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Short communication

Inhibition of porcine endogenous retrovirus (PERV) replication by HIV-1 gene expression inhibitors

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ABSTRACT

Porcine endogenous retrovirus (PERV) is persistently integrated into the host genomic DNA as a provirus and released from a variety of porcine cells. PERV infects a certain range of human cells, which is a major concern in xenotransplantation. Therefore, the use of viral gene expression inhibitors could be envisaged, if they reduce PERV production from porcine organs and minimize viral transmission to human recipients. In the present study, four HIV-1 gene expression inhibitors were examined for their inhibitory effect on PERV replication in porcine cells constitutively producing the virus. Among the compounds, the fluoroquinolone derivative K-37 and the bacterial product EM2487 displayed potent and selective inhibition of PERV replication in the cells mediated by the suppression of viral mRNA synthesis. Thus, retroviral gene expression inhibitors may be able to reduce the risk of PERV transmission.

Xenotransplantation, the grafting of cells, tissues, or organs into different species, is a possible solution to overcome the extreme shortage of human allografts for transplantation (Cooper and Keogh, 2001). Among the animals, non-human primates and pigs are considered to be suitable donors for xenotransplantation. The use of non-human primates as organ donors is associated with a high risk of transmitting various infectious pathogens to humans (Allan, 2003). Apart from immunological rejection, pigs may be more suitable donors than non-human primates because of the resemblance of their organ sizes and a lower risk of transmitting various infectious pathogens. However, porcine endogenous retrovirus (PERV) is still a major obstacle to successful xenotransplantation with sufficient safety. PERV is a type C retrovirus persistently integrated into the host genomic DNA as a provirus. Multiple copies of PERV proviral DNA exist in all of the breeds examined to date (Louz et al., 2008). PERV is classified into three subtypes, such as PERV-A, -B, and -C, based on the divergence of its envelope genes.

It has been demonstrated that PERV particles are released from a variety of porcine cells and infect a certain range of human cells (Martin et al., 1998; Patience et al., 1997; Wilson et al., 1998). There are a number of patients who received porcine tissues, such as pancreatic islet cells, skin, liver, and kidney; nevertheless PERV infection has not been observed in these individuals (Heneine et al., 1998; Paradis et al., 1999; Patience et al., 1998). However, long-lived microchimerism was found in some patients treated by extracorporeal splenic perfusion, which might increase a potential risk of PERV infection through the activation of viral replication (Paradis et al., 1999). An immunosuppressive treatment upon organ transplantation may also increase a risk of PERV transmission. The use of antiretrovirals would be the first option to minimize the possibility of PERV transmission to recipients, if they could have an inhibitory effect on PERV replication without serious side effects. Among the antiretrovirals, zidovudine (AZT) and didanosine (ddI) proved to be active against PERV replication in cell cultures (Powell et al., 2000; Qari et al., 2001). We have previously demonstrated that the acyclic nucleoside phosphonate tenofovir (PMPA), an HIV-1 reverse transcriptase (RT) inhibitor, selectively inhibits PERV replication in human cells (Shi et al., 2007). However, such RT inhibitors cannot suppress the production of PERV from the porcine cells in which its proviral DNA is integrated. Therefore, it would be very useful if an inhibitor of PERV gene expression could be identified. In the present study, we have examined four inhibitors of HIV-1 gene expression for their antiviral activity against PERV replication in porcine cells persistently infected with the virus and found that the fluoroquinolone derivative K-37 (Baba et al., 1998) and the bacterial product EM2487 (Baba et al., 1999) are potent and selective inhibitors of PERV replication.

K-37 and the nuclear factor κB (NF-κB) inhibitor cepharanthine (Okamoto et al., 1998) were provided by Daiichi Pharmaceutical

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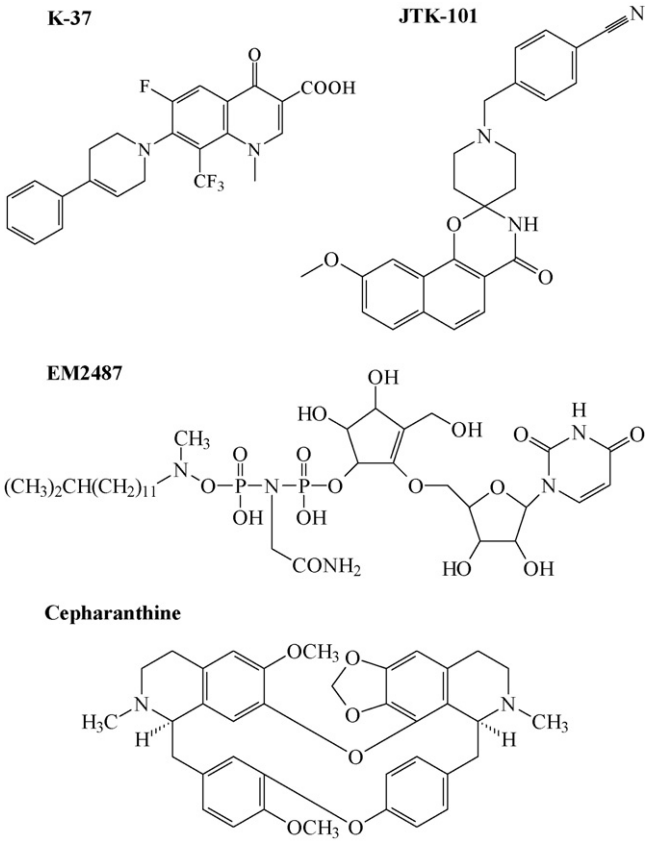

Fig. 1. Chemical structures of test compounds.

Co. (Tokyo, Japan) and Kaken Shoyaku (Mitaka, Japan), respectively. JTK-101 (Wang et al., 2007) was synthesized by Japan Tobacco Co. (Takatsuki, Japan). EM2487 was provided by Esai Co. (Tsukuba, Japan). These compounds (Fig. 1) were chosen for this study, because their antiviral activity against HIV-1 replication in chronically infected cells had been demonstrated (Baba et al., 1998, 1999; Okamoto et al., 1998; Wang et al., 2007). All compounds were dissolved in dimethyl sulfoxide (DMSO) at 10 mM or higher concentrations to exclude any antiviral or cytotoxic effect of DMSO and stored −20 °C until use. The porcine embryonic kidney cell line PK15, which produces PERV particles, was obtained from the American Type Culture Collection. The cells were maintained in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate, and antibiotics.

The activity of the compounds against persistent PERV infection was based on the inhibition of PERV particle production from PK15 cells. PK15 cells were seeded in a 24-well plate (2.5×10^4 cells/well). After incubation for 16 h at 37 °C, the culture supernatants were removed and the cell monolayer was washed by phosphate-buffered saline (PBS), and then 2 ml of fresh medium containing various concentrations of the test compounds was added to each well. After a 48-h incubation period, the culture supernatants were collected and filtered (0.45 μm pore size). Then the filtrates were mixed with 22% (w/v) polyethylene glycol 6000 solution. After incubation for 5 h at 4 °C with continuous stirring, the mixture was centrifuged at 15,000 rpm for 15 min at 4 °C. The pellets contained PERV particles released from PK15 cells. The inhibition of PERV particle production was determined by the decrease of PERV reverse transcriptase activity using a commercial RT assay kit (Roche, Mannheim, Germany). The pellets obtained above were resuspended in lysis buffer supplied by the assay kit and subjected to reverse transcription reaction for 2 h, according to the Manufacturer's instructions, except that $MgCl_2$ in the reaction mixture was replaced by $MnCl_2$ (Phan-Thanh et al., 1992). All experiments were carried out in duplicate.

The antiviral activity of test compounds was also determined by the inhibition of PERV mRNA expression in PK15 cells. PK15 cells were seeded and cultured in the medium containing test compounds in the same manner as described for the antiviral assay. After a 48-h incubation, the culture supernatants were removed, and the cells were extensively washed with PBS, trypsinized, and washed again with PBS. Total RNA was extracted from the cells with RNeasy Mini Kit (Qiagen) and subjected to real-time RT-PCR analysis. The PERV mRNA level was determined using the sense primer (5′-AGCTCCGGGAGGCCTACTC-3′), the antisense primer (5′-ACAGCCGTTGGTGTGGTCA-3′), and the Taqman® probe (5′-FAM-CCACCGTGCAGGAAACCTCGAGACT-TAMRA-3′). The primer pair amplifies a region of the *pol* gene of PERV (Paradis et al., 1999). The nucleotide sequences used for the construction of the primers and probe were based on the reports by B. Bartosch, R.A. Weiss and Y. Takeuchi (GeneBank accession numbers: AY099323 and AY099324). The final concentrations of the primer pairs and probe were 200 and 100 nM, respectively. The Taqman® PCR reagent kit and Taqman® Multiscribe™ reverse transcription reagent kit (Applied Biosystems, Roche, Branchburg, NJ) were used according to the Manufacturer's instructions. Each sample was run in triplicate. Nonspecific inhibition of host cellular mRNA synthesis by the test compounds was determined with the Taqman 18S rRNA reagent kit (Applied Biosystems).

Cytotoxicity of the test compounds was determined by a tetrazolium dye method (Tetracolor One®, Seikagaku Corporation, Tokyo, Japan) (Yamamoto et al., 2001). PK15 cells were seeded and cultured in the medium containing test compounds in the same manner, as described in the antiviral assay. After a 48-h incubation, 1.5 ml of the culture supernatants were removed and 25 μl of the dye was added to each well. After a 4-h incubation at 37 °C, the specific (450 nm) and reference (630 nm) absorbances were monitored for each well by a microplate reader.

When four HIV-1 gene expression inhibitors were examined for their inhibitory effect on PERV replication in PK15 cells, K-37 and EM2487 displayed dose-dependent reduction of PERV RT activity in culture supernatants (Fig. 2A and C). K-37 and EM2487 did not show a direct inhibitory effect on PERV RT activity (data not shown). These compounds did not display apparent cytotoxicity to PK15 cells at concentrations up to 1 and 10 μM, respectively, indicating that K-37 and EM2487 are selective inhibitors of PERV replication in porcine cells. In contrast, JTK-101 and cepharanthine did not show any activity against PERV replication at the highest concentration tested (1 μM) (Fig. 2B and D). Since PERV proviral DNA is integrated in the genome of the host cells, the compounds were also examined for their inhibitory effect on viral mRNA synthesis in PK15 cells. As shown in Fig. 3, dose-dependent suppression of PERV mRNA synthesis was observed for K-37 and EM2487 but not for JTK-101 or cepharanthine. These results are in accordance with those obtained in the RT assay (Fig. 2). The 50% effective concentration (EC_{50}) of K-37 for PERV replication and its 50% inhibitory concentration (IC_{50}) for viral mRNA synthesis were 0.35 ± 0.04 and 0.34 ± 0.05 μM, respectively (Table 1). On the other hand, its 50% cytotoxic concentration (CC_{50}) was 4.63 ± 1.62 μM, suggesting that K-37 is a selective inhibitor of PERV gene expression. Similarly, the EC_{50}, IC_{50}, and CC_{50} of EM2487 were 5.44 ± 1.40, 4.36 ± 0.30, and >10 μM, respectively.

K-37 is a potent and selective inhibitor of HIV-1 replication in both acutely and chronically infected cells at submicromolar concentrations (Baba et al., 1998). K-37 could inhibit Tat-dependent transactivation, yet it was not an inhibitor of Tat itself or its cofactor CDK9/cyclin T1. Since PERV does not generate Tat protein, it is apparent that the anti-PERV activity of K-37 is not due to the inhi-

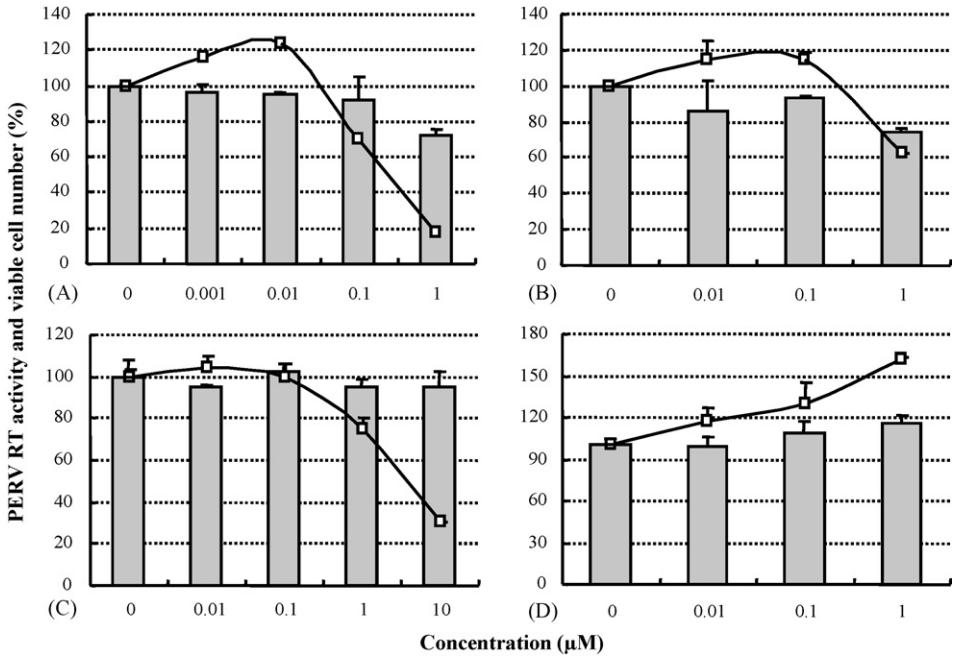

Fig. 2. Inhibitory effects of the test compounds on PERV replication in PK15 cells. PK15 cells were cultured in the presence of various concentrations of (A) K-37, (B) JTK-101, (C) EM2487 and (D) cepharanthine. After a 48-h incubation period, the culture supernatants were collected and mixed with 22% (w/v) polyethylene glycol 6000 solution for 5 h. PERV particles were harvested by centrifugation of the mixture. The viral pellets were resuspended in lysis buffer and subjected to RT assay (lines). The viable cell number was determined by a tetrazolium dye cell proliferation assay (bars). Both the RT activity and cell proliferation assays were performed in duplicate. The data represent means plus ranges. Representative results for two independent experiments are shown.

bition of Tat functions. Furthermore, K-37 was reported to inhibit the gene expression of human T-lymphotropic virus type 1 (HTLV-1) in persistently infected cells (Wang et al., 2002a). Although the target molecule of K-37 still remains to be determined, the present observations for PERV suggest that K-37 may interact with a cellular factor or factors that play an important role in retroviral gene expression. It is assumed that K-37 inhibits an early stage of transcriptional elongation of viral RNA (Okamoto et al., unpublished observations). EM2487 is a substance produced from a *Streptomyces* species and a potent and selective inhibitor of HIV-1 replication in acutely and chronically infected cells (Baba et al., 1999). Like K-37, EM2487 could inhibit HTLV-1 gene expression without affecting host cellular functions (Wang et al., 2002b). The chemical structures of K-37 and EM2487 are totally different from each other

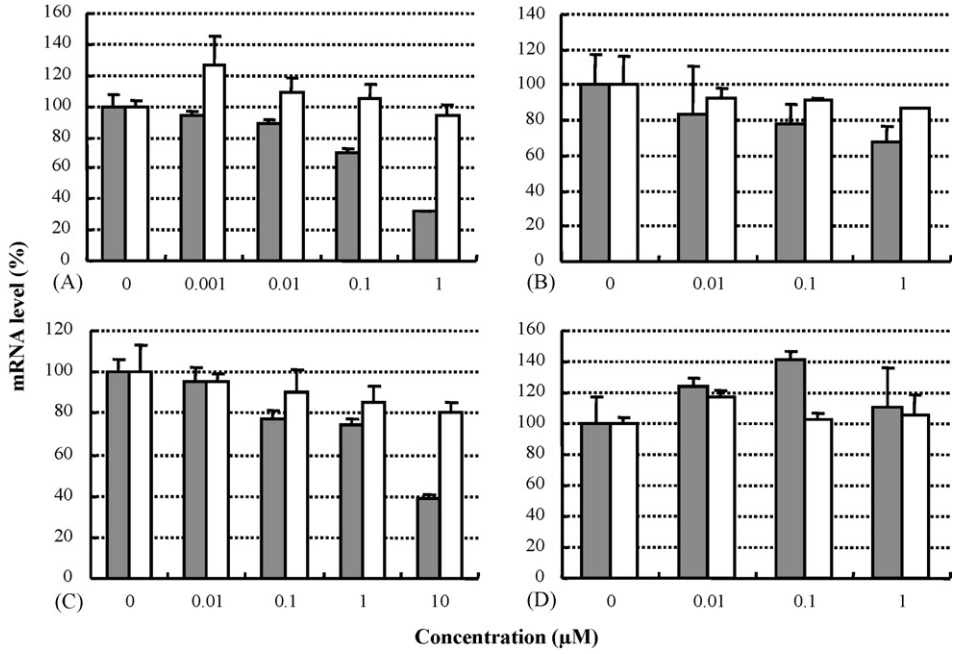

Fig. 3. Inhibitory effect of the test compounds on PERV mRNA synthesis in PK15 cells. PK15 cells were cultured in the presence of various concentrations of (A) K-37, (B) JTK-101, (C) EM2487 and (D) cepharanthine. After a 48-h incubation, the cells were collected, and total RNA was extracted. Quantitative real-time RT-PCR was performed to determine the amount of PERV mRNA in PK15 cells using a primer pair and probe specific to the PERV *pol* gene (gray columns). The inhibitory effect of the test compounds on host cellular mRNA synthesis was determined by quantitative RT-PCR for 18S mRNA (white columns). All experiments were performed in triplicate. The data represent means plus standard deviations. Representative results for two independent experiments are shown.

Table 1
Inhibitory effect of test compounds on PERV antigen production and mRNA synthesis in PK15 cells[a].

Compounds	PERV[b]			HIV-1[c]		HTLV-1[d]	
	EC_{50} (μM)	IC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)	EC_{50} (μM)	CC_{50} (μM)
K-37	0.35 ± 0.04	0.34 ± 0.05	4.63 ± 1.62	0.033 ± 0.012	2.1 ± 0.3	0.44 ± 0.13	5.7 ± 1.0
EM2487	5.44 ± 1.40	4.36 ± 0.30	>10	0.075 ± 0.032	12.5 ± 4.1	3.6 ± 0.6	30.6 ± 3.5
JTK-101	>1	>1	2.18 ± 0.63	0.0014 ± 0.0005	3.8 ± 0.2	N.D.[e]	N.D.
Cepharanthine	>1	>1	4.39 ± 1.99	0.028 ± 0.016	1.3 ± 0.3	>3.0	3.0 ± 0.4

[a] Each experiment was carried out in duplicate or triplicate, and all data represent means ± ranges for two independent experiments.
[b] EC_{50}: 50% effective concentration based on the inhibition of PERV antigen production (RT) in culture supernatants of PK15 cells. IC_{50}: 50% inhibitory concentration based on the inhibition of PERV mRNA synthesis. CC_{50}: 50% cytotoxic concentration based on the inhibition of host cell proliferation.
[c] EC_{50}: 50% effective concentration based on the inhibition of HIV-1 antigen production (p24) in chronically infected cells. CC_{50}: 50% cytotoxic concentration based on the inhibition of host cell proliferation. Data are taken from the reports by Wang et al. (2007) for K-37 and JTK-101, Baba et al. (1999) for EM2487, and Baba et al. (2001) for cepharanthine.
[d] EC_{50}: 50% effective concentration based on the inhibition of HTLV-1 antigen production (p19) in infected cells. CC_{50}: 50% cytotoxic concentration based on the inhibition of host cell proliferation. Data are taken from the reports by Wang et al. (2002a) for K-37 and cepharanthine and Wang et al. (2002b) for EM2487.
[e] Not determined.

(Fig. 1), nevertheless they appear to share some common properties in antiretroviral activity and mechanism of action.

JTK-101 is a novel naphthalene derivative that inhibits HIV-1 replication in cell cultures (Wang et al., 2007). This compound was found to be highly active against HIV-1 in chronically infected cells but much less active in acutely infected cells. Studies of its mechanism of action suggested that JTK-101 exerted its anti-HIV-1 activity through the inhibition of CDK9/cyclin T1. Cepharantine is a plant alkaloid that has been shown to inhibit HIV-1 replication in a certain chronically infected cell line at low concentrations through the inhibition of NF-κB (Okamoto et al., 1998). Cepharanthine could also suppress stimulation-induced production of proinflammatory cytokines in human macrophages (Okamoto et al., 2001). This compound did not inhibit PERV replication in PK15 cells or even slightly enhanced it at the highest concentration tested (Fig. 2D).

The viral gene expression inhibitors K-37 and EM2487 may be able to keep PERV silent in porcine organs thereby reducing the risk of PERV transmission to recipients, which is never attainable with RT inhibitors. On the other hand, RT inhibitors are capable of inhibiting De Novo infection of recipients with PERV derived from porcine organs. Thus, an ideal strategy to prevent PERV transmission to organ recipients may be the combined treatment with an RT inhibitor for recipients and a gene expression inhibitor for donor organs. Unfortunately, the current gene expression inhibitors, such as K-37 or EM2487, may be toxic to human recipients at concentrations that completely suppress PERV production from donor cells or organs. Therefore, the optimization of their chemical structures would be required for the inhibition of PERV replication in vivo without generating serious side effects. Although the risk of PERV transmission upon xenotransplantation is supposed to be lower than initially thought, optimized retroviral gene expression inhibitors may be worth further pursuing for their potential efficacy in the clinical setting.

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