Susceptibility of the Porcine Endogenous Retrovirus to Reverse Transcriptase and Protease Inhibitors

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Porcine xenografts may offer a solution to the shortage of human donor allografts. However, all pigs contain the porcine endogenous retrovirus (PERV), raising concerns regarding the transmission of PERV and the possible development of disease in xenotransplant recipients. We evaluated 11 antiretroviral drugs licensed for human immunodeficiency virus type 1 (HIV-1) therapy for their activities against PERV to assess their potential for clinical use. Fifty and 90% inhibitory concentrations (IC50s and IC90s, respectively) of five nucleoside reverse transcriptase inhibitors (RTIs) were determined enzymatically for PERV and for wild-type (WT) and RTI-resistant HIV-1 reference isolates. In a comparison of IC50s, the susceptibilities of PERV RT to lamivudine, stavudine, didanosine, zalcitabine, and zidovudine were reduced >20-fold, 26-fold, 6-fold, 4-fold, and 3-fold, respectively, compared to those of WT HIV-1. PERV was also resistant to nevirapine. Tissue culture-based, single-round infection assays using replication-competent virus confirmed the relative sensitivity of PERV to zidovudine and its resistance to all other RTIs. A Gag polyprotein-processing inhibition assay was developed and used to assess the activities of protease inhibitors against PERV. No inhibition of PERV protease was seen with saquinavir, ritonavir, indinavir, nelfinavir, or amprenavir at concentrations >200-fold the IC50s for WT HIV-1. Thus, following screening of many antiretroviral agents, our findings support only the potential clinical use of zidovudine.
of sensitivity of PERV to zidovudine and poor or no susceptibility to all other drugs.

RT susceptibility analysis by an enzymatic assay. The susceptibility of PERV RT to the RTIs was evaluated enzymatically in the Amp-RT assay to determine the 50% and 90% inhibitory concentrations (IC50s and IC90s, respectively) (20, 52). Results obtained in triplicate were compared with those of reference wild-type (WT) and drug-resistant HIV-1 isolates using methodologies previously described (15, 49). The RTIs tested were nevirapine; the triphosphorylated deoxynucleoside analogs of zidovudine (AZT-TP), lamivudine (ddC-TP), stavudine (ddA-TP), and 2'-3'-didehydro-3'-dideoxy-3'-thiacytidine; 3TC-TP, zalcitabine (dideoxycytidine [ddC]-TP), stavudine (2',3'-didehydro-3'-deoxynucleoside; d4T-TP), and the active form of didanosine (dideoxyadenine [ddA]-TP). The ratios of nucleoside analogs to their corresponding deoxynucleoside triphosphates (dNTPs) in the RT reaction of the Amp-RT assay varied, with 15 μM dNTP being used for reaction mixtures containing AZT-TP or d4T-TP, 5 μM dCTP being used for mixtures containing 3TC-TP or ddC-TP, and 5 μM dATP being used for mixtures containing dd-ATP along with 20 μM concentrations of each of the other three dNTPs. For nevirapine reactions, a 20 μM dNTP mixture containing nevirapine completely inhibits WT HIV-1 RT activity (IC50, 4 μM) (49). In contrast to what was observed with WT HIV-1, no inhibition of PERV RT was observed in this test, indicating lack of activity of nevirapine on PERV RT (data not shown).

Two sources of PERV were used, the first from culture supernatant of a porcine embryonic kidney cell line (PK15) and the second from PERV-infected human embryonic kidney cell line 293 (PERV-293) (33). PK15 and PERV-293 cells, which release both PERV-A and -B (23), were maintained in minimal essential medium by using standard tissue culture techniques. Three HIV-1 isolates derived from molecular infectious clones containing WT RT, HIV-1SUM8 (Q151M) (38, 47) and HIV-1SUM13 (A62V, V75I, F77L, F116Y, Q151M) (48). Table 1 also shows that, in comparison to WT HIV-1, both PERV isolates had reduced susceptibilities to all five nucleoside RTIs. However, the level of resistance varied among the drugs. No activity was seen with lamivudine, while high-level resistance was observed with stavudine. The susceptibility of PERV to both zalcitabine and didanosine was also reduced compared to that of WT HIV-1. The highest activity against PERV RT was observed with zidovudine, with which there was only a threefold difference in IC50 from that for WT HIV-1. This level of resistance was lower than that of HIV-1SUM8 (Q151M) has low-level resistance, and HIV-1SUM13 (A62V, V75I, F77L, F116Y, Q151M) has higher levels of resistance (40, 48). No significant differences were found in the drug susceptibilities of PERV-PK15 and PERV-293, except for d4T-TP, with which a twofold difference in IC50 was observed. This likely reflected assay variability rather than inherent phenotypic changes between the two viral preparations. Similarly, changes were also seen for the WT HIV-1 isolates, with IC50 for 3TC-TP and ddA-TP differing by 1.5-fold and 2.7-fold, respectively.

We also tested PERV RT for susceptibility to nevirapine, a nonnucleoside inhibitor of HIV-1 RT. One Amp-RT assay with 50 μM nevirapine was used in this screening. This concentration of nevirapine completely inhibits WT HIV-1 RT activity (IC50, 4 μM) (49). In contrast to what was observed with WT HIV-1, no inhibition of PERV RT was observed in this test, indicating lack of activity of nevirapine on PERV RT (data not shown).

<table>
<thead>
<tr>
<th>Virus (mutation[s])</th>
<th>AZT-TP</th>
<th>ddC-TP</th>
<th>3TC-TP</th>
<th>ddA-TP</th>
<th>d4T-TP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50</td>
<td>IC90</td>
<td>IC50</td>
<td>IC90</td>
<td>IC50</td>
</tr>
<tr>
<td>WT HIV-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-1SUM8</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>HIV-1SUM13</td>
<td>0.5</td>
<td>0.9</td>
<td>0.5</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>MDR HIV-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-1SUM8 (Q151M)</td>
<td>25.5</td>
<td>45.4</td>
<td>18.3</td>
<td>27.5</td>
<td>3.3</td>
</tr>
<tr>
<td>HIV-1SUM13 (A62V, V75I, F77L, F116Y, Q151M)</td>
<td>18.3</td>
<td>45.4</td>
<td>7.5</td>
<td>8.2</td>
<td>6.0</td>
</tr>
<tr>
<td>PERV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PERV-PK15</td>
<td>1.5</td>
<td>3.4</td>
<td>2.0</td>
<td>4.3</td>
<td>&gt;10</td>
</tr>
<tr>
<td>PERV-293</td>
<td>1.5</td>
<td>3.4</td>
<td>2.0</td>
<td>4.3</td>
<td>&gt;10</td>
</tr>
</tbody>
</table>

*IC50s and IC90s were determined by using the Amp-RT assay. Numbers in parentheses indicate fold resistance compared to the mean IC50s for WT HIV-1.
TABLE 2. Susceptibility of PERV to different RTIs and comparison with that of WT HIV-1

<table>
<thead>
<tr>
<th>Virus</th>
<th>Zidovudine</th>
<th>Zalcitabine</th>
<th>Lamivudine</th>
<th>Didanosine</th>
<th>Stavudine</th>
<th>Nevirapine</th>
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</thead>
<tbody>
<tr>
<td>HIV-1&lt;sub&gt;LA1&lt;/sub&gt;</td>
<td>0.015</td>
<td>1.3</td>
<td>0.12</td>
<td>2.2</td>
<td>1.5</td>
<td>0.03</td>
</tr>
<tr>
<td>PERV-B</td>
<td>0.061 (4.1)</td>
<td>28 (21.5)</td>
<td>&gt;50 (&gt;417)</td>
<td>&gt;50 (&gt;23)</td>
<td>30 (20)</td>
<td>&gt;50 (&gt;1,667)</td>
</tr>
</tbody>
</table>

*IC<sub>50</sub>s were determined by culture-based infectivity assays. Numbers in parentheses indicate fold resistance compared to the IC<sub>50</sub> for WT HIV-1. IC<sub>50</sub>s (in micromolar units) were calculated from duplicate results.

RT susceptibility analysis by cell culture-based infectivity assays. Susceptibility of PERV and HIV-1 to RTIs was evaluated on the CD4-expressing human rhabdomyosarcoma cell line (RD-CD4) (7). This cell line is able to support the infection and replication of both PERV and HIV-1. In situ focus-forming assays were carried out using PERV-B lacZ pseudotype (see below) and the HIV-1<sub>LA1</sub> isolate. For IC<sub>50</sub> and IC<sub>90</sub> determinations for WT HIV-1, reduction in the number of foci producing p24 was quantitated (41), while for PERV a lacZ pseudotype was generated and reduction in the number of foci producing β-galactosidase was quantitated (44).

To generate PERV-B lacZ pseudotypes, the human embryonic kidney cell line 293/PERV-B, persistently infected with PERV-B (43), was transduced with the MFGnslacZ vector using a helper-free vector bearing gibbon ape leukemia virus envelopes (35). MFGnslacZ is a murine leukemia virus (MuLV)-based vector containing functional long terminal repeats and a Ψ packaging signal sequence, as well as a lacZ marker gene. It encodes β-galactosidase and no MuLV gag, pol, or env gene products (13). A cell population of which the majority of cells (>95%) contain MFGnslacZ was established and named 293/PERV-B/lacZ. These cells produce a mixture of replication-competent PERV-B and PERV pseudotypes which contain the MFGnslacZ genome (45). This viral mixture is referred to as PERV-lacZ pseudotypes. After overnight incubation of these cells, the supernatant containing PERV-lacZ was filtered (filter pore size, 0.45 μm) and used for drug susceptibility testing.

RD-CD4 cells were seeded in 24-well plates at 8 × 10<sup>4</sup> cells/well in Dulbecco modified Eagle medium with 10% fetal calf serum and incubated overnight. The culture medium was replaced with 450 μl of HIV-1 or PERV-lacZ pseudotype supernatants which had been diluted in Opti-MEM (Gibco-BRL) to have approximately 150 and 60 focus-forming units of virus per well, respectively. After incubation for 1 h, virus inocula were removed and 1 ml of medium containing RTIs was added. Three days later, cells were fixed and stained for expression of β-galactosidase (44) or HIV-1 p24 (HIV-1<sub>LA1</sub>) (41).

The data from the culture-based assays are shown in Table 2 and are in agreement with the data from the enzymatic assays. This testing also demonstrated that, with the exception of zidovudine, which had an IC<sub>50</sub> close to that for WT HIV-1, all other drugs had little or no activity against PERV. The level of resistance of PERV to zalcitabine and didanosine was higher in these assays than in the enzymatic assays.

Protease susceptibility analysis. Susceptibility of PERV to PIs was assessed in a Gag polyprotein-processing inhibition assay, as previously described for MuLV (2), in which the presence of cleaved PERV Gag proteins in PERV-293 cells was determined by Western blot analysis following PI treatment. PIs tested included indinavir, nelfinavir, saquinavir, ritonavir, and amprenavir. For WT HIV-1 PI tests, OM-10.1 cells were used. These cells contain a single integrated WT HIV-1 provirus which is induced after treatment with 20 μl of tumor necrosis factor alpha per ml (4). HIV-1 production in 3 × 10<sup>5</sup> OM-10.1 cells was induced at the time of addition of PIs (0.001 to 10 μg/ml). Cells were incubated for 24 h and harvested. One to 2 μg of each PI per ml was added to 3 × 10<sup>6</sup> PERV-293 cells in 10 ml of growth medium. After incubation for 3 days, PERV-293 cells were harvested by trypsinization. Aliquots of harvested OM-10.1 and PERV-293 cells were checked for viability by trypan blue exclusion. Protein concentration of homogenized lysates was determined with a BCA protein assay kit (Pierce Chemical Co., Rockford, Ill.).

Ten micrograms of OM-10.1 and 15 μg of PERV-293 whole-cell lysate proteins were electrophoresed and electroblotted (27). HIV-1 p24 monoclonal antibody (16), which reacts with p24, p55, and several intermediates, was used (1:600 dilution) to react with the OM-10.1 blots. Anti-simian sarcoma-associated virus p29 polyclonal serum (Quality Biotech, Camden, N.J.), which cross-reacts with the processed PERV Gag p30 and its precursor p55, was used (1:200 dilution) to react with PERV-293 blots (27). Proteins were visualized by chemiluminescence using an ECL Western blot detection reagent (Amersham Pharmacia Biotech, Piscataway, N.J.).

As expected, WT HIV-1 was found to be sensitive to all five PIs tested. No p24 Gag protein was detected in OM-10.1 cell cultures treated with 1 μg of PIs per ml, while untreated cells had a detectable p24 band (Fig. 1). In addition, accumulation of the unprocessed p55 and other intermediates (p48 and p42) was also observed in these lysates (Fig. 1). No p24 or its precursors (p55, p48, and p42) were detected in the cells which were not stimulated with tumor necrosis factor alpha (data not shown). In contrast, no difference in p30 reactivity was seen among PERV-293 cells that were treated with a concentration up to 25 μg/ml (the highest concentration tested) of indinavir, nelfinavir, saquinavir, ritonavir, or amprenavir, which represented a 233- to 6,520-fold increase in the IC<sub>50</sub> reported for WT HIV-1 (3, 9). Representative results are shown for nelfinavir and indinavir in Fig. 1. Thus, our results indicate that PERV is resistant to all five PIs.

Sequence comparison. To better understand the basis of the susceptibility of PERV to the antiretrovirals tested in this study, we compared the amino acid sequences of the pol regions of PERV derived from PK15 cells (GenBank accession numbers U77599 and AF038601) and HIV-1 (subtype B) (accession number M38432). Alignment was performed with the multiple alignment construction and analysis workbench pro-
gram (38), with introduction of minimal gaps to facilitate optimal alignment. The PERV sequence analyzed corresponded to the HIV-1 protease codons 29 to 99 and RT codons 1 to 291. A portion of the alignment of HIV-1 RT, which harbors codons associated with drug resistance, with PERV RT is shown in Fig. 2. HIV-1 and PERV were found to share only about 22.5% amino acid residues in protease or RT, indicating that these proteins are structurally diverse. The high level of divergence between both sequences restricts the comparison between RTI resistance-related mutations of HIV-1 and PERV to only those which are conserved among all retroviruses. Mutations at two such codons, Q151M (in the conserved LPOG motif), and M184V (in the polymerase active-site YMDD motif) are associated with resistance to multiple nucleoside analogs and to lamivudine, respectively, in HIV-1 and other lentiviruses. PERV RT, as in WT HIV-1, has a Q at the codon corresponding to 151, which does not support a role of this residue in the observed resistance of PERV to several nucleoside analogs. However, PERV has a V instead of an M at the codon corresponding to 184, which may explain the observed resistance to lamivudine. Verifying the possible role of this residue in PERV’s resistance to lamivudine will require drug susceptibility analysis of site-specific mutants containing either an M or a V residue at this site.

The observed susceptibility of PERV to zidovudine may not be surprising since this nucleoside analog has been shown to have a broad range of activity against several retroviruses (25, 28), including MuLV and feline leukemia virus, two C-type viruses which are closely related to PERV (36, 46). Our sequence analysis indicated that both MuLV (accession number U13766) and feline leukemia virus (accession number AF052723) share significant homology (79 and 70% amino acid residues, respectively) with PERV RT. Accordingly, isolates with lacZ pseudotypes containing Moloney MuLV Gag-Pol were tested by our culture-based infectivity assay and showed a pattern of drug sensitivity similar to that of PERV (data not shown).

As expected, PERV showed no susceptibility to HIV-1-specific PIs, despite the structural diversity of these compounds and the use of concentrations that were several hundredfold higher than those for WT HIV-1 (6, 12, 29). The lack of susceptibility may be explained by the structural differences between PERV and HIV-1 protease, which were found to share only ~22% of their amino acid residues.

Structure-dependent binding may also explain the resistance of PERV to nevirapine, which binds specifically to a hydrophobic cavity adjacent to the polymerase active site in HIV-1 RT and requires close contact with a tyrosine residue at codon 181 (21, 42). The observed resistance of PERV RT to nevirapine was, therefore, expected and may very likely be due to the absence of this pocket, resulting from significant structural differences.

Our data do, however, demonstrate that some nucleoside RTIs were active against PERV RT. Zidovudine had the highest level of activity against PERV, with IC50s only ~3-fold those for WT HIV-1 but well within the achievable concentration of zidovudine in vivo, which has a maximum concentration of drug in serum of 3.4 μM (14). These in vitro data are

![FIG. 1. Susceptibility of HIV-1 and PERV to PIs by a Gag protein-processing inhibition assay. (A) Immunoblot of HIV-1-infected OM-10.1 cells treated with different concentrations of indinavir and nelfinavir (first lane, no treatment). Positions of Gag proteins are indicated. (B) Immunoblot of PERV-293 cells untreated (first lane) and treated with different concentrations of nelfinavir and indinavir.](https://jvi.asm.org/)

![FIG. 2. Alignment of portions of HIV-1 and PERV RT amino acid sequences. Identical residues are indicated with an asterisk. Four D residues (codons 110, 113, 185, and 186) and the LPOG motif (codons 149 to 152) conserved among the retroviruses are underlined (34a). The HIV-1 polymerase active-site YMDD (codons 183 to 186) is in bold. Codons associated with resistance of HIV-1 to multinucleoside analogs (Q151M), nevirapine (Y181C), lamivudine (M184V), and zidovudine (T215Y/F) are shaded.](https://jvi.asm.org/)
promising and support the clinical use of zidovudine in PERV-infected persons. Prophylactic use of zidovudine has also been successful in reducing transmission of HIV-1 in infants born to HIV-1-infected mothers as well as in recipients of needle-stick injuries from HIV-1-infected source patients (reviewed in references 18 and 53). Therefore, our data on zidovudine may also support evaluating the use of this drug as a prophylactic in persons who will be transiently exposed to pig tissues, such as in extracorporeal perfusions with pig livers or hepatocytes. The need for such a prophylactic will, however, depend on the risks of PERV transmission from these procedures. The assays developed and used in this study also provide tools for identifying novel Pi5 or RT5s that are active against PERV.

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