Original Article

Pentagalloylglucose Blocks the Nuclear Transport and the Process of Nucleocapsid Egress to Inhibit HSV-1 Infection

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SUMMARY: Herpes simplex virus type 1 (HSV-1), a widespread virus, causes a variety of human viral diseases worldwide. The serious threat of drug-resistance highlights the extreme urgency to develop novel antiviral drugs with different mechanisms of action. Pentagalloylglucose (PGG) is a natural polyphenolic compound with significant anti-HSV activity; however, the mechanisms underlying its antiviral activity need to be defined by further studies. In this study, we found that PGG treatment delays the nuclear transport process of HSV-1 particles by inhibiting the upregulation of dynein (a cellular major motor protein) induced by HSV-1 infection. Furthermore, PGG treatment affects the nucleocapsid egress of HSV-1 by inhibiting the expression and disrupting the cellular localization of pEGFP-UL31 and pEGFP-UL34, which are indispensable for HSV-1 nucleocapsid egress from the nucleus. However, the over-expression of pEGFP-UL31 and pEGFP-UL34 could decrease the antiviral effect of PGG. In this study, for the first time, the antiviral activity of PGG against acyclovir-resistant virus was demonstrated in vitro, and the possible mechanisms of its anti-HSV activities were identified based on the inhibition of nuclear transport and nucleocapsid egress in HSV-1. It was further confirmed that PGG could be a promising candidate for HSV therapy, especially for drug-resistant strains.

INTRODUCTION

Herpes simplex virus type 1 (HSV-1), belonging to the *Herpesviridae* family, the *Alphaherpesvirinae* subfamily and genus *Simplexvirus*, was the first human herpes virus to be discovered (1). HSV-1 is a linear double-stranded DNA virus that primarily affects the mucocutaneous surfaces but can also infect the visceral organs or the central nervous system (2). Many diseases can be caused by HSV-1 infections, such as cold sores, oral ulcers, genital herpes, hepatitis, meningoencephalitis, esophagitis, and pneumonia (3). In the United States, approximately 50–70% of healthy adults and 20–40% of healthy children are infected with HSV-1, and these proportions are even higher in some of the developing countries (4). HSV-1 also establishes life-long latent infections in the ganglia of sensory nerves and can reactivate when the immune system is deficient or has been stimulated (5). However, the serious cases are the one in which HSV-1 infections cause herpetic lesions that can be persistent, extensive, and are more easily disseminated with increased recurrences in immunocompromised patients, such as patients with HIV infection or recipients of organ transplants (6,7). Despite intensive efforts, current therapeutic strategies remain inadequate to control HSV-1 infections (8). In the absence of an efficient vaccine, acyclovir (ACV) and its derivatives

are used for the standard therapy for HSV infections; however, problems related to toxicity and drug resistance have long been reported and have created an obstacle for the treatment of HSV-1 (9). Therefore, novel antiviral agents, exhibiting different mechanisms of action, are urgently needed to treat infections with HSV-1.

Pentagalloylglucose (PGG) is an active compound present in many traditional medicinal herbs. It exhibits multiple biological activities, including anti-inflammatory (10), anti-mutagenic (11), anti-cancer (12), antioxidant (13), anti-bacterial (14), and antiviral effects. Many studies have reported that PGG can inhibit the replication of HSV-1 (15), respiratory syncytial virus (16), hepatitis B virus (17), and influenza virus (18). The antiviral mechanisms of PGG include direct inactivation, inhibition of virus adsorption on host cells and viral gene expression, as well as protein translational inhibition of the replicated viral genome (15). The present study investigated the PGG-induced inhibition of HSV-1 nuclear transport and nucleocapsid egress as novel means of inducing antiviral activity in vitro.

MATERIALS AND METHODS

Chemicals and reagents: The PGG used in this study was isolated from *Phyllanthus emblica*, it had a purity of >99% and the structure of which has been identified as published previously (19). ACV (acycloguanosine) and 2-(2,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). TRIzol Reagent and Lipofectamine 2000 were purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin were bought from Gibco-BRL (Gland Island, NY, USA). PGG and ACV were dissolved in

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dimethylsulfoxide (DMSO) before use, and the final concentrations of DMSO were less than 0.01%. Restriction enzymes were purchased from Takata Bio (Shiga, Japan).

Cells and viruses: African green monkey kidney cells (Vero; ATCC CCL81) were cultured in DMEM supplemented with 10% heat-inactivated FBS. The maintenance medium used for virus dilutions was DMEM supplemented with 2% heat-inactivated FBS. HSV-1/F (ATCC VR-733) was preserved in our lab. HSV-1/Blue, a TK mutant derived from HSV-1 (KOS) (20), two ACV-resistant clinical HSV-1 strains (HSV-1/106 and HSV-1/153) were a kind gift from Tao Peng (Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences). All viruses were propagated in Vero cells and stored at -80°C until further use.

MTT assay: The MTT assay was performed according to the standard protocol. Briefly, Vero cells were cultured in 96-well plates. After the cell confluence reached 90%, various concentrations of compound were added to the plate, with each concentration having eight replicates. After 48 h of incubation, 10 μL MTT solution (5 mg/mL) was added to each well, and the plate was incubated for 4 h in the dark. Then, the MTT solution was discarded, and 100 μL DMSO was added to each well. Plates were incubated for 15 min at room temperature with gently shaking. The optical density (OD) at 570 and 630 nm was measured for each well with an enzyme immunoassay reader (Bio-Rad, Hercules, CA, USA). The 50% cytotoxicity concentration (CC_{50}) was defined as the concentration to reduce 50% cell viability.

Plaque formation assay: Vero cells were seeded in 24-well plates, to 70%–80% confluence. Then, the cells were infected with different HSV-1 strains (30 to 40 PFUs each well). After 2 h, the virus suspension was discarded and then replenished with 1 mL of overlay medium (1:1 mixture of sodium carboxymethylcellulose [NaCMC]: DMEM with 2% FBS) containing various concentrations of the compound. At 72 h post-infection (p.i.), the medium was discarded, and the plates were washed with phosphate buffered saline (PBS) three times, before fixing with 10% paraformaldehyde for 15 min. Lastly, the plates were stained with 1% crystal violet for 20 min and washed with PBS. The images of the monolayers were acquired and plaques were enumerated in each well, then the plaque reduction ratio was calculated.

Quantitative real-time PCR (qPCR): Vero cells were cultured in 6-well plates to 70%–80% confluence, and then the cells were infected with HSV-1 at indicated conditions, as described in the figure legends. After different time durations p.i., the total RNA from the infected cells was extracted using TRIzol (Invitrogen). The extracted RNA was reverse-transcribed into cDNA using a PrimeScript RT reagent kit (Takara Bio). The qPCR assays were conducted using SsoFast EvaGreen Supermix (Bio-Rad) according to the manufacturer's instructions. The specific primer pairs used for *ICP0* were (F: 5'-TTA CGT GAA CAA GAC TAT CAC GG-3' and R: 5'-TCC ATG TCC AGG ATG GGC-3'), for *ICP4* were (F: 5'-GGC CTG CTT CCG GAT CTC-3' and R: 5'-GGT GAT GAA GGA GCT GCT GTT-3'), for *UL54* were (F: 5'-TGG CGG ACA TTA AGG ACA TTG-3' and R:

5'-TGG CCG TCA ACT CGC AGA-3'), for *Dynein* were (F: 5'-AGC TGG AAG GCC TGT GGA TGC-3' and R: 5'-TTC GCC AGG ACC CCT TCG CT-3'), and for *GAPDH* were (F: 5'-CAC CAC CAA CTG CTT AGC C-3' and R: 5'-CAG TGG ATG CAG GGA TGA TG-3'). The relative expression of each gene was normalized to that of the housekeeping gene *GAPDH*.

Plasmid construction: To construct an pEGFP-UL31 and pEGFP-UL34 over-expression plasmids, the HSV-1 *UL31* and *UL34* coding sequence, without the stop codon, were cloned from the total cDNA of HSV-1 infected Vero cells with the following primers: *UL31* (F: 5'-CCC AAG CTT GGG ATG TAT GAC ACC GAC CCC-3'; R: 5'-CGG GAT CCC GCG GCG GAG GAA ACT CGT C-3') and *UL34* (F: 5'-CCC AAG CTT GGG ATG GCG GGA CTG GGC AAG-3'; R: 5'-CGG GGT ACC CCT AGG CGC GCG CCA GCA CC-3'). The PCR fragment was then digested with *HindIII* and *BamHI* for *UL31* PCR fragment or *HindIII* and *KpnI* for *UL34* PCR fragment and inserted into the multiple cloning site of the pEGFP-N1 vector plasmid (Clontech, Palo Alto, CA, USA). The successful constructions of the recombinant plasmids were confirmed by DNA sequencing analysis.

Indirect immunofluorescence assay: To quantify HSV-1 entry and trafficking to the nucleus, Vero cells were cultured in glass-bottom dishes overnight to achieve 40–50% confluence. Next, the cells were infected with HSV-1 (multiplicity of infection [MOI] = 50) and incubated at 4°C for 1 h to allow virus attachment to the cell monolayer. After that, the infected cells were incubated at 37°C for 10 min to maximize virus penetration. Then, the cells were treated with or without PGG (10 μM) for further 4 h to allow HSV-1 trafficking to the nucleus. The cells were later fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 for 5 min, blocked in 5% bovine serum albumin (BSA) for 1 h, stained with a primary antibody against HSV-1 VP5 (1:1000) (Abcam, Cambridge, UK) in 5% BSA/PBS for 1 h, and stained with an Alexa Fluor 647-conjugated secondary antibody (1:1000) (Invitrogen) in 5% BSA/PBS for 1 h. The cells were stained with 5 μM TRITC-phalloidin (Sigma-Aldrich) for 30 min and 1 mg/mL DAPI/PBS (Biotium, Hayward, CA, USA) for 15 min to label the F-actin and the nuclei, respectively. The cells were washed with PBS 3 times and images were acquired by a confocal laser scanning microscope (LSM 510; Zeiss, Jena, Germany). To observe the effect of PGG on the localization of EGFP-UL31 and EGFP-UL34, Vero cells were seeded in glass-bottom dishes and cultured overnight to achieve 40–50% confluence. Then, the cells were transfected with the pEGFP-UL31 or pEGFP-UL34 using Lipofectamine 2000 according to the manufacturer's instructions. The cells were treated with or without PGG (10 μM) for 24 h to allow protein expression. The cells were later fixed and stained with DAPI/PBS for 15 min to label the nuclei. The cells were washed as described above, and images were obtained by LSM.

Western blot analysis: To detect the influence of PGG on the expression of EGFP-UL31 and EGFP-UL34, Vero cells grown in 25-cm² culture flasks were transfected with pEGFP-UL31 and pEGFP-UL34 using Lipofectamine 2000 according to the manufacturer's

instructions. Next, the cells were treated with or without PGG (10 μ M). After 24 h, the cells were harvested and lysed in RIPA buffer (Beyotime, Jiangsu, China) for 30 min on ice, and the protein concentrations were measured with an enhanced BCA protein assay kit (Beyotime). The cell lysates were mixed with 5 $\times$ SDS-PAGE sample buffer (Beyotime) and boiled for 10 min. Then, the protein samples were subjected to 8–12% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Immobilon; Millipore, Bedford, MA, USA). After blocking with 5% w/v nonfat dry milk (Difco, Detroit, MI, USA) at room temperature for 1 h, the membranes were incubated with primary antibodies against enhanced green fluorescent protein (EGFP; Cell Signaling, Danvers, MA, USA) at 4°C, overnight. The membranes were subsequently incubated with secondary antibodies diluted in TBS-T containing 5% w/v nonfat dry milk at room temperature for 1 h. The membranes were washed 3 times with TBS-T for 5 min each. The protein bands were then detected with enhanced chemiluminescence reagent (Millipore) and imaged using autoradiography. Amounts of each loading protein was normalized to the corresponding level of the GAPDH control.

Statistical analysis: Results were calculated as the mean $\pm$ SEM, and statistical significance were determined by the Student's *t* test. *P* values (*P*) < 0.05 were considered statistically significant.

RESULTS

Cytotoxicity and anti-HSV activity of PGG: PGG, a hydrolyzable polyphenol, was isolated from the branches and leaves of *Phyllanthus emblica*, and its structure was identified as previously described (21). Based on the MTT assay, the CC₅₀s of PGG and control compound ACV were 401.15 $\pm$ 4.51 μ M and 2826.11 $\pm$ 26.38 μ M, respectively (Table 1). To fully analyze the anti-HSV activity of PGG; HSV-1/F, a standard experimental strains, HSV-1/Blue, a TK mutant derived from HSV-1 (KOS), and two ACV-resistant clinical HSV-1 strains (HSV-1/106 and HSV-1/153) were used. As shown in Table 1, the 50% effective concentrations (EC₅₀) for PGG and ACV to inhibit HSV-1/F were 3.13 $\pm$ 0.55 μ M and 4.30 $\pm$ 0.27 μ M, respectively. The EC₅₀ of PGG for HSV-1/153, HSV-1/106, and HSV-1/Blue were 3.19 $\pm$ 0.83 μ M, 3.26 $\pm$ 0.67 μ M, and 3.49 $\pm$ 0.36 μ M, respectively. However, the EC₅₀ of ACV for all three resistant strains were more than 20 μ M. These results indicate that PGG may have a different anti-HSV mechanism than that of ACV. As shown by previous work, the PGG can affect the attachment of HSV-1 as well as a strong direct inactivation activity. Moreover, PGG can affect the cytoskeleton and induce autophagy. Therefore, in the present study, we studied

to find some other mechanisms involved in the anti-HSV activity of PGG. To avoid the cytotoxicity of PGG, a concentration of 10 μ M (CC₁₀ was 23.55 $\pm$ 3.56 μ M) was selected for further experiments.

PGG treatment inhibits the nucleus transport of HSV-1: To investigate whether PGG treatment could inhibit the nucleus transport of HSV-1, Vero cells were infected with HSV-1 at an MOI of 50, and PGG-treatment was performed immediately after the HSV-1 penetration into the cells. As shown in Fig. 1A, at 4 h p.i., we can easily see that almost all the HSV-1 particles in the virus control groups have reached inside the nucleus. However, when the cells were treated with PGG, the HSV-1 particles were mostly located in the cytoplasm. To confirm the inhibitory effects of PGG treatment on the process of nuclear transfer, qPCR were carried out to detect the expression of the immediately-early genes. As shown in Fig. 1B, at 2 h, 4 h, and 6 h p.i., there was a significant decrease in the gene expression of *ICP0*, *ICP4*, and *UL54* in the PGG-treated group. From these results, we can conclude that PGG treatment can inhibit the nuclear transport of HSV-1. As reported previously, HSV-1 particles were transported along the microtubules of the cells, driven by the microtubule motor protein dynein, and the biphasic dynamics of F-actin were also required for efficient viral infection. As shown in our previous work, the PGG can affect the actin skeleton dynamics by downregulating of cofilin1. However, whether PGG treatment could affect the expression or organizations of tubulin and dynein needs to be further confirmed. From qPCR and immunofluorescence, we found that the expression and organization of tubulin were not affected by the PGG treatment (data not shown). However, as shown in Fig. 2, the cellular expression level of dynein was significantly increased after the HSV-1 infection, but the up-regulation of dynein was significantly decreased in the PGG treatment groups. From all of these data, we can infer that the inhibitory effects of PGG treatment on the nuclear transport were partly owing to its influence on the expressions of the motor protein dynein.

PGG treatment inhibits the nucleocapsid egress of HSV-1: Previous work has reported that the nuclear egress of HSV-1 capsid was significantly inhibited by the PGG treatment. To confirm this data, we detected the extracellular virus titers of HSV-1 infected cells in the virus or PGG treatment groups at 18 h, 20 h, and 22 h p.i. As shown in Fig. 3A, we found that the extracellular virus titers showed a significant decrease after the PGG treatment, and this was consistent with our previous work. Numerous studies have reported that the late-gene *UL31* and *UL34* expedite the process of capsid egress from nucleus. Next, we detected the effect of PGG treatment on the expression of *UL31* and *UL34* genes. As indicated in Fig. 3B and C, there was a

Table 1. Cytotoxicity and anti-viral activity of pentagalloylglucose (PGG) and acyclovir (ACV)

Compound	CC ₅₀ (μ M)	EC ₅₀ (μ M)				Therapeutic index (TI)			
		HSV-1/F	HSV-1/153	HSV-1/106	HSV-1/Blue	HSV-1/F	HSV-1/153	HSV-1/106	HSV-1/Blue
PGG	401.15 $\pm$ 4.51	3.13 $\pm$ 0.55	3.19 $\pm$ 0.83	3.26 $\pm$ 0.67	3.49 $\pm$ 0.36	128	126	123	115
ACV	2826.11 $\pm$ 26.38	4.30 $\pm$ 0.27	>20	>20	>20	657	<141	<141	<141

Fig. 1. (Color online) Nuclear transport of HSV-1 was inhibited by PGG treatment. (A) Vero cells were grown to 40–50% confluence in a glass-bottom dishes and infected with HSV-1 (MOI = 50) at 4°C for 1 h to allow virus attachment to the cell monolayer. The infected cells were then incubated at 37°C for 10 min to maximize virus penetration. After that, the cells were treated with or without PGG (10 μ M) and incubated at 37°C in a humidified atmosphere with 5% CO₂ for more 4 h to allow HSV-1 trafficking to the nucleus. The cells were then stained for VP5 (green), F-actin (red), and nuclei (blue), and images were obtained by confocal microscopy. (B) PGG significantly decreased the expression of immediately-early genes of HSV-1. Vero cells were cultured in 6-well plates to 80% confluence and were infected HSV-1 as described in the section (A). Various hours (1 h, 2 h, 4 h, and 6 h) post-infection (p.i.), total RNAs were extracted from the infected cells and reverse-transcribed to cDNAs. The relative expression levels of the immediately-early gene (ICP0, ICP4, and UL54) were estimated by qPCR. Data are the mean values of at least three independent experiments $\pm$ SEM. *, $P < 0.05$: significant difference compared to the virus control group.

significant decrease in the expression level of *UL31* and *UL34* in the PGG-treated group compared with the virus groups. To directly detect the influence of PGG treatment on the expression or localization of pUL31 and pUL34, two expression plasmids pEGFP-UL31-N1 and pEGFP-UL34-N1 (EGFP fused to *UL31* and *UL34*), were constructed, and the cellular expression or localization of EGFP-UL31 or EGFP-UL34 was detected using immunofluorescence. As shown in Fig. 4, we found that the expression of pEGFP-UL31 (Fig. 4A) as well as pEGFP-UL34 (Fig. 4B) was significantly decreased after PGG treatment, and the mean EGFP fluorescence intensity of the PGG treated group was decreased by about 70% and 55%, respectively. At the

same time, we also observed that the cellular localization of pEGFP-UL34 was influenced by PGG treatment. In the control group, EGFP-UL34 fusion protein was uniformly and continuously distributed around the nucleus, but in the PGG treatment group, the EGFP-UL34 fusion protein was clumped together and discontinuously distributed around the nucleus (Fig. 4B). Furthermore, qPCR and western-blotting were performed to detect the impact of PGG on the expression of *UL31* and *UL34*. As shown in Fig. 5A and B, we found that PGG treatment can significantly downregulate the expression of pEGFP-UL31 and pEGFP-UL34. To further confirm the results that PGG inhibited the nuclear egress in HSV-1 by decreasing the expression and locali-

Fig. 2. Effect of PGG on the expression of microtubule motor protein dynein. Vero cells were cultured and infected as described in Fig. 1B. The total RNA was isolated and reverse-transcribed to cDNAs, and the relative expression level of dynein to that of cells infected with the virus for 1 h, was estimated by qPCR. Data are the mean values of at least three independent experiments $\pm$ SEM. *, $P < 0.05$: significant difference compared to the virus control group.

Fig. 3. Nucleocapsid egress of HSV-1 was inhibited by PGG treatment. (A) To detect the direct influence of PGG on the nucleus egress of HSV-1, PGG were treated in the time of nucleus egress (at 12 h). Vero cells were cultured in 6-well plates to 80% confluence and infected with HSV-1 (MOI = 10) for 12 h. Then the medium was replaced with PGG medium (10 μ M). After 6 h, 8 h, and 10 h of PGG treatment, the medium of each group were collected, and the viral titers were determined by a 50% tissue culture infectious dose (TCID₅₀) assay on Vero cells. (B and C) Vero cells were cultured, infected, and treated as section (A). After 6 h, 8 h, and 10 h of PGG treatment, the total RNA was extracted from each group and reverse-transcribed to cDNAs. The relative expression level of UL31 (B) and UL34 (C) was detected by qPCR. The levels of the cells infected with virus at 18 h.p.i. was estimated as 1.0. Data are the mean values of at least three independent experiments $\pm$ SEM. *, $P < 0.05$: significant difference compared to the virus control group.

Fig. 4. (Color online) PGG affects the expression and intracellular localization of EGFP-UL31 and EGFP-UL34. Vero cells were seeded in glass-bottom dishes overnight to achieve 40–50% confluence. Then, the cells were transfected with the pEGFP-UL31 or pEGFP-UL34 plasmid using Lipofectamine 2000 according to the manufacturer's instructions. After that, the cells were treated with or without PGG (10 μ M) for 24 h to allow protein expression. The cells were fixed and stained with DAPI/PBS for 15 min to label the nuclei. The images were obtained by confocal microscopy.

zation of pUL31 and pUL34, we conducted an overexpression experiment. As shown in Fig. 5C, we clearly found that overexpression of pEGFP-UL31 or pEGFP-UL34 could abate some of the antiviral effects of PGG.

DISCUSSION

PGG, a hydrolyzable polyphenol, has potent antiviral activity. In a previous study, Pei et al. showed that PGG exhibited strong antiviral activity with an EC_{50} value of $4.12 \pm 0.67 \mu$ M, and from a time-of-addition assay, they found that PGG displayed antiviral activity during the whole HSV-1 life cycle. Moreover, they also found that PGG can inhibit the DNA replication, gene expression, and protein synthesis in HSV-1. In addition, PGG can inhibit the HSV-1-induced actin-skeleton rearrangements by downregulating the expression of cofilin1, a

key regulator of actin cytoskeleton dynamics. Pei et al. found that autophagy induced following PGG treatment also contributed to its anti-HSV activity (15). PGG can also prevent the adsorption and release of HSV-1 and influenza A virus (18). However, although many antiviral activities of PGG have been explored, more works are required to fully explore the antiviral mechanisms of PGG and to better promote its clinical application.

Data presented in this study suggest for the first time that PGG effectively inhibits the proliferation of clinical ACV-resistant HSV-1 viruses, and it does so with an EC_{50} value of 3.13 to 3.49 μ M better than that of ACV. This result indicates that PGG can be employed as an anti-HSV drug, especially for drug-resistant virus therapy. Data presented here showed that PGG inhibits the replication of HSV-1 via two new mechanisms: preven-

Fig. 5. Overexpression EGFP-UL31 or EGFP-UL34 abated the antiviral effect of PGG. (A) Vero cells were seeded in 6-well plates and transfected with the pEGFP-UL31 or pEGFP-UL34. After that, the cells were treated with or without PGG ($10 \mu\text{M}$) for 20 h, then the total RNA from each group was extracted and reverse-transcribed to cDNAs. The relative expression levels of UL31 and UL34 to that of PGG minus control were estimated using qPCR. (B) Inhibitory effects of PGG on the protein expressions of the transfectants with pEGFP-UL31 or pEGFP-UL34 plasmid were detected by western-blotting performed as described in the Materials and Methods section. (C) Vero cells were seeded in 24-well plates and transfected with the pEGFP-N1, pEGFP-UL31, or pEGFP-UL34. After that, the cells were infected with HSV-1 (30–40 PFUs/each well) and treated with or without PGG ($10 \mu\text{M}$). Then the cells were used for plaque formation assay as described previously. The data are the mean values of at least three independent experiments $\pm$ SEM. *, $P < 0.05$: significant difference compared to the virus control group.

tion of viral nuclear transport and suppression of nucleocapsid egress. From immunofluorescence, we directly detected that in 4 h p.i. in the PGG treatment group, the viral particles were tethered in the cytoplasm. The delay in the nuclear transport directly induces the downregulation of the expression of HSV-1 genes. Nuclear transport is a process of viral particles transfer from the cell membrane to the nucleus. During this process, the cytoplasmic microtubules in host cells act as the piping system for viral delivery (22). There is a variety of host proteins participating in the process of cellular transportation, and each protein has its own driving direction (23). Dynein is responsible for the retrograde transport process, which is from the cell membrane to the nucleus (24). Therefore, dynein is very important in the import of HSV-1 particles into nucleus. In this study, using immunofluorescence, no changes in the expression or organization of microtubules have been found. However, results from qPCR found that the expression of dynein increases significantly after the infection of HSV-1, but this increase is fully inhibited by the PGG treatment. Thus, from these results, it is concluded with confidence that PGG treatment inhibits the import of HSV-1 into nucleus partly by downregulating

the major cellular motor protein, dynein, which is significantly upregulated after HSV-1 infection. However, the regulatory mechanism involved in the HSV-1 infection induced upregulation of dynein is poorly understood, and this is an interesting phenomenon, worth to investigate further.

A previous study have reported that PGG treatment can inhibit the nucleus egress of HSV-1, but the underlying mechanism remained unclear (15). Nucleocapsid egress of HSV has evolved a unique mechanism. Firstly, the nucleocapsids bud through the inner nuclear membrane into the particular space. Secondly, enveloped nucleocapsids fuse with the outer nuclear membrane to release nucleocapsids into the cytoplasm (25,26). Two conserved viral proteins, which are encoded by the *UL31* and *UL34* genes, form a complex termed the nuclear egress complex and are associated with the nucleocapsids egress process (27–29). The absence of either molecule prevents HSV-1 nucleocapsid egress from the nucleus (30). Data presented in this study showed that PGG treatment significantly inhibits the expression of *UL31* and *UL34* genes, and PGG treatment affects the cellular localization of EGFP-UL31 and EGFP-UL34. Furthermore, overexpression of

pEGFP-UL31 and pEGFP-UL34 could abolish the antiviral activity of PGG. All of these results indicate that PGG inhibits the nucleocapsids egress process of HSV-1 by downregulating the expression of *UL31* and *UL34* or by disrupting the correct cellular localization of EGFP-UL31 and EGFP-UL34 proteins. In the present study, we found that PGG treatment could significantly downregulate the expression of EGFP-UL31 and EGFP-UL34 fusion protein. One possible reason is that PGG could directly interact with *UL31* or *UL34* gene, and thereby, inhibits the transcription of these two genes. Alternatively, PGG could directly interact with the mRNA of these two genes and consequently promote the mRNA degradation. Nevertheless, all of these speculations require further experiments to be confirmed.

In conclusion, this study has demonstrated for the first time that PGG possesses antiviral activity against ACV-drug resistant viruses in vitro. Meanwhile, PGG exhibits its antiviral activities by disturbing the process of import of HSV-1 particles into nucleus as well as by inhibiting nucleocapsids egress. Taken together, the present study has further confirmed that PGG could be a promising candidate for HSV therapy, especially for the drug-resistant strains.

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Conflict of interest None to declare.

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