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Research Article

Susceptibility of recombinant porcine endogenous retrovirus reverse transcriptase to nucleoside and non-nucleoside inhibitors

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Abstract. Transplantation of organs, tissues or cells from pigs to humans could be a potential solution to the shortage of human organs for transplantation. Porcine endogenous retroviruses (PERVs) remain a major safety concern for porcine xenotransplantation. Thus, finding drugs that could be used as virological prophylaxis (or therapy) against PERV replication would be desirable. One of the most effective ways to block retroviral multiplication is to inhibit the enzyme reverse transcriptase (RT) which catalyzes the reverse transcription of viral RNA to proviral double-stranded DNA. We report here the cloning and expression of PERV RT and its susceptibility to several inhibitors. Our data demonstrate PERV susceptibility in vitro to the triphosphorylated nucleoside analog of zidovudine (AZT) and to ddGTP and to a lesser extent to ddTTP but almost no susceptibility to the non-nucleoside RT inhibitors tested.

Key words. Porcine endogenous retrovirus; reverse transcriptase; inhibitors.

Xenotransplantation, the grafting of organs, tissues or cells between species, is a potential solution to the shortage of human organs for transplantation. Pigs are considered the most likely donors for humans because their organs resemble those of humans in size and function. Some of the immunologic hurdles to xenotransplantation, including hyperacute rejection (HAR), may be overcome through the cloning of genetically modified piglets [1–5]. One of the dangers of transplanting animal cells into humans is, however, the possible introduction of agents that could infect the human recipient and spread to other humans [6, 7]. The use of pathogen-free pigs can prevent infection by many known pathogens including porcine influenza and porcine cytomegalovirus [8]. However porcine endogenous retroviruses (PERVs) remain a potential concern due to their integration as proviruses in the pig genome and their vertical transmission with the host DNA. The pig genome contains approximately 50 copies of integrated PERVs belonging to the groups of gamma and beta retroviruses [4, 9–13]. Infectious retroviral PERV particles recovered from pig tissues such as peripheral blood mononuclear cells, endothelial cells or pancreatic islets are capable of infecting various human cell lines in vitro [14–18]). Recent data suggest that human tissues are infectable by a novel hybrid of PERV types A and C [19]. Fortunately, retrospective surveys of approximately 200 patients treated with living porcine tissues has not revealed evidence of in vivo PERV infection

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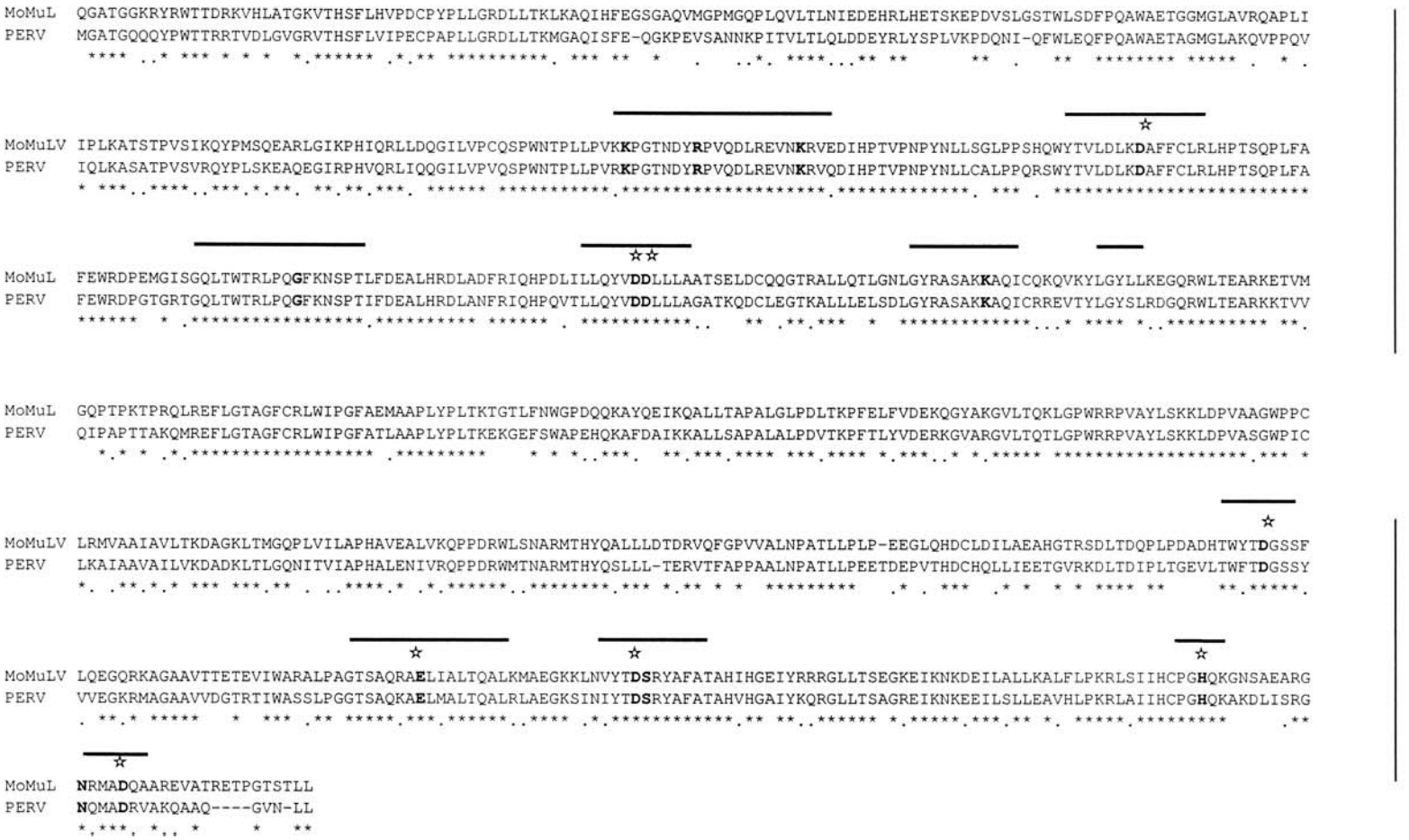


Figure 1. Sequence comparison of PERV and MoMuLV RTs. The conserved domains found in all RNA-dependent polymerases are overlined. The invariant residues are in bold. The catalytically essential residues of the polymerase and RNase H active sites are highlighted by stars.

[20–22]. However, since some patients showed persistent microchimerism or the presence of porcine DNA in circulation up to 8.5 years after exposure to porcine tissues, activation of latent PERV infection cannot be excluded in the long term. Observations [23] that pig pancreatic islet cells transplanted into immunodeficient mice can transmit PERV to murine tissues have not yet been reproduced and are potentially confounded by the presence of endogenous murine viruses.

It would be desirable to identify drugs that could be used as virological prophylaxis (or therapy) against PERV replication. Inhibition of reverse transcriptase (RT) is one of the most effective ways to block retroviral replication. In this study, we cloned and expressed a PERV RT and evaluated its susceptibility to inhibitors that are known to be active against HIV-1 RT. These inhibitors include three nucleoside-based chain terminators and six non-nucleoside RT inhibitors (NNRTIs). Our data provide evidence of sensitivity to the triphosphorylated nucleoside analog of zidovudine (AZT), to ddGTP and to a lesser extent to ddTTP but almost no susceptibility to the NNRTIs tested.

The cloned PERV-MSL RT has polymerase and RNase H activities in vitro

PERV-MSL (porcine endogenous retrovirus from miniature swine lymphocytes) is a type C retrovirus of 8132 bp with greatest nucleic acid sequence similarity to gibbon ape leukemia virus and murine leukemia virus [9]. The *pol* open reading frame (ORF) (nt 2160–5741; accession number AF038600) encodes a 1194-amino-acid polyprotein. The highly conserved sequence of RTs, YXDD, is encoded by nucleotides 3177–3189. The sequence of the PERV RT domain is shown in figure 1 and compared with that of MoMuLV (Moloney murine leukemia virus). The polymerase domain in the N-terminal half of the protein contains the three catalytically essential D residues and all the conserved motifs identified by Poch et al. [24] and Xiong and Eickbush [25]. The C-terminal half of the RT contains the RNAse H domain and the conserved motifs identified by McClure [26] and Malik and Eickbush [27]. As all the conserved domains of PERV RT are found between nt 2307 and nt 4511, we cloned this region in an *Escherichia coli* expression vector. The recombinant protein was expressed, purified and assayed for polymerase and RNase H activities as previously described [28]. Divalent cations (Mg^{2+} or Mn^{2+}) were required for RT activity.

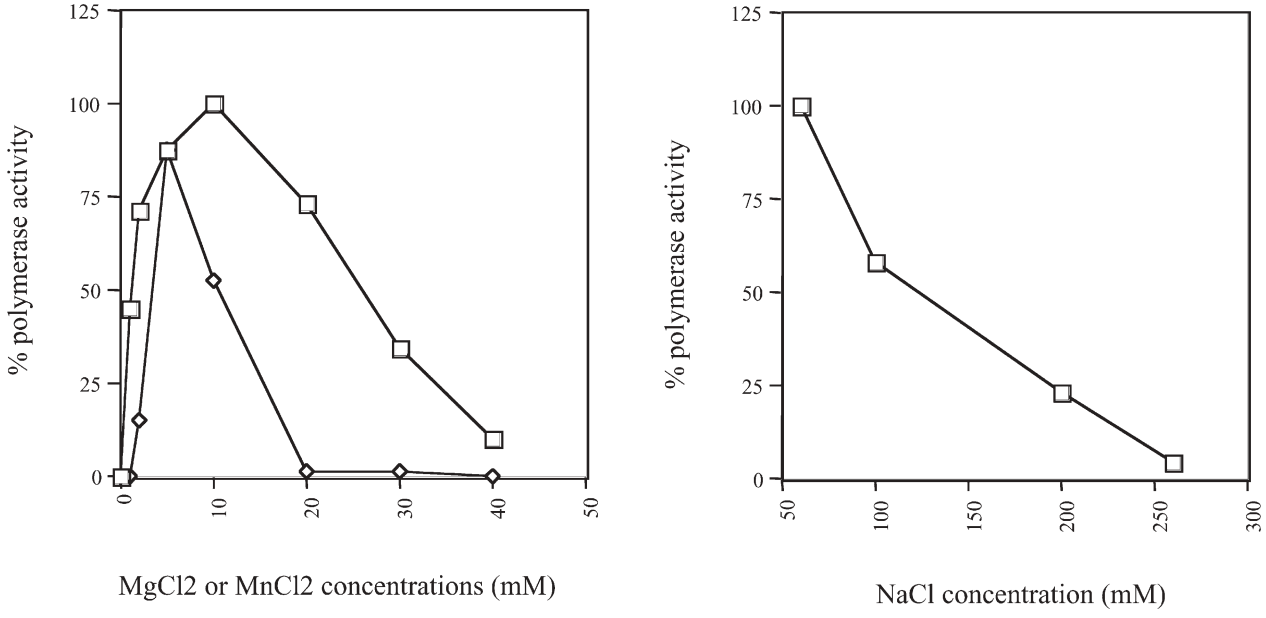
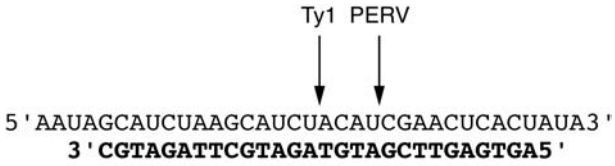


Figure 2. Effect of $MgCl_2$, $MnCl_2$ and NaCl on polymerase activity of recombinant PERV RT. Enzymatic activities are expressed as percentages of the maximal activity (100%) observed. 100% activity is equal to 24 pmol dGTP incorporated/pmol RT in 30 min. In the same experimental conditions, 100% activity of HIV RT was observed at 5–10 mM $MgCl_2$ and was equal to 30 pmol dGTP incorporated/pmol RT in 30 min. Polymerase activity was tested by following the poly$(rC)_n$-oligo$(dG)_{12-18}$-directed incorporation of [α^{32}P]dGTP. The assay mix (20 µl) contained final concentrations of 50 mM Tris-HCl pH 7.8, 12 µM dGTP, 8 mM 2-mercaptoethanol, 1 µCi of [α^{32}P]dGTP, 200 nM of poly$(rC)_n$-oligo$(dG)_{12-18}$, 50 nM of RT and the indicated concentrations of $MgCl_2$, $MnCl_2$ or NaCl. Incubation was for 30 min at 37 °C. Incorporation of [α^{32}P]dGTP into high-molecular-mass polydG was determined by scintillation counting. Aliquots of the reaction mixture were spotted onto DE81 filters (Whatman). The filters were washed three times in 5% Na_2HPO_4 to remove unincorporated [α^{32}P]dGTP, washed once in deionized water, ethanol washed, dried and counted.



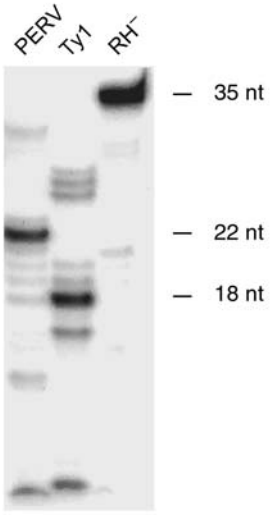


Figure 3. RNase H activity of PERV RT. A 35-nt RNA-28-nt DNA heteroduplex was incubated with PERV or Ty1 RT to induce RNase H cleavage. The assay mix (20 µl) contained the final concentration of 50 mM Tris-HCl pH 7.8, 10 mM $MgCl_2$, 8 mM 2-mercaptoethanol and PERV or Ty1 RT. The lane marked RH– is a control made with an RNase H–Ty1 enzyme. The RNA template was radiolabeled at the 5′ end to allow analysis of the cleavage products on a denaturing 15% polyacrylamide gel. The site of the main cleavage by the PERV or Ty1 RT is indicated by an arrow above the RNA sequence.

Maximal activity was observed at 10 mM for Mg^{2+} and 5 mM for Mn^{2+} and was slightly higher for Mg^{2+} (fig. 2). Similar to other retroviral RTs, the polymerase activity of the PERV protein was inhibited by increasing concentrations of NaCl. The temperature optimum was observed at 37 °C. To test the RT inhibitors described below, the reactions were therefore done at 37 °C in the presence of 10 mM Mg^{2+} with no added NaCl.

The RNase H activity of PERV RT was examined by monitoring the cleavage of a 28-nt DNA-35-nt RNA primer template used previously to analyze the interdependence between the RNase H and polymerase active sites of HIV-1 and Ty1 (a yeast LTR retrotransposon) reverse transcriptases [28]. The 28–35 primer template contains a recessed 3′ terminus of DNA primer which binds the polymerase active site of RT (fig. 3). The 5′ end of the RNA was radiolabeled to allow the demonstration of the RNase H cleavage products by gel electrophoresis. Efficient cleavage of the primer template was obtained with the recombinant PERV RT (lane PERV in fig. 3). The RNase H cleavage was specific for the RNA phosphodiester bond between nt 18 and 19 downstream from the nucleotide complementary to the 3′-terminal nucleotide of the primer indicating that the distance between the positions of the RNase H and DNA polymerase active sites is 18 nt. In comparison, the Ty1 enzyme cleaved the RNA between positions 14 and 15 (lane Ty1 in fig. 3) and the HIV enzyme between positions 18 and 19 [see fig. 5 in ref. 28].

Inhibition of DNA polymerase activity of PERV RT by nucleoside and non-nucleoside analogs

We evaluated the susceptibility of PERV RT to three nucleoside analogs (ddTTP, ddGTP and AZT-TP) and six non-nucleoside side-chain-modified analogs of 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine) (HEPT) (fig. 4) [29].

RTs are known to be sensitive to dideoxynucleoside analogs which act as chain terminators because the 3′-OH group of the sugar moiety is missing. AZT-TP is a potent inhibitor of HIV-1 which provides a 0.5–0.6 log inhibition in clinical trials. It has been used successfully for the treat-

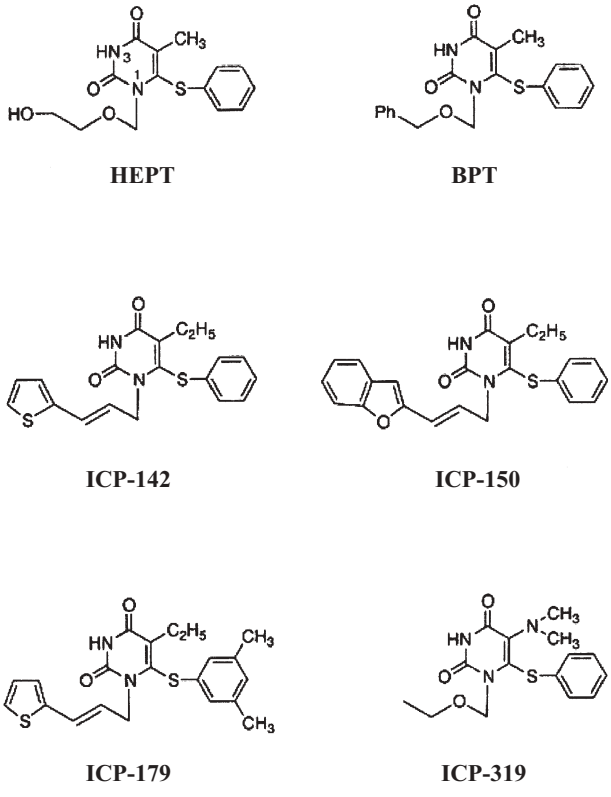


Figure 4. Structure of HEPT and five analogs examined for their inhibitory effect on PERV RT.

ment of AIDS patients in combination with other RT inhibitors and protease inhibitors. The sensitivity of PERV RT was examined in comparison to that of HIV-1 RT which served as a standard. Figure 5 shows that, in comparison to HIV-1 RT, PERV RT has reduced susceptibility to all three chain terminators. ddTTP was a weak inhibitor of PERV polymerase activity. The same inhibition of PERV polymerase activity (IC_{50} = 0.5 µM) was observed with AZT-TP and ddGTP. This result is in line with observations published by Qari et al. [30] and Stephan et al. [31] showing that AZT-TP has a high level of activity against PERV RT in PERV-infected cells.

Since long-term administration of nucleoside RT inhibitors leads to the emergence of resistant viral strains,

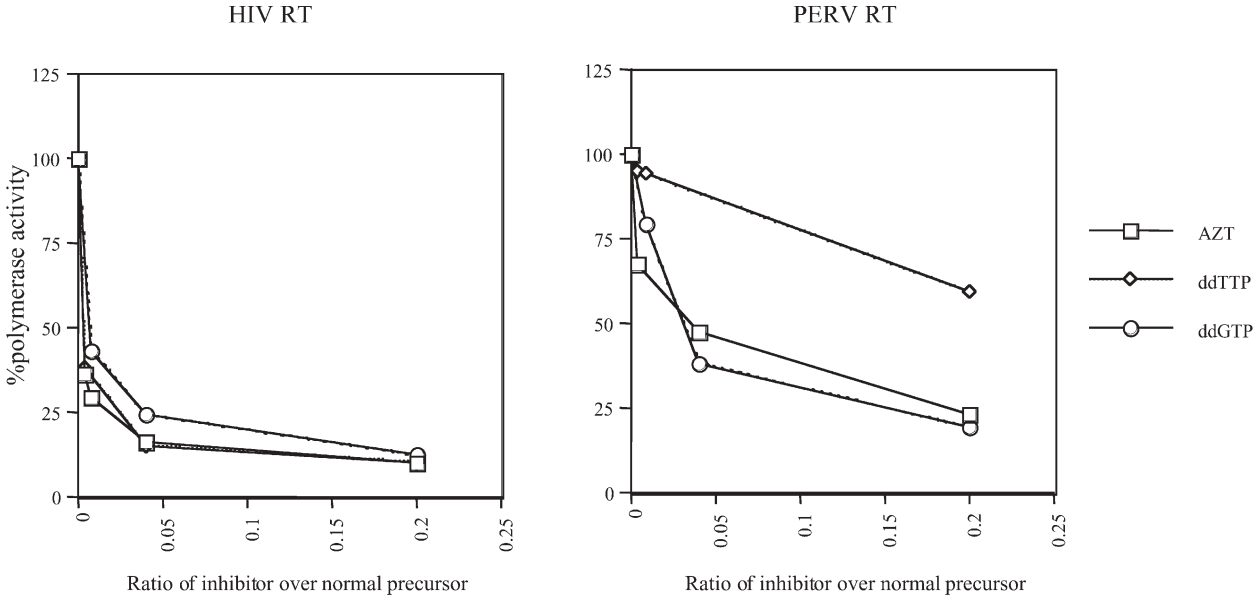


Figure 5. Effect of three nucleoside analogs on PERV and HIV-1 RT polymerase activity. The three nucleoside analogs, ddGTP, ddTTP and AZT-TP, which act as chain terminators, were tested at increasing concentrations of each analog. The polymerase activity was assayed with poly(rC)$_n$-oligo(dG)$_{12-18}$ when ddGTP was tested and poly(rA)$_n$-oligo(dT)$_{12-18}$ when ddTTP or AZT-TP were tested. The assays were carried out with a constant level (12 µM) of normal substrate (dGTP or dTTP) and increasing levels of inhibitor (ddGTP, ddTTP or AZT-TP). Enzymatic activities are shown as percentage of the control reaction with no inhibitor present.

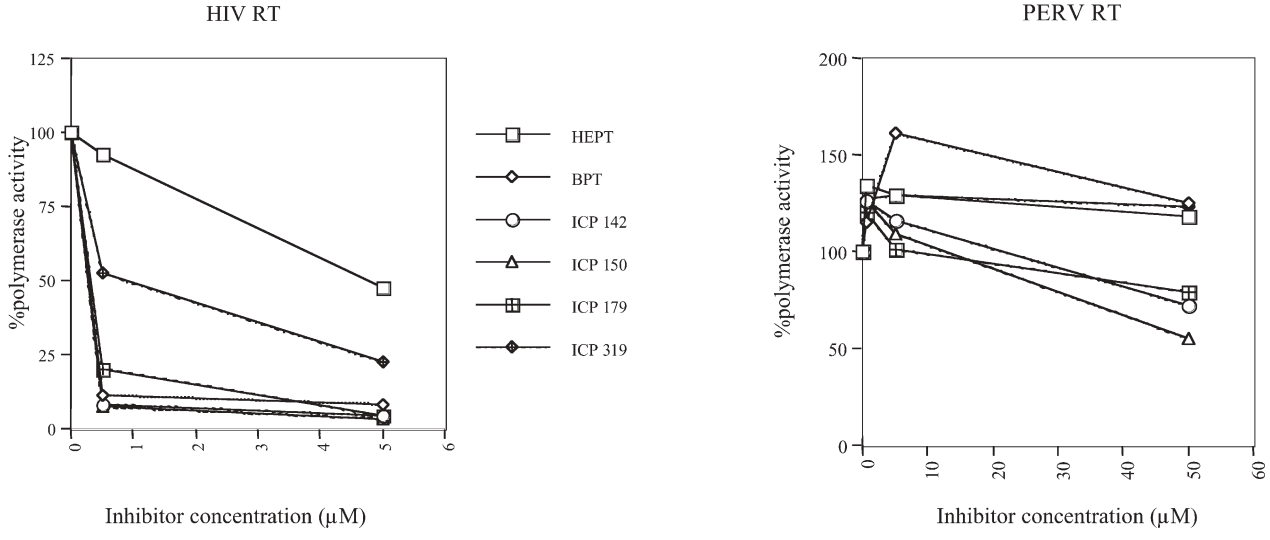


Figure 6. Effect of HEPT analogs on PERV and HIV-1 RT polymerase activity. The polymerase activity tested at increasing concentrations of HEPT analogs was assayed with poly(rC)$_n$-oligo(dG)$_{12-18}$ as described in the legend to figure 2. Enzymatic activities are shown as a percentage of the control reaction with no inhibitor present. Note that the inhibitor is ten times more concentrated in the experiment with PERV RT.

Table 1. Anti-RT activity of HEPT analoges expressed as the concentration in (μm) of compound required to achieve 50% inhibition of the RT polymerase activity in vitro or 50% ingibition of HIV-1 multiplication in CEM-SS or MT4 infected cells [29].

HEPT analog	HIV RT			PERV RT
	in vitro	in vivo [29]		in vitro
		CEM-SS/LAI	MT-4/IIIB	
ICP142	0.03	0.008	0.08	>50
ICP179	0.03	0.028	0.04	>50
ICP150	0.05	0.038	>1	50
BIPT	0.1	0.09	0.74	>50
ICP319	0.5	0.46	n.d.	>50
HEPT	5	2	11	>50

non-nucleoside inhibitors might be used along with nucleoside analogs in a combination therapy approach. Analogs of HEPT are potent non-competitive RT inhibitors which bind to a hydrophobic pocket near the polymerase active site in HIV-1 RT [32]. We evaluated the susceptibility of PERV RT to HEPT and five analogs. The inhibitory effect of these molecules was also evaluated on the polymerase activity of HIV-1 RT. The data shown in figure 6 and table 1 demonstrate that the six NNRTIs tested have almost no activity against PERV RT ($IC_{50} > 50$ μM) whereas four HEPT analogs were active against HIV-1 RT with IC_{50}s of 0.03–0.1 μM. Similarly, Qari et al. [30] observed the lack of activity of nevirapin, a strong NNRTI of HIV-1 RT, on PERV RT ex vivo. As suggested by those authors, the resistance of PERV RT to NNRTIs could be due to structural differences between the HIV-1 and PERV enzymes and to the absence of the hydrophobic pocket adjacent to the polymerase active site in PERV RT. In table 1, we compare the effect of the HEPT analogs in HIV-1-infected cells published by Pontikis et al. [29] with our in vitro results. The relative trend in inhibition activity is the same in our in vitro assay and in the HIV-I-infected cells, thus validating the in vitro assays to assess the strength of RT inhibitors.

The cloning and expression of PERV RT described here provide tools for identifying new inhibitors that are active against PERV. A chemical mass screening assay which uses scintillation proximity technology was recently developed to detect new HIV RT inhibitors [33] and should be used to identify new molecules against potential PERV infection.

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