PHYTOTHERAPY RESEARCH
Phytother. Res. **25**: 975–982 (2011)
Published online 7 January 2011 in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.3368

In vitro Anti-Herpes Simplex Virus Activity of 1,2,4,6-Tetra-*O*-galloyl-β-D-glucose from *Phyllanthus emblica* L. (Euphorbiaceae)

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In this study, 1,2,4,6-tetra-*O*-galloyl-β-D-glucose (1246TGG), a polyphenolic compound isolated from traditional Chinese medicine *Phyllanthus emblica* L. (Euphorbiaceae), was found to inhibit herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) infection at different magnitudes of activity *in vitro*. Further studies revealed that 1246TGG directly inactivated HSV-1 particles, leading to the failure of early infection, including viral attachment and penetration. 1246TGG also suppressed the intracellular growth of HSV-1 within a long period post-infection (from 0 h p.i. to 12 h p.i.), while it might exert an antiviral effect mainly before 3 h p.i. It inhibited HSV-1 E and L gene expressions as well as viral DNA replication but did not affect the RNA synthesis of IE gene in our study. Also, in the presence of 1246TGG, the synthesis of viral protein was reduced. Taken together, it was suggested that 1246TGG might exert anti-HSV activity both by inactivating extracellular viral particles and by inhibiting viral biosynthesis in host cells. These results warrant further studies on the antiviral mechanisms of 1246TGG and suggest that it might be a candidate for HSV therapy. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: anti-herpes simplex virus activity; HSV; 1,2,4,6-tetra-*O*-galloyl-β-D-glucose; polyphenol; *Phyllanthus emblica* L. (Euphorbiaceae).

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are enveloped DNA viruses belonging to the Herpesviridae family that cause lifelong infections. HSV-1 usually causes orofacial infections and encephalitis, and HSV-2 is mostly associated with genital and newborn infections, although either virus can cause genital herpes (Kalinyak *et al.*, 1977; Corey *et al.*, 1983). In the absence of an efficient vaccine, synthetic acyclic purine-nucleoside analogs such as acyclovir (ACV) targeting viral kinase and DNA polymerase have been the standard therapy for HSV infections. Yet, the widespread use of ACV has led to the emergence of resistant strains since the 1980s (Burns *et al.*, 1982; Crumpacker *et al.*, 1982; Sibrack *et al.*, 1982). Especially among bone-marrow-transplant recipients and patients with AIDS, resistant HSV isolates frequently occur after therapeutic treatment with ACV (Whitley *et al.*, 1998). Mutant viruses resistant to one nucleoside drug are commonly resistant to other analogs, increasing the difficulty of disease management. In recent years, a range of promising drugs with novel molecular targets has been developed (Coen and Schaffer, 2003). Among these, docosanol, a saturated fatty alcohol inhibiting virus fusion, is the first topical antiviral approved for over-the-counter use in recurrent herpes labialis (Leung and Sacks, 2004).

Polyphenols are secondary metabolites widely distributed in medical plants and plant-derived foods, and could be promising anti-HSV agents (Khan *et al.*, 2005). A variety of flavonoids, phenolic acids and related derivatives have been found to possess anti-HSV activity recently (Lin *et al.*, 2000; Lyu *et al.*, 2005; Uozaki *et al.*, 2007; Isaacs *et al.*, 2008). Previously an *in vitro* cell-based assay was conducted to screen compounds isolated from different traditional Chinese medicines for their antiviral activity, and one compound, 1,2,4,6-tetra-*O*-galloyl-β-D-glucose (1246TGG) from *Phyllanthus emblica* L. (Euphorbiaceae), was found to be a potent anti-HSV agent. Here, the anti-HSV activity of 1246TGG is reported and possible antiviral mechanisms of 1246TGG were also explored.

MATERIALS AND METHODS

Chemicals. 1246TGG was isolated and its structure was identified by the team at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences using procedures described in a previous paper (Zhang *et al.*, 2002). Briefly, the EtOH extract of the

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Received 02 September 2010
Revised 25 October 2010
Accepted 31 October 2010

fresh leaves and branches of *P. emblica* (15 kg) was suspended into water and then extracted with Et_2O. The Et_2O layer was partitioned between hexane and MeOH, and the MeOH layer was further chromatographed successively over Sephadex LH-20, silica gel, MCI-gel CHP 20P and Chromatorex ODS to afford the desired compound (75 mg, purity >95%): pale amorphous powder, 1H-NMR (CD3OD, 300 MHz): 4.16 (1H, t, J=9.6 Hz, H-3), 4.19 (1H, m, H-5), 4.29 (1H, dd, J=4.2, 12.3 Hz, H-6a), 4.49 (1H, dd, J=1.8, 12.3 Hz, H-6b), 5.37 (1H, dd, J=8.4, 9.6, H-2), 5.38 (1H, dd, J=9.6, 9.9 Hz, H-4), 6.05 (1H, d, J=8.4 Hz, H-1), 7.05, 7.07, 7.11, 7.12 (each 2H, s, galloyl-H). Its structure was identified by comparison of the physical and spectral data with literature values and 1H-1H COSY spectrum (Fig. 1). 1246TGG was dissolved in dimethyl sulfoxide (DMSO) before use. The final concentration of DMSO was less than 0.2%.

Acyclovir, purchased from Sigma (purity ≥99%), was dissolved in maintenance medium before use.

Cells and viruses. African green monkey kidney cells (Vero, ATCC CCL81), provided by Wuhan Institute of Virology, Chinese Academy of Sciences, were cultured in growth medium (Dulbecco's modified Eagle medium, DMEM; Gibco) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 0.22% sodium bicarbonate (Sigma) and 50 μg/mL gentamycin (Gibco). The constituents of the maintenance medium were the same as those of the growth medium except that only 2% FBS was added. The cells were cultured at 37 °C in a humid atmosphere with 5% CO_2. HSV-1 F (ATCC VR-733), obtained from Hong Kong University, and HSV-2 strain 333, obtained from Wuhan Institute of Virology, Chinese Academy of Sciences, were propagated in Vero cells and stored at −80 °C until use. Virus titers were determined by a plaque assay on Vero cell monolayers (Russell, 1962).

XTT assay. The XTT (sodium 3′-[1-(phenylaminocarbonyl)-3, 4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulphonic acid; Invitrogen) assay was performed according to the protocol. Briefly, Vero cells were seeded onto 96-well tissue culture plates (Corning). After a 24 h period of incubation at 37 °C in a humid atmosphere with 5% CO_2, various concentrations of compound were added. After another 72 h of incubation, the XTT solution and phenazine methosulphate (PMS) were added. The absorbance was assayed with a microplate reader (Bio-Rad 550) at dual wavelengths of 480 nm and 630 nm. The 50% cytotoxicity concentration (CC_{50}) was defined as the concentration reducing 50% cell viability.

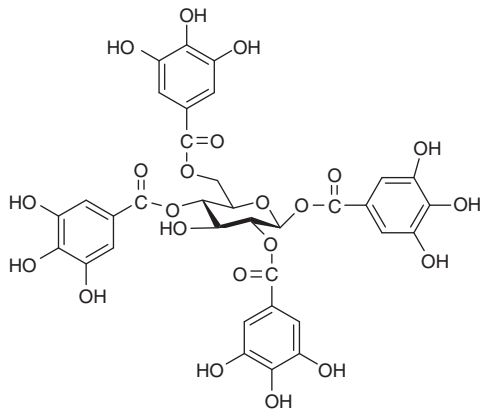

Figure 1. Structure of 1,2,4,6-tetra-*O*-galloyl-β-D-glucose (1246TGG) from *Phyllanthus emblica* L. (Euphorbiaceae).

Vialight assay. Intracellular adenosine triphosphate (ATP) levels of cells in culture were detected using the Vialight HS kit (Lonza) according to the protocol. Briefly, Vero cells were seeded onto 96-well tissue culture plates (Corning) and incubated at 37 °C in a humid atmosphere with 5% CO_2 overnight. Various concentrations of 1246TGG were added and the cells were allowed to incubate for another 72 h. For the Vialight assay, 100 μL of nucleotide releasing reagent (NRR) was added to each well. Then 5 min later, 180 μL of cell lysate was transferred to a luminescence compatible plate (Berthold), which was then placed in a luminometer (Berthold) to initiate the detecting program. The luminometer was previously primed with ATP monitoring reagent (AMR) and programmed to dispense 20 μL into each well taking an immediate 1 s integrated reading.

Plaque assay. Antiviral activity was determined by plaque assay using monolayer cultures of Vero cells in 24-well culture plates (Corning). Virus suspension containing either HSV-1 or HSV-2 (30 plaque forming units (PFU)/well) was added to the cell wells and incubated at 37 °C with 5% CO_2 for 2 h. The virus inoculum was then removed and overlay medium (maintenance medium containing 1% methylcellulose and various concentrations of tested compound) was added to each well. After another 72 h of incubation, the cell monolayers were fixed with 10% formalin and stained with 1% crystal violet. Plaques were counted and the percentage of inhibition was calculated according to Nitta *et al.* (1994). The concentration reducing plaque numbers by 50% was calculated by regression analysis of the dose–response curves generated from the plaque assay and was defined as 50% inhibitory concentration (IC_{50}). The selectivity index (SI) was calculated as the ratio of CC_{50} to IC_{50}.

Viral attachment and penetration assay. The attachment assay was performed primarily as described by Su *et al.* (2008). The Vero cells were grown in a 24-well culture plates and pre-chilled at 4 °C for 1 h. The cell monolayer was then infected with HSV-1 (30 PFU/well) in the presence or absence of compound at indicated concentrations and incubated at 4 °C for another 80 min. The infected cell monolayer was then washed three times with cold PBS and treated for plaque assay.

The penetration assay was performed as described previously (Cheng *et al.*, 2005). The Vero cells were grown in a 24-well culture plates and pre-chilled at 4 °C for 1 h. The cell monolayer was then infected with HSV-1 (30 PFU/well) and incubated at 4 °C for another 2 h to allow virus attachment to the cell monolayer. After that, various concentrations of tested compound were added. The infected cell monolayer was then incubated at 37 °C for 10 min to maximize virus penetration. PBS at pH 3 was added for 1 min to inactivate unpenetrated virus, and PBS at pH 11 was then added immediately to neutralize the acidic PBS. The neutral PBS was removed and the cell monolayer was treated for plaque assay.

Table 1. Cytotoxic effect, antiviral activity and selective index of 1246TGG from *Phyllanthus emblica* L. (Euphorbiaceae)

	Cytotoxicity[a] (μM)	Antiviral activity[b] (μM)			
		HSV-1		HSV-2	
Compound	CC_{50}	IC_{50}	SI[c]	IC_{50}	SI[c]
1246TGG	>253.63	10.77±0.61	>23.55	24.73±1.08	>10.26
ACV	>887.94	4.16±1.02	>213.45	3.32±0.44	>267.45

The value represents mean ± SD of three independent experiments.

[a]Cytotoxic effect was determined by the XTT assay, CC_{50} was the concentration reducing 50% cell viability.

[b]Antiviral activity was determined by the plaque reduction assay, IC_{50} was the concentration that inhibited 50% of HSV multiplication.

[c]SI (selective index) was calculated as the ratio of CC_{50} to IC_{50}.

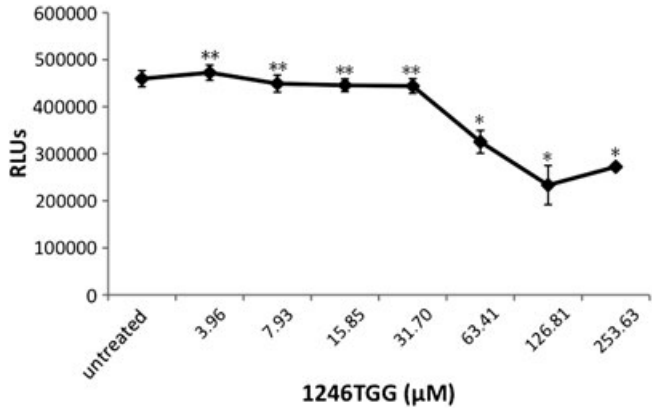

Figure 2. Cytotoxic effect of 1246TGG examined using the Vialight kit. Vero cells were incubated with 1246TGG at various concentrations for 72 h and subjected to ATP assay. Each value represents the mean of four parallel wells with its respective standard deviation indicated. $*p < 0.01$, $**p > 0.05$ (compared between cell control and 1246TGG treated groups).

Virucidal assay. For the virucidal assay (Cheng *et al.*, 2004), HSV-1 suspension (30 PFU/well) was pre-incubated with or without various concentrations of compound at 37 °C for 2 h and was added to Vero cells for another 2 h of incubation. Then virus inoculum was removed and replaced by overlay medium. The following treatment was as described for the plaque assay. An additional virucidal assay was conducted according to Uozaki *et al.* (2007) with minor modifications. Briefly, a series of HSV-1 preparations (approximately 10^6 PFU in 900 μL maintenance medium) received 100 μL 1246TGG solutions at various concentrations and were incubated at 4 °C for 10 min. The viruses were then diluted with maintenance medium and subjected to titration using the plaque assay.

Addition time assay. For the addition time assay, Vero cells were grown in a 24-well culture plates. The cell monolayer was then infected with HSV-1 at a multiplicity of infection (MOI) of 1. The virus inoculum was then removed 2 h post-infection (p.i.) and replaced by maintenance medium. Maintenance medium containing the desired concentration of compounds was added to each well at 3 h, 6 h, 9 h and 12 h post-infection. Meanwhile, compounds were also added simultaneously with the inoculation of virus (0 h p.i.). At 24 h p.i., the cell cultures were harvested, subjected to three cycles of freezing–thawing and virus titration.

For the cell pretreatment assay, the Vero cell monolayer was incubated with maintenance medium containing the desired concentration of compounds for 2 h. Compounds were then removed and the cells were infected with HSV-1 (MOI=1). At 24 h p.i., the cell cultures were harvested, subjected to three cycles of freezing–thawing and virus titration.

Real-time polymerase chain reaction. For the viral DNA synthesis assay, the Vero cells were grown in 12-well culture plates and infected with HSV-1 (MOI=20) at 37 °C for 2 h. After the removal of virus inoculum, the cells were overlaid with maintenance medium containing either 1246TGG at a concentration of 25 μg/mL (31.70 μM) or ACV at a concentration of 5 μg/mL (22.20 μM). Infected cultures were harvested and subjected to three cycles of freezing–thawing at 24 h p.i. before viral DNA extraction.

For the viral RNA synthesis assay, the Vero cells were grown in a 12-well culture plates and infected with HSV-1 at a MOI of 20 at 37 °C for 2 h. Following removal of virus inoculum, the infected cells were overlaid with maintenance medium containing either 1246TGG at a concentration of 25 μg/mL (31.70 μM) or ACV at a concentration of 5 μg/mL (22.20 μM). The maintenance medium was aspirated and the cells were washed with PBS before total RNA extraction. Real-time PCR was conducted to determine the expression levels of HSV-1 immediate early (IE) gene U_L54, early (E) gene U_L52 and late (L) gene U_L27 at 3 h, 6 h and 9 h p.i., respectively.

Viral DNA and total RNA of infected cells were extracted using commercial kits, UNIQ-10 Viral DNA Kit (Sangon) and TRIzol reagent (Invitrogen), respectively. RNA concentrations were measured using a spectrophotometer (260 nm/280 nm). 1 μg of eluted RNA was subjected to reverse transcription by the RevertAidTM M-MuLV Reverse Transcriptase (Fermentas) at 42 °C for 60 min with oligo (dT)18 primer in a total volume of 20 μL.

A real-time PCR assay was used for quantitation of the obtained viral DNA and cDNA. The primer pairs for U_L54 were U_L54F (5′ TGG CGG ACA TTA AGG ACA TTG 3′) and U_L54R (5′ TGG CCG TCA ACT CGC AGA 3′); for U_L52 were U_L52F (5′ GAC CGA CGG GTG CGT TAT T 3′) and U_L52R (5′ GAA GGA GTC GCC ATT TAG CC 3′); for U_L27 were U_L27F (5′ GCC TTC TTC GCC TTT CGC 3′) and U_L27R (5′ CGC TCG TGC CCT TCT TCT T 3′); and for GAPDH were GAPDH-F (5′ CCC ACT CCT CCA CCT TTG AC 3′) and GAPDH-R (5′ TCT TCC TCT TGT GCT CTT GC 3′). The real-time PCR was performed by

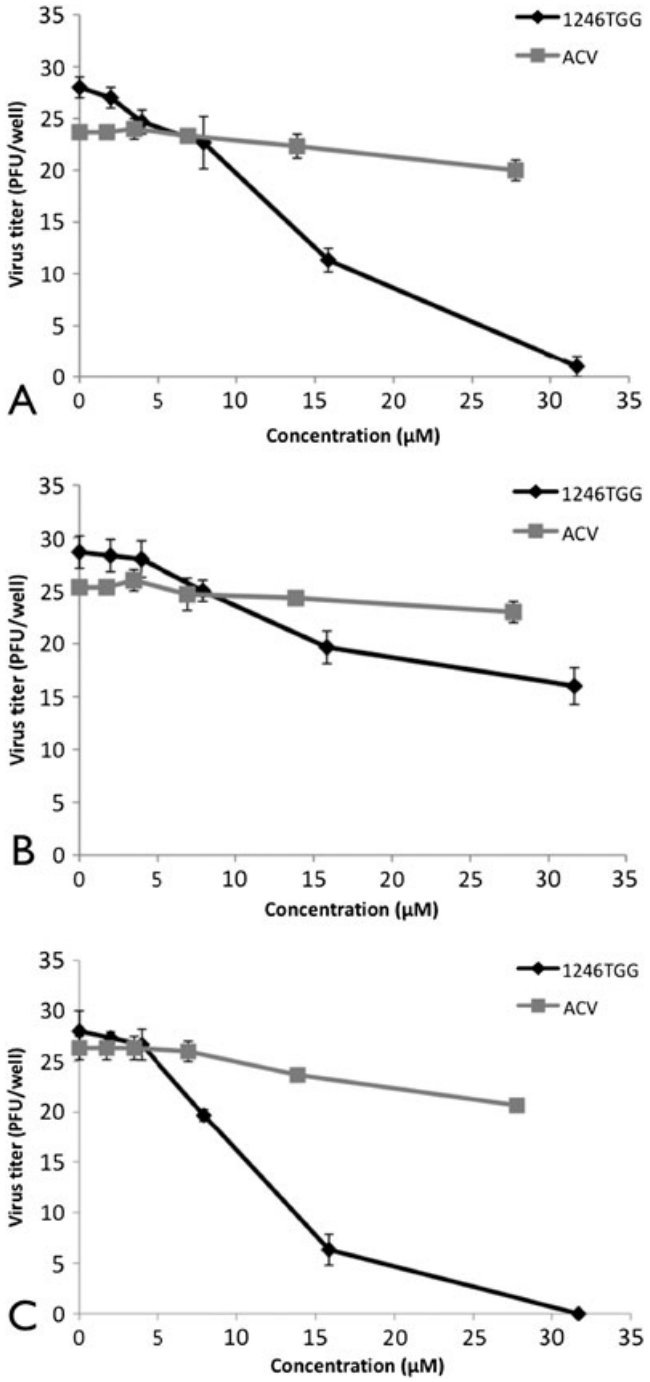

Figure 3. Effects of 1246TGG on HSV-1 attachment (A), penetration (B) and on virus inactivation (C). Each point represents the mean of triplicate wells with its respective standard deviation indicated.

using the Chromo 4 system (Bio-rad) in a total volume of 20 μL containing 1 μL of either DNA or cDNA template, 10 μL of SYBR Green Realtime PCR Master Mix (Toyobo) and 0.2 μM of each primer. After initial denaturation at 95 °C for 1 min, the amplification was carried out through 40 cycles, each consisting of denaturation at 95 °C for 15 s, annealing at 58 °C for 15 s and polymerization at 72 °C for 40 s, followed by a final extension at 72 °C for 2 min. The PCR amplification product of each gene was extracted using a gel extraction kit II (U-gene), diluted serially and used as a standard for quantitative analysis. The initial copy number of the tested gene was calculated using the formula: $CT = -k \log X_0 + b$, where k, X_0 and b refer to slope rate, initial copy number and constant, respectively.

Indirect immunofluorescence and confocal microscopy. Vero cells were grown on coverslips and infected with HSV-1 at a MOI of 20 at 37 °C for 2 h. Following removal of virus inoculum, infected cells were overlaid with maintenance medium with or without 25 μg/mL (31.70 μM) of 1246TGG and incubated for 9 h or 20 h post-infection. The cells were fixed for 15 min with 4% paraformaldehyde (PFA) and permeabilized with Triton X-100, both in PBS, and subsequently incubated with a gB-specific monoclonal antibody (mouse Mab [10B7] to HSV1/HSV2 gB; Abcam) at dilutions of 1:5000 for 60 min and Qdot® 565 Goat F(ab')2 anti-mouse IgG conjugate (H + L) solution (Invitrogen) for 60 min. After each step the slides were washed repeatedly with PBS, and finally they were preserved with PBS. Fluorescence was recorded in a confocal laser scan microscope (LSM 510; Zeiss).

RESULTS

Cytotoxicity and anti-HSV activity of 1246TGG

1246TGG, the acyl glucose containing four galloyl groups at the glucose core, was isolated from *P. emblica* and its structure was identified (Fig. 1). As determined by XTT assay (Table 1), the CC_{50} of 1246TGG and the control compound ACV were higher than 253.63 μM and 887.94 μM, respectively. The anti-HSV activities were evaluated using the plaque assay. As shown in Table 1, 1246TGG inhibited the multiplication of HSV-1 and HSV-2 with IC_{50} values of 10.77 μM and 24.73 μM, respectively. The selective index (SI) of 1246TGG against HSV-1 and HSV-2 were higher than 23.55 μM and 10.26 μM, respectively. For ACV, the clinical drug for the treatment of HSV infection, the IC_{50} values against HSV-1 and HSV-2 were 4.16 μM and 3.32 μM, respectively, and the SI values were higher than 213.45 μM and 267.45 μM, respectively.

To confirm whether 1246TGG exerted minor cellular toxicity that was not readily detected by the XTT assay, the Vialight kit was used to monitor intracellular ATP levels under treatment with 1246TGG. As shown in Fig. 2, 1246TGG at concentrations ranging from 253.63 μM to 63.41 μM resulted in certain changes of cellular ATP levels, while no significant changes were found when the cells were treated with 1246TGG at concentrations lower than 63.41 μM. Taken the XTT and ATP assays together, the concentration of 1246TGG that reached CC_{50} was higher than 253.63 μM and the completely non-toxic concentration would be lower than 63.41 μM. For further studies, the concentrations of 1246TGG used were kept below 63.41 μM.

Effects of 1246TGG on early stages of HSV-1 infection

As shown in Fig. 3A, 1246TGG at a concentration of 31.70 μM almost completely inhibited HSV-1 attachment, while in the penetration assay it was relatively inactive but still displayed inhibitory effects to a certain extent (Fig. 3B). 1246TGG displayed a similar effect in

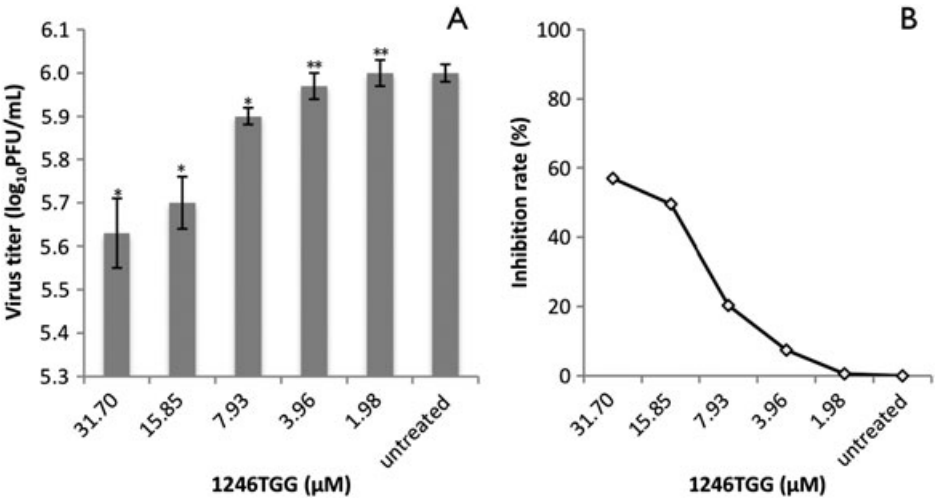

Figure 4. Virucidal activity of 1246TGG against HSV-1. Approximately 10^6 plaque forming units of HSV-1 in 900 μL maintenance medium received 100 μL 1246TGG at various concentrations for 10 min incubation at 4 °C. The viruses were then subjected to titration using the plaque assay. Each value represents the mean of four parallel wells with its respective standard deviation indicated. $*p < 0.01$, $**p > 0.05$ (compared between control and tested compound).

the virucidal assay (Fig. 3C) as in the attachment assay (Fig. 3A). The IC_{50} values of 1246TGG in attachment, penetration and virucidal assays, as calculated, were 11.29 μM, 53.13 μM and 9.53 μM, respectively. ACV, a purine-nucleoside analog that would not affect such stages of HSV-1 infection, was used as a reference compound and was comparatively inactive in these assays (Fig. 3A, B, C). The IC_{50} values of ACV in attachment, penetration and virucidal assays were all higher than 1000 μM as calculated.

An additional virucidal assay was conducted in which more viruses (approximately 10^6 PFU) were used and a shorter incubation time (10 min) was allowed before virus titration. As shown in Fig. 4, pretreatment with 1246TGG at concentrations ranging from 31.70 μM to 7.93 μM all significantly reduced the infectivity of HSV-1. Now that the attachment and penetration assay both involve incubation of HSV-1 and 1246TGG for a certain time, the effects of 1246TGG on HSV-1 attachment and penetration could also be ascribed to its virucidal activity. Taking these together, it was suggested that 1246TGG could block HSV-1 infection at the early stage probably by inactivating extracellular viral particles directly.

Effects of 1246TGG on HSV-1 growth

The addition time assay was conducted to examine the anti-HSV-1 activity of 1246TGG added at different times during the viral growth cycle. As shown in the results, 1246TGG at concentrations of 31.70 μM and 15.85 μM could significantly inhibit HSV-1 growth when added from 0 h p.i. to 12 h p.i., with inhibitory rates over 50% when added as late as 12 h p.i. (Fig. 5A). ACV, the viral polymerase inhibitor, also showed antiviral effects when added during this time period (from 0 h p.i. to 12 h p.i.; Fig. 5B). Nevertheless, a comparison between 1246TGG and ACV revealed their difference of action (Fig. 5C). The antiviral effect of 1246TGG declined significantly when added at 3 h p.i. compared with that when added at 0 h p.i., with a $5.21\log_{10}$ decrease of virus yield. For ACV, the time period during which its antiviral effect declined most significantly was between 3 h p.i. and 6 h p.i. ($4.30\log_{10}$ decrease of virus yield). These results indicated that unlike ACV, 1246TGG might suppress HSV-1 growth mainly before 3 h post-infection.

Meanwhile, Vero cells were pretreated with 1246TGG 2 h before HSV-1 infection and subjected to virus titration at 24 h post-infection. However, no decrease of virus yields was found (Fig. 5A), suggesting that the antiviral effect of 1246TGG did not involve possible interactions between the compound and host cell before virus entry.

Effects of 1246TGG on HSV-1 RNA and DNA synthesis

HSV-1 infected cells were treated with 1246TGG at 2 h p.i. and RNA levels of IE (U_L54), E (U_L52) and L (U_L27) genes were examined at 3 h, 6 h, and 9 h p.i., respectively. As shown in Fig. 6A, 1246TGG did not affect the expression of U_L54 ($p > 0.05$), but significantly reduced the expression of both U_L52 and U_L27 ($p < 0.01$). ACV was used as a reference compound here and it showed similar effects. Thus, it was demonstrated that 1246TGG could significantly decrease RNA synthesis of HSV-1 E and L genes, and that although 1246TGG might inhibit HSV-1 growth mainly before 3 h p.i., it did not suppress the RNA synthesis of IE genes.

To determine whether 1246TGG is able to affect viral DNA replication, viral DNA was isolated 24 h p.i. and the copy number of U_L52 was examined. As shown in Fig. 6B, in the presence of 1246TGG, the HSV-1 DNA replication was significantly reduced ($p < 0.01$). ACV, which targets viral DNA polymerase, was used as a positive control here. Thus, it was clear that 1246TGG could fundamentally suppress HSV-1 DNA replication.

Effects of 1246TGG on HSV-1 protein synthesis

HSV-1 infected cells were treated with 1246TGG, stained with glycoprotein B (gB) antibody and subjected to

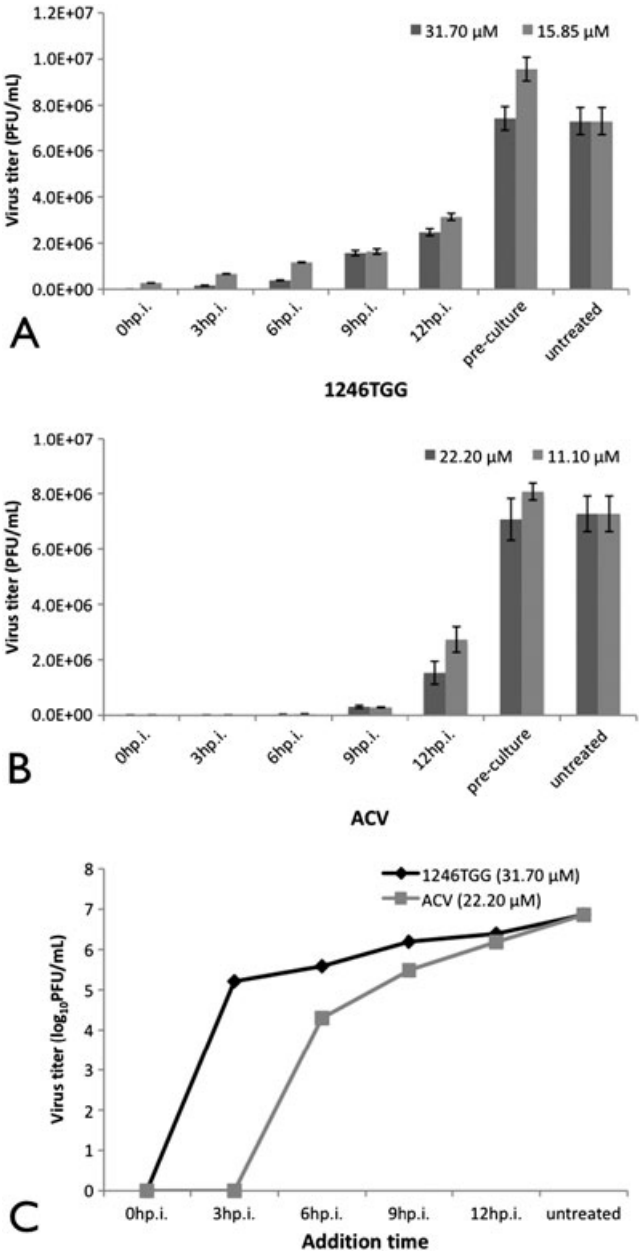

Figure 5. Anti-HSV-1 activity of 1246TGG in addition time assay. The Vero cell monolayer was infected with HSV-1 (MOI, 1). The virus inoculum was removed and maintenance medium containing various concentrations of 1246TGG (A) or ACV (B) was added at 0 h, 3 h, 6 h, 9 h and 12 h post-infection. For the pre-culture assay, the desired compounds were incubated with Vero cells for 2 h and were removed before HSV-1 infection. At 24 h post-infection, the cell cultures were harvested, subjected to three cycles of freezing–thawing and virus titration. The data represent the mean of three independent experiments. (C) Comparison of antiviral effects between 1246TGG and ACV.

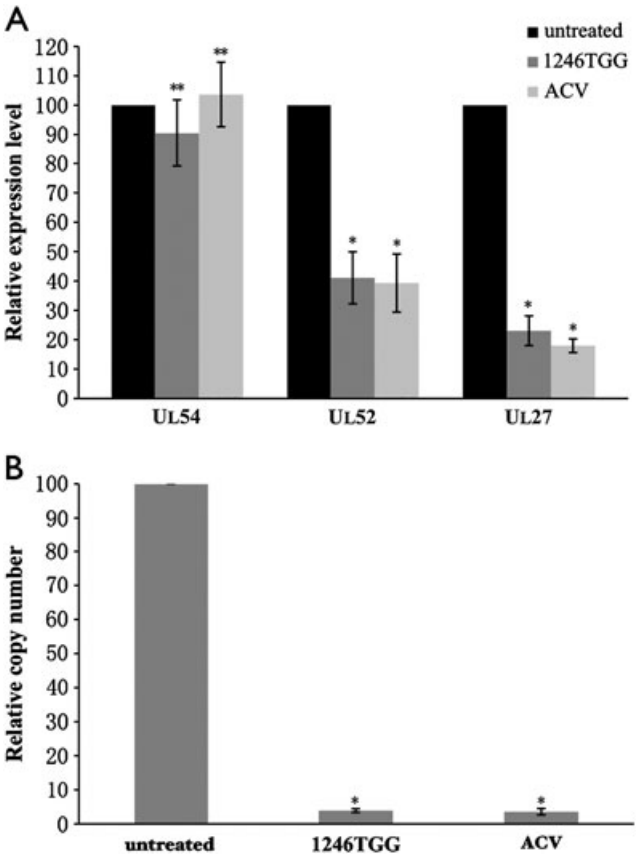

Figure 6. Effect of 1246TGG on HSV-1 gene expression (A) and DNA synthesis (B). Vero cells were infected with HSV-1 at a MOI of 20 at 37 °C for 2 h. Following removal of the virus inoculum, the infected cells were overlaid with maintenance medium containing either 1246TGG at a concentration of 25 μg/mL (31.70 μM) or ACV at a concentration of 5 μg/mL (22.20 μM). Real-time PCR was conducted to determine the expression levels of HSV-1 IE (U_L54), E (U_L52) and L (U_L27) genes at 3 h, 6 h and 9 h post-infection, respectively. For the DNA synthesis assay, the infected cultures were harvested and subjected to three cycles of freezing–thawing at 24 h post-infection before viral DNA extraction. Real-time PCR was conducted to determine the copy number of U_L52. Each value represents the mean of three independent experiments with its standard derivation indicated. $*p < 0.01$, $**p > 0.05$ (compared between control and tested compound).

confocal laser scan microscope observation. At 9 h p.i., gB had already been synthesized and mainly localized in cytoplasm (Fig. 7E), while in the 1246TGG treated group, the synthesis of gB was repressed (Fig. 7A), which is in accord with the results of the RNA synthesis assay (Fig. 6A). At 20 h p.i., the fluorescence in the untreated group (Fig. 7G) was relatively weaker compared with that at 9 h p.i. (Fig. 7E) due to the release of progeny viruses. In the 1246TGG treated group, fluorescence could barely be observed at this time point (Fig. 7C). These results demonstrated that 1246TGG could reduce HSV-1 protein synthesis.

DISCUSSION

Phyllanthus emblica L. (Euphorbiaceae) is a shrub or tree distributed in subtropical and tropical areas of China, India, Indonesia and the Malay Peninsula. The root, leaves and bark have been used for treating eczema, wart, diarrhea and headache after a fever in the Southwest of China, and the root is also used as an astringent and hematostatic agent in Nepal (Xia *et al.*, 1997). In the present study, 1246TGG, an acyl glucose isolated from *P. emblica*, was found to inhibit both HSV-1 and HSV-2 infection at different magnitudes of activity for the first time.

As revealed by the XTT assay, 1246TGG showed low cytotoxicity towards Vero cells, with a CC_{50} value higher than 253.63 μM. Minor cellular toxicity was further examined by monitoring cellular ATP levels, which indicated that despite the barely changed cell viability as determined in the XTT assay, there were still changes in ATP levels when the cells were treated with 1246TGG at concentrations ranging from 253.63 μM to 63.41 μM.

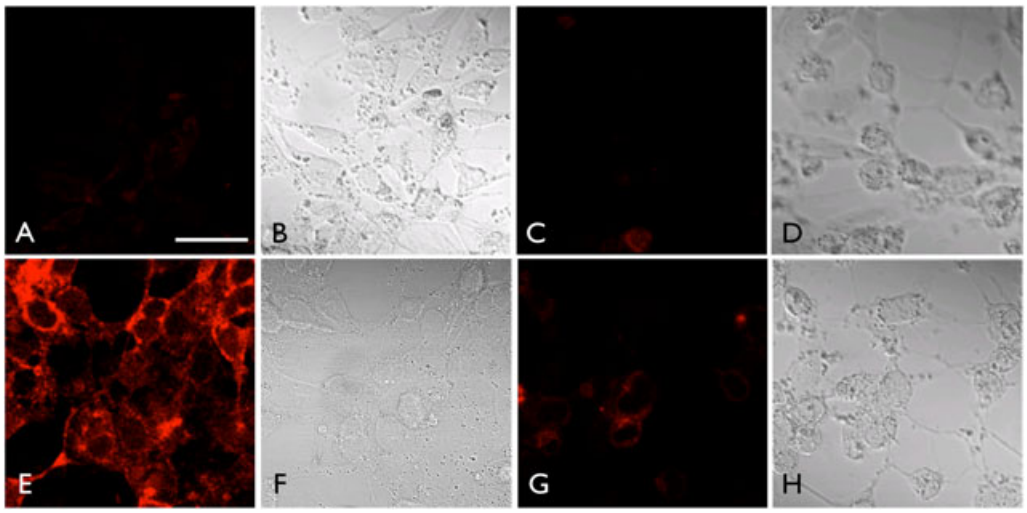

Figure 7. Effect of 1246TGG on viral protein synthesis. The cells were infected with HSV-1 at a MOI of 20 at 37 °C for 2 h. Following removal of virus inoculum, the infected cells were overlaid with maintenance medium with (A, B, C, D) or without (E, F, G, H) 25 μg/mL (31.70 μM) of 1246TGG and incubated until 9 h (A, B, E, F) or 20 h (C, D, G, H) post-infection before being stained with glycoprotein B-specific monoclonal antibody. Glycoprotein B (red) was then visualized in a confocal laser scan microscope (LSM 510, Zeiss; bar, 40 μm). (A, C, E, G): fluorescence image. (B, D, F, H): bright field image. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr

Thus, concentrations below 63.41 μM were used for further antiviral studies.

1246TGG displayed anti-HSV-1 activity at early stages of infection, including suppressing viral attachment, penetration and inactivating viral particles. Nevertheless, based on the prime property of polyphenols to bind with proteins, together with relevant reports on polyphenols' ability to inactivate viral particles (Song *et al.*, 2005; Uozaki *et al.*, 2007; Isaacs *et al.*, 2008), we still maintain that 1246TGG might exert antiviral activity by inactivating extracellular viral particles directly. This possibility was further confirmed by an additional virucidal assay where more viruses were treated with 1246TGG and a shorter incubation time was allowed (Fig. 4), and by the cell pretreatment assay in which Vero cells were precultured with 1246TGG before HSV-1 infection but no decrease of virus yields was observed (Fig. 5A). The inactivation effect could probably be the consequence of interactions between 1246TGG and certain glycoproteins on the surface of the viral envelope; virus incubated with 1246TGG would be inactivated and lose the ability to complete the process of attachment and penetration.

It was found that treatment with 1246TGG within a period of 12 h post-infection could inhibit HSV-1 infection, although the addition of 1246TGG at 12 h post-infection resulted in a relatively weaker inhibitory effect. The results also suggested that 1246TGG exerts an antiviral effect mainly before 3 h p.i., which differs from the viral polymerase inhibitor ACV. To further explore the effects of 1246TGG on the intracellular biosynthesis of HSV, the expression levels of HSV-1 IE, E and L genes as well as the viral DNA synthesis were examined. As the results showed, the expression of IE gene (U_L54) was not affected, while the expression levels of both E (U_L52) and L (U_L27) genes were significantly reduced. HSV-1 DNA replication was also suppressed in the presence of 1246TGG. Besides, the synthesis of viral gB was obviously inhibited, which coincided with the decrease of U_L27 RNA synthesis. These results suggest that, apart from blocking HSV-1 infection by inactivating viral particle directly, 1246TGG could also inhibit the intracellular biosynthesis of HSV-1, including DNA replication, RNA and protein synthesis.

Since 1246TGG was found to suppress HSV-1 growth mainly before 3 h p.i. but did not decrease RNA synthesis of viral IE gene, and that the expression of downstream genes (E and L genes) were inhibited in the presence of 1246TGG, it is possible that 1246TGG might affect the expression or function of HSV-1 IE genes on post-transcriptional levels and thus block the subsequent expressions of E and L genes. Nevertheless, the possibility that 1246TGG directly targets HSV-1 DNA replication should not be excluded. Recently, eugeniin, a compound that shares a common structure with 1246TGG, was isolated from *Geum japonicum* and *Syzygium aromaticum*, and showed anti-HSV activity (Kurokawa *et al.*, 1998, 2001). It was suggested that eugeniin might be an inhibitor of HSV-1 DNA polymerase, thus one of the major target sites of eugeniin is viral DNA synthesis. This result correlates with ours that treatment of 1246TGG significantly inhibited HSV-1 DNA replication. Yet, whether 1246TGG directly targets viral DNA polymerase remains to be confirmed. Besides, another analog of 1246TGG, 1,3,4,6-tetra-*O*-galloyl-β-D-glucose from *Phyllanthus urinaria*, was also reported to inhibit HSV infection, but no further antiviral actions were explored (Yang *et al.*, 2007).

To summarize, it was found that 1246TGG possesses significant anti-HSV activity at non-toxic concentrations. The possible antiviral actions of 1246TGG involve at least two pathways, blocking infection extracellularly by inactivating free viral particles and inhibiting intracellular biosynthesis of virus, which differ from that of ACV. Thus, it probably can be used as a supplement to the treatment of ACV or other nucleoside analogs in the future. Nevertheless, further studies are still needed to elucidate the detailed

antiviral mechanisms and to investigate the *in vivo* antiviral activity of 1246TGG.

Acknowledgement

This study was supported by the Joint Funds of National Science Foundation of China (U0632010), the State Key Laboratory of Phytochemistry and Plant Resources in West China, Chinese Academy of Sciences (P2008-KF07, P2008-ZZ08) and '211 grant of MOE'.

Conflict of Interest

The authors have declared that there is no conflict of interest.

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