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Herpes simplex virus 1 (HSV-1) is a common human pathogen that causes lifelong latent infection of sensory neurons. Non-nucleoside inhibitors that can limit HSV-1 recurrence are particularly useful in treating immunocompromised individuals or cases of emerging acyclovir-resistant strains of herpesvirus. We report that chebulagic acid (CHLA) and punicalagin (PUG), two hydrolyzable tannins isolated from the dried fruits of Terminalia chebula Retz. (Combretaceae), inhibit HSV-1 entry at nontoxic doses in A549 human lung cells. Experiments revealed that both tannins targeted and inactivated HSV-1 viral particles and could prevent binding, penetration, and cell-to-cell spread, as well as secondary infection. The antiviral effect from either of the tannins was not associated with induction of type I interferon-mediated responses, nor was pretreatment of the host cell protective against HSV-1. Their inhibitory activities targeted HSV-1 glycoproteins since both natural compounds were able to block polykaryocyte formation mediated by expression of recombinant viral glycoproteins involved in attachment and membrane fusion. Our results indicated that CHLA and PUG blocked interactions between cell surface glycosaminoglycans and HSV-1 glycoproteins. Furthermore, the antiviral activities from the two tannins were significantly diminished in mutant cell lines unable to produce heparan sulfate and chondroitin sulfate and could be rescued upon reconstitution of heparan sulfate biosynthesis. We suggest that the hydrolyzable tannins CHLA and PUG may be useful as competitors for glycosaminoglycans in the management of HSV-1 infections and that they may help reduce the risk for development of viral drug resistance during therapy with nucleoside analogues.

Herpes simplex virus 1 (HSV-1) is an alphaherpesvirus that typically causes mucocutaneous lesions in oral, perioral, and other mucosal sites in the body (58). The virus commonly uses the oropharyngeal mucosa as a port of entry, and after primary infection, establishes a lifelong latent state in the host’s trigeminal ganglia sensory neurons. Sporadic recurring infections occur when HSV-1 is reactivated by various stimuli, such as sunlight, immunosuppression, menstruation, fever, or stress (23). Although primary or reactivated HSV-1 infections can be subclinical or manifested by mild and self-limited diseases, severe cases of this viral infection may lead to complications such as keratoconjunctivitis, meningitis, and encephalitis (3, 5). Importantly, corneal HSV-1 infection can lead to stromal keratitis, which remains one of the leading causes of blindness in developing countries (37). More aggressive diseases due to HSV-1 are common in immunocompromised individuals (3, 5, 23). To date, no treatment has been identified that eradicates or resolves latent infections by this ubiquitous pathogen.

HSV-1 viral entry into cells is initiated by interaction of viral envelope glycoproteins (gB and gC) with host cell surface proteoglycans (PGs) conjugated to glycosaminoglycans (GAGs) containing heparan sulfate (HS) or chondroitin sulfate (CS) moieties. These initial interactions are sufficient for viral adsorption but not viral entry (67). Subsequently, higher affinity interaction of gD with its receptors including herpesvirus entry mediator (HVEM; a member of tumor necrosis factor receptor family), nectin-1 and nectin-2 (two members of the immunoglobulin superfamily), and/or 3-O-sulfated HS, leads to fusion of the viral membrane with either the plasma or endosomal membranes of the cell through further interactions with gB, gH, and gL (29, 57, 67). Initial interaction of HSV-1 with GAGs ensures a highly efficient infection, but infection of cells deficient in HS or CS can still be achieved via the high-affinity receptors, albeit at lower efficiency. After transport of the viral capsid to the nucleus, where the HSV-1 genome is released, viral products are then expressed in a sequential and coordinated fashion and are divided into three groups of virus-specific proteins designated as immediate-early (α) (ICP0 and...
ICP4)-, early (β) (ICP8, UL42, and thymidine kinase)-, and late (γ) (gB, gC, gD, and gH)-phase proteins (73). Although cellular innate immunity is activated upon virus infection, HSV-1 can produce one or more proteins that counteract the host antiviral response (46, 49, 50).

Most anti-herpes drugs target the viral DNA polymerase and include nucleoside or pyrophosphate analogues. Acyclovir (ACV), a guanosine analogue, has been the most important clinical drug for the prophylactic or therapeutic treatment of HSV infections, and represents the gold standard for anti-HSV therapy (4, 62). However, extensive use of this drug has led to clinical problems with the emergence of ACV-resistant virus strains, particularly in immunocompromised patients, including those who have had transplantation surgery or have been infected by the human immunodeficiency virus (HIV) (9, 21, 24, 48, 70, 79). Subsequent management of ACV-resistant patients using a different class of DNA polymerase-targeting inhibitor, such as foscarnet, has also been hindered by drug resistance (21, 59). There is a need to identify alternative antiviral therapies with different modes of action to improve the treatment and control of HSV infections, especially in immunocompromised individuals.

Terminalia chebula Retz. (T. chebula), a member of the Combretaceae family, is a traditional medicinal plant that is native to India and Southeast Asian countries. The dried ripened fruit of T. chebula (Fructus Chebulae), often referred to as “myrobalsans,” contains antioxidants (15) and is commonly used as a broad-spectrum medicinal agent for the treatment of dysentery, asthma, cough, sore throat, bloody stools, and diseases of the heart and bladder (30). T. chebula is rich in tannins, which are polyphenolic secondary metabolites found in higher plants (27, 32, 36). Tannins are characterized by their relatively high molecular mass (500 to 20,000 Da) and the unique ability to form insoluble complexes with proteins, carbohydrates, nucleic acids, or alkaloids (27, 55, 63). The hydrolyzable class of tannins possesses structures that generally consist of gallic or ellagic acid esters conjugated to a sugar moiety (28, 36). These polyphenols have high affinity for proteins and polysaccharides and are thought to be the major bioactive compounds found in the leaves and the fruit of T. chebula.

Antiviral activities from hydrolyzable tannins are well documented and are generally thought to target viral adsorption to the host cell membrane (for HSV and HIV), as well as reverse transcriptase activity of HIV (reviewed in references 8 and 63). We have previously identified several tannins of various plant sources that exhibit potent antiviral activities against HSV-1 and HSV-2. These include 1,3,4,6-tetra-O-galloyl-B-d-glucose (77), casuarin A (10), ent-epiafzelechin-(4α-R8)-epiafzelechin (17), excoecarianin (16), geraniin (77), hippomannin A (76), prodelphinidin B-2 3’-O-gallate (11), prodelphinidin B-2 3,3’- di-O-gallate (12), pterocarnin A (13), and putranjivain A (14). Studies from other laboratories have also reported a series of tannins and related compounds capable of inhibiting HSV infections (25, 56, 64, 71, 72). These earlier reports provide strong precedent for our studies and suggest that the tannins constitute an excellent focus for antiviral discovery, particularly in the field of HSV therapeutics.

Identification of multiple drugs that can act on different phases of the viral life cycle can be particularly useful in managing HSV-1 infection or reactivation in either immunocompromised individuals or cases of ACV resistance. To pursue this goal, we extended our previous studies and concentrated our efforts on four chemically defined hydrolyzable tannins (Fig. 1), including chebulagic acid (CHLA), chebulinic acid (CHLI), punicalagin (PUG), and punicalin (PUN), which are present in T. chebula (39, 40, 78). Although an effect against HSV-1 has been previously reported for CHLA, the mechanism of its activity was not elucidated (71). In the present study we report that two of the tannins tested, specifically CHLA and PUG, were found to be most effective against HSV-1. Detailed studies into their inhibitory action revealed that both drugs specifically target HSV-1 particles, block virus entry into the cell, inhibit cell-to-cell spread of the virus, and reduce secondary infection from released virions. The antiviral mechanism is attributed to the binding of CHLA and PUG to viral glycoproteins that interact with cell surface GAGs. Their ability to effectively control viral entry and spread, underscore the potential of these two hydrolyzable tannins for treating HSV-1 infection and/or recurrence.

MATERIALS AND METHODS

Chemicals and reagents. Dulbecco modified Eagle medium (DMEM) and fetal calf serum (FCS) were supplied by Wisent Scientific (St-Bruno, Quebec, Canada). Gentamicin and amphotericin B (Fungizone) were purchased from Gibco-Invitrogen (Carlsbad, CA). ACV (acyclovir) was obtained from Calbiochem (EMD Biosciences, Darmstadt, Germany). Foscarnet (FOS; sodium phosphonoformate tribasic hexahydrate), dimethyl sulfoxide (DMSO), and an in vitro toxicity assay kit (XTT-based) were purchased from Sigma (St. Louis, MO).

Test compounds. Fructus Chebulae and dried leaves from T. chebula were commercially obtained from Uchida Wakanyaku Co. (Tokyo, Japan) and an herbal market in Ping-Tung, Taiwan, respectively. Prior to extraction, both materials were anatomically authenticated by C-C. Lin. CHLA, CHLI, and PUG were extracted from Fructus Chebulae, and PUN was derived from the leaves of T. chebula. The tannins were isolated and purified as described previously (39, 40). Before use, the structure of each compound was further confirmed by HPLC/UV/ESI-MS analyses, and their purities were checked by using high-pressure liquid chromatography with photodiode array detection (HPLC-PDA) as previously reported (34, 35). CHLA, CHLI, PUG, PUN, and ACV were dissolved in DMSO. FOS was dissolved in sterile double-distilled H2O. All compounds were diluted with cell culture medium before use. The final concentration of DMSO in the drug solution was below or equal to 1% at the effective doses used.

Plasmids. The pCAGGS/MCS vector and its derivative plasmids expressing HSV-1 gB (pPEP98), gD (pPEP99), gH (pPEP100), and gL (pPEP101) (54) were generously provided by Patricia G. Spear and Richard Longnecker (Northwestern University, Chicago, IL).

Cells and viruses. Vero (African green monkey kidney cells; ATCC CCL 81), HEL (human embryonic lung fibroblast; ATCC CCL 137), and A549 (human lung carcinoma; ATCC CCL-185) cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and cultured in DMEM supplemented with 10% FCS, 50 μg of gentamicin/ml, and 0.5 μg of amphotericin B/ml at 37°C in a 5% CO2 incubator. Mouse L cells (provided by Bruce W. Banfield, Queen’s University, Kingston, Ontario, Canada) and its mutant derivative cell lines gro2C (26) (obtained from Gary H. Cohen and Roselyn J. Eisenberg, University of Pennsylvania, Philadelphia, PA) and sog9 (6) were cultured as described above. Sog9/EXT1 cells were established as previously described (45) by transfecting sog9 cells with plasmid expressing the exostosin-1 (EXT1) gene and selecting in medium containing 700 μg of G418/ml. HSV-1 KO5 strain (a gift from James R. Smiley, University of Alberta, Edmonton, Alberta, Canada), HSV-1 KO5 strain with green fluorescent protein tag (HSV-1-GFP; provided by Karen L. Mossman, McMaster University, Hamilton, Ontario, Canada) (47), and vesicular stomatitis virus with green fluorescent protein tag (VSV-GFP; Indiana serotype, a gift from Brian D. Lichty, McMaster University, Hamilton, Ontario, Canada) (69) were propagated in Vero cells. HSV-1-GFP and VSV-GFP exhibit similar infectivity as their nontagged wild-type counterparts. Virus titers were determined by standard plaque assay. Overlay media containing 0.1% Gamunex (purified clinical human IgGs, provided by Andrew C. Issekutz, Dalhousie Uni-
versity, Halifax, Nova Scotia, Canada) or 2% methylcellulose were used for determination of virus titer for HSV-1 and VSV, respectively. The basal medium for the antiviral assays consisted of DMEM plus 2% FCS with antibiotics.

**Cytotoxicity assay.** The cytotoxic effects of CHLA, CHLI, PUG, and PUN on the different cell types used in the present study were measured by the calorimetric XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-5-phenylamino)-carbonyl]-2H-tetrazolium hydroxide) assay as described previously with some modifications (16). Briefly, cells were seeded in 96-well plates (10⁴ cells per well) and incubated overnight to form a monolayer. Increasing concentrations of the test compounds were then applied to the culture wells in triplicate. After incubation at 37°C for 72 h, the medium on the plate was discarded, and the cells were washed twice with phosphate-buffered saline (PBS). A volume of 100 μl of assaying solution from the in vitro XTT-based toxicology assay kit was added to each well. The plates were incubated for another 3 h to allow XTT formazan production. The absorbance was determined with an ELx800 Microplate reader (Bio-Tek Instrument, Inc., Winooski, VT) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The data were calculated as percentage of surviving cells using the following formula: cell viability (%) = (A_t/A_s) × 100%, where “A_t” and “A_s” refer to the absorbances of the test compounds and the solvent control (DMSO), respectively. The concentration of 50% cellular cytotoxicity (CC50) of the test compounds was determined as the drug concentration that yielded 50% cell death as previously described (19).

**Antiviral plaque assay and drug dose-response analysis.** A549 cells seeded in 12-well plates (5 × 10⁵ cells per well) were treated with serial dilutions of the test compounds for 15 min at 37°C and then challenged with HSV-1 (50 PFU/well) for 1 h. The inocula and drugs were subsequently removed from the wells, and the cells were washed with PBS twice and overlaid again with different dilutions of the test compounds. After further incubation for 48 h, the supernatant was removed, and the wells were fixed with methanol and stained with Giemsa stain solution (Sigma). Viral inhibition (%) was calculated as follows: [%1 – (number of plaques)_{exp}/(number of plaques)_{control}] × 100%, where “(number of plaques)_{exp}” indicates the plaque counts from virus infection with test compound treatment and “(number of plaques)_{control}” indicates the number of plaques derived from virus-infected cells with control treatment (HSV-1 with DMSO only) (10). The 50% effective concentration (EC50) for antiviral activity was defined as the concentration of antiviral compound that produced 50% inhibition of the virus-induced plaque formation (19).

For dose-response determination, A549 cells seeded in 96-well plates were infected with HSV-1-GFP (MOI = 1) in the presence or absence of the test compounds at various concentrations (0, 10, 20, 40, and 60 μM) for 24 h. The plates were then scanned by the Typhoon 9410 variable mode imager (Amer sham Biosciences; Baie d’Urfe, Quebec, Canada), and the data were analyzed by using ImageQuant TL software (Amersham Biosciences). Viral infection (%) was calculated as follows: (fluorescence_{exp} – fluorescence_{cell control})/(fluorescence_{virus control} – fluorescence_{cell control}) × 100%, where “fluorescence_{exp}” indicates the GFP expression value from the virus-infected wells with drug treatment, “fluorescence_{cell control}” signifies the GFP expression value of the cell control (DMSO only), and “fluorescence_{virus control}” indicates the GFP expression value derived from virus-infected cells with control treatment (HSV-1-GFP with DMSO only). Values were obtained from three independent experiments with each sample assay performed in triplicate. A standard curve was also generated to ensure linear correlation between virus infection and GFP expression at the multiplicity of infection (MOI) assessed.

**Assays for effect of tannin treatment at different times.** The effect of drug addition over time was assessed according to a previously reported method with some modifications (41). To assess the effect of pretreating cells with tannins, A549 cell monolayers seeded in 12-well plates were treated with CHLA (60 μM) and PUG (40 μM) for 24 h (long term) or 1 h (short term) and then washed with PBS before challenge with HSV-1 (50 PFU/well) in DMEM containing 2% FCS. To study the effect of adding tannins and virus concurrently, A549 cells were

![Chemical structures of chebulagic acid (CHLA), chebulinic acid (CHLI), punicalagin (PUG), and punicalin (PUN). Components of the tannins including galloyl, hexahydroxydiphenoyl (HHDP), gallagyl, and chebuloyl units are indicated.](http://jvi.asm.org/Download/4388/lin_et_al_.figure1.jpg)
fluorescent plaques at 48 h p.i. plates were scanned by a Typhoon 9410 variable mode imager to visualize twice and subsequently infected with VSV-GFP at an MOI of 0.01 for 1 h before interferon (IFN-α; H9262) were washed and subsequently overlaid with media containing CHLA (60 μM) or PUG (40 μM). For the continuous drug treatment, cells were pretreated for 1 h with the tannins, challenged with HSV-1 in the presence of the drugs, and overlaid with media containing the test compounds after viral entry. For all of the above experiments, viral plaques were stained and counted following a total incubation of 48 h postinfection (p.i.). DMSO (0.1%) treatment was included as control in each condition. 

VSV plaque reduction assay for host innate immunity. To evaluate whether CHLA and PUG induced host innate immune response, a VSV plaque reduction assay was performed. Briefly, A549 cells were seeded in 12-well plates (5 × 10^4 cells per well) and then pretreated with CHLA (60 μM), PUG (40 μM), or alpha interferon (IFN-α) from human leukocytes (1,000 U/ml; Sigma) or with medium and DMSO (0.1%) controls for 24 h. Cell monolayers were washed with PBS twice and subsequently infected with VSV-GFP at an MOI of 0.01 for 1 h before the overlay media containing 2% FCS and 2% methyleneblue were added. The infected cell monolayers were then incubated in the presence of CHLA (60 μM), PUG (40 μM), heparin (100 μg/ml), or DMSO (0.1%) for an additional 20 min at 37°C to facilitate HSV-1 penetration. At the end of the incubation, extracellular virus was inactivated by citrate buffer (pH 3.0) for 1 h and then cells were washed with PBS and overlaid with DMEM containing 2% FCS. After 48 h of incubation at 37°C, viral plaques were stained and counted.

Effect of tannin addition on viral RNA expression at different times postinfection. The effects of tannin addition on HSV-1 RNA expression within the cell was performed by reverse transcriptase PCR (RT-PCR) analysis. A549 cells were infected with HSV-1 (MOI = 1) for 1 h and then treated with low pH citrate buffer (pH 3.0) to inactivate viral particles. Cells were then overlaid with medium containing CHLA (60 μM), PUG (40 μM), or DMSO control (0.1%). At 4, 8, and 12 h p.i., total cellular RNA was isolated using TRIzol Reagent (Invitrogen), treated with DNase I (Qiagen, Inc., Mississauga, Ontario, Canada) to remove genomic DNA, and purified by phenol-chloroform according to the protocols of the manufacturer. Aliquots of 1 μg of RNA were used to synthesize cDNA using SuperScript III reverse transcriptase (Invitrogen). The following primers were used for the following RT-PCR amplification: TK, and late (gD) genes and also against the cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene: ICP27 forward primer, 5'-GCCCGCAGA GACTCGGAGTCGTTGAC-3'; ICP27 reverse primer, 5'GCCGCGGAC CGCCTGGTAAGC-3'; gD reverse primer, 5'-AGGTATCGCGCGGCCGGGT-3' (533 bp); gD forward primer, 5'-ATGGAGGACCCTGACTCTAC-3'; gD reverse primer, 5'-CTCGGTTCGTCAGGATAAAG-3' (250 bp); GAPDH forward primer, 5'-GCCCTTCGACACACCTGTCCG-3'; and GAPDH reverse primer, 5'-AGCCG TGTTTACACCTGTCC-3' (349 bp). For comparison, A549 cells were also infected with HSV-1 (MOI = 1) in the presence of CHLA (60 μM), PUG (40 μM), or DMSO (0.1%) at time 0 (co-incubation). After incubation for 1 h at 37°C, the inocula and tannins were removed, and the cells were washed with PBS before being overlaid with DMEM containing 2% FCS. Again, at 4, 8, and 12 h p.i., total cellular RNA was harvested and subjected to RT-PCR analysis as described above.

Effect of tannins on HSV-1 secondary infection and cell-to-cell spread. To assess the effect of the tannins on secondary viral infections, confluent A549 cells were seeded in 12-well plates and then infected with HSV-1-GFP (200 μl/well) for 1 h. The cells were then washed twice with ice-cold PBS to remove unattached virus, incubated at 37°C for 1 h. Photomicrographs were taken at 24 h p.i. for comparison, A549 cells were also infected with HSV-1 (MOI = 1) in the presence of CHLA (60 μM), PUG (40 μM), or DMSO (0.1%) at time 0 (co-incubation). After incubation for 1 h at 37°C, the inocula and tannins were removed, and the cells were washed with PBS before being overlaid with DMEM containing 2% FCS. Again, at 4, 8, and 12 h p.i., total cellular RNA was harvested and subjected to RT-PCR analysis as described above.

Viral penetration assay. The viral penetration assay was performed as previously described (41) with minor modifications. A549 cell monolayers grown in 12-well plates were prechilled at 4°C for 1 h and subsequently incubated with HSV-1 (100 μl/well) for 3 h at 4°C for allowing viral adsorption. The infected cell monolayers were then incubated in the presence of CHLA (60 μM), PUG (40 μM), heparin (100 μg/ml), or DMSO (0.1%) for an additional 20 min at 37°C to facilitate HSV-1 penetration. At the end of the incubation, extracellular virus was inactivated by citrate buffer (pH 3.0) for 1 h and then cells were washed with PBS and overlaid with DMEM containing 2% FCS. After 48 h of incubation at 37°C, viral plaques were stained and counted.

Effect of tannin addition on viral RNA expression at different times postinfection. The effects of tannin addition on HSV-1 RNA expression within the cell was performed by reverse transcriptase PCR (RT-PCR) analysis. A549 cells were infected with HSV-1 (MOI = 1) for 1 h and then treated with low pH citrate buffer (pH 3.0) to inactivate viral particles. Cells were then overlaid with medium containing CHLA (60 μM), PUG (40 μM), or DMSO control (0.1%). At 4, 8, and 12 h p.i., total cellular RNA was isolated using TRIzol Reagent (Invitrogen), treated with DNase I (Qiagen, Inc., Mississauga, Ontario, Canada) to remove genomic DNA, and purified by phenol-chloroform according to the protocols of the manufacturer. Aliquots of 1 μg of RNA were used to synthesize cDNA using SuperScript III reverse transcriptase (Invitrogen). The following primers were used for the following RT-PCR amplification: TK, and late (gD) genes and also against the cellular glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene: ICP27 forward primer, 5'-GCCCGCAGA GACTCGGAGTCGTTGAC-3'; ICP27 reverse primer, 5'GCCGCGGAC CGCCTGGTAAGC-3'; gD reverse primer, 5'-AGGTATCGCGCGGCCGGGT-3' (533 bp); gD forward primer, 5'-ATGGAGGACCCTGACTCTAC-3'; gD reverse primer, 5'-CTCGGTTCGTCAGGATAAAG-3' (250 bp); GAPDH forward primer, 5'-GCCCTTCGACACACCTGTCCG-3'; and GAPDH reverse primer, 5'-AGCCG TGTTTACACCTGTCC-3' (349 bp). For comparison, A549 cells were also infected with HSV-1 (MOI = 1) in the presence of CHLA (60 μM), PUG (40 μM), or DMSO (0.1%) at time 0 (co-incubation). After incubation for 1 h at 37°C, the inocula and tannins were removed, and the cells were washed with PBS before being overlaid with DMEM containing 2% FCS. Again, at 4, 8, and 12 h p.i., total cellular RNA was harvested and subjected to RT-PCR analysis as described above.

Effect of tannins on HSV-1 secondary infection and cell-to-cell spread. To assess the effect of the tannins on secondary viral infections, confluent A549 cells were seeded in 12-well plates and then infected with HSV-1-GFP (200 μl/well) for 1 h. The cells were then washed twice with ice-cold PBS to remove unattached virus, incubated at 37°C for 1 h. Photomicrographs were taken at 24 h p.i. for comparison, A549 cells were also infected with HSV-1 (MOI = 1) in the presence of CHLA (60 μM), PUG (40 μM), or DMSO (0.1%) at time 0 (co-incubation). After incubation for 1 h at 37°C, the inocula and tannins were removed, and the cells were washed with PBS before being overlaid with DMEM containing 2% FCS. Again, at 4, 8, and 12 h p.i., total cellular RNA was harvested and subjected to RT-PCR analysis as described above.

Viral fusion cell association assay. To examine whether the compounds interacted with HSV-1 glycoproteins to inhibit glycoprotein-mediated fusion events, a virus-cell free fusion assay was performed (54). A549 cells were seeded in six-well dishes and transfected with plasmid DNA expressing the individual HSV-1 glyco-
Infection using the ImageQuant TL software. The data were expressed as the percent viral titration for 48 h, fluorescent plaques were scanned as described above and counted before overlaying them with DMEM containing 2% FCS. After further incubation at 37°C, the virus-drug mixture was removed, and the cells were washed with PBS. Growth of virus in the host cell was assessed, and similar results were observed (data not shown).

Antiviral effects were evaluated by plaque assay to determine the effective concentration that achieved 50% inhibition (EC₅₀) against HSV-1 infection. The SI was also assessed, and similar results were observed (data not shown). Given their higher SI values, CHLA and PUG were chosen for subsequent analyses.

To obtain a more accurate dose-response curve for these two hydrolyzable tannins, A549 cells were infected with HSV-1-GFP (MOI = 1) in the presence of the tannins, and fluorescent signals were quantified. The HSV-1-GFP was susceptible to the antiviral effects of the tannins. Both CHLA and PUG displayed anti-HSV-1 activity in a dose-dependent manner (Fig. 2), and the concentrations of CHLA at 60 μM and PUG at 40 μM, which provided near complete protection against the virus infection at an MOI of 1, were chosen for all subsequent experiments.

Antiviral activities of CHLA and PUG depend upon the presence of HSV-1, and inhibition is not due to activation of host cell innate immunity. To better understand the antiviral mechanism and the stage of HSV-1 infection affected by these two T. chebula tannins, we added the compounds at different times of the virus life cycle (pre-entry, entry, and post-entry). In order to study pre-entry, more specifically the effect of the compounds on the cell itself prior to virus addition, A549 cells were pretreated with CHLA and PUG for long-term (24 h) or short-term (1 h) periods and then washed prior to HSV-1 infection. For effects on the viral entry stage, virus and the drugs were simultaneously applied to the cells. To investigate events after virus entry, A549 cells were first infected with HSV-1 for 1 h and then treated with the tannins. For comparison, the tannins were also maintained throughout the experimental period.

Pretreating A549 cells with CHLA and PUG (both long term and short term) did not protect against HSV-1 infection.

### RESULTS

#### Inhibition of HSV-1 infection by the hydrolyzable tannins

CHLA, CHLI, PUG, and PUN (Fig. 1) have been reported to exhibit antiviral activities. We investigated whether these hydrolyzable tannins could inhibit HSV-1 infection. In order to ensure that the tannin concentrations were not cytotoxic, a toxicity analysis was carried out in A549 cells by using a XTT cell viability assay. Our results indicated that these four tannins did not have apparent cytotoxic effects below 100 μM in A549 cells, while a dose-dependent cytotoxic effect was seen when concentration >100 μM was used (data not shown). The 50% cellular toxicity indices (CC₅₀) of CHLA, CHLI, PUG, and PUN were 316.87 ± 9.01, 330.83 ± 9.07, 318.84 ± 4.98, and 310.85 ± 1.99 μM, respectively (summarized in Table 1). For comparison, toxicity in primary human fibroblast (HEL) cells was also assessed, and similar results were observed (data not shown).

We then evaluated the antiviral effects of these four natural compounds against HSV-1 infection by using a plaque assay. ACV and FOS were used as positive controls, and DMSO was included as a negative control. FOS is the treatment of choice in the clinical setting if resistance develops against ACV. All four tannins could inhibit viral plaque formation, after inoculation of 50 PFU, in a dose-dependent manner, and their 50% effective concentration (EC₅₀) values were 17.02 ± 2.82 (CHLA), 20.85 ± 2.40 (CHLI), 10.25 ± 1.13 (PUG), and 21.33 ± 1.77 μM (PUN) (Table 1). The selectivity index (SI), which measures the preferential antiviral activity of a drug in relation to its cytotoxicity, was calculated according to their CC₅₀ and EC₅₀. The SIs of CHLA, CHLI, PUG, and PUN were 18.62, 15.87, 31.11, and 14.57, respectively (Table 1).

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<th>Compound</th>
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<th>Mean EC₅₀ (μM) ± SEM</th>
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<td>&gt;2,000</td>
<td>183.37 ± 25.18</td>
<td>&gt;10.91</td>
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*The values shown are means from three independent experiments with each treatment performed in triplicate.

### Table 1. Cytotoxicity and anti-HSV-1 activity of CHLA, CHLI, PUG, and PUN in A549 cells

![Graph showing dose-response for inhibition of HSV-1 infection in A549 cells by CHLA and PUG.](http://jvi.asm.org)

**FIG. 2.** Dose response for inhibition of HSV-1 infection in A549 cells by CHLA and PUG. A549 cells were seeded into 96-well plates and then infected with HSV-1-GFP (MOI = 1) in the presence of the tannins, and fluorescent signals were quantified. The HSV-1-GFP was susceptible to the antiviral effects of the tannins. Both CHLA and PUG displayed anti-HSV-1 activity in a dose-dependent manner (Fig. 2), and the concentrations of CHLA at 60 μM and PUG at 40 μM, which provided near complete protection against the virus infection at an MOI of 1, were chosen for all subsequent experiments.

Antiviral activities of CHLA and PUG depend upon the presence of HSV-1, and inhibition is not due to activation of host cell innate immunity. To better understand the antiviral mechanism and the stage of HSV-1 infection affected by these two T. chebula tannins, we added the compounds at different times of the virus life cycle (pre-entry, entry, and post-entry). In order to study pre-entry, more specifically the effect of the compounds on the cell itself prior to virus addition, A549 cells were pretreated with CHLA and PUG for long-term (24 h) or short-term (1 h) periods and then washed prior to HSV-1 infection. For effects on the viral entry stage, virus and the drugs were simultaneously applied to the cells. To investigate events after virus entry, A549 cells were first infected with HSV-1 for 1 h and then treated with the tannins. For comparison, the tannins were also maintained throughout the experimental period.

Pretreating A549 cells with CHLA and PUG (both long term and short term) did not protect against HSV-1 infection.
Both tannins were effective in preventing plaque formation when added during virus adsorption, immediately after viral entry, and throughout multiple cycles of virus replication (Fig. 3). The data indicate that HSV-1 infection is severely impaired only if the drugs are present at the time of infection or during viral spread and that it is unlikely that the antiviral activity is due to direct effects on the cells (such as masking cellular receptors or entry factors for HSV-1). To confirm that neither of the tannins activated host cell innate immunity and induced production of antiviral cytokines such as interferons (IFNs), a VSV plaque reduction assay was performed. VSV replication is extremely sensitive to cellular IFN production. IFN-α, a potent inhibitor of VSV, was included as a positive control. In line with the above observation, neither CHLA nor PUG pretreatments protected A549 cells from VSV infection, whereas IFN-α treatment produced an intact cell monolayer (Fig. 4). Moreover, neither of the tannins induced IFN-stimulated genes in the A549 cells (data not shown). These results suggest that the anti-HSV-1 activities produced by CHLA and PUG (i) are unlikely to be mediated by effects through binding to the cellular receptors for HSV-1 or in triggering antiviral innate immunity and (ii) absolutely require the presence of HSV-1.

CHLA and PUG block HSV-1 entry by inactivating virus particles and preventing virus attachment and penetration into A549 cells. In order to evaluate the antiviral mechanism of CHLA and PUG, we investigated their effects on the virus particles themselves. Tannin compounds were preincubated with virus particles and then diluted to a subtherapeutic concentration prior to infection. Both CHLA and PUG could interact with virus particles, irreversibly, to prevent infection (Fig. 5A). This suggests that CHLA and PUG can bind to virus particles and neutralize virus infectivity.

We next assessed the ability of CHLA and PUG to affect viral attachment and penetration. The attachment assay was carried out at 4°C, which allows for virus binding but prevents entry (41). Using ELISA to detect bound virus on the adherent cells, both tannin compounds were observed to inhibit HSV-1 attachment to the A549 cell surface in a dose-dependent manner (Fig. 5B). An alternative binding assay was also performed using virus-specific antibodies to detect bound HSV-1 particles by flow cytometry. Again, CHLA and PUG could prevent HSV-1 binding to the surface of the target A549 cells (Fig. 5C). Heparin, a competitive HSV binding inhibitor, was included as a positive control in both experiments. These results suggest that CHLA and PUG might interact with viral glycoprotein(s) and/or cellular receptor(s) during the virus attachment phase. To further assess the effects of CHLA and PUG on virus penetration step, HSV-1 particles were allowed to first bind to A549 cells at 4°C and were subsequently allowed to fuse with and penetrate the host cell membrane by shifting the temperature to 37°C in the presence or absence of the tannins. As shown in Fig. 5D, CHLA retained most of its antiviral activity even during the viral penetration phase and PUG could completely abrogate virus penetration into the A549 cells, resulting in a protected monolayer. In contrast, heparin, which is effective at blocking HSV-1 adsorption, did not prevent subsequent virus penetration (42). The data indicate that CHLA and PUG impair viral receptor attachment and penetration functions during the HSV-1 infection.

CHLA and PUG do not affect HSV-1 transcription after entry but limit secondary viral infection and cell-to-cell transmission. The observation in Fig. 3 that CHLA and PUG inhibited HSV-1 plaque formation when treatment was initiated immediately after the virus had entered the cell suggested that the two tannins may block HSV-1 replication cycle or inhibit...
HSV-1 secondary infection and/or cell-to-cell spread in the ensuing incubation period. To specifically address these possibilities, we first evaluated the effects of CHLA and PUG on HSV-1 mRNA expression after virus entry. A549 cells were infected with HSV-1 for 1 h; extracellular virus was then inactivated by citrate buffer treatment and washed away; and CHLA, PUG, or DMSO was subsequently added to the cells. For comparison, CHLA and PUG were also added simultaneously with HSV-1. Total cellular RNA was isolated from all samples at various time points after viral infection. Our results clearly indicated that CHLA and PUG did not affect HSV-1 mRNA expression after virus penetration, since levels of immediate-early (ICP27), early (TK), and late (gD) viral gene transcripts were unaffected by the compounds (Fig. 6A).
the other hand, both tannins suppressed HSV-1 mRNA synthesis when added together with the virus at the same time (Fig. 6B). These findings suggest that neither CHLA nor PUG inhibit HSV-1 transcription following penetration of the host cell.

We next examined whether CHLA and PUG inhibited HSV-1 secondary infection and/or cell-to-cell spread. A fluorescent plaque assay was performed using HSV-1-GFP. After viral inoculation of A549 cells, CHLA and PUG were added to the overlay media at 12 or 24 h p.i. in the presence of the tannins was applied. Total cellular RNA was isolated for RT-PCR analysis as in panel A. Representative data shown are from one of two independent experiments.

FIG. 6. CHLA and PUG do not affect HSV-1 transcription, following entry into the host cell. (A) A549 cells were infected with HSV-1 (MOI = 1) for 1 h, treated with low-pH citrate buffer (pH 3.0) to inactivate noninternalized extracellular viral particles, and subsequently overlaid with medium containing CHLA (60 μM), PUG (40 μM), or DMSO control (0.1%). At 4, 8, and 12 h p.i., total cellular RNA was isolated, subjected to first-strand synthesis by reverse transcription, and then amplified by standard PCR procedures with primers against HSV-1 immediate-early (ICP27), early (TK), and late (gD) gene products. GAPDH was included as a control. (B) A549 cells were coincubated with HSV-1 (MOI = 1) and CHLA (60 μM), PUG (40 μM), or DMSO control (0.1%) for 1 h. Cells were washed with PBS before overlay media without the tannins was added. Total cellular RNA was isolated for RT-PCR analysis as in panel A. Representative data shown are from one of two independent experiments.

Several HSV-1 glycoproteins are known to interact with cell surface GAGs. To further explore the virus-host interactions that are being targeted by the tannins, we used a series of cell lines known to possess defects in surface HS and CS synthesis. The relative infectivities of HSV-1 (KOS) are ca. 10% for HS-deficient gro2C cells and 0.5% for HS/CS-deficient sog9 cells compared to parental mouse L cells (6). Stable expression of the EXT1 gene in sog9 cells (sog9-EXT1) restores HS biosynthesis and susceptibility to HSV-1 infection (43). To evaluate the effects of the drugs in the presence or absence of GAG expression, each cell line was infected with different dilutions of HSV-1 sufficient to achieve 200 PFU/well (MOI = 0.0004) in the presence of the tannins. CHLA and PUG effectively protected the parental mouse L cells and sog9-EXT1 cells from infection, but antiviral effects were diminished in HS-deficient gro2C cells, and almost completely abolished in HS/CS-deficient sog9 cells (Fig. 9). Similar results were obtained in experiments using different MOIs (data not shown). These observations strongly suggest that CHLA and PUG target interactions between HSV-1 glycoproteins and
GAGs. CHLA inhibition also appeared to be more sensitive to cell surface deficiency in GAGs compared to that of PUG. Taken together, the data indicate that CHLA and PUG function as GAG competitors to inhibit the initial events of HSV-1 infection (adsorption and penetration) and the cell-to-cell spread of virus. The interaction of HSV-1 glycoproteins with cellular GAGs plays a critical role in viral infections, and the hydrolyzable tannins could offer a primary means of defense against HSV-1 infections.

DISCUSSION

There is currently no cure that completely resolves latent infections caused by alpha-herpesviruses. Therefore, the development of small molecules capable of inhibiting infection by reactivated virus represents an attractive therapeutic strategy, particularly in immunocompromised individuals who are often at risk of generating ACV-resistant HSV-1 strains. In a search for such molecules, we report that CHLA and PUG, two hydrolyzable tannins isolated from the fruits of *T. chebula*, effectively inhibited HSV-1 infection in A549 cells without significantly reducing cell viability. In addition, our results suggest that CHLA and PUG specifically targeted HSV-1 particles by binding to viral glycoproteins that interact with cellular GAGs, rendering the virus incapable of adsorbing, penetrating, and spreading throughout the cell monolayer. These features underscore the potential of tannins as HSV-1 entry inhibitors.

Our data show that entry events, including primary and secondary infection, viral attachment and/or penetration, and cell-to-cell spread are inhibited only when the tannins and HSV-1 glycoproteins are in contact with each other. Pretreatment of host cells with the tannins, followed by washes to remove unadsorbed compounds, had no effects upon HSV-1 replication. This indicated that masking cell surface receptors or entry factors for HSV-1 by the tannins is unlikely. Viral binding assays using ELISA and flow cytometry analyses revealed that the tannins blocked viral attachment to the host cell. While CHLA and PUG could inactivate the HSV-1 particles, we do not believe that a direct lysis effect of the viral membrane is responsible for their effects, since HSV-1 infection of GAG-deficient mutant cell lines was still observed, even in the presence of these compounds (Fig. 9). Given their large molecular weights (CHLA, 954; and PUG, 1,084) and high affinity for proteins and sugars, the two hydrolyzable tannins are thought to bind to HSV-1 glycoproteins on the infectious virions making them inert, impairing glycoprotein function, and preventing successful attachment and entry of the host cell. These tannins could also bind to viral glycoproteins on the infected-cell surface, rendering them unavailable to mediate the cell-to-cell spread of virus.

HSV-1 entry into epithelial cells, which express the cellular receptors (HS, HVEM, nectin-1, and nectin-2) for HSV (67), requires an ordered and concerted effort from the viral glycoproteins. Specifically, the glycoproteins gB, gC, gD, gH, and gL interact with host cell receptors and are involved in penetration of the plasma membrane through a membrane fusion

FIG. 7. CHLA and PUG can limit HSV-1 secondary infection and cell-to-cell spread of the virus. A549 cells were infected with HSV-1-GFP (200 PFU/well) for 1 h and then treated with citrate buffer (pH 3.0) to inactivate noninternalized extracellular viral particles. Cells were overlaid with medium alone (A) or medium containing 0.1% neutralizing antibody (B). After a p.i. incubation period of 12 h (A) or 24 h (B), the infected cells were treated with CHLA (60 μM), PUG (40 μM), heparin (100 μg/ml), or DMSO (0.1%), before further incubation for a total of 48 h after the initial infection. Over the course of infection subsequent to the drug addition, the plates were scanned and quantified for fluorescent viral plaques (A) or photographed using an inverted fluorescence microscope at ×100 magnification (B). (A) Number of fluorescent plaques counted between 24 and 48 h p.i. with drug treatment initiated at 12 h after viral challenge. The data shown are means ± the SEM of three independent experiments with each treatment performed in duplicate. (B) Comparison of viral plaque size between 24 and 48 h p.i. with drug treatment initiated at 24 h after viral challenge. Scale bars, 100 μm. Representative images are from one of two independent experiments.
process (29, 57, 67). While viral entry and spread require a particular combination of viral surface proteins, several HSV-1 glycoproteins are repeatedly involved in both processes. Importantly, gB and gD function in viral binding and fusion and are also engaged during cell-to-cell transmission in cultured epithelial cells (22, 29, 33, 38, 57, 60, 61, 67). The associations between viral glycoproteins that mediate HSV-1 attachment and entry represent a complex scenario when considering CHLA and PUG and their mechanism of action. The candidate targets of the tannins likely involve viral glycoproteins that interact with host cell surface GAGs and participate in adsorption, membrane fusion, and cell-to-cell spread. The observation that both tannins blocked virus attachment to cells, as did heparin, suggests that interaction of gC and gB with heparan sulfate proteoglycans (HSPGs) is targeted. However, the drugs also prevented virus internalization into cells in the postbinding phase. At this point, the interaction with HSPGs should be irrelevant, since virions now interact with a gD receptor and become resistant to removal by heparin (42) (Fig. 5D). One explanation is that the tannins bind to gB and block its interaction with HSPG while also interfering with its subsequent role in membrane fusion during virus entry (in which gC is not involved). Alternatively, the tannins may be impeding the activity of additional glycoproteins (gD and/or gH/gL) or working by some other mechanism(s) to prevent successful entry into the A549 cells. Finally, there is the possibility that viral glycoproteins may still be accessible to the tannins, even when these viral proteins are bound to the host cell or are expressed in the intercellular junctions. This could explain why the considerably larger heparin (molecular weight, 17,000 to 19,000) can bind free gB but is unable to interact efficiently with the shielded glycoprotein that is needed in order to inhibit viral penetration or cell-to-cell transmission. Additional binding experiments using glycoprotein-deficient HSV-1 mutants,
as well as soluble recombinant HSV-1 glycoproteins could help elucidate the targeting specificity of the tannins. We speculate that the two natural compounds can bind to all GAG-interacting glycoproteins, including gB, gC, and/or gD, and neutralize their functions. The ability of CHLA and PUG to effectively block virus membrane penetration, as well as virus attachment, could explain their higher efficacy in restricting the spread of HSV-1 compared to the inhibitory effects of heparin.

In the case of HSV-1, the interactions between several of its glycoproteins and cell surface GAGs are critical for ensuring efficient viral entry, as well as viral spread (6, 26, 44, 52, 53, 65, 68). CS can confer susceptibility to HSV-1 infection in the absence of HS (7, 26), but HS is still the preferred substrate for viral attachment. GAG deficiency renders cell surfaces relatively resistant to HSV-1 binding and residual infectivity relies on stable attachment receptors (6). Earlier studies have shown that the HS/CS-deficient sog9 cells are insensitive to inhibitory effects mediated by soluble HS on HSV-1 infection (6). We observed that the absence of HS in gro2C cells weakened the tannins’ inhibitory effects, the absence of HS and CS on sog9 cells whereas the absence of HS in gro2C cells weakened the tannins’ inhibitory effects, the absence of HS and CS on sog9 cells whereas the absence of HS in gro2C cells weakened the tannins’ inhibitory effects of recombinant HIV gp120 to CD4 and to exert inhibitory effects on HIV-1 RT and integrase (1, 51, 74). The ability of these natural agents to inhibit both HSV-1 and HIV-1 underscores their potential value in the treatment of AIDS patients who also exhibit HSV-1-related symptoms.

Use of these tannins could improve the prognosis of anti-HSV-1 therapy in immunosuppressed individuals and help to reduce the risk of ACV resistance by lowering the ACV dose required. Since Fructus Chebulae contains both CHLA and PUG, inclusion of purified extracts from this plant in topical creams or microbicides would be a feasible method for controlling recurrent HSV-1 infections. Future studies will determine whether these natural products are effective against additional members of the herpesvirus family and other enveloped viruses. Our preliminary studies have shown that both CHLA and PUG inhibit the growth of HSV-2 and human cytomegalovirus (L.-T. Lin and T.-Y. Chen, unpublished data). Other viruses known to use GAGs as entry factors, such as measles virus and human respiratory syncytial virus, are also inhibited by these tannins, reinforcing our discovery that these compounds act as GAG competitors that inhibit viral glycoprotein-cell receptor interactions (Lin and Chen, unpublished). Further studies with the tannins derived from T. chebula may provide new ways to inhibit recurrent HSV-1 infections and control the reemergence of this virus in immunocompromised patients.

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We have declared that no competing interests exist.

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