Human immunodeficiency virus type 1 transcription is regulated by thieno[3,4-*d*]pyrimidine

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Abstract. In the present study, the effect of thieno[3,4-*d*] pyrimidine (TEP) on the transcription of human immunodeficiency virus type 1 (HIV-1) was investigated. To the best of the authors' knowledge, this is the first study describing the effect of TEP on the transcription of HIV-1. The present results identified a marked decrease in the production of the HIV-1 genome in 293T cells after treatment with TEP. The treatment of HIV-1infected 293T cells with TEP led to the upregulation of retinoblastoma binding protein 4 (RbAp48) mRNA and protein. The activity of long terminal repeats (LTRs) was decreased by 19, 24, 29, 34, 38, 41, 52, 63, 76 and 92% in treatments with concentrations of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5 μM TEP, respectively. The p65 translocation to the nucleus was markedly reduced in 293T cells treated with TEP for 48 h. A marked decrease was observed in the production of HIV-1 in 293T cells with the increase in concentration of pRbAp48. In 293T cells, RbAp48 and TEP decreased tumor necrosis factor-α and phorbol 12-myristate 13-acetate-induced activity of LTR. Therefore, the present study suggested that TEP inhibited transcription of HIV-1 through upregulation of RbAp48 expression and activation of the NF-κB pathway. Therefore, TEP may be used for the treatment of HIV-1 infection.

Introduction

Human immunodeficiency virus type 1 (HIV-1) infection is followed by the integration of viral DNA with the host genome leading to formation of proviral DNA (1). Viral replication in the human body is a complex process regulated by several hosts as well as viral factors (1). Transcription of HIV-1 is facilitated by the 5' long terminal repeat (LTR) through the formation of an integrated provirus (2). The process of HIV transcription is suppressed by the inactivation of host chromatin through the expression of inhibitors and remodeling molecules (3). Therefore, the organization of chromatin material plays a vital role in the expression of the HIV-1 genes involved in transcription (4).

Retinoblastoma binding protein 4 (RbAp48) maintains chromatin organization and is associated with chromatin assembly factor 1 (5). It acts as a histone de-acetylation and nucleosome remodeling agent, thereby inhibiting the process of viral transcription (6). A previous study identified that RbAp48 interacts with H3-H4 histones, which makes it an important component of various complexes that are involved in chromatin remodeling (7). RbAp48 is also involved in several other cellular processes, such as the maintenance of pluripotent stem cells and the induction of apoptosis in some selected tissues (8,9). Higher expression of RbAp48 has been shown to induce autoimmune exocrinopathy and inhibit the process of transcription (10). Therefore, transcription of genes is regulated by RbAp48 through several pathways. In HIV infection, one of the most important steps is transcription of genes to multiply and spread the virus. In the present study, the effect of thieno[3,4-*d*]pyrimidine (TEP) on the transcription of viral genes and its association with RbAp48 was investigated. The present study demonstrated that TEP inhibited HIV-1 infection through the upregulation of RbAp48 expression and activation of the NF-κB pathway. TEP also suppressed tumor necrosis factor-α (TNF-α) and phorbol 12-myristate 13-acetate (PMA)-mediated upregulation of HIV-1 transcription, thereby acting as an anti-HIV agent.

Materials and methods

Chemicals and reagents. TEP was purchased from Sigma Aldrich; Merck KGaA.

Plasmid constructs. The pRbAp48 vector and the pCTL (control) vector were purchased from OriGene Technologies, Inc. pRbAp48 was knocked down using short hairpin RNA (shRNA; (Takara Bio, Inc.) using the following gene sequences: Forward, 5'-CGAGGAAUACAAAAUAUGGTT-3' and reverse, 5'-CCAUAUUUUGCUCGTT-3' (10).

Cell culture and transfections. 293T and TZM-bl cell lines were supplied by American Type Culture Collection. The cells were cultured in DMEM (Thermo fisher Scientific, Inc.) mixed with 10% FBS (Thermo Fisher Scientific, Inc.). The medium

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also contained antibiotics, 100 U/ml penicillin-100 μg/ml streptomycin (Thermo Fisher Scientific, Inc.). The cell culture was performed under an atmosphere of 5% CO_2 and 95% air at 37˚C. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for the transfection of plasmids into 293T and TZM-bl cells.

Viral stock production and infection. For the production of viral stocks, H9/HTLV-IIIB cells (obtained from Zhejiang University, Hangzhou, China) were used according to a previously published procedure (11). Detection of antigens corresponding to viral p24 was performed using an ELISA kit (cat. no. ab218268; Abcam) according to the manufacturer's instructions. For infection with HIV-1, CEM cells (American Type Culture Collection) at a concentration of $2.5x10^6$ cells/well were cultured at one multiplicity of infection using spinoculation for 2.5 h at 1,000 x g at a temperature of 25˚C (12). The free virus was removed from the cell cultures by centrifugation at 25˚C for 10 min at 300 x g. TZM-bl cells were subjected to infection for determination of infectious viruses quantitatively.

Analysis of luciferase activity. 293T cells at a density of $2x10^5$ cells/well were distributed into a 96-well plate. After overnight incubation the cells were subjected to firefly luciferase construct and pRbAp48 construct transfection. The negative control cells were transfected with pGL3-vector (Promega Corporation) and empty pCTL vector (OriGene Technologies, Inc.). The cells, after treatment with concentrations of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5 μM TEP, were exposed to 25 ng/ml PMA (Sigma Aldrich; Merck KGaA) and 20 ng/ml TNF-α. The activity of luciferase was determined after 48 h of transfection.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA from the cells was obtained using the RNeasy mini kit (Qiagen GmbH), according to the manufacturer's instructions. The total RNA (1 μg) samples were used for the preparation of cDNA by employing oligo(dT) primers and SuperScript III RT (Invitrogen; Thermo Fisher Scientific, Inc.). Detection of HIV-1 DNA was carried out using the known primers for the LTR (13). Determination of HIV-1 mRNA was also performed using previously published procedures (14,15). The ABI 7500 Sequencing Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used for the amplification process. The amplification was carried out using cDNA (1 ng), forward and reverse primers (125 nM) and 2X SYBR®Premix Ex Taq™ (25 μl; Takara Bio, Inc.) in 50 μl reaction. The reactions were carried out for 1 min at 95˚C, 40 cycles for 15 sec at 95˚C, 20 sec at 56˚C and 30 sec at 70˚C. The quantification cycle (Cq) of each sample was recorded as a quantitative measure of the amount of PCR product in the sample. The relative mRNA expression levels of the genes were calculated following normalization to β-actin mRNA using the $2^{-\Delta\Delta Cq}$ method (16). The primer sequences were as follows: RbAp48 forward, CGA GGAAUACAAAAUAUGGTT and reverse, CCAUAUUUU GUAUUCCUCGTT; HIV-1 forward, 5'-TAGCAGTGGCGC CCGA-3' and reverse, 5'-TCTCTCTCCTTCTAGCCTCC GC-3'; β-actin forward, 5'-TCCTCTCCCAAG TCCACACA GG-3' and reverse, 5'-GGGCACGAAGGCTCATCATTC-3'.

Western blotting. Lysates of the cells were prepared using a radioimmunoprecipitation assay buffer mixed with phenyl methylsulfonyl fluoride (Beyotime Institute of Biotechnology). The lysates were centrifuged for 10 min at 12,000 x g at a temperature of 4˚C. Concentrations of the proteins were determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology). The proteins (20 μg samples/lane) were separated by SDS PAGE on 12% gels and subsequently electro-transferred using electro-blotting apparatus (Bio Rad Laboratories, Inc.) onto nitrocellulose membranes. The membranes were blocked with tris-buffered saline with 1/1,000 tween containing 5% non-fat milk for 1 h at room temperature. Subsequently the membranes were incubated overnight with antibodies against RbAp48 (cat. no. ab79416; 1:1,000; Abcam), NF-κB (cat. no. 545380-34-5; 1:1,000; Merck KGaA) and GAPDH (cat. no. D16H11; 1:1,000; Cell Signaling Technology, Inc.) at 4˚C. After washing, membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.). After incubation, membrane washing was performed with TBS and Tween-20 followed by enhanced chemiluminescence reagent (EMD Millipore) treatment. For analysis, Gel Pro Analyzer software version 4.0 (Media Cybernetics, Inc.) was used.

Electrophoretic mobility shift assay (EMSA). The 293T cells were subjected to incubation in6-well plates containing DMEM for 24 h with 20 ng/ml TNF-α. The medium was then replaced with new medium containing concentrations of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5 μM TEP and cells were incubated for 48 h. Commercially available NE-PERTM nuclear and cytoplasmic extractions kit (cat. no. 78833; Thermo Fisher Scientific, Inc.) were used for extraction of nuclear fractions, according to the manufacturer's protocol. NF-κB was probed in nuclear and cytoplasmic extracts using the Light Shift Chemiluminescent EMSA kit (Thermo Fisher Scientific, Inc.). The nuclear extract samples (10 μg) were subjected to incubation at room temperature with wild probes for 45 min in 20 μl reaction buffer. The buffer contained 1X binding buffer, magnesium chloride (5 mM), propantriol (2.5%), poly (dI.dC; 55 ng/μl) and NP-40 (0.08%). Electrophoresis of the binding reactions was performed in a polyacrylamide gel (8%) for 1 h at 110 V in Tris-borate-EDTA (TBE) buffer (0.5X) after the addition of 4 μl loading buffer. The transfer unit was sandwiched with nylon membrane (+vely charged) and gel, which was subsequently placed into ice-cold 0.5X TBE buffer for 1 h at 370 V. The membranes were blocked with tris-buffered saline with 1/1,000 tween containing 5% non-fat milk for 1 h at room temperature. Then, membrane incubation was performed with peroxidase conjugated streptavidin horseradish antibody (1:300; cat. no. 7074; Thermo Fisher Scientific, Inc.) for 20 min at 4˚C. The membrane washing with 1X buffer was followed by equilibration and incubation with substrate. The VersaDoc MP 5000 imaging system (Bio-Rad Laboratories, Inc.) was used for the visualization of reaction bands.

Statistical analysis. The values are presented as the mean ± SEM. The experiments were carried out in triplicates independently. The data were compared using Student's t-test and one way ANOVA followed by tukey's post hoc multiple

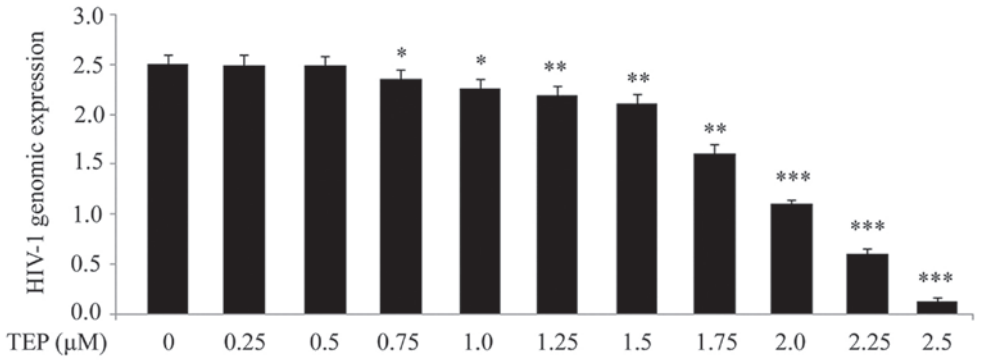

Figure 1. Effect of TEP on HIV-1 production in 293T cells. TEP at concentrations of 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5 μM was added to 293T cells after 48 h HIV infection (multiplicity of infection of 1 by spinoculation at 1,200 x g for 2 h at 25˚C). The cell supernatants were collected after 48 h of TEP treatment to examine the HIV-1 genome expression by reverse transcription-quantitative PCR. *P<0.05, **P<0.02 and ***P<0.01 vs. untreated cells. TEP, thieno[3,4-*d*]pyrimidine; HIV-1, human immunodeficiency virus type 1.

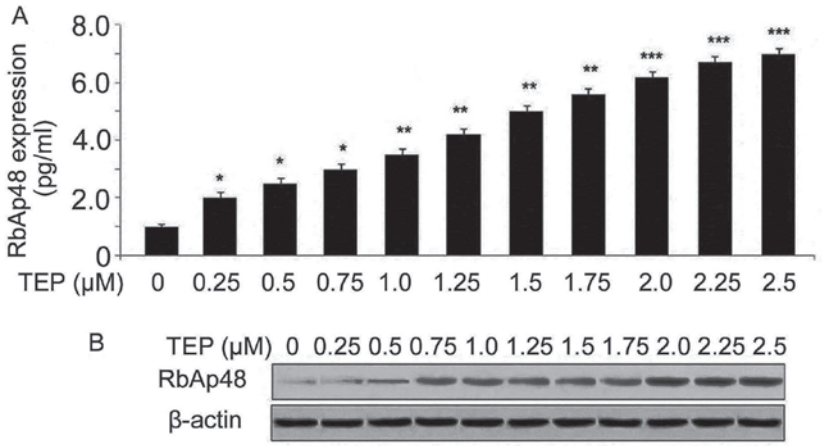

Figure 2. Effect of TEP on RbAp48 expression. 293T cells were treated with 0.25-2.5 μM TEP after 48 h of HIV infection (multiplicity of infection of 1 by spinoculation at 1,200 x g for 2 h at 25˚C). (A) Reverse transcription-quantitative PCR analysis was used to examine the effect of TEP on expression of RbAp48 mRNA. β-actin was used as an internal loading control. (B) Western blotting was used to analyze the RbAp48 protein expression in 293T cells. GAPDH was used as a control. *P<0.05, **P<0.02 and ***P<0.01 vs. untreated cells. TEP, thieno[3,4-*d*]pyrimidine; RbAp48, retinoblastoma binding protein 4.

comparison test. P<0.05was considered to indicate a statistically significant difference. Analysis of the data obtained was performed using SPSS version 18.0 (SPSS, Inc.).

Results

TEP inhibits HIV-1 production in 293T cells. In order to determine whether TEP can affect HIV-1 production, 293T cells were treated with 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5 μM of TEP on day 2 after HIV-1 infection. A marked decrease was observed in the production of the HIV-1 genome in 293T cells with increasing concentrations of TEP ≤2.5 μM (Fig. 1). At higher concentrations of TEP there was no further decrease in HIV-1 replication was observed (data not shown). There was also no significant change in HIV-1 production in 293T cells at concentrations <0.25 μM by TEP (data not shown). RT-qPCR analysis demonstrated a significant decrease (P<0.05) in HIV-1 genomic expression from 0.75 μM concentration of TEP compared with the untreated cells. No significant change in the production of the HIV-1 genome in 293T cells was induced by TEP at concentrations of 0.25 and 0.5 μM.

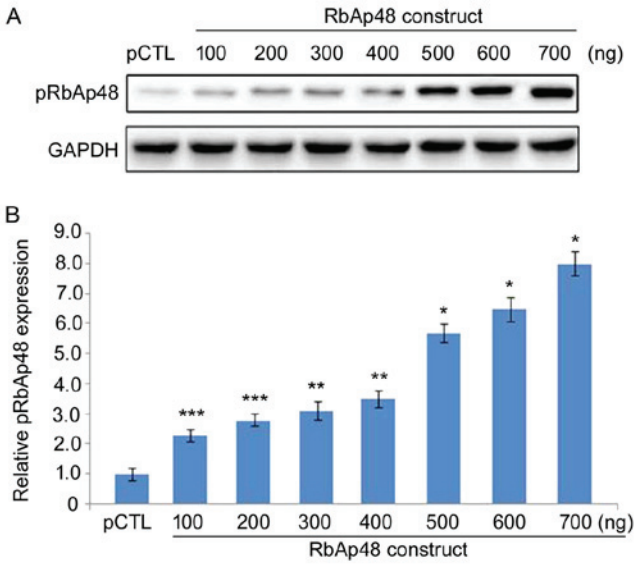

Figure 3. Effect of pRbAp48 transfection on protein expression. (A) Cells were transfected with 100, 200, 300, 400, 500, 600 and 700 ng pRbAp48 to analyze pRbAp48 protein expression at 48 h by western blot analysis. (B) The data were subsequently quantified. *P<0.05, **P<0.02 and ***P<0.01 vs. pCTL. RbAp48, retinoblastoma binding protein 4.

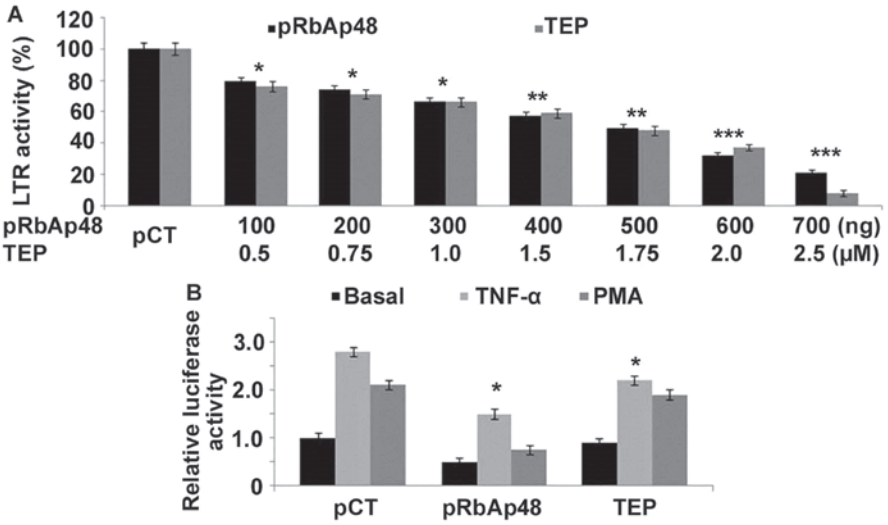

Figure 4. Effect of TEP and pRbAp48 on LTR activity. (A) Cells were treated with 100, 200, 300, 400, 500, 600 and 700 ng pRbAp48 to analyze the LTR activity after 48 h. (B) TNF-α and PMA-stimulated 293T cells were incubated with pRbAp48 (700 ng) and TEP (2.5 μM) for determination of the luciferase activity. The experiments were performed in triplicates. The 0 ng pRbAp48 group was transfected with the negative control, pCTL. *P<0.05, **P<0.02 and ***P<0.01 vs. respective control cells. TEP, thieno[3,4-*d*]pyrimidine; TNF-α, tumor necrosis factor-α; RbAp48, retinoblastoma binding protein 4; LTR, long terminal repeat; PMA, phorbol 12-myristate 13-acetate.

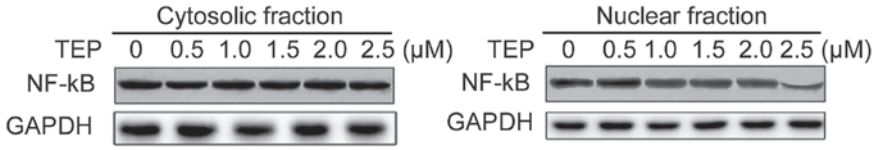

Figure 5. Effect of TEP on NF-κB translocation. 293T cells after incubation with TEP were analyzed for NF-κB translocation. Western blotting was used to determine expression of NF-κB in cytosolic and nuclear fractions. TEP, thieno[3,4-*d*]pyrimidine.

TEP increases expression of RbAp48 in 293T cells. The expression levels of RbAp48 mRNA and protein in 293T cells following HIV-1 infection were examined using RT-qPCR and western blotting assays, respectively. TEP treatment of HIV-1 infected 293T cells led to upregulation of RbAp48 mRNA and protein in a concentration-dependent manner (Fig. 2). The level of RbAp48 mRNA at 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 1.75, 2.0, 2.25 and 2.5 μM TEP on day 2 after HIV-1 infection increased to 2.0-, 2.5-, 3.0-, 3.5-, 4.2-, 5.0-, 5.6-, 6.2-, 6.7- and 7-fold, respectively, compared with the untreated cells (Fig. 2A). Western blotting also demonstrated that the RbAp48 protein level increased in 293T cells with TEP treatment (Fig. 2B).

pRbAp48 transfection increases the pRbAp48 protein level in 293T cells. The pRbAp48 protein level was markedly upregulated in 293T cells following pRbAp48 transfection in a dose-dependent manner (Fig. 3). Transfection with 100, 200, 300, 400, 500, 600 and 700 ng pRbAp48 markedly upregulated the pRbAp48 protein expression. The pRbAp48 protein expression was not elevated in 293T cells transfected with the negative control, pCTL. These results demonstrated successful transfection of pRbAp48 in 293T cells.

TEP and pRbAp48 inhibit LTR activity in 293T cells. pRbAp48 transfection of 293T cells decreased the LTR activity in a concentration-dependent manner. When the cells were transfected with 100, 200, 300, 400, 500, 600 and 700 ng pRbAp48, the activity of LTR was decreased by 21, 26, 34, 43, 51, 68 and 79%, respectively, in comparison with cells transfected with the empty vector (Fig. 4A). These concentrations of pRbAp48 were selected based on preliminary screening (data not shown). TEP treatment of 293T cells also decreased LTR activity in a concentration-dependent manner. The LTR activity was decreased by 24, 29, 34, 41, 52, 63 and 92% in 293T cells upon treatment with 0.5, 0.75, 1.0, 1.5, 1.75, 2.0 and 2.5 μM TEP, respectively (Fig. 4A). The effect of RbAp48, TEP and pCTL on TNF-α and PMA-induced activity of LTR was analyzed by determining the relative luciferase activity. In 293T cells RbAp48 and TEP decreased TNF-α and PMA-induced LTR activity in comparison with pCTL (Fig. 4B).

TEP reduces nuclear translocation of NF-κB via stabilization of IκBα. The NF-κB translocation to the nucleus was markedly reduced in 293T cells after incubation for 48 h with TEP compared with the untreated cells (Fig. 5). With the increase in concentration of TEP from 0.5 to 2.5 μM a marked decrease was observed in NF-κB nuclear translocation.

HIV-1 expression in 293T cells is inhibited by RbAp48. Transfection of shRNA to knockdown RbAp48 or sh-NC as control into 293T cells was followed by HIV 1 infection. RT-qPCR analysis on days 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 demonstrated a marked downregulation in RbAp48 expres-

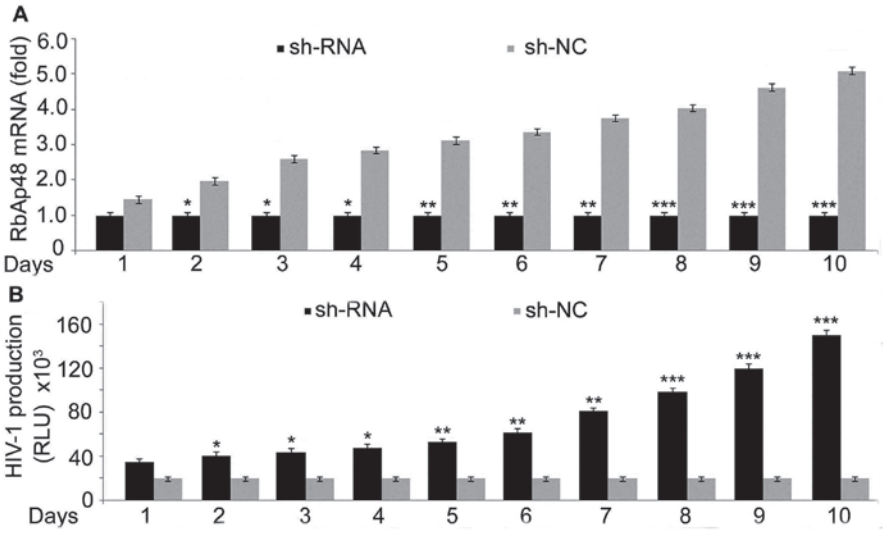

Figure 6. Effect of RbAp48 on HIV-1 production. (A) sh-RbAp48 (1 μg) or sh-NC transfection into 293T cells was followed by administration of HIV-1 (multiplicity of infection of 1 by spinoculation at 1,200 x g for 2 h at 25˚C). Reverse transcription-quantitative PCR was used to examine the expression of RbAp48 mRNA on days 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10. (B) Detection of viral production was analyzed using a commercially available ELISA kit for HIV-1 p24. *P<0.05, **P<0.02 and ***P<0.01 vs. respective sh-NC transfected cells. RbAp48, retinoblastoma binding protein 4; HIV-1, human immunodeficiency virus type 1; sh, short hairpin; NC, negative control; RLU, relative light units.

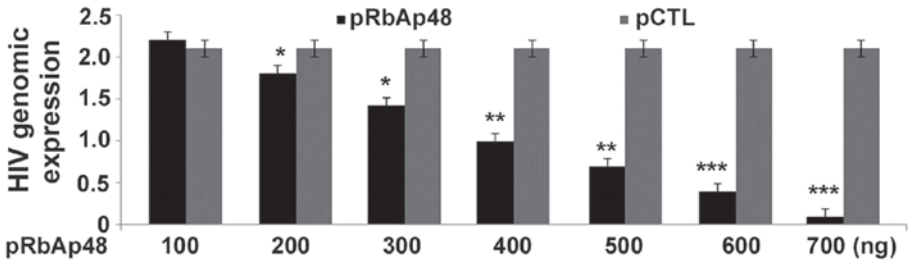

Figure 7. Effect of RbAp48 on HIV-1 production in 293T cells. pRbAp48 at concentrations of 100, 200, 300, 400, 500, 600 and 700 ng were transfected into 293T cells after HIV-1 infection (multiplicity of infection of 1 by spinoculation at 1,200 x g for 2 h at 25˚C). The cell supernatants were collected after 48 h of pRbAp48 transfection to examine HIV-1 genome expression by reverse transcription-quantitative PCR. *P<0.05, **P<0.02 and ***P<0.01 vs. pCTL-transfected cells. RbAp48, retinoblastoma binding protein 4; HIV-1, human immu-nodeficiency virus type 1.

sion (Fig. 6A). Knockdown of RbAp48 expression by sh-RNA in 293T cells caused a marked increase in HIV-1 production compared with the control cells (Fig. 6B). These results suggested that RbAp48 expression inhibits HIV production.

RbAp48 transfection inhibits HIV-1 production in 293T cells. In order to confirm whether RbAp48 transfection can influence the HIV-1 production, 293T cells after HIV-1 infection were administered various concentration of pRbAp48 (RbAp48 construct) or pCTL as a control vector. A marked decrease was observed in the production of the HIV-1 genome in 293T cells with upregulated pRbAp48 (Fig. 7). RT-qPCR analysis showed a decrease in HIV-1 genomic expression at 100, 200, 300, 400, 500, 600 and 700 ng pRbAp48 compared with the cells transfected with pCTL.

Discussion

HIV-1 infection leads to the upregulation of several anti-HIV compounds such ascaveolin-1 which in turn inhibits replication of HIV-1 (17,18). Infection with HIV-1 has been demonstrated to induce the expression of NF-Iβ, which subsequently downregulates HIV-1 replication (17,18). The present study investigated the effect of TEP on transcription of HIV-1 in 293T cells. Initial screening identified a significant decrease in HIV-1 replication with TEP treatment in HIV-1 infected 293T cells. The present findings suggested that TEP possess anti-HIV activity and requires further evaluation to understand the underlying mechanism of its action.

The transcription of the viral genome is inhibited by overexpression of RbAp48 through histone de-acetylation and nucleosome remodeling (5,6). Therefore, the present study examined if TEP interferes with the expression of the RbAp48 molecule in 293T cells. The results from the present study demonstrated that TEP treatment of HIV-1 infected 293T cells upregulated the RbAp48 mRNA and protein levels in a concentration-dependent manner. A marked increase in the level of RbAp48 mRNA and protein was observed in HIV-1 infected 293T cells treated with TEP compared with the control cells. Therefore, the present results suggested that TEP exhibited anti-HIV effects in 293T cells through the upregulation of RbAp48 expression.

Replication of viral particles is regulated by the transcription of a provirus, which comprises an important phase

in the life cycle of HIV-1 (19). The transcription process is controlled by the HIV-1 LTR through a well-organized pathway involving participation of several factors (20). Many molecules bind to the HIV-1 5' LTR and inhibit viral transcription (19). The molecules which bind and inhibit viral transcription by targeting HIV-1 LTR include transcriptional repressor protein YY1, transcription factor LSF, zinc finger protein ZBRK1 and scaffold/matrix-associated region-1 binding protein (21,22). In the present study, the effect of exogenously transfected pRbAp48 and TEP treatment on LTR activity in HIV-infected 293T cells was also analyzed. The present study demonstrated that both pRbAp48 transfection and TEP treatment significantly decreased LTR activity in a concentration-dependent manner in HIV-1 infected 293T cells. The activity of LTR was lowest in 293T cells treated with 2.5 μM TEP. It is known that the triggering of LTR activity and activation of the NF-κB signaling pathway is promoted by the administration of PMA and TNF-α (23). In addition, PMA and TNF-α also play an important role in the NF-κB signaling pathway activation (23). In the present study, the effect of RbAp48 and TEP on TNF-α- and PMA-induced LTR activity in 293T cells was investigated. The present results demonstrated that in 293T cells transfected with RbAp48 or treated with TEP, TNF-α- and PMA-induced LTR activity was decreased. TEP treatment of 293T cells suppressed the activity of luciferase mediated by LTR following PMA- or TNF-α stimulation as well as in basal states. A previous study identified inhibition of HIV genome transcription and the induction of latency upon activation of NF-κB p50 in HIV-infected cells (24). The results from the present study demonstrated that nuclear translocation of p65 was markedly reduced in HIV-infected 293T cells treated with TEP compared with untreated cells.

In summary, the present study suggested that TEP inhibits transcription of HIV-1 through upregulation of RbAp48 expression and activation of the NF-κB pathway. Therefore, TEP may be used for the development of a HIV treatment strategy either in combination with other drugs or by structural modification. However, further studies are required to fully elucidate the role of TEP in inhibition of HIV replication.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WZ designed the study and wrote the manuscript. WX, YW and JZ performed the experiments and compiled the data. JC analyzed the data and performed literature survey. All the authors approved the manuscript for publication.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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