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Susceptibility of porcine endogenous retrovirus to anti-retroviral inhibitors

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Abstract

Background—Porcine endogenous retrovirus (PERV) is an endogenous retrovirus that poses a risk of iatrogenic transmission in the context of pig-to-human xenotransplantation. The lack of a means to control PERV infection in the context of pig-to-human xenotransplantation is a major concern in the field. In this study, we set out to evaluate the ability of currently licensed anti-HIV drugs, and other types of anti-retroviral compounds, to inhibit PERV infection in vitro.

Methods—We used target cells stably expressing one of the known PERV viral receptors, an infectious molecular clone, PERV-A 14/220, and at least one drug from each class of anti-retroviral inhibitors as well as off-label drugs shown to have anti-viral activities. The susceptibility of PERV-A 14/220 LacZ to the anti-retroviral drugs was determined from infected cells by histochemical staining.

Results—We extend the results of previous studies by showing that, in addition to raltegravir, dolutegravir is found to have a potent inhibitory activity against PERV replication (IC_{50} 8.634 $\pm$ 0.336 and IC_{50} 3.06 $\pm$ 0.844 nM, respectively). The anti-HIV drug zidovudine (AZT) showed considerable anti-PERV activity with IC_{50} of 1.923 $\pm$ 0.691 μ M as well.

Conclusions—The study results indicate that some of the licensed antiretroviral drugs may be useful for controlling PERV infection. However, the efficacy at nanomolar concentrations put forward integrase inhibitors as a drug that has the potential to be useful in the event that xenotransplantation recipients have evidence of PERV transmission and replication.

Keywords

anti-retroviral; porcine endogenous retrovirus; xenotransplantation

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Introduction

Due to anatomical and physiological similarities with humans and well-established animal husbandry practices, pigs have been championed as the one potential source animal of xenotransplantation products such as cells, tissues, organs, and extracorporeal support. Through the use of genetic modification of pigs, some immunologic challenges have been addressed and considerable progress has been made to alleviate biological incompatibilities between pig and humans [1–4]. However, against the backdrop of these promising developments, there is the inherent risk that porcine endogenous retroviruses (PERV) may be transmitted from the transplanted pig tissue to the human recipient, and this risk has impeded development of xenotransplantation products. Despite this risk, the study of pig-to-human xenotransplantation is ongoing in some parts of the world [3,5,6].

Porcine endogenous retroviruses are endogenous retroviruses and are members of the genus gammaretroviruses. Endogenous retroviruses are remnants of ancient viral infections found in the genomes of most, if not all, mammalian species.

Data from animal studies [7–11], and human xenotransplantation clinical trials and reports [5, 9, 12–16], showed lack of viral replication and transmission of PERV *in vivo*. However, the possibility of cross-species transmission of PERV could not be ruled out due to tangible *in vitro* data from our laboratory [17–19] and others [7, 20, 21] showing that exposure of human cells to pig-derived biological materials (i.e., plasma, blood, endothelial cells) results in PERV transmission *in vitro*. Further, our laboratory has demonstrated in certain animal models, evidence of *in vivo* infection without viral replication [8, 22]. These findings suggest the potential risk of PERV transmission associated with pig-to-human xenotransplantation. Moreover, in a long-term study of mini-pigs, Wood et al. produced striking data showing that several adolescent mini-pigs released viruses that were initially capable of only infecting pig cells but later acquired the ability to also infect human cells [23]. These results suggest that recombinant replication competent PERVs that are able to infect human cells could arise anytime during the life of pigs destined for clinical xenotransplantation or potentially after transplantation into a human recipient. Therefore, it is prudent to evaluate the potential of available anti-retroviral drugs for their ability to block or limit PERV infection or replication. Prior studies demonstrated that reverse transcriptase inhibitors had some activity against PERV [24, 25]. However, additional classes of anti-retroviral drugs have been licensed as the initial studies were performed. Very recently, a study focused on the susceptibility of PERV integrase to the anti-HIV drug showed that raltegravir is a potent drug candidate in controlling the risk of zoonotic PERV infection during pig-to-human xenotransplantation [26]. Screening combinations of different anti-retroviral agents are commonly employed to overcome development of anti-retroviral resistance to single-drug therapy [27, 28]. Therefore, we set out to evaluate the use of different commercially available anti-HIV drugs in controlling the risk of zoonotic PERV infection.

Materials and methods

Cells

The target cells, SIRC/HuPAR-2eGFP cells (rabbit corneal fibroblasts, ATCC-CRL60) stably expressing a fusion protein derived from the cDNA encoding the human PERV-A receptor type 2, HuPAR2 and the reporter gene, eGFP (Sirc-HuPAR2-eGFP) were a gift from Dr. Daniel Salomon [29]. HuPAR2 was recently renamed SLC52A1 [30]. These cells were supplemented in the same manner and cultured under the same condition as previously described [31]. SIRC/ HuPAR-2eGFP cells support similar levels of PERV infectivity titers as the natural host, pig, ST cells, making them a suitable model to make the assessment described here [31].

Viruses

PERV-A 14/220 is a biological viral isolate known for its high titer on human cells [32]. This viral isolate is the product of a recombination between PERV-A and PERV-C that retains the tropism of PERV-A [33].

Generation of PERV-A 14/220/LacZ and Infectivity Assay

To create PERV-A 14/220 LacZ pseudotypes, we cotransfected 293T cells with pMDG VSV-G encoding the vesicular stomatitis virus G (VSV-G) envelope glycoprotein, pMLV Moloney murine leukemia virus (MoMuLV) *gag-pol*-expressing the core and enzymatic proteins, and the beta-gal-expressing plasmid pRT43.2TnsI β -gal [34, 35] by calcium phosphate precipitation using ProFection mammalian transfection system (catalog #E1200, Promega, Madison, WI). Seventy-two hours post-transfection, supernatant containing the VSV-G pseudo-typed retroviral vector was harvested, filtered through 0.45 μ m filter, diluted 1:10 with complete medium, and adjusted to a final concentration of 8 μ g/ml polybrene. The treated, diluted supernatant was used to transduce 293 cells chronically infected with PERV-A 14/220 [32, 33]. The PERV-A 14/220 LacZ cells were expanded in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD, USA) and supplemented with 10% FBS (HyClone, Logan, Utah, USA), 1% glutamine, 1% penicillin/streptomycin, and 1% sodium pyruvate (Lonza, Walkersville, MD, USA).

To prepare a stock of viral pseudotypes for this study, the 293 PERV-A 14/220 LacZ cells were trypsinized and grown over night to subconfluence in ten T125 flasks. Upon reaching 100% cell confluence, supernatant was collected, filtered with 0.45 μ m filter, and transferred to the Amicon Ultra centrifuge column to concentrate virus with a gradual centrifugation process according to the manufacturer's instruction (Amicon Ultra 15, Millipore Billerica, MA, USA). Before being used, the concentrated (20 : 1) viruses from different columns were first pooled into one 50-ml tube as a lot before being aliquoted (0.250 ml) into 1-ml tubes and frozen in -80 °C until use. Forty-eight hours after freezing, virus samples from the lots were tittered on 293 cells. Infectivity titers were determined after histochemical staining and microscopic enumeration of foci of cells expressing β -galactosidase as previously described [36].

Anti-retroviral treatment, infection, and maintenance of cell culture

We set out to evaluate the susceptibility of PERV-A14/220 to nine types of drugs and compounds known for their anti-viral effects (Table 1).

We reconstituted each drug following the manufacturer's instructions and prepared serial logarithmic dilutions to reach final concentrations to test ranging from 0.001 to 100 μ M. In those cases in which an organic solvent was required for dissolution of a drug, dimethyl sulfoxide (DMSO) was used to final concentrations of 1%, to minimize cytotoxic effects. As an initial screen for cytotoxic effects, we evaluated cell viability after treatment using trypan blue exclusion assay (Trypan Blue Solution, Life Technology, and Grand Island, NY). Those anti-viral drugs showing reduction in cell viability (more than 25% relative to untreated cells) at lower concentrations were discontinued from further evaluation.

For those compounds that showed <25% cytotoxicity relative to controls, we evaluated their anti-viral activities by measuring levels of PERV replication in target cells in the presence of an anti-viral compound compared with untreated controls. Therefore, to determine the efficacy of the anti-virals in protecting target cells from infection by PERV, 1×10^5 SIRC HuPAR-2eGFP cells/well were seeded in 12-well plates, grouped by treated and untreated, and incubated overnight at 37 °C. After first treating the cells with the indicated anti-viral dose for two hours, equal doses of PERV-A 14/220 LacZ viruses (approximately 500 blue-forming units (BFU) per 1 ml) were added to each well in the presence of eight micrograms of polybrene (to both treated and untreated cells) and then incubated for 24 h. Twenty-four hours after viral exposure, medium was removed and replaced with fresh medium containing the corresponding drug or control medium without any drug added.

To evaluate whether competitive receptor inhibition by riboflavin occurs, the same number of target cells specified above was treated with different concentrations of riboflavin for 0, 5, 30, 60, or 120 min before exposure to the same amount of virus mentioned above.

Forty-eight hours after drug treatment, the cells were washed with PBS, fixed with 2% paraformaldehyde, washed again, and viral infectivity was determined using beta-galactosidase assay as previously described [37]. Each anti-viral drug was assessed over a range of doses in at least three independent experiments. Each experiment was performed using triplicate wells for each drug concentration tested as well as controls. Blue-forming units (BFU) in anti-retroviral treated cells were determined by microscopic enumeration. The data are represented as relative infectivity (%) of treated cells compared to untreated samples (100%). To determine the half-maximal inhibitory concentration (IC_{50}), the relative infectivity rate was then subtracted from 100% to find PERV susceptibility to anti-retroviral treatment. Then, based on the relative infectivity data, the half-maximal inhibitory concentration 50 (IC_{50}) was calculated using GraphPad Prism 6 software (GraphPad Software Inc. La Jolla, CA, USA). The IC_{50} value was estimated by interpolation of the dose–response curve.

As a control for potential drug-mediated cytotoxicity, cell proliferation was determined using the WST-1 kit, according to the manufacturer's instructions (Roche Diagnostics

GmbH, Mannheim, Germany). No difference was observed between control and drug-treated cells (data not shown).

Results and discussion

In the current study, we evaluated the susceptibility of PERV virions to anti-HIV drugs. The results of our analysis are shown in Fig. 1 and Table 2. We first evaluated five reverse transcriptase (RT) inhibitors (RTI) that employ different mechanisms to inhibit viral replication: nucleoside-analog RT inhibitors, AZT and tenofovir; a nucleotide-analog RT inhibitor, adefovir; and non-nucleoside RT inhibitors, nevirapine and efavirenz. Previously, PERV RT was evaluated enzymatically for susceptibility to RT inhibitors. But, in this study, we evaluated the susceptibility of PERV virions to reverse transcriptase inhibitors in a replication assay. Interestingly, the IC_{50} measured in our study with AZT, $1.92 \pm 0.62 \mu M$, was strikingly similar to that observed in the RT enzyme inhibition assays by Qari et al. [24] that has IC_{50} of, $1.5 \mu M$. We also found comparable results to Qari et al., with respect to PERV resistance to the non-nucleoside inhibitor nevirapine. These observations agreed with previous data in which comparing susceptibility of different retroviruses, non-nucleoside reverse transcriptase inhibitors such as nevirapine were considered as highly selective for HIV-1 [38, 39]. Another study that evaluated the susceptibility of PERV in an extended time (three weeks) of treatment of infected cell culture with Zidovudine reported inhibition of replication with a 5-fold lower concentration ($0.25 \mu M$) [25] compared with our results (IC_{50} of $1.923 \pm 0.62 \mu M$). The discrepancy in the IC_{50} results might be associated with the viral isolates, methods of infection, cell types, or assays used to measure susceptibility. The other reverse transcriptase inhibitor tested for the first time for PERV control is tenofovir. Tenofovir showed better efficacy among the tested reverse transcriptase inhibitors in controlling PERV infection with IC_{50} $0.7 \mu M \pm 0.08$. The susceptibility of PERV to tenofovir is comparable to another gammaretrovirus, xenotropic murine retrovirus (XMRV), as reported previously [39].

Our analysis of PERV susceptibility to the non-nucleoside RTI efavirenz was impeded by occurrence of cytotoxicity at minimal concentrations ($1 \mu M$) used for the initial screening of the drugs (data not shown).

We also tested the susceptibility of PERV to the nucleotide reverse transcriptase inhibitor (NRTI) adefovir. Adefovir is a prodrug that after intracellular conversion to adefovir diphosphate attains activity against DNA viruses such as HBV as well as retroviruses such as HIV-1 and FIV (reviewed; [40]). In this study, adefovir demonstrated moderate inhibition of PERV replication with IC_{50} of $8.67 \mu M \pm 1.65$ (Fig. 1 and Table 2). Prior studies of adefovir inhibition of PERV replication reported IC_{50} of $3.4 \mu M$ [41] an inhibitory activity over 50% higher than in the current study.

Nevirapine is another reverse transcriptase inhibitor tested for PERV inhibitory activity. While a 15–20% reduction in replication was observed compared to untreated cells, there was no dose-dependent increase in inhibitory effects on viral replication with increasing drug concentration ($IC_{50} > 50 \mu M$) (Fig 1).

HIV integrase inhibitors, raltegravir and dolutegravir, were evaluated for their ability to inhibit PERV replication. Integrase is a retroviral enzyme that catalyzes the insertion of the double-stranded DNA intermediate of the viral genome into the host cell genome. Raltegravir treatment resulted in inhibition of PERV replication at the nanomolar level with IC_{50} 8.634 ± 0.336 nM. This result is comparable to a very recently reported work in which raltegravir could inhibit PERV infection at nanomolar level [26]. These two results showing the potent anti-PERV activities of raltegravir correspond with its anti-HIV activity shown previously [42]. Raltegravir had an IC_{50} of 2–7 nM against a recombinant HIV-1 integrase *in vitro* and an IC_{95} of 33 nM in a cell-based assay (reviewed in [43]). In human clinical trials, the plasma concentration of raltegravir 12 h after a directly observed dose was above the HIV 95% inhibitory concentration (IC_{95}) of 33 nM [44]. Comparison of the integrase catalytic domain (DDE-motif) of HIV-1 and PERV C/A 14-220 demonstrated complete conservation (Fig. 2), most likely accounting for the similar patterns of susceptibility to raltegravir. According to a recent report, all retroviruses, including primate and non-primate lentiviruses, a betaretrovirus, a gammaretrovirus, and the alpharetrovirus Rous sarcoma virus (RSV) were susceptible to inhibition by raltegravir, a first-in-class inhibitor of viral integrase [42]. Therefore, in light of these studies, it is not surprising that we observed anti-retroviral activity by Raltegravir against PERV. The other IN inhibitor, dolutegravir, also showed anti-PERV potency at nanomolar level (IC_{50} 3.06 ± 0.844 nM). Dolutegravir the third FDA licensed drug in its class, HIV-1 and HIV-2 IN inhibitors, was tested for the first time here for the ability to inhibit simple gammaretroviruses [45].

In light of the emergence of drug-resistant HIV-1, there has been an effort to evaluate other drugs that could be used to inhibit HIV-1. Studies have demonstrated that two FDA-approved anti-cancer drugs decitabine and gemcitabine have anti-HIV [46] and anti-FeLV [47] activity *in vitro*. Our attempt to evaluate their activity against PERV infection was hampered by their cytotoxic effect on target cell viability, although these drugs were evaluated previously for the control of another retrovirus, FeLV, without any deleterious effect on feline target cells [24]. Presumably, the difference in species of origin of the cells and the tissue culture cell line type might explain this discrepancy. Additionally, we also took this opportunity to assess whether riboflavin, the natural ligand for the PERV-A receptor on human cells (SCL52A, a riboflavin transporter [30]) could act as a competitive inhibitor of PERV entry. However, no inhibition of PERV infection was seen following the treatment of cells with riboflavin (data not shown).

In summary, we have shown that PERV infection is inhibited by raltegravir, AZT, and adefovir at doses comparable to those required to inhibit HIV-1 and other related gammaretroviruses, such as XMRV and FeLV [39, 47]. It is noteworthy that at the highest concentration of these drugs, as shown in Fig. 1, the viability of the cells exposed to the drugs is similar to the untreated cells (data not shown), indicating that the IC_{50} values obtained for the tested drugs were not influenced by drug-mediated toxicity.

Our studies with zidovudine agree with those of a previous study [24] that supported the clinical use of RTI zidovudine (AZT) in cases where PERV infections arise and as a prophylactic in patients who will be transiently exposed to pig tissues and cells (i.e., extracorporeal perfusions with pig livers or hepatocytes). However, we also demonstrate

here that among the different anti-HIV-1 drugs evaluated, the IN inhibitors, dolutegravir and raltegravir are the most potent inhibitors of PERV replication. The results demonstrate that IN inhibitors as a class might be useful in the event that xenotransplantation recipients have evidence of PERV transmission and replication. However, the recent report of using genome editing to inactivate multiple PERV loci may pave the way to a long-term strategy to minimize the risk of pig-to-human xenotransplantation [48].

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Abbreviations

PERV	porcine endogenous retroviruses
HuPAR-2	human PERV-A receptor-2
eGFP	enhanced green fluorescent protein
VSVG	vesicular stomatitis virus G
MLV	murine leukemia virus
BFU	blue-forming units
FBS	fetal bovine serum
HIV-1	human immunodeficiency virus-1
DMSO	dimethyl sulfoxide
RT	reverse transcriptase
AZT	zidovudine
RAL	raltegravir

Fig. 1.

Dose-response curves of PERV-A 14/220 infectivity after treatment with anti-retroviral drugs in SIRC HuPAR2 cells exogenously expressing viral receptor. (A) Reverse transcriptase inhibitors; (B) Integrase inhibitors. Each data point represents the mean $\pm$ SE of three independent replicates. The molarity of the drug concentration range analyzed is indicated along the x-axis. Control = cells without drug treatment.

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HIV IN   (47) GSVDS-PGIWQLDCTH-----LEGKVLVAVHVASGYIEAEVIPAEETGQE
C/A IN   (1)  -RGSHPGAHWEVDFTFVKPAKYGNKYLLVFVDTFSGWVEAYPTKKETSTV
Con      (1)          T          K LV V   SGW EA   ET

                                     116
HIV IN   (96) TAYFLLK--LAGRWPKTVHTDNGSNFTSTTVKQACWAGIKQEPGIPYN
C/A IN   (50) VAKKILEEIPPRFGIPKVIKSDNGPAFVAQVSQGLAKILGIDWKLHCAYR
Con      (51)  A IL          K I SDNG  F A   A   GI   Y

               146       152       159
HIV IN   (145) PQSQGVIESMINKELKKIIGVVRDQAEHLKTAVQMAVFIHNKKRKGIGGY
C/A IN   (100) PQSSGVVERMRTIKET-----
Con      (101) PQS G IE MNK IK

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Fig. 2.

Alignment of portions of HIV-1 integrase (IN) sequence and PERV-IN. Adapted from Koh, Y. et al. [42], whereby the HIV-1IN sequences were compared with other different retroviruses to analyze the contribution of potential amino acids that confer different susceptibilities to raltegravir (RAL). Green indicates residues that when changed can confer resistance to RAL for HIV-1 (not tested for PERV); black fonts indicate residues with unknown effects on potential HIV resistance; red indicates active sites (D64, D116, E152); and blue highlights a conserved DNA-binding residue. Alignments were made using Vector NTI advance 11.5 sequence analysis and data management software (Life Technologies; Grand Island, NY). Amino acid identities between the two integrases are shown in the bottom consensus (con) sequences. Numbers above the alignment indicate HIV-1 amino acid positions; C/A denotes the 2 classes of PERV, PERV-A and PERV-Accession number for PERV-IN: AY570980.1; accession number for HIV-1 IN: 1BIZ_B.

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Table 1
Anti-retroviral drugs and compounds evaluated for their anti-PERV activity

Anti-retrovirals		Chemical name and manufacturer	Mechanism of action
1	Zidovudine/AZT	3'-azido-3'-deoxythymidine) (SIGMA-ALDRICH; St. Louis, MO)	NRTI
2	Tenofovir	R)-1-6-amino-9H-purin-9-yl)propan-2-yl)oxy)methylphosphonic acid; Selleckchem.com Houston, TX	NRTI
3	Adefovir	(P-[[2-(6-Amino-9H-purine-9-yl)ethoxy]methyl]phosphonic acid, 9-(2-phosphonyl-methoxyethyl)adenine)(SML 0240 SIGMA)	NRTI
4	Nevirapine	(11-Cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido[3,2-b:2',3'-e][1,4]diazepin-6-one); (SML-0097; SIGMA)	NNRTI
5	Efavirenz	(4S)-6-Chloro-4-(2-cyclopropylethynyl)-1,4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one (SML-0536; SIGMA)	NNRTI
6	Raltegravir	NIH-ARP Cat #11680; AIDS Research and Reference Reagent Program, Germantown, MD	Integrase inhibitor Anti-cancer Anti-cancer Competitive inhibitor/ Receptor blocker
7	Dolutegravir	GSK1349572; (Cat S2667) Selleckchem.com; Houston, TX,	
8	Gemcitabine hydrochloride	2'-Deoxy-2',2'-difluorocytidine (G6423; SIGMA)	
9	Decitabine	5-Aza-2'-deoxycytidine (A3656-50 mg; SIGMA)	
10	Riboflavin	Lactoflavin, Vitamin B-12 (R4500-SIGMA)	

NRTI, Nucleoside reverse transcriptase inhibitor; NNRTI, Nucleotide reverse transcriptase inhibitor.

Table 2
Half maximal inhibitory concentration (IC₅₀) of anti-HIV drugs to in vitro porcine endogenous retrovirus replication

Inhibitor classes	Inhibitors	Inhibitory concentration (IC ₅₀) (±SE)
NRTI ^a	AZT, Zidovudine	1.923 ± 0.621 µM
	Tenofovir	0.7 ± 0.08 µM
NtARTI ^b	Adefovir	8.676 ± 1.12 µM
NNRTI ^c	Nevirapine	> 50 µM
Integrase Inhibitor	Raltegravir, integrase inhibitor	8.634 ± 0.336 nM
	Dolutegravir	3.06 ± 0.844 nM

Target cells were first treated with different concentration of anti-retroviral and then exposed to replicating porcine endogenous retroviruses.

^aNARTI, nucleoside-analog reverse transcriptase inhibitor;

^bNtARTI, Nucleotide-analog reverse transcriptase inhibitor;

^cNNRTI, non-nucleoside reverse transcriptase inhibitor.