is currently being evaluated in clinical trials in patients with AIDS. In this study, we investigated the efficacy of PMEA and a related analog, 9-(2-phosphonylmethoxypropyl)diaminopurine (PMPDAP), against HIV-1 isolates exhibiting various degrees of resistance to zidovudine (azidothymidine [AZT]). HIV isolates highly (~50 to 200-fold) resistant to AZT were found to be about two- to eightfold less susceptible to PMEA. A comparable degree of cross-resistance to PMPDAP, a structurally related analog of PMEA, was also observed. However, the 50% effective dose values of PMEA or PMPDAP against a panel of HIV isolates showing intermediate levels (~8 to 25-fold) of AZT resistance was indistinguishable from the 50% effective dose values of PMEA (0.7 to 1.7 versus 2 μM) or PMPDAP (0.4 to 1.4 versus 0.8 to 1 μM) against HIV isolates from patients who had not previously used AZT.

In addition, we were unable to generate PMEA- (or PMPDAP)-resistant HIV-1 variants by >30 serial passages of the virus in the presence of increasing concentrations of PMEA. Careful analysis of HIV-1 isolates from patients previously treated with AZT for cross-resistance to PMEA are needed to evaluate the significance of these observations.

Several dideoxynucleosides have been shown to inhibit the replication of human immunodeficiency virus (HIV), the etiologic agent of AIDS. Three compounds, 3'-azido-3'-deoxythymidine (AZT or zidovudine) (19, 25), 2',3'-dideoxycytidine (ddC or didanosine) (1, 18), and 2',3'-dideoxyctydidine (ddC or zalcitabine) (5, 26), have been approved for antiretroviral therapy in AIDS. Administration of AZT, ddC, or ddI has been shown to result in clinical and immunologic improvement and confers increased survival on HIV-infected patients with advanced immunodeficiency. None of these agents is curative, and their long-term use is restricted by either transient or incomplete activities and development of resistances. Recently, a new class of compounds—the phosphonamidethyler analogs of purine and pyrimidine bases—have been shown to exert broad-spectrum antiviral activity against several DNA and RNA viruses, including HIV (7). Of these, the adenine analog 9-(2-phosphonylmethoxyethyl)adenine (PMEA) has been studied extensively and found to be active against HIV and other retroviruses, including HIV type 2 (HIV-2), simian immunodeficiency virus, feline immunodeficiency virus, and Moloney murine sarcoma virus (2, 3). PMEA is also active against various herpesviruses, including herpes simplex virus types 1 and 2, cytomegalovirus, and Epstein-Barr virus (7-10). Thus, PMEA is of interest both as a potential antiretroviral drug for HIV-1 infections and for the treatment of some of the opportunistic infections associated with AIDS. A related phosphonate analog, 9-(2-phosphonylmethoxypropyl)diaminopurine (PMPDAP), exhibits greater anti-HIV activity, although it is less effective against herpesviruses. The chemical structures of PMEA and PMPDAP are shown in Fig. 1.

AZT is considered the standard antiretroviral therapy for initiating treatment of patients with HIV infection. Widespread use of AZT monotherapy has led to the emergence of AZT-resistant HIV, and several lines of evidence indicate that late decreases in CD4 cell counts in patients receiving AZT may be due in part to the development of viral resistance (4, 23). Furthermore, primary infections with AZT-resistant HIV are being documented (11). It is therefore important to evaluate the efficacy of new anti-HIV agents for efficacy against AZT-resistant HIV isolates. In this study, we investigated the patterns of susceptibility of various AZT-resistant HIV isolates to the acyclic nucleoside phosphonates PMEA and PMPDAP.

The human T-lymphocytic cell lines H9 and MT-2, as well as the various HIV isolates used in this study, were obtained from the National Institutes of Health AIDS Research and Reference Reagent Repository (Ogden Bioservices, Rockville, Md.). The various HIV isolates used in this study included pairs of predrug (AZT-susceptible) isolates (H112-2 and G762-3) and postdrug (AZT-resistant) isolates G910-6 and G691-2 from patients who underwent long-term AZT monotherapy (14, 15), as well as a panel of four isolates with an intermediate degree of resistance to AZT (1391-4, 1495-2, 1391-1, and G910-11) (16). All of the viruses were propagated in H9 or MT-2 cells maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamate.

Susceptibilities of AZT-resistant HIV-1 mutants to acyclic nucleoside phosphonates. We first examined HIV-1 isolates G910-6 and G691-2, reported to be highly resistant to AZT, and the corresponding AZT-susceptible predrug isolates H112-2 and G762-3 for in vitro susceptibility to AZT, ddI, or PMEA in MT-2 cells by using a tetrazolium (XTT) dye conversion assay (12, 24). The various virus isolates were tested in a cell culture inhibition assay, and the 50% inhibitory concen-
PMEA

![Structure of PMEA](image)

PMPDAP

![Structure of PMPDAP](image)

FIG. 1. Structures of PMEA, PMPDAP, and AZT.

Concentrations of the drugs were determined. Under the experimental conditions used, all of the HIV-infected MT-2 cells succumb to virus infection and no viable cells remain at the end of 7 days. Thus, the drug concentration in which the viable cell numbers of HIV-infected cultures correspond to 50% of the viable cell numbers in uninfected cultures is the 50% effective antiviral dose (ED50). AZT-susceptible isolates H112-2 and G762-3 were both readily inhibited by PMEA (ED50 ~ 2 ± 0.2 μM) (Fig. 2C). Surprisingly, AZT-resistant isolates G-910-6 and G691-2 were four- to eightfold less susceptible to PMEA, with ED50 values of 8 ± 2 and 16 ± 6 μM, respectively. As previously described, compared with H112-2 and G762-3, both G-910-6 and G691-2 were highly resistant to AZT, with ED50 values exceeding an AZT concentration of 20 μM (Fig. 2A). By contrast, the mean ED50 of AZT against susceptible isolates H112-2 and G762-3 in three independent experiments was ~0.05 μM. The susceptibility of the AZT-resistant isolates to ddl was unaffected, and the ED50 values ranged from 12 to 14 μM for the AZT-susceptible and AZT-resistant isolates (Fig. 2B).

The XTT assay is an indirect way to measure the antiviral activities of various compounds, and it measures the protection of HIV-induced MT-2 cells from virus-induced cell killing. We therefore investigated the effects of different compounds on virus yields from HIV-infected MT-2 cells. For these studies, MT-2 cells were infected with various virus stocks by using an inoculum standardized to contain 1 reverse transcriptase (RT) cpm per cell. Virus-infected cells were seeded at a concentration of 0.2 × 10⁶/ml in media containing various concentrations of AZT, ddl, or PMEA. After 5 days of incubation, the RT activities of the culture supernatants were determined and the drug concentrations yielding half-maximal RT activity were calculated. RT assays were performed in accordance with previously described procedures (20), with the following modifications. Primarily, [3H]TTP was used in place of [32P]TTP and incorporation of radioactive TTP was monitored by liquid scintillation in an LKB Betaplate reader instead of autoradiography. The results were similar to those obtained with the XTT assay, and the ED50 values of PMEA against AZT-resistant isolates G910-6 and G691-2 were about four- to fivefold greater than the values against AZT-susceptible isolates H112-2 and G762-3 (data not shown).

FIG. 2. Susceptibilities of paired AZT-susceptible and AZT-resistant HIV-1 isolates to AZT, ddl, and PMEA. Two pairs of HIV isolates obtained from two patients prior to and after AZT monotherapy were tested for susceptibility to AZT, ddl, and PMEA in MT-2 cells by XTT assay. The viable cell numbers expressed as percentages of uninfected control cultures were used as a measure of antiviral drug efficacy. H112-2 and G762-3 were pre-AZT isolates, and G910-6 and G691-2 were post-AZT isolates. Data from a representative experiment are shown, and the ED50 values in the text represent means of three independent experiments.
TABLE 1. Susceptibilities of various AZT-resistant HIV isolates to PMEA and PMPDAP in peripheral blood mononuclear cell cultures

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mutation(s)</th>
<th>Mean ED50 (µM) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AZT</td>
<td>PMEA</td>
</tr>
<tr>
<td>AZT susceptible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H112-2</td>
<td>None (wild type)</td>
<td>0.04 ± 0.001</td>
</tr>
<tr>
<td>G762-3</td>
<td>None (wild-type)</td>
<td>0.04 ± 0.001</td>
</tr>
<tr>
<td>AZT resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G910-6</td>
<td>D-67→N, K-70→R, T-215→F, K-219→Q</td>
<td>6 ± 0.4</td>
</tr>
<tr>
<td>Intermediately AZT resistant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1391-4</td>
<td>67 (m→w), 215 (m→w), 70 (w→m)</td>
<td>0.3 ± 0.02</td>
</tr>
<tr>
<td>1391-1</td>
<td>215</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>G910-11</td>
<td>215</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>1495-2</td>
<td>70,215</td>
<td>0.3 ± 0.1</td>
</tr>
</tbody>
</table>

* The genotypes of AZT-resistant and-susceptible isolates have been determined by nucleotide sequencing (11, 13). The intermediate AZT resistance panel has been analyzed for mutations at RT codons 67, 70, 215, and 219 by PCR (12). The prevalence of wild-type (w) or mutant (m) sequences at each codon is indicated when a mature of viruses was present.

* Each value represents the mean of three independent measurements.

Previous studies have identified several mutations in the RT gene of G910-6 and G691-1, and the presence of multiple mutations has been shown to correlate with a high degree of AZT resistance (14). We therefore tested a panel of genotypically defined HIV-1 isolates that exhibit various degrees of AZT resistance to identify the RT mutations that determine cross-resistance to PMEA. These experiments were carried out in peripheral blood mononuclear cell cultures. Peripheral blood mononuclear cells were isolated from heparinized blood samples collected from healthy volunteers by Ficoll-Hypaque density gradient centrifugation and maintained in lymphocyte stimulation medium (RPMI containing 20% fetal bovine serum, 5 μg of phytohemagglutinin-P per ml, and 1 ng of interleukin-2 per ml) for 48 to 72 h prior to infection. The cells were infected with the various HIV isolates (at a multiplicity of 0.1) and maintained in the absence or presence of increasing concentrations of AZT, PMEA, or PMPDAP for 5 to 7 days.

The extent of virus replication was monitored by determining the RT activity in the culture supernatants, and the data were used to calculate the ED50 values of the different drugs. Table 1 shows that the ED50 values of AZT were ~0.04 µM for susceptible isolates H112-2 and G762-3 and ~5 µM for resistant isolates G910-6 and G691-1. The ED50 values against HIV isolates in the intermediate AZT-resistant panel ranged from 0.3 to 1.1 µM. The ED50 value of PMEA against the AZT-susceptible isolates was ~2 µM. Consistent with the results obtained with MT-2 cells, highly AZT-resistant viruses G910-6 and G691-2 showed some reduction (about four- to eightfold) in their susceptibility to PMEA, compared with the corresponding predrug isolates H112-2 and G762-3. However, all four isolates with intermediate AZT resistance showed full susceptibility to PMEA (ED50 0.7 to 1.7 µM). Essentially similar results were obtained with PMPDAP. The highly resistant AZT strains were ~4- to 10-fold less susceptible to PMPDAP than were the corresponding AZT-susceptible isolates, while strains with intermediate AZT resistance retained their susceptibility to PMPDAP (Table 1).

In other studies, we have tried to isolate HIV variants capable of replicating in the presence of high concentrations of PMEA. MT-2 cells were infected with HIV at a multiplicity of 1 RT cpn per cell and incubated in the presence of PMEA (1, 2, or 4 µM) or AZT (0.05, 0.1, or 0.2 µM) at concentrations corresponding to 0.5, 1, or 2 ED50 units of the drugs. At weekly intervals, one-half of the culture was removed and replaced with an equal volume of the culture medium containing the appropriate drug concentrations. The culture supernatants were harvested when the cultures exhibited extensive syncytia, clarified by centrifugation, and used to initiate the next round of infection in fresh MT-2 cells. In each successive passage, a drug concentration that was equal to or twice as high as the drug concentration in the previous passage was used. The ED50 values of the respective drugs against the viruses isolated from different passages were determined by RT assays. HIV with reduced (~10-fold) susceptibility to AZT was isolated after about 10 passages (data not shown). By contrast, we were not able to isolate viruses resistant to PMEA, even after 30 passages over a period of 1 year, suggesting that HIV does not readily acquire resistance to acyclic nucleoside phosphonates. It is interesting to speculate why we were unable to isolate PMEA-resistant HIV-1 variants. It is possible that the drug concentrations used in the selection procedure were insufficient to exert pressure for selection of variants. Alternatively, development of resistance to PMEA might require interplay between mutations at different sites on the RT-encoding gene.

In this study, eight clinical isolates with different degrees of susceptibility to AZT were tested for susceptibility to the acyclic nucleotide phosphonates PMEA and PMPDAP. Only two isolates, G910-6 and G691-2, highly (~50- to 200-fold) resistant to AZT, showed partial resistance to both PMEA and PMPDAP. The cross-resistance observed does not seem to be related to experimental variations, since these isolates did not show reduced susceptibility to ddI. In earlier studies, AZT-resistant HIV isolates were found to exhibit cross-resistance to other nucleoside analogs which contain a 3'-azido group (e.g., 3'-azido-2',3'-dideoxyuridine and 3'-azido-2',3'-dideoxyguanosine) but retain their susceptibility to other 2',3'-dideoxynucleosides such as ddI, ddC, 3'-fluoro-2'-deoxythymidine, and 2',3'-dideoxy-2',3'-dideoxythymidine (21). Mauers et al. (17) recently reported data from a large, multicenter study which demonstrated cross-resistance to ddI and ddC in AZT-resistant HIV from patients receiving prolonged AZT monotherapy. The extent of cross-resistance was found to correlate with the degree of AZT resistance, and an average twofold decrease in ddI-ddC susceptibility was observed for each
10-fold decrease in AZT susceptibility. Interestingly, the highly (>100-fold) AZT-resistant viruses G910-6 and G691-2 both displayed approximately two- to eightfold decreases in susceptibility to PMEA and PMPDAP but showed no decreased susceptibility to ddI. The reduced susceptibility of AZT-resistant HIV to both PMEA and PMPDAP suggests that the acyclic phosphonil groups on these molecules are responsible for this cross-resistance. This is surprising in view of the fact that the 3'-azido group and the phosphonyl groups have distinct chemical structures and show no obvious similarities.

The acquisition of AZT resistance by HIV following therapy is often a consequence of specific mutations in the viral RT (reviewed in reference 21). The genotypes of the different AZT-resistant isolates used in this study at the different RT loci that influence AZT susceptibility have been previously described. G910-6 and G691-2, the highly AZT-resistant viruses, both carry mutations at four of the five distinct loci in HIV RT implicated in AZT resistance (11) and also show cross-resistance to PMEA and PMPDAP. By contrast, the different viruses in the intermediate AZT resistance panel displayed no cross-resistance to the phosphonates. Of these, 1391-1 and G910-11 both carry a single mutation at codon 215, 1495-2 carries mutations at both codons 70 and 215, while 1391-4 contains mixtures of wild-type DNA and DNA carrying mutations at codons 67, 70, and/or 215. These results suggest that a single mutation at codon 215, 67, or 70 or a double mutation at codons 70 and 215 may not be sufficient to determine cross-resistance to phosphonates. It is not known whether a single mutation at codon 219 can confer cross-resistance to phosphonates. Further studies using viruses engineered by site-specific mutagenesis are required to identify which of the various mutations might be required to determine resistance to these acyclic nucleoside phosphonates.

Both AZT and PMEA are phosphorylated in vivo into biologically active AZTTP and PMEApp, respectively, which inhibit HIV reverse transcription by competing with corresponding deoxynucleoside triphosphates and cause chain termination when incorporated into the transcripts. It is therefore reasonable to presume that the mutant RT from highly AZT-resistant HIV fails to recognize PMEApp efficiently. Direct enzymological studies of this question are in progress. However, biochemical studies are complicated because of the parallel between inhibition of RT activity by AZTTP and resistance of HIV-1 to the parent drug has not been demonstrated (21). Other explanations that may account for this cross-resistance include possible changes in the transport and/or phosphorylation of PMEA (as well as PMPDAP and other deoxynucleosides) in cells infected with AZT-resistant HIV, and these need to be investigated.

PMEA is undergoing phase II/III clinical trials in patients with advanced HIV infection (6), and bis-pivaloyloxymethyl PMEA is being evaluated as an oral produg of PMEA in preclinical studies (13, 22). Since some of the patients being studied are likely to have had prior AZT therapy, in vitro testing of the susceptibility of AZT-resistant isolates to PMEA should be carried out to correlate the clinical activity of the drug. That may be an important consideration for any phase III/II study, since HIV isolates from patients with prior AZT therapy may vary widely in susceptibility to the test compounds and compromise the results of trials which carry a virological end point.

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