Herpes simplex virus (HSV) is a single large double-strand DNA-enveloped virus, which causes localised skin infection of the mucosal epithelia of the genitalia, the oral cavity, the pharynx, the oesophagus and the eye, depending upon the type of virus involved. After primary infection, HSV establishes latency in sensory and autonomic neurones that innervate the mucosal tissue where primary infection takes place (Baringer & Swoveland, 1973). Proper stimuli will reactivate HSV to cause recurrence (Roizman & Sears, 1996).

Immunocompromised patients, including recipients of organ transplantation or acquired immunodeficiency syndrome (AIDS) patients, are either at high risk for increased severity of HSV infection (Pass et al., 1978) or in danger of recurrence of HSV infection (Logan et al., 1971). HSV has been shown to be a factor for the spreading of human immunodeficiency virus (HIV) and causes severe disease in AIDS patients (Mann et al., 1984). In addition, the immediate early gene of HSV has also been shown to be able to stimulate the activation of genes belonging to HIV (Ostrove et al., 1987), varicella-zoster virus (Fehrer et al., 1988) or human papillomavirus type 18 (Gius & Laimins, 1989).

To date, only acyclovir, valacyclovir, penciclovir and famciclovir, which target viral DNA replication, have been successfully developed for anti-HSV therapies. Although these drugs have been successfully used in treating active infection, they fail to modulate the recurrence of latent virus and become ineffective when resistance mutations occur (Coen, 1994). Consequently, there is a need to search for new and more effective antiviral agents as an effort to prevent and/or to treat HSV infection in the future.

Recently, many pure compounds from plants have been shown to possess antiviral activity against HSV. These pure compounds include alkaloids (Martin, 1987), flavonoids (Lin et al., 1999), saponins (Sindambiwe et al., 1998), quinines (Anderden et al., 1991), terpenes (Bourne et al., 1999), lignans (Charlton, 1998), tannins (De Bruyne et al., 1999), polysaccharides (Bourne et al., 1999), steroidal glycoside (Ikeda et al., 2000), thiosulfinates (Tsai et al., 1985) and proteins (Aoki et al., 1995).

As an effort to search new alternative antiviral compounds a series of experiments were conducted to investigate the antiviral properties of prodelphinidin B-2 3′-O-gallate against HSV-2 in vitro.

**Antiviral properties of prodelphinidin B-2 3′-O-gallate from green tea leaf**

Hua-Yew Cheng1, Chun-Ching Lin1* and Ta-Chen Lin2

1Graduate Institute of Pharmaceutical Science, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China
2Department of Pharmacy, Ta-Jen Institute of Technology, Ping-Tung, Taiwan, Republic of China

*Corresponding author: Tel: +886 73 121 101; Fax: +886 73 135 215; E-mail: aalin@ms24.hinet.net

Prodelphinidin B-2 3′-O-gallate, a proanthocyanidin gallate isolated from green tea leaf, was investigated for its anti-herpes simplex virus type 2 properties in vitro. Prodelphinidin B-2 3′-O-gallate exhibited antiviral activity with IC50 of 5.0 ±1.0 µM and 1.6 ±0.3 µM for XTT and plaque reduction (PRA) assays, respectively. Cytotoxicity assay had shown that prodelphinidin B-2 3′-O-gallate possessed cytoxic effect toward Vero cell at concentration higher than its IC50. The 50% cytotoxic concentration for cell growth (CC50) was 33.3 ±3.7 µM. Thus, the selectivity index (SI) (ratio of IC50 to CC50) for XTT assay and PRA was 6.7 and 20.8, respectively. Prodelphinidin B-2 3′-O-gallate significantly reduced viral infectivity at concentrations 10 µM or more. Result of time-of-addition studies suggested that prodelphinidin B-2 3′-O-gallate affected the late stage of HSV-2 infection. In addition, it was also shown to inhibit the virus from attaching and penetrating into the cell. Thus, prodelphinidin B-2 3′-O-gallate was concluded to possess antiviral activity with mechanism of inhibiting viral attachment and penetration, and disturbing the late stage of viral infection.

**Keywords:** prodelphinidin B-2 3′-O-gallate, anti-HSV-2 activity, viral attachment, viral penetration, late stage of viral infection
Materials and methods

Test compounds

Prodelphinidin B-2 3′-O-gallate (Figure 1) was isolated from fresh green tea leaves as described in literature (Nonaka et al., 1983). Briefly, fresh green tea leaves were extracted four times with 80% aqueous acetone at room temperature. The acetone was then removed by evaporation under reduced pressure (ca. 40°C) and the resulting aqueous solution was further filtrated to remove chlorophylls. After removing chlorophylls, the filtrate was extracted with seven successive equal volumes of ethyl acetate. The resulting ethyl acetate layer was concentrated to dryness (ca. 40°C) and then chromatographed on Sephadex LH-20 to give four fractions. Fraction IV was further divided by Diaion HP-20 chromatography into three fractions, Fraction IV-a, IV-b and IV-c. Fraction IV-b was then repeatedly chromatographed over Diaion HP-20 and Sephadex LH-20 to give prodelphinidin B-2 3′-O-gallate. The structure of prodelphinidin B-2 3′-O-gallate was identified according to the NMR and physical data as described by Nonaka et al. (1983).

Acyclovir (ACV) was purchased from Sigma Company (USA). Prodelphinidin B-2 3′-O-gallate and ACV were dissolved in sterile de-ionized water and stored at 4°C for up to 2 weeks. Both compounds were stable under test conditions.

Viruses and cells

All cell culture reagents and medium were purchased from Gibco BRL (Grand Island, NY, USA). African green monkey kidney cells (Vero) (ATCC CCR-81) were obtained from the hospital of Kaohsiung Medical University (Kaohsiung, Taiwan). Cells were propagated in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 5% fetal calf serum (FCS), 200 U/ml penicillin G sodium, 200 mg/ml streptomycin sulfate and 0.5 mg/ml amphotericin B. Overlay medium for the plaque assay of HSV-2 consisted of DMEM plus 2% FCS, 1% methylcellulose and antibiotics as described above.

HSV-2 strain 196 was kindly provided by Dr. Lien-Chai Chiang (Department of Microbiology, College of Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan). Its titre was determined by plaque assay and was expressed as plaque forming units (PFU) per ml. Virus stocks were stored at ~80°C until use.

Antiviral assays

XTT assay

The antiviral activity of prodelphinidin B-2 3′-O-gallate was assayed using XTT (Sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid) as described by Weislow et al. (1989). Briefly, 10⁴ cells/well were seeded into 96-well culture plates (Falcon). After 4 h of incubation at 37°C with 5% CO₂, cells were infected with HSV-2 at multiplicity of infection (MOI)=0.5 and then various concentrations of prodelphinidin B-2 3′-O-gallate were added. The infected cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for another 72 h. The medium was then aspirated and XTT reagent was added. The plate was reincubated for an additional 2 h to allow the production of formazan. Optical densities were measured with EIA reader (Lab Systems) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The antiviral activity of prodelphinidin B-2 3′-O-gallate was determined as described by Pauwels et al. (1988). Its minimal concentration required to inhibit 50% HSV-2 growth (IC₅₀) was evaluated according to Weislow et al. (1989).

Plaque reduction assay

Vero cells were seeded into 24-well culture plates (Falcon) at a density of 10⁵ cells/well and incubated at 37°C with 5% CO₂ until reaching at least 95% confluency. Cell monolayer was then infected with 100 PFU HSV-2 in the absence or presence of prodelphinidin B-2 3′-O-gallate and further incubated at 37°C for 1 h with 5% CO₂. After 1 h of adsorption, cell monolayer was overlaid with overlay medium. Optical densities were measured with EIA reader (Lab Systems) at a test wavelength of 492 nm and a reference wavelength of 690 nm. The antiviral activity of prodelphinidin B-2 3′-O-gallate was determined as described by Pauwels et al. (1988). Its minimal concentration required to inhibit 50% HSV-2 growth (IC₅₀) was evaluated according to Weislow et al. (1989).

Plate reduction assay

Vero cells were seeded into 24-well culture plates (Falcon) at a density of 10⁵ cells/well and incubated at 37°C with 5% CO₂ until reaching at least 95% confluency. Cell monolayer was then infected with 100 PFU HSV-2 in the absence or presence of prodelphinidin B-2 3′-O-gallate and further incubated at 37°C for 1 h with 5% CO₂. After 1 h of adsorption, cell monolayer was overlaid with overlay medi-

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regression analysis of the dose-response curves generated from the data (Logu et al., 2000).

**Cytotoxicity assay**

Cellular viability was assayed with XTT (Scudiero et al., 1988). It was performed with the procedures similar to XTT assay, except that HSV-2 was not inoculated. The concentration of 50% cellular cytotoxicity (CC50) of tested compounds was calculated according to Wesslow et al. (1989).

**Evaluation of selectivity index**

The selectivity index (SI) of prodelphinidin B-2 3'-O-gallate against HSV was evaluated as the ratio of CC50 to IC50.

**Virucidal assay**

Virucidal activity of prodelphinidin B-2 3'-O-gallate was evaluated as described by Barluzzi et al. (1999). Briefly, a virus suspension containing 2×10⁵ PFU HSV-2 was mixed with or without various concentrations of prodelphinidin B-2 3'-O-gallate for 6 h at room temperature (about 26°C). The sample was then diluted and its residual infectivity was determined using the plaque-forming assay.

**Time of addition studies**

The antiviral activity of test samples was evaluated at various time periods up to 24 h according to procedures described by Bouwstra et al. (2001). Vero cells were seeded into 12-well culture plates (Nunc) at a density of 2×10⁵ cells/well and incubated at 37°C with 5% CO₂ for 24 h. Cell monolayer was then infected with 2×10⁵ PFU HSV-2/well. 5 µM of prodelphinidin B-2 3'-O-gallate was added into wells concurrent with HSV-2 infection (0 h) or at intervals of 2, 4, 7 and 12 h post-infection. After 24 h of infection, infected cells were scrapped and viruses were released from cells by freeze-thawing three times. Cell pellets were removed by centrifugation at 3000g for 10 min. The supernatants were divided into small quantity and then stored at –80°C to inactivate unpenetrated virus. PBS at pH 11 was then added immediately to neutralize acetic PBS (pH 3). The neutral PBS was removed and the cell monolayer was overlaid with overlay medium. After a further 48 h of incubation at 37°C, the cells were fixed and stained. Plaques were counted and the percentage of inhibition of penetration was calculated.

**Penetration assay**

The penetration assay of HSV into Vero cells was performed according to procedures in the literature (Rosenthal et al., 1985; Albin et al., 1997; Logu et al., 2000) with minor modifications. Vero monolayer was grown in 24-well culture plates and pre-chilled at 4°C for another 3 h to allow the attachment of HSV-2 to monolayer. After 3 h of incubation, 5 mM prodelphinidin B-2 3'-O-gallate was added. The control group contained no prodelphinidin B-2 3'-O-gallate. The infected cell monolayer was then incubated at 37°C to maximise the penetration of virus. At 10-min intervals, the infected cell monolayer was treated with PBS at pH 3 for 1 min to inactivate unpenetrated virus. PBS at pH 11 was then added immediately to neutralize acetic PBS (pH 3). The neutral PBS was removed and the cell monolayer was overlaid with overlay medium. After a further 48 h of incubation at 37°C, the cells were fixed and stained. Plaques were counted and the percentage of inhibition of penetration was calculated.

**Results**

**Antiviral properties of prodelphinidin B-2 3'-O-gallate**

Anti-HSV-2 activity of prodelphinidin B-2 3'-O-gallate was investigated using XTT and plaque reduction assays. Prodelphinidin B-2 3'-O-gallate had been shown to possess antiviral activity (Table 1). Its IC50 in XTT and plaque reduction assays was 5.0 ±1.0 mM and 1.6 ±0.3 mM, respectively.

Since antiviral agents should be non-toxic to eukaryotic cells, the cytotoxic effect of prodelphinidin B-2 3'-O-gallate to Vero cells was thus evaluated to ensure that it showed no cytotoxic effect on cell viability at a concentration that blocked HSV-2 infection. As determined by XTT assay, prodelphinidin B-2 3'-O-gallate exhibited cellular cytotoxic effect at concentrations higher than its IC50. The CC50 of prodelphinidin B-2 3'-O-gallate and ACV in XTT assay were 6.7 and >3250, whereas in plaque reduction assay was 20.8 and >3333, respectively.
This result demonstrated that prodelphinidin B-2 3′-O-gallate exhibited antiviral activity at the concentration that was non-toxic to cells.

**Effect of prodelphinidin B-2 3′-O-gallate on viral infectivity**

Although results generated from XTT and plaque reduction assays showed that prodelphinidin B-2 3′-O-gallate was a HSV-2 inhibitor, they were unable to provide any information on the mechanism of action of this compound. Therefore, a series of experiments was carried out to investigate the antiviral properties of prodelphinidin B-2 3′-O-gallate. HSV-2 was incubated with various concentrations of prodelphinidin B-2 3′-O-gallate for 6 h at room temperature (26°C). Its residual infectivity was then quantitated by plaque assay on Vero cells. Prodelphinidin B-2 3′-O-gallate showed no effect on viral infectivity at concentrations of 5 µM or less (Figure 2). However, viral residual infectivity was decreased at concentrations 10 µM or higher of prodelphinidin B-2 3′-O-gallate. Results showed that 10 and 20 µM of prodelphinidin B-2 3′-O-gallate inactivated about 2 and 5 log 10 PFU of viral infectivity, respectively. Thus, the concentration of prodelphinidin B-2 3′-O-gallate at 5 mM or less was selected and used in the following mechanistic studies.

**Time course studies of prodelphinidin B-2 3′-O-gallate**

A time-of-addition experiment was performed to investigate the effect of prodelphinidin B-2 3′-O-gallate in the viral life cycle. Compounds were added at 0, 2, 4, 7 and 12 h p.i. and the infected cell was harvested at 24 h p.i. Virus yield was determined by plaque forming assay. When 5 mM of prodelphinidin B-2 3′-O-gallate was incubated concurrently with viruses, 97.4% of the virus yield was inhibited (Figure 3). The inhibition percentage of virus yield was decreased as the addition of prodelphinidin B-2 3′-O-gallate was delayed. Prodelphinidin B-2 3′-O-gallate only inhibited 66.8% of virus yield when added 4 h post-infection. Nevertheless, it remained active even when added 12 h after infection with 69.0% of the virus yield inhibited. This result indicates that prodelphinidin B-2 3′-O-gallate affected the late stage (12 h or later) of HSV-2 infection.

**Effect of prodelphinidin B-2 3′-O-gallate on the viral attachment and penetration**

According to the result of time course studies, prodelphinidin B-2 3′-O-gallate was shown to affect the late stage of HSV-2 infection. However, the different inhibitory effect of prodelphinidin B-2 3′-O-gallate on virus yield was observed when it was added at 0 or 4 h p.i. This observation suggested that prodelphinidin B-2 3′-O-gallate might disturb any event(s) within the first 4 h of HSV-2 infection, including viral attachment, viral penetration, the entering of viral DNA into cell nucleus and so on. This is in addition to the late event(s) of HSV-2 infection. Thus, the effect of prodelphinidin B-2 3′-O-gallate on viral attachment and penetration was investigated.
Antiviral properties of prodelphinidin B-2 3′-O-gallate

The results of the effect of prodelphinidin B-2 3′-O-gallate on the attachment between HSV-2 and cells are shown in Figure 4. The attachment between HSV-2 and cells was inhibited by prodelphinidin B-2 3′-O-gallate in a dose-dependent manner. Prodelphinidin B-2 3′-O-gallate inhibited 95% of the HSV-2 attached to the cells at the concentration of 1 mM. In contrast, ACV, which is commonly known to be only active in affecting the HSV replication, failed to significantly inhibit any plaque forming up to 10 mM. Therefore, it was concluded that prodelphinidin B-2 3′-O-gallate inhibited HSV-2 to attach to Vero cells and its inhibitory effect was dependent to dose levels.

Besides the attachment, the effect of prodelphinidin B-2 3′-O-gallate on penetration of HSV-2 into cell was also investigated. Our studies revealed that prodelphinidin B-2 3′-O-gallate possessed activity in preventing the penetration of HSV-2 into cells (Figure 5). The inhibitory effect on viral penetration was observed as early as 10 min after prodelphinidin B-2 3′-O-gallate was added. The inhibition percentage of penetration by 5 µM prodelphinidin B-2 3′-O-gallate in the first 10 min was 100%.

Discussion

Condensed tannins or proanthocyanidins are widely distributed in nature and are, in many cases, the active compounds of the medicinal plants from which they can be isolated (Haslam, 1996). In our studies, prodelphinidin B-2 3′-O-gallate, a proanthocyanidin gallate extracted from the fresh green tea leaves, was evaluated for its antiviral properties. Previous study had shown that prodelphinidin B-2 3′-O-gallate exhibited anti-HSV-1 activity (Takechi et al., 1985). Nevertheless, no further studies have been reported in elucidating its antiviral mechanism. This is the first report on the antiviral properties of prodelphinidin B-2 3′-O-gallate.

The present study demonstrated that prodelphinidin B-2 3′-O-gallate suppressed HSV-2 multiplication in Vero cell
without significantly reducing the cell viability and growth. Results generated from cytotoxicity assay showed that prodelphinidin B-2 3′-O-gallate did not decrease Vero cell viability at concentrations that exhibited antiviral activity. The morphology and characteristics of Vero cells treated with prodelphinidin B-2 3′-O-gallate and ACV, or untreated were similar, suggesting that inhibitory effect of prodelphinidin B-2 3′-O-gallate on HSV-2 multiplication was not related to the pH, osmolarity or other physiology variables (data not shown). In addition, the SI values for XTT and plaque reduction assays were more than 6 and 20, respectively. Thus, it was concluded that the inhibitory action of prodelphinidin B-2 3′-O-gallate on HSV-2 multiplication was not related to its cytotoxic effect toward cells.

Results of the vincucidal assay showed that prodelphinidin B-2 3′-O-gallate was not related to its virucidal ability. The inhibitory effect of prodelphinidin B-2 3′-O-gallate on virus yield was decreased as the addition time was delayed. However, prodelphinidin B-2 3′-O-gallate remained active in inhibiting viral replication even when added 12 h p.i. These observations suggested that prodelphinidin B-2 3′-O-gallate affected the late stage of HSV-2 infection. Mechanistic studies showed that prodelphinidin B-2 3′-O-gallate inhibited HSV-2 from attaching and penetrating into cells. Attachment of HSV to cell is dependent on the presence of glycoprotein D (gD) (Rajcani & Vojvodova, 1998). Fusion of the membrane between virus envelope and plasma membrane of the target cell requires glycoproteins B, D, H and L, or a combination of all of them (Roizman & Sears, 1996; Spear, 1993). According to our results on the viral attachment and penetration assay, prodelphinidin B-2 3′-O-gallate was suggested to affect the attachment and penetration between viruses and cells possibly through the disturbance of viral glycoproteins.

Many hydrolysable tannins and condensed tannins have been tested for anti-HSV activity (Fukushi et al., 1989; Takechi et al., 1985). The mechanism of antiviral activity of tannic acid was found to inhibit the virus adsorption to the cells (Fukushi et al., 1989). Nevertheless, no further studies have been reported in elucidating the antiviral mechanism of tannins. In our studies, prodelphinidin B-2 3′-O-gallate was found to inhibit anti-HSV-2 activity in several mode of actions. These are: (1) prodelphinidin B-2 3′-O-gallate remained active in inhibiting the viral replication even when added 12 h after infection, which indicated that this compound affects the late stage of viral infection (Figure 3). (2) Prodelphinidin B-2 3′-O-gallate inhibited the viral attachment to cells in a dose-dependent manner (Figure 4). (3) Prodelphinidin B-2 3′-O-gallate blocked viral penetration into cells (Figure 5).

As suggested by Casadevall & Whitely (1997), future antiviral herpesviruses agents will probably target enzymes or viral factors essential for infection or inhibiting other steps of the viral infection cycle, such as viral entry, protein synthesis or capsid assembly. Since prodelphinidin B-2 3′-O-gallate was shown to inhibit the attachment and penetration of HSV-2, and to disturb the late event(s) of HSV-2 infection, as well as to inactive the HSV-2 infectivity at high concentrations, its broad spectrum of mechanism of action merit further investigation.

References


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