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Zinc ionophores pyrithione inhibits herpes simplex virus replication through interfering with proteasome function and NF-κB activation

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ABSTRACT

Pyrithione (PT), known as a zinc ionophore, is effective against several pathogens from the *Streptococcus* and *Staphylococcus* genera. The antiviral activity of PT was also reported against a number of RNA viruses. In this paper, we showed that PT could effectively inhibit herpes simplex virus types 1 and 2 (HSV-1 and HSV-2). PT inhibited HSV late gene (Glycoprotein D, gD) expression and the production of viral progeny, and this action was dependent on Zn^{2+}. Further studies showed that PT suppressed the expression of HSV immediate early (IE) gene, the infected cell polypeptide 4 (ICP4), but had less effect on another regulatory IE protein, ICP0. It was found that PT treatment could interfere with cellular ubiquitin-proteasome system (UPS), leading to the inhibition of HSV-2-induced IκB-α degradation to inhibit NF-κB activation and enhanced promyelocytic leukemia protein (PML) stability in nucleus. However, PT did not show direct inhibition of 26S proteasome activity. Instead, it induced Zn^{2+} influx, which facilitated the dysregulation of UPS and the accumulation of intracellular ubiquitin-conjugates. UPS inhibition by PT caused disruption of IκB-α degradation and NF-κB activation thus leading to marked reduction of viral titer.

1. Introduction

Herpes simplex virus types 1 and 2 (HSV-1 and 2), members of *Herpesviridae* family, are the most prevalent human pathogens. HSV-1, usually establishing latency in the trigeminal ganglion (near the ears) causes recurring outbreaks around the mouth of facial region. Differently, HSV-2 recurring outbreaks will occur in the genital region, and it always establishes latency in sacral ganglion (lower base of the spine) (Ryan and Ray, 2010). Following infection of permissive cells, a cascade of herpesvirus proteins, encoded by immediate-early (IE), early, and late genes, are produced (Honess and Roizman, 1974). IE genes, which encode proteins that regulate the expression of early and late viral genes, are the first to be expressed following infection. Early gene allows the synthesis of enzymes involved in DNA replication and the production of certain envelope glycoproteins and the late genes predominantly encode viral structural proteins (Weir, 2001). HSV has evolved complex mechanisms to develop lytic or latent infection and evade host inhibitory factors by modulating host's cellular machinery to its favor. Infected cell polypeptides 0 and 4 (ICP0 and ICP4) are two important HSV IE proteins and play critical roles during viral infection (Everett, 1984a,b; O'Hare and Hayward, 1985a,b). ICP0, an important activator of viral genes, is required growth during low-multiplicity infection, and its gene activation function correlated with its E3-ubiquitin ligase function that disrupted intranuclear nuclear domain 10 (ND10) and target proteasome-degradation of certain cellular proteins (Everett, 2006). ICP4 was a major transcriptional activator for the viral early and late genes and essential for viral growth (DeLuca et al., 1985).

Proteasomes, located in both nucleus and cytoplasm, are very large protein complexes that mediate intracellular protein proteolysis and maintain cellular metabolic homeostasis (Peters et al., 1994). Ubiquitin, a small regulatory protein that is found in almost all eukaryotic organisms and evolutionarily conserved, is conjugated to proteins destined for proteasomal degradation (Hershko and Ciechanover, 1998). Attachment of ubiquitin moieties to proteasome substrates occurs by the sequential enzymatic reactions which are catalyzed by E1 ubiquitin activating enzyme, E2 ubiquitin conjugating enzymes and E3 ubiquitin ligases. The ubiquitin-proteasome system (UPS), degrading both functional proteins and aberrantly folded polypeptides, is a major intracellular proteolytic system that regulates cell cycle progression, apoptosis, inflammatory responses and antigen presentation (Goldberg, 2007; Pickart, 2004). Evidence has shown the importance of active proteasome in the effective replication of certain viruses. Some viruses can hijack host cellular UPS to facilitate their propagation events, such as capsids translocation, viral gene expression, replication and immune evasion (Banks et al., 2003). HSV ICP0 contains a typical RING-finger domain which has been demonstrated to have

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E3-ubiquitin ligase activity (Boutell et al., 2002; Van Sant et al., 2001). Many studies focus on the interaction of ICP0 and cellular transcriptional factors or regulators. It has been reported that ICP0 can stimulate the proteasome-dependent proteolysis of cellular proteins, such as promyelocytic leukemia protein (PML) (Boutell et al., 2003; Chelbi-Alix, 1999; Everett et al., 2006), Sp100 (Chelbi-Alix, 1999), CENP-A/B/C (Everett et al., 1999; Lomonte and Morency, 2007; Lomonte et al., 2001), and E2FBP1 (Fukuyo et al., 2011), etc. to impair host's antiviral responses, and to facilitate productive replication and reactivation from latent infection (Everett, 2000). Consequently, HSV has evolved a regulatory machinery to utilize host's UPS to facilitate its infection. Recently, it was reported that cellular proteasome function played a vital role in HSV capsid postpenitration steps (Delboy et al., 2008), and this process was abrogated when ICP0-null mutant virus was used (Delboy and Nicola, 2011). The evidence suggests that cellular UPS is important for HSV life cycle and productive infection.

Pyrithione (PT), a zinc ionophore, commonly exists as a centrosymmetric dimmer to chelate Zn^{2+} via its oxygen and sulfur centers. Zinc pyrithione is well-known for its use in treating dandruff and seborrhoeic dermatitis (Faergemann, 2000). It has also been reported as an NF-κB inhibitor, showing potent inhibitory effect on PMA- and TNF-α-induced NF-κB activation (Kim et al., 1999). PT was also used as an antibacterial and antifungal agent, and evidence has shown that PT is effective in inhibiting infection by a number of RNA viruses (Krenn et al., 2009), such as human rhinovirus, coxsackievirus and mengovirus, all of which belong to *Picornaviridae* family. In the current study, we reported that PT exhibited potent inhibitory activity against both HSV-1 and HSV-2 infections. Mechanistic study showed that the antiviral activity of PT was dependent on its dysregulation of cellular UPS, preventing the degradation of IκB-α, an endogenous NF-κB inhibitor, and intranuclear PML bodies induced by viral infection. We provided evidence that the viral inhibitory activity of PT required the presence of Zn^{2+}. We concluded that PT inhibited viral gene expression and replication through induction of zinc influx and dysregulation of UPS. The current study provided a mechanistic insight to the roles of Zn^{2+} in inhibition of viral infection and suggested a novel strategy for the inhibition of herpesvirus infection.

2. Materials and methods

2.1. Reagents, cell lines, plasmids, and viruses

Sodium pyrithione, EDTA, EDTA-Ca, EDTA-Mg, EDTA-Zn, TPEN and ATP were purchased from Sigma-Aldrich (St. Louis, MO, USA). MG-132, BAY11-7082, PMA and IκB-α antibody were from Beyotime (Haimen, Jiangsu, China). Alexa Fluor 488 goat anti-mouse IgG (H + L), DAPI and FluoZin-3 AM were from Life Technologies (Carlsbad, CA, US). IRDye 680 goat-anti-rabbit and IRDye 800 goat-anti-mouse were obtained from LI-COR (Lincoln, NE, USA). Antibodies specific for gD-1/2, ICP0-1, ICP4-1, GAPDH, β-catenin, p65, PML and p53, and RIPA lysis buffer were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-ubiquitin antibody was obtained from eBioscience (San Diego, CA, USA). Anti-GFP antibody was from ZSGB-Bio Ltd. (Beijing, China). Recombinant human TNF-α was from R&D Systems (Minneapolis, MN, USA). Bz-VGR-AMC, Suc-LLVY-AMC and Z-LLE-AMC were synthesized by GL Biochem Ltd. (Shanghai, China).

HEC-293T, Vero, Hela and HEC-1-A cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Vero-ICP10P, an HSV-2 infection indicator cell line, was generated from Vero cells stably-transfected with HSV-2 ICP10 promoter-driven luciferase reporter gene cassette.

NF-κB-luc was purchased from Clontech (Palo Alto, CA, USA). Ub-G76V-GFP (Plasmid #11941) was from Addgene (Cambridge, MA, USA). HSV-1 (HF) and HSV-2 (G) were propagated and titrated on Vero cells as described previously (McLean et al., 1994).

2.2. In vitro antiviral assay

The *in vitro* antiviral activity of PT was determined via In-cell Western assay or using Vero-ICP10P cell line. Briefly, 2×10^4 cells were dispersed into each well of 96-well plates. After 24 h culture, the cell were either mock-treated or pre-treated with PT for 30 min, and then infected with HSV-1 (HF) or HSV-2 (G) (moi = 1) by directly adding the virus into the culture medium. Viral protein expression levels were determined via In-cell Western as described below.

To titrate the infectious virions, HSV-2-infected HEC-1-A cells were frozen and thawed with three cycles in 200 μl cultural medium to release the virions. Viral titration was determined by counting the numbers of plaques on Vero cells or using Vero-ICP10P cells.

Vero-ICP10P cells were seeded at a density of 2×10^4 per well into 96-well plate. Fifty microliters of virions-containing medium were dispensed onto Vero-ICP10P cells and the relative luminescence unit (RLU) was determined after 24 h using Bright-Glo luciferase assay system (Promega, Madison, WI, USA). The viral titration was calculated from the standard curve.

2.3. In vitro cytotoxicity assay

The *in* vitro cytotoxicity of PT was measured using a commercial CCK-8 kit (Dojindo, Kumamoto, Japan) via colorimetric method according to the manufacturer's instructions. Briefly, 2×10^4 cells per well were dispersed into 96-well plates and cultured for 24 h before serially diluted concentrations of PT were added in triplicate. After 24 h culture, 10 μl CCK-8 reagent was dispensed into each well, and the plates were incubated at 37 °C for 3 h. Absorbance at 450 nm was measured using a TECAN Infinite M200 microplate reader (Männedorf, Switzerland). Cell viability was plotted as the percent viable cells of the mock-treated control cells.

2.4. Western blot and In-cell Western

Cells were lysed using RIPA lysis buffer on ice for 30 min and then centrifuged at 12,000*g* for 10 min at 4 °C. The supernatants were collected and total protein concentrations were determined using BCA protein assay kit (Pierce, Rockford, IL, USA). After separated using SDS-PAGE, the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked and then inoculated in primary antibodies for 2 h at room temperature (RT). The membranes were incubated in IRDye IgG (1:10,000) for 1 h at RT and visualized under LI-COR Odyssey Infrared Imager (LI-COR).

In-cell Western was performed in 96-well plate. The cells were fixed with 4% paraformaldehyde for 20 min at RT and permeabilized by five washes in 0.1% Triton-X 100 in PBS with 5 min for each wash. Cell monolayers were blocked for 90 min and then incubated with primary antibodies diluted into blocking buffer (1:200) for 2 h at RT. After washing with PBS-T buffer, the cell layers were stained in IRDye IgG (1:1500) for 1 h. The plate was rinsed and scanned in Odyssey Infrared Imager. Relative protein expression level was normalized against β-catenin.

2.5. Immunofluorescence staining and microscopy

HEC-1-A cells were seeded onto Φ10 mm glass coverslips in 24-well plates. After treatment, cells were rinsed with PBS and then

fixed with 4% paraformaldehyde for 15 min at RT. The cells were then permeabilized with 0.2% Triton X-100 for 15 min followed by PBS washing twice. The coverslips were blocked with 1% BSA in PBS for 30 min at RT. Target proteins were immunolabeled using the respective primary antibodies and followed by Alexa Fluor 488 IgG. Nuclei were visualized by staining with DAPI. Images were acquired using an Olmpus FluoView FV10i confocal microscope (Tokyo, Japan).

2.6. Cell transfection and luciferase assay

HEC-1-A cells cultured in 96-well plate were transiently transfected with 100 ng/well luciferase reporter plasmid using Lipofectamine 2000 transfection reagent (Life Technologies). The cells were further cultured for 24 h and processed for RLU determination as described above.

2.7. RNA extraction and real-time PCR

Total RNA was extracted using TRIzol reagent (Life Technologies) according to manufacturer's protocol. cDNA was reverse-transcribed using ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan). Real-time PCR was performed in triplicate on ABI Prism 7300 Sequence Detection System using the SYBR Green PCR Master Mix (Life Technologies). The sequences of primer pairs were listed in Table 1. Messenger RNA transcription levels were standardized against housekeeping gene GAPDH.

2.8. Cellular 26S proteasome activity determination

HEC-1-A cells were lysed on ice for 30 min and centrifuged at 12,000*g* for 10 min 4 °C. The cell extract supernatant was collected for cellular 26S proteasome activity measurement as described (Luo et al., 2003). Briefly, 20 μg total cellular protein diluted in Assay Buffer (20 mM Tris, pH 8.0, 1 mM ATP and 2 mM $MgCl_2$) was mixed with 75 μM substrates and serially diluted concentrations of PT to a final volume of 100 μl. The substrates Bz-VGR-AMC, Suc-LLVY-AMC and Z-LLE-AMC were used to determine the proteasome trypsin-like, chymotrypsin-like and PDGH activities, respectively. The mixtures were incubated at 30 °C for 1 h. 26S proteasome activity was measured using Hitachi F7000 spectrofluorometer (Tokyo, Japan) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm.

2.9. Intracellular Zn^{2+} detection

HEC-1-A cells grown in black opaque 96-well plates were loaded with 5 μM FluoZin-3 AM in PBS for 30 min at 37 °C. The cells were then washed with serum-free medium to remove non-specific dye staining on the cell surface and subsequently incubated in the complete medium to allow de-esterification at 37 °C for 30 min. Serially diluted concentrations of PT were added to the medium and the plate was incubated at 37 °C for 30 min. Intracellular zinc ion concentrations were measured by spectrofluorometer using an excitation wavelengths of 494 nm, and an emission wavelength of 516 nm.

Table 1
Primer pairs used in this study.

Gene name	Sequence	
	Forward	Reverse
HSV-1 ICP4	GGCCTGCTTCCGGATCTC	GGTGATGAAGGAGCTGCTGTT
HSV-1 gD	AGCAGGGGTTAGGGAGTTG	CCATCTTGAGAGAGGCATC
HSV-2 gD	CCAAATACGCCTTAGCAGACC	CACAGTGATCGGGATGCTGG
IL-1β	AAGCTGAGGAAGATGCTG	ATCTACACTCTCCAGCTG
IL-6	TCTCCACAAGCGCCTTCG	CTCAGGGCTGAGATGCCG
IL-8	ATTGAGAGTGGACCACACTG	ACTACTGTAATCCTAACACCTG
TNF-α	CCTGCCCCAATCCCTTTATT	CCCTAAGCCCCCAATTCTCT
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

2.10. Statistics

Statistical analysis was performed using two-tailed student *t*-test. Statistical significance: $^{*}p < 0.05$, $^{**}p < 0.01$.

3. Results

3.1. PT inhibited HSV gD expression and viral replication with low cytotoxicity

We investigated the PT inhibitory effect on HSV replication by monitoring gD expression, a viral late gene product. It was shown that PT was effective in inhibiting the expression of both HSV-1 and HSV-2 gD in HEC-1-A cells with IC_{50} values in the sub-microgram levels (Fig. 1A and B, respectively). To rule out the cell specific phenomenon, we also investigated the inhibitory effects of PT in other two HSV permissive cells, Vero and Hela cells and observed that PT also blocked HSV-1 and HSV-2 gD expression in these two cell lines (Fig. 1C). The production of HSV-1 and HSV-2 infectious virions in HEC-1-A cells in the presence of serially diluted concentrations of PT was determined by freezing and thawing infected HEC-1-A cells and titrating the released infectious virions by counting the numbers of plaques on Vero cells (HSV-1 or HSV-2) or using a Vero-ICP10P luciferase reporter system (HSV-2 only) as described. As shown in Fig. 1D–F, PT inhibited HSV-1 and HSV-2 replication in a dose-dependent manner, paralleling to PT inhibition of HSV-2 gD expression. In addition, PT effect on HSV-2 gD mRNA transcription was analyzed by real-time PCR and the inhibition was in parallel to that of the viral protein expression (Fig. 1G). Together, the data suggested that PT inhibited HSV at post-entry steps by modulating structural protein expression, such as gD.

The cytotoxicity of PT was investigated on three cell lines used in the inhibitory activity analyses above. As shown in Fig. 1H, PT showed low cytotoxicities to HEC-1-A, Vero and Hela cells with 50% cytotoxicity concentrations (CC_{50}) greater than 380 μg/ml, presenting appreciable Therapeutic Indexes (TIs) and suggesting that the viral inhibitory activity was not due to cytotoxicity.

3.2. Zn^{2+} was essential for PT antiviral activity

Previous studies have shown that Zn^{2+} played a vital role in PT bioactivities (Kim et al., 1999; Krenn et al., 2009). Therefore, we investigated the importance of Zn^{2+} in PT inhibition of HSV gene expression and replication. We found that PT lost its antiviral activity in FBS-free medium (Fig. 2A). The specific role of Zn^{2+} was illustrated by the treatment of metal ion chelator EDTA and divalent ion-EDTA replacement. In the presence of EDTA, EDTA-Ca or EDTA-Mg, the PT inhibition of gD expression was partially abrogated since Zn^{2+} in the culture medium would compete for binding EDTA-Ca or EDTA-Mg to form higher affinity EDTA-Zn, thus depleting the free Zn^{2+}. Only EDTA-Zn treatment maintained PT inhibitory property against viral replication, in contrast to the treatment with EDTA-Ca^{2+} and EDTA-Mg^{2+} which partially reversed the PT inhibition of gD expression (Fig. 2B). The specific requirement for Zn^{2+} was further demonstrated by using TPEN, a Zn^{2+}-specific chelator, to mitigate the PT inhibition of gD expression in a dose-dependent manner (Fig. 2C), suggesting that Zn^{2+} was required for PT antiviral activity.

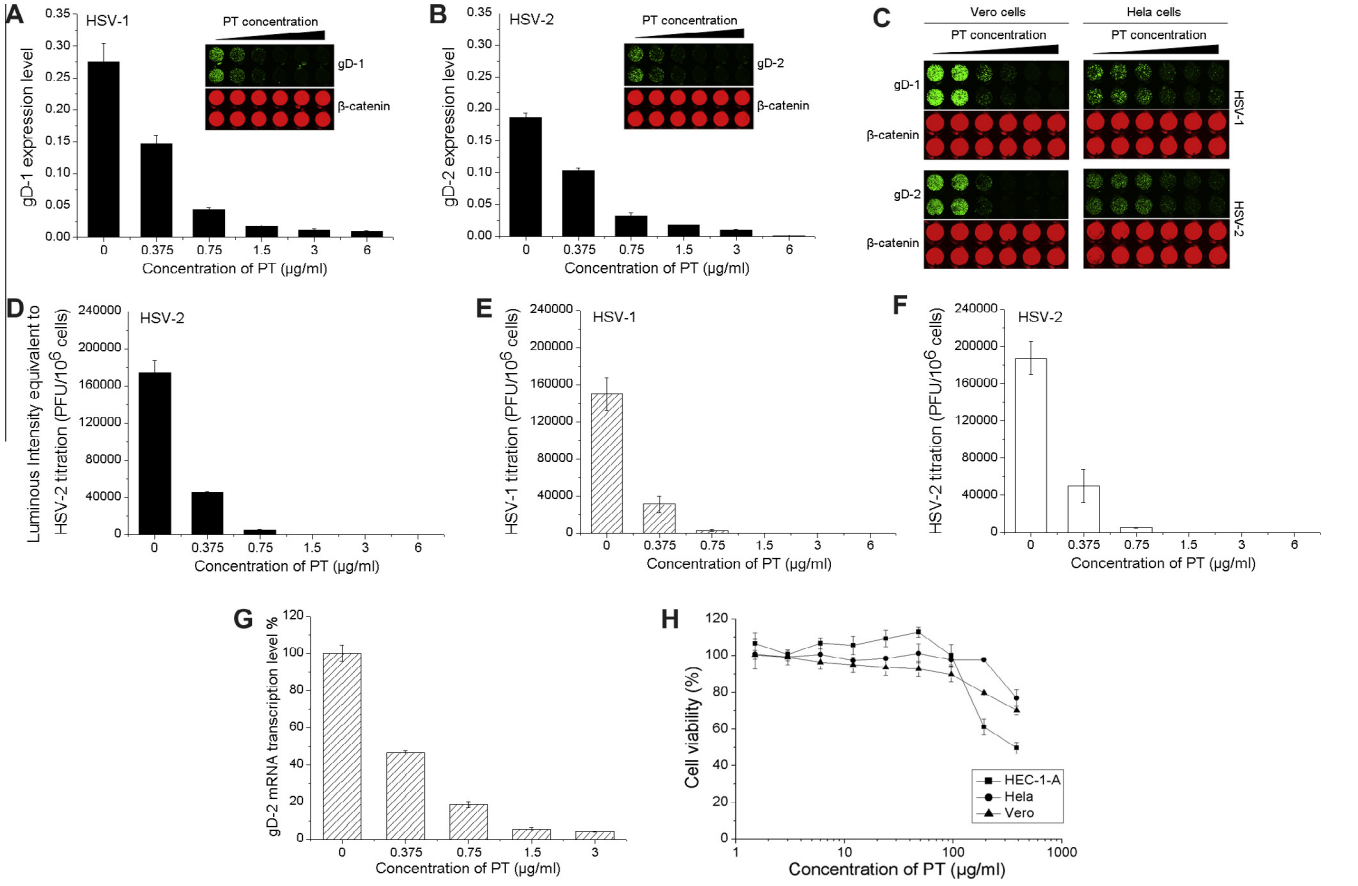

Fig. 1. PT inhibited HSV gD expression and viral replication. (A–C) PT inhibited HSV-gD protein expression in HEC-1-A cells (A and B), Vero and Hela cells (C). Cells were treated as described in Section 2. gD-1 (A) and gD-2 (B) expression level was determined via In-cell Western 24 h p.i. as described. (D–F) PT inhibited the formation of the infectious virus particles. HEC-1-A cells treated with the same condition were frozen and thawed with three cycles. Viral titration was determined via using Vero-ICP10P cells (D, for HSV-2 titration) or counting the numbers of plaques on Vero cells (E and F, for HSV-1 and HSV-2 titration, respectively). (G) PT interfered with HSV-2 gD mRNA transcription. The levels of gD-2 mRNA were quantified by real-time PCR 12 h p.i. (H) PT showed low cytotoxicity on HEC-1-A, Vero and Hela cells. Cells were treated with PT for 24 h, and cytotoxicity was determined using CCK-8 kit. All experiments were performed two or three times. A representative result was shown.

3.3. The effect of PT on HSV-1 IE genes expression

Since the HSV early and late gene expression is modulated by its IE genes, we further investigated the effect of PT on the expression of two important HSV-1 IE genes, ICP0 and ICP4. As shown in Fig. 3A, PT showed minimal inhibitory effect on ICP0 expression, while significantly inhibited ICP4 expression 8 and 12 h postinfection (p.i.). ICP4 mRNA transcriptional level was also analyzed via real-time PCR 4, 6, 8 and 12 h p.i., and the results were consistent with the reduced protein expression (Fig. 3B), suggesting that PT inhibition of HSV late gene expression was associated with its downregulation of the expression of viral IE protein ICP4.

3.4. PT inhibition of NF-κB activation contributed to its antiviral activity

HSV-1 infection will activate NF-κB pathway, an essential step for effective viral gene expression and replication (Gregory et al., 2004; Patel et al., 1998). Using a luciferase reporter driven by IκB-α binding element, it was found that PT inhibited HSV-2-induced NF-κB activation in HEC-1-A cells in a dose-dependent manner (Fig. 4A). Further analysis showed that PT inhibited PMA or TNF-α mediated NF-κB activation as shown in Fig. 4B, suggesting that PT may specifically target at NF-κB pathway to inhibit viral replication. In addition, PT effect on HSV-2-induced p65 nuclear translocation was investigated by immunofluorescence staining. As a result, PT attenuated HSV-2-mediated p65 nuclear translocation (Fig. 4C), further supporting the evidence that PT inhibited HSV-2-induced NF-κB activation. HSV infection was previously reported to induce the upregulation of cytokines/chemokines via NF-κB pathway in various cell types (Li et al., 2005; Melchjorsen et al., 2006). Whether PT could inhibit HSV-2-induced upregualtion of cytokines/chemokines expression was also investigated. As shown in Fig. 4D, PT potently inhibited the upregualtion of IL-1β, IL-6 and TNF-α expression in HSV-2-infected HEC-1-A cells, demonstrating that PT could interfere with NF-κB-mediated cytokines/chemokines production caused by HSV-2 infection.

3.5. PT increased intracellular ubiquitin-conjugate level by mediating Zn^{2+} influx

As shown in Fig. 5A, PT treatment could potently augment ubiquitin-conjugate levels in HEC-1-A. It could also enhance the accumulation of poly-ubiquitinated proteins in Vero and Hela cells (data not shown). We also evaluated whether PT treatment could increase the fluorescence intensity of Ub-G76V-GFP fusion protein. Ub-G76V-GFP plasmid encodes ubiquitin fusion degradation-targeted GFP with a mutation (G76V) that renders an uncleavable ubiquitin moiety. This fusion protein is destined to rapid degradation by the UPS, allowing convenient measurement of UPS activity *in vivo*. As shown in Fig. 5B, PT inhibited Ub-G76V-GFP proteolysis and increased its intracellular accumulation, which could be

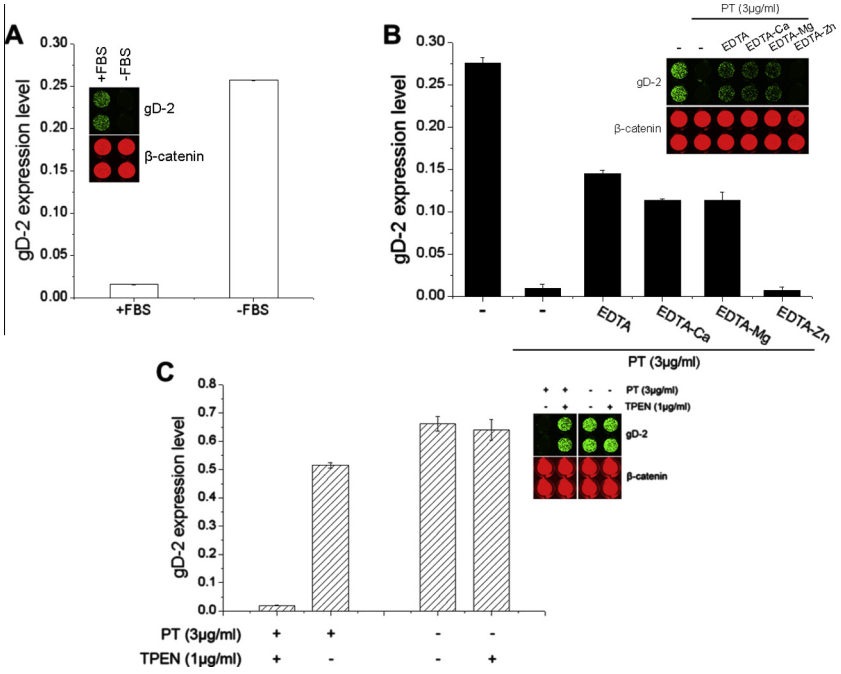

Fig. 2. Zn^{2+} was essential for PT antiviral activity. (A) HEC-1-A cells cultured in complete or serum-free medium were infected with HSV-2 (moi = 1) in the presence of PT (3 μg/ml). (B) HEC-1-A cells were incubated with EDTA, EDTA-Ca, EDTA-Mg or EDTA-Zn (10 μM each) in the presence of PT (3 μg/ml) for 30 min and then infected with HSV-2 (moi = 1). The EDTA compounds and PT were present during virus infection. (C) HEC-1-A cells were either mock-treated or treated with PT (3 μg/ml) in the presence or absence of TPEN (1 μg/ml), and infected with HSV-2 (moi = 1). gD-2 expression level was determined 24 h p.i. All experiments were performed two or three times, and a representative result was shown.

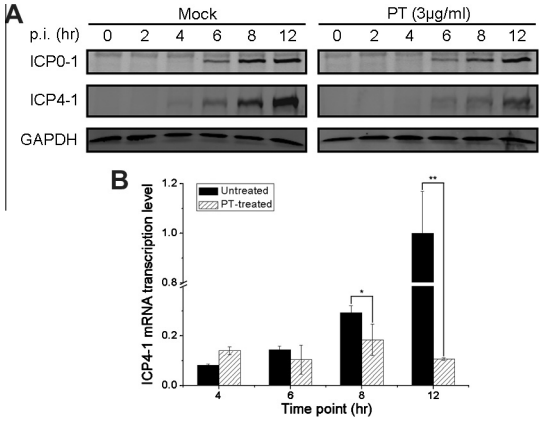

Fig. 3. The effect of PT on HSV-1 IE gene expression. (A) HEC-1-A cells were either mock-treated or treated with PT (3 μg/ml) and then infected with HSV-1 (moi = 1). Cells were lysed at each time point. ICP0 and ICP4 were visualized via Western blot. (B) HEC-1-A cells were either mock-treated or pre-treated with PT (3 μg/ml) for 30 min and then infected with HSV-1 (moi = 1). ICP4-1 mRNA transcription levels 4, 6, 8 and 12 h p.i. were determined via real-time PCR analysis.

reversed by EDTA treatment, demonstrating that PT effect on proteasome activity was Zn^{2+}-dependent. Further evidence showed that PT induced the accumulation of polyubiquitinated p53 protein (Fig. 5C), a well-known proteasome-regulated protein, suggesting that PT suppressed proteasome-dependent proteolysis, thus increased polyubiquitinated p53 protein level.

However, PT did not exhibit any inhibitory effect on cellular 26S proteasome function in a cell-free system with concentrations that inhibited HSV infection (Fig. 6A). Krenn et al. (2009) reported that PT treatment increased the intracellular Zn^{2+} level in Hela cells. In HEC-1-A cells, it was also illustrated that PT treatment would augment cellular uptake of Zn^{2+} in a dose-dependent manner as determined by fluorescent FluoZin-3 AM probes (Fig. 6B). We proposed that the intracellular Zn^{2+} transported by PT was a direct antagonist against cellular proteasome. In Fig. 6C, it was shown that the addition of 200 μM Zn^{2+} in culture medium would increase the level of high molecule-weight ubiquitin-conjugates, implicating the direct effect of Zn^{2+} on cellular UPS.

3.6. PT inhibits viral replication through dysregulation of the proteasomal function

Previous studies indicated that NF-κB was essential for HSV-1 gene expression and replication (Gregory et al., 2004; Patel et al., 1998). The degradation of IκB-α, an endogenous NF-κB inhibitor, was responsible for the NF-κB activation. IκB-α was degraded by 26S proteasome following its covalent conjugation with ubiquitin (Chen et al., 1995; Palombella et al., 1994). As shown in Fig. 7A, PT inhibited the degradation of ubiquitin-conjugates induced by HSV-2 infection in a dose-dependent manner. The amount of the accumulated ubiquitin-conjugates was also associated with IκB-α level, and negatively correlated with HSV gD expression representing the viral replication, suggesting that PT inhibition of NF-κB was mediated by preventing the degradation of IκB-α through dysregulation of UPS.

We further investigated whether Zn^{2+} was required for the PT dysregualtion of cellular proteasome activity. As shown in Fig. 7B, the addition of EDTA abrogated the PT inhibition of proteasome activity, and thus restored the viral replication to normal level (as indicated by the level of gD expression). In considering the earlier data on the requirement for Zn^{2+} in the viral inhibitory activity, we concluded that PT inhibition of HSV replication was mediated by Zn^{2+} influx into the intracellular compartments, which interferes with the cellular UPS.

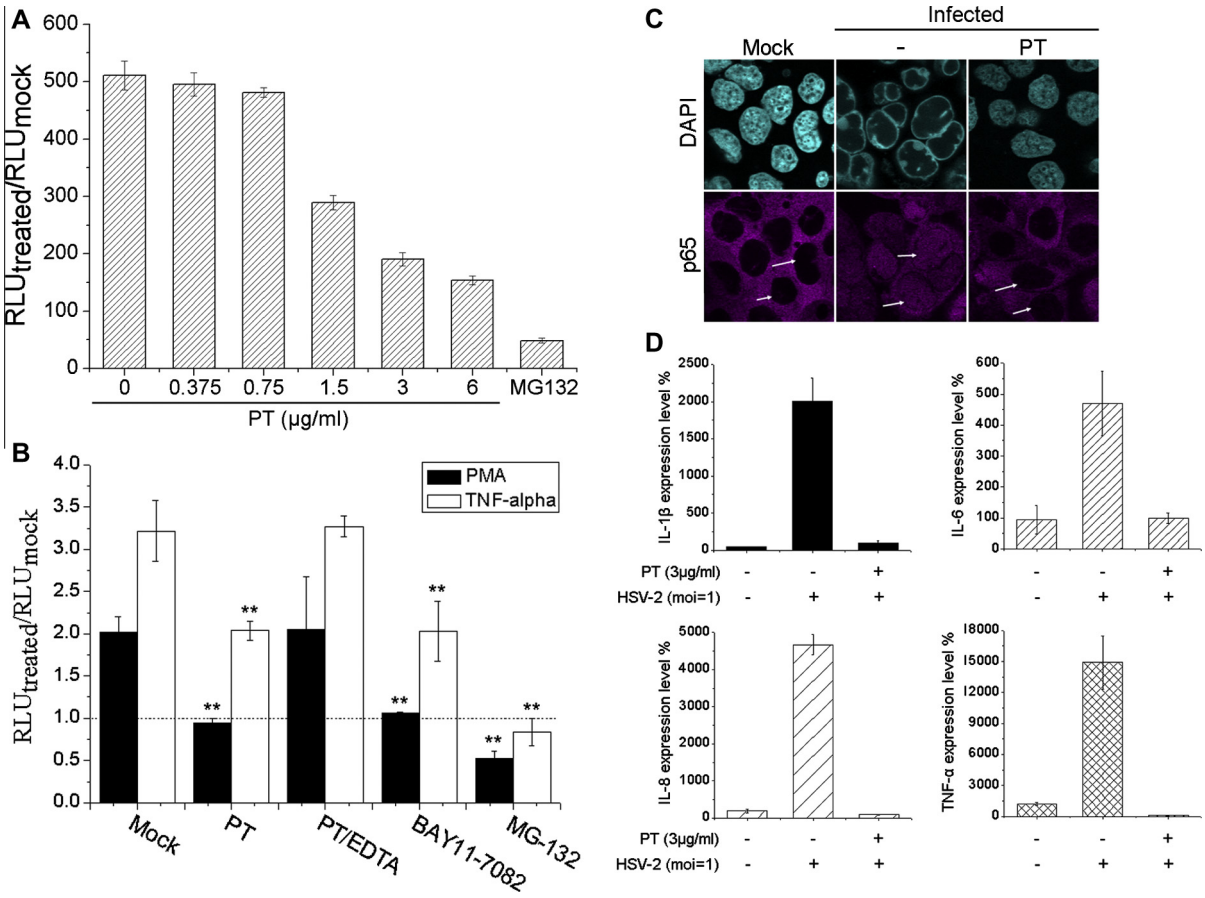

Fig. 4. PT inhibiting NF-κB activation might contribute to its antiviral activity. (A and B) PT inhibited HSV-2-induced and PMA-/TNF-α-induced NF-κB activation. HEC-1-A cells transfected with NF-κB luciferase reporter plasmid were either mock-infected or infected with HSV-2 (moi = 1) in the presence or absence of PT (A), or incubated with PT (3 μg/ml) minus or plus 10 μM EDTA for 30 min and then treated with PMA (5 μg/ml) or TNF-α (100 ng/ml) (B). BAY11-7082 (2 μg/ml) or/and MG132 (5 μg/ml) was set as positive control. The RLU was determined after 6 h (for PMA- or TNF-α-treated cells) or 24 h (for HSV-2-infected cells) and expressed as percentage of that in the mock-treated cells. (C) PT interfered with HSV-2-mediated p65 nuclear translocation. Cells were either mock-infected or infected with HSV-2 (moi = 1) in the presence or absence of PT (3 μg/ml). p65 localization was determined via immunofluorescence staining 24 h p.i. (D) PT inhibited NF-κB-mediated upregulation of cytokines/chemokines caused by HSV-2. Cells were either mock-infected or infected with HSV-2 (moi = 1) in the presence or absence of PT (3 μg/ml). IL-1β, IL-6, IL-8 and TNF-α expressions were determined via real-time PCR 24 h p.i. All experiments were performed three times. A representative result was shown.

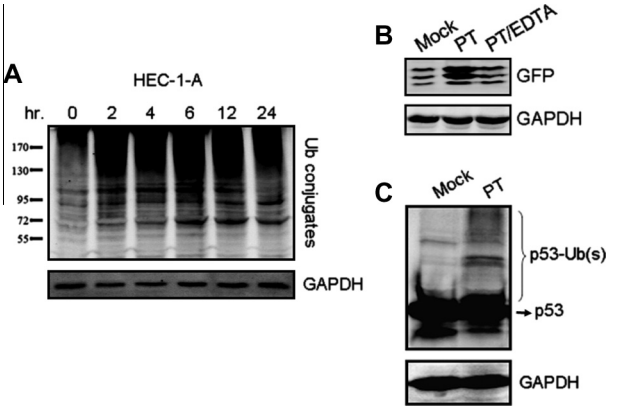

Fig. 5. PT increased the amount of intracellular ubiquitin-conjugates. (A) PT increased the level of ubiquitin-conjugates. HEC-1-A cells were incubated with PT (3 μg/ml) for indicated time. Ubiquitin-comjugates were visualized via Western blot. (B) PT treatment increased the accumulation of short-lived GFP. Hela cells transiently transfected with Ub-G76 V-GFP expression construct were treated with vehicle, PT (3 μg/ml) or PT plus EDTA (10 μM). GFP levels were determined via Western blot after 8 h. (C) PT treatment increased the ubiquitinated-p53 level. HEC-1-A cells were either mock-treated or treated with PT (3 μg/ml). p53 and its ubiquitinated forms were determined via Western blot.

3.7. PT stabilized nucleus-localized PML and inhibited PML degradation by HSV-2 infection

Earlier studies demonstrated that PML nuclear bodies (PML-NBs) played a significant role against viral infection (Everett and Chelbi-Alix, 2007; Regad and Chelbi-Alix, 2001). Everett *et al.* reported that HSV-1 infection led to proteasome-dependent PML degradation and delocalization which was mediated by viral ICP0 (Chelbi-Alix, 1999; Everett et al., 1998). In the current study, we investigated whether PT inhibited HSV-2-induced PML degradation. As shown in Fig. 8A, PT treatment resulted in enlarged PML-NB size and increased PML-NB numbers in both uninfected cells and HSV-2-infected cells, which suggested that PT was capable of stabilizing the PML-NB complexes through dysregulating proteasome activity. Consistently, the effect of PT on stabilizing nucleus-localized PML-NBs and inhibiting HSV-2-induced PML proteolysis was attenuated after the addition of EDTA (Fig. 8B).

4. Discussion

PT has been reported to inhibit the infection of human rhinovirus, coxsackievirus, and mengovirus, all of which are RNA viruses belonging to the *Picornaviridae* family (Krenn et al., 2009). In this paper, we reported that PT also exhibited inhibitory activity

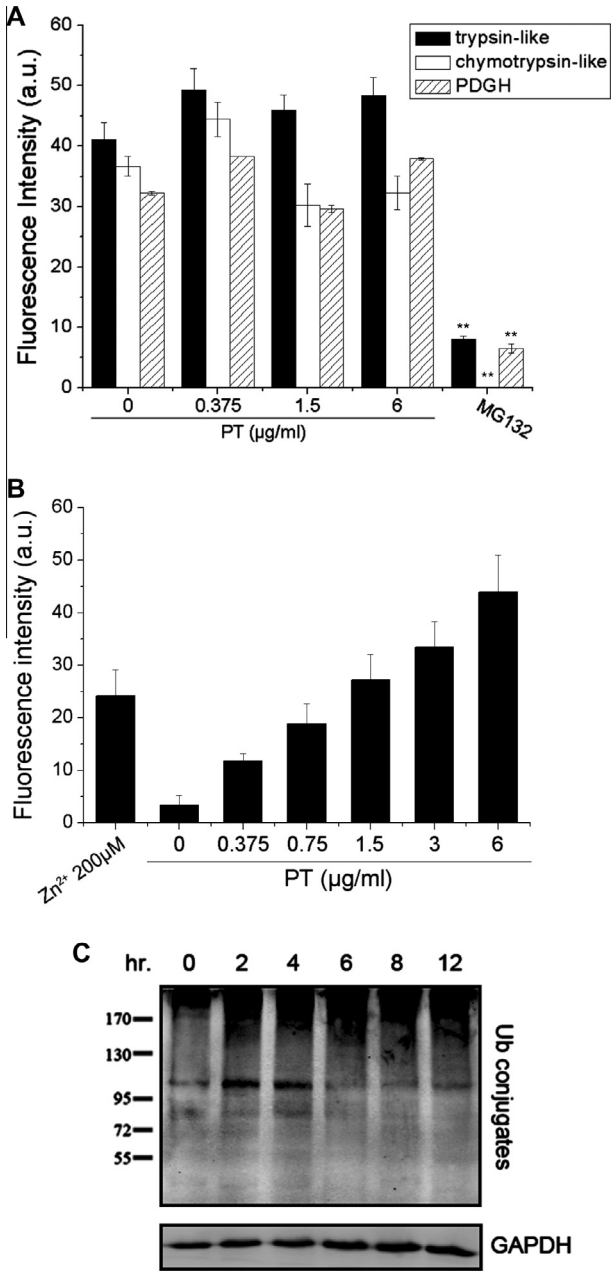

Fig. 6. PT induced zinc influx that might contribute to its dysregulation of UPS. (A) PT did not inhibit 26S proteasome activity in cell-free system. The effect of PT on 26S proteasome activity was measured using the fluorogenic substrates as described. MG132 was set as a positive control. (B) PT increased the Zn^{2+} influx. Intracellular Zn^{2+} was detected using FluoZin-3. (C) Free Zn^{2+} increased intracellular ubiquitin-conjugates. HEC-1-A cells were treated with Zn^{2+} (200 μM) and lysed at indicated time point and ubiquitin-conjugates were visualized via Western blot.

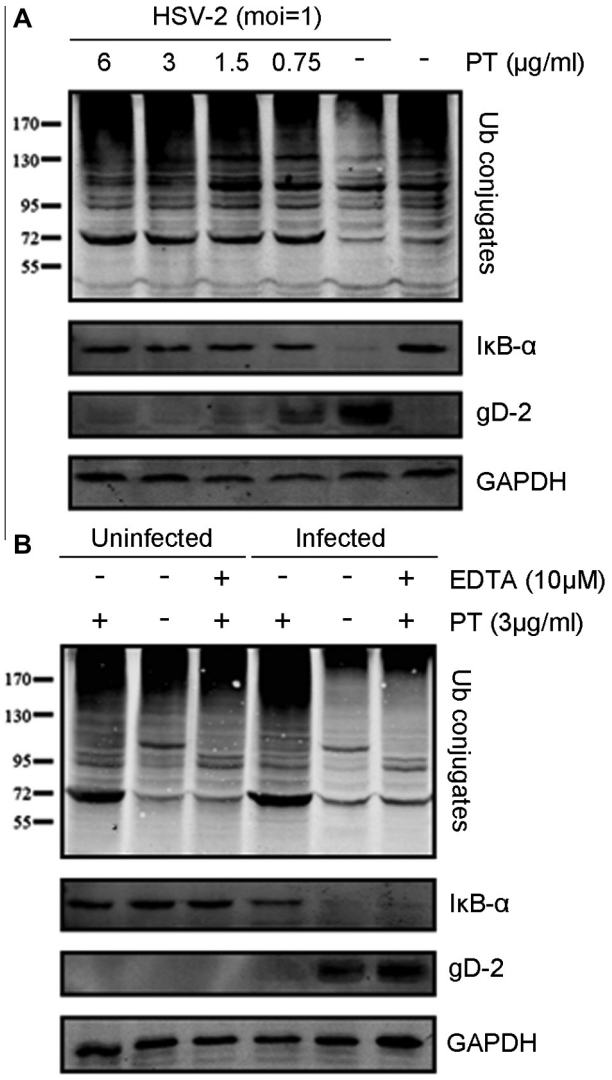

Fig. 7. PT inhibits viral replication through the dysregulation of the proteasome function. (A) PT inhibited HSV-2-induced degradation of high molecule-weight ubiquitin-conjugates and IκB-α. HEC-1-A cells were either mock-infected or infected with HSV-2 (moi = 1) in the presence of serially diluted concentrations of PT. (B) Deletion of Zn^{2+} could attenuate PT inhibiting the degradation of ubiquitin-conjugates and IκB-α. Cells were either mock-treated or treated with PT (3 μg/ml) prior to infected with HSV-2 (moi = 1) in the presence or absence of EDTA (10 μM). Ubiquitin-conjugates, IκB-α and gD-2 were determined 24 h p.i. via Western blot.

against HSV-1 and HSV-2 at non-cytotoxic concentrations. HSV-1 and HSV-2, members of *Herpesviridae* family, are large DNA viruses. To our knowledge, it was the first such a report describing PT as an antiviral agent against DNA viruses.

PT is a zinc ionophore that can transport extracellular zinc ions into intracellular compartments (Andersson et al., 2009; Krenn et al., 2009; Magda et al., 2008). Previous study has shown that PT inhibited NF-κB activation in a Zn^{2+}-dependent manner (Kim et al., 1999). In this study, we demonstrated that PT anti-HSV activity was Zn^{2+}-dependent as this inhibitory activity could be blocked by the addition of Zn^{2+} chelators, such as EDTA and TPEN. It was consistent with previous reports that the presence of Zn^{2+} played a vital role in PT bioactivity.

In this study, we showed that PT inhibited HSV late gene expression by inhibiting the transcription and expression of IE genes, mainly ICP4 and, to a less extent, ICP0 (Fig. 3A). ICP0 and ICP4 are two important IE regulatory proteins of HSV that play a vital role in viral replication, cell growth and apoptosis (Everett, 1984a,b; O'Hare and Hayward, 1985a,b). ICP4 is a major regulator of viral transcription that is essential for HSV replication and latent infection (DeLuca and Schaffer, 1985; Everett, 1984b; Gelman and Silverstein, 1985; Godowski and Knipe, 1986; O'Hare and Hayward, 1985a,b). We demonstrated that PT inhibited ICP4 expression and consequently caused the silence of viral early and late genes expression, leading to the reduced production of viral progeny.

Evidence has shown that JNK/p38 MAPK/AP-1 pathway was important for HSV-1 gene expression and replication (Karaca et al., 2004; McLean and Bachenheimer, 1999; Zachos et al.,

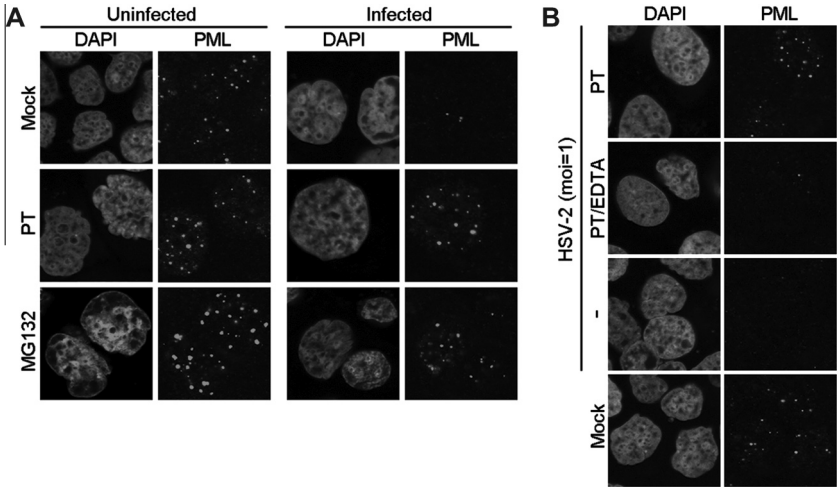

Fig. 8. PT stabilized nucleus-localized PML and inhibited PML degradation by HSV-2 infection, which was dependent on zinc ions. (A) HEC-1-A cells were either mock-treated or pre-treated with PT (3 μg/ml) or MG132 (5 μg/ml) for 30 min and then mock-infected or infected with HSV-2 (moi = 1). (B) Cells either mock-treated or treated with PT (3 μg/ml) were infected with HSV-2 in the presence or absence of EDTA (10 μM). Cells were fixed 16 h p.i. and PML was determined via immunofluorescence staining.

1999). Therefore, we also investigated PT effect on AP-1 transcriptional activation relating to its antiviral property. PT did not inhibit virus-induced AP-1 activation (data not shown). We also analyzed PT effects on AP-1 activation induced by PMA and TNF-α, and the results showed that PT had no effect on PMA- or TNF-α-induced AP-1 activation (data not shown).

Several reports have shown that HSV-1 infection caused the activation of NF-κB which was contributed by IKKα/β activation, and it was essential for viral gene expression and HSV replication (Gregory et al., 2004; Patel et al., 1998). Kim et al. (1999) found that PT could inhibit NF-κB activation via the enhancement of Zn^{2+} influx. In the present study, we also determined that PT treatment inhibited IκB-α binding site-driven luciferase expression and p65 nuclear translocation caused by viral infection (Fig. 4A and C). Also, PT inhibited HSV-2-induced upregulation of certain NF-κB-mediated cytokines/chemokines to the basal level (Fig. 4D). In conclusion, we proposed that the anti-HSV activity of PT is mediated through NF-κB pathway.

NF-κB activation needs IKK activation by a variety of extracellular signals, in turn, phosphorylation and ubiquitination of IκB-α, and eventual degradation by cellular proteasome (Perkins, 2007). It was observed that PT-treated cells accumulated high molecule-weight ubiquitin-conjugates (Fig. 5A), indicating that PT stabilized the multi-ubiquitinated proteins or blocked the degradation of these proteins. This conclusion is in line with the observation of PT inhibition of IκB-α degradation, allowing the suppression of NF-κB activation during HSV infection, thus contributing to the inhibition of viral replication. However, it was not surprising that no significant PT effect on proteasome activity was observed in a cell-free system since PT acted as an ionophore for Zn^{2+} influx into intracellular compartments, and Zn^{2+} is required for inhibition of proteasome activity, as we showed that Zn^{2+} alone could directly inhibit 26S proteasome cleavage activity (Fig. 6). Earlier study showed that in *Drosophila*, Zn^{2+} induced reversible dissociation of subunit Rpn10/p54 of the 26S proteasome (Kiss et al., 2005). The dissociation of Rpn10/p54, the ubiquitin receptor subunit of the proteasome, induced extensive rearrangements within the lid subcomplex of the regulatory compartment in the 26S complex and resulted in the loss of the peptidase activity. Our observations were consistent with the direct effect of Zn^{2+} on the proteasome. However, Zn^{2+} may also impact on other targets as suggested by our experimental observation. We showed that PT treatment interfered with the proteasome-dependent IκB-α degradation, thus inhibited NF-κB activation eventually. In addition, Hayakawa et al. (2003) reported that PDTC, another zinc ionophore, could attenuate NF-κB activation through blocking SCF complex, IκB-specific E3 ubiquitin ligase. SCF ubiquitin ligase complex contains Skp1, a member of the Rbx1/Roc1 family of RING finger proteins (Jin and Harper, 2002). Therefore, we speculated that dysregualtion of intracellular zinc homeostasis would disrupt certain E3 ubiquitin ligase activation. The specific roles of Zn^{2+} require further investigation.

UPS has been demonstrated to be important for the replication of several viruses, and many viruses have evolved to hijack host cellular proteasome to facilitate their effective infection (Kanlaya et al., 2010; Karpe and Meng, 2012; Klinger and Schubert, 2005; Neznanov et al., 2008; Raaben et al., 2010; Satheshkumar et al., 2009; Si et al., 2008; Teale et al., 2009; Thomas et al., 1999; Widjaja et al., 2010). HSV ICP0 with RING-finger domain acted as ubiquitin E3 ligase and induced degradation of cellular component proteins, such as PML and Sp100 (Boutell et al., 2002; Parkinson and Everett, 2000). Recently, Delboy *et al.* reported that cellular proteasome activity facilitated the postpenetration steps of HSV, and inhibition of proteasome pathway using specific inhibitors such as MG-132 or lactacystin would reduce HSV capsid transportation to nucleus (Delboy et al., 2008), and further demonstrated that this effect was dependent on the presence of ICP0 (Delboy and Nicola, 2011). In this study, we reported that proteasome activity affected HSV-2-induced NF-κB activation and viral gene expression through regulating the degradation of an endogenous NF-κB inhibitor, IκB-α, implicating a distinct antiviral mechanism for proteasome inhibitors.

PML has been shown to be a repressor of HSV-1 infection, and it is degraded by proteasome via interacting with ICP0 (Everett et al., 2006). It was implied that impaired proteasome activity would retain PML in nucleus that might contribute to PT antiviral activity. We showed that HSV-2-induced PML-NBs degradation and delocalization could be interrupted by PT and MG-132. Therefore, we speculated that the PT inhibition of HSV replication may act on multiple targets during the viral life cycle.

Other zinc ionophores have been reported to possess antiviral activities. PDTC, widely used as an antioxidant (Hayakawa et al., 2003) or potent inhibitor of NF-κB activation (Schreck et al., 1992; Ziegler-Heitbrock et al., 1993), has been reported to inhibit the infection of several viruses, such as influenza virus (Uchide et al., 2002), rhinovirus (Gaudernak et al., 2002), poliovirus (Krenn

et al., 2005), coxsackievirus (Lanke et al., 2007; Si et al., 2005) and mengovirus (Lanke et al., 2007). Our recent research found that PDTC could also inhibit HSV-1 and HSV-2 replication, also through disrupting proteasome activity in a Zn^{2+}-dependent manner (Qiu et al., 2013). In addition, Krenn et al. (2009) found that another zinc ionophores, hinokitiol, inhibited rhinovirus, coxsackievirus and mengovirus infections in the presence of Zn^{2+}. Although these compounds are structurally different, their antiviral different, they appeared to function by importing Zn^{2+} into intracellular compartments as a common mechanism for their antiviral effects. The current study has furthered our understanding of the mechanisms on the well recognized antiviral activity of Zn^{2+} and Zn^{2+} ionophores and may facilitate efforts to identify new antiviral targets.

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