Evaluation of Anti-HSV-2 Activity of Gallic Acid and Pentyl Gallate

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The synthetic *n*-alkyl esters of gallic acid, also known as gallates, are widely employed as antioxidants by food and pharmaceutical industries. Besides the antioxidant activity, other biological activities have been described for this group of molecules, mainly anticancer, antibacterial and antifungal properties. In the present study, the anti-herpes simplex virus (HSV)-2 activity of gallic acid and pentyl gallate was evaluated followed by the determination of the site of antiviral activity of these compounds. Our results demonstrated that both compounds reduced HSV-2 replication in a concentration-dependent manner when either incubated with the virus prior to the addition of the mixture to cells, or added to and incubated with cells after their infection. In summary, the anti-HSV-2 activity of gallic acid and pentyl gallate was ascribed to their virucidal effect on virus particles, a change that was likely accompanied by partial inhibition of the virus attachment to cells and its subsequent cell-to-cell spread activity. This suggests that these compounds can be regarded as promising candidates for development as topical anti-HSV-2 agents.

Key words antiviral; herpes simplex virus-2; gallic acid; pentyl gallate

Herpes simplex virus (HSV) is a DNA-containing enveloped virus that causes common viral infections in humans worldwide leading to a variety of diseases. HSV-1 and HSV-2 can be distinguished on the basis of clinical manifestations (the former is more frequently associated with oral cold sores, while the later causes genital ulcers) and biochemical and serological examinations. In most cases, HSV infection is usually benign or asymptomatic in immunocompetent individuals; however, in patients with an immature or compromised immune system, the infection can be serious and sometimes life-threatening.[1,2)]

Several nucleoside analogues have been approved for clinical use. Among those, acyclovir is widely used for the systemic treatment of HSV infections. It is a highly selective antiviral agent because it is specifically phosphorylated by viral thymidine kinase in infected cells. However, acyclovir-resistant HSV infection in immunocompromised patients such as transplanted patients and patients with AIDS has recently been observed. Therefore, it is desirable to develop new anti-HSV agents in order to substitute or complement the antiviral drugs available.[3,4)]

The synthetic *n*-alkyl esters of gallic acid (GA), also known as gallates, especially propyl, octyl and dodecyl gallates, are widely employed as antioxidants by food and pharmaceutical industries.[5,6)] Besides the antioxidant activity, other biological activities have been described for this group of molecules, mainly anticancer,[7—10)] antibacterial and antifungal properties.[11—16)] There are few reports about the antiviral activity of these compounds. In 1988, the potent inhibition of HSV-1 and HSV-2 by methyl gallate was described.[17)] In 2000, as part of the screening of phenolic compounds against HIV-1 integrase, gallic acid was found to be active.[18)] More recently, the anti-HSV activity of several gallates was described by our research group, which proposed various structure–activity relationships regarding the antiviral, antioxidant and genotoxic effects.[19)] Furthermore, the pronounced anti-HSV-1 activity of octyl gallate, and its inhibitory effect against RNA viruses were also recently described.[20,21)] In the present study, the anti-HSV-2 activity of gallic acid and pentyl gallate was evaluated followed by the determination of the site of antiviral activity of these compounds.

MATERIALS AND METHODS

Compounds GA and pentyl gallate (PG) (Fig. 1) were synthesized as previously described.[19)] The compounds (50 mM) were dissolved in dimethyl sulfoxide, stored at −20 °C protected from light, and further diluted in culture medium prior to use.

Cells and Virus African green monkey kidney cells (GMK AH1) were grown in Eagle's minimum essential medium (EMEM, Gibco BRL, Grand Island, NY, U.S.A.) supplemented with 2% fetal calf serum (FSC), 0.05% primaton substance (Kraft Inc., Norwich, CT, U.S.A.), 100 U/ml

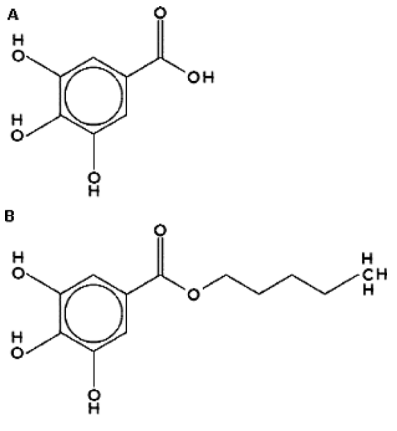

Fig. 1. Chemical Structures of Gallic Acid (A) and Pentyl Gallate (B)

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penicillin and 100 μg/ml streptomycin. Cell cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere. The virus strain used was HSV-2 strain 333.[22] Stock viruses were prepared as previously described.[23] After one cycle of freezing/thawing the fluids were titrated on the basis of plaque forming units (PFU) count as previously described[24] and stored at −70 °C until use.

The HSV-2 glycoproteins gB and gC were isolated as previously described[25] and extracellular methyl-[^{3}H]-thymidine labeled HSV virions were purified by centrifugation through a three-step discontinuous sucrose gradient as previously described.[25,26] The specific activity obtained was 3.83×10^{-2} counts per minute [cpm]/PFU.

Cytotoxicity Evaluation Confluent GMK-AH1 cells were exposed to different concentrations of both compounds for 72 h at 37 °C. After incubation, cell viability was assessed by using the tetrazolium-based CellTiter 96 kit (Promega, Madison, WI, U.S.A.) according to the manufacturer's protocol. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration (μM) that reduced cell viability by 50% when compared to untreated controls.

Viral Plaque Assays Viral plaque assays were performed as previously described.[27] For the viral plaque number reduction assay, different concentrations of compounds and approximately 200 PFU of HSV-2 in serum-free EMEM were incubated for 10 min at room temperature prior to their addition to GMK AH1 cells. Following 2 h of viral infection at 37 °C, cells were washed and overlaid with EMEM containing 1% methylcellulose, 2% FCS and antibiotics. Alternatively, different concentrations of compounds in the methylcellulose overlay medium were added to cells after the 2 h period of virus infection in the absence of the compounds. After 2 d of incubation at 37 °C, the viral plaques were visualized by staining with crystal violet. The 50% inhibitory concentration (IC_{50}) was defined as the concentration (μM) that inhibited 50% of viral plaque formation when compared to untreated controls.

For the viral plaque size reduction assay, different concentrations of the compounds were added to GMK AH1 cells 2 h after their infection with 200 PFU of HSV-2 and incubated with cells for the entire period of plaque development. Images of twenty viral plaques formed in the presence or absence (control) of each compound were captured using a DC300 digital camera (Leica, Heerbrugg, Switzerland) attached to a Diavert microscope (Leitz, Wetzlar, Germany). The area of each plaque was determined using the IM500 image software (Leica, Cambridge, U.K.).

Binding of Purified Virions and Isolated Viral Glycoproteins to Cells The effect of compounds on the binding of purified HSV-2 to GMK AH1 cells was evaluated as previously described.[27] Briefly, confluent monolayers were washed with PBS-A (PBS supplemented with 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and blocked with PBS-A containing 1% bovine serum albumin (BSA) for 1 h at room temperature. Different concentrations of the compounds and purified virions were incubated for 15 min at 4 °C prior to their addition to and incubation with cells under moderate agitation for 2 h at 4 °C. The cells were extensively washed and lysed with PBS-A containing 5% SDS and finally transferred to scintillation vials for quantification of radioactivity.

The effect of compounds on the binding of viral gC and gB glycoproteins to GMK AH1 cells was evaluated as previously described.[28] Briefly, purified gC or gB were preincubated for 15 min at 4 °C with different concentrations of compounds. Then, mixtures containing 0.87 μg of glycoprotein were added to cells and left for attachment for 1 h at 4 °C. Bound glycoproteins were detected by an ELISA-based procedure.[28]

Virus Inactivation Assay The assay followed the procedures previously described.[29] Mixtures of each compound and 4.0×10^4 PFU of HSV-2 in serum-free EMEM were coincubated for 20 min at 37 °C in a water bath prior to the dilution of the mixture to non-inhibitory concentrations of compound (1 : 100), and the residual infectivity was determined by plaque number reduction assay, as described above.

Antiviral Activity of Compounds in the Presence of Protein-Rich Solutions The effect of protein-rich solutions on the inactivation of HSV-2 by compounds was evaluated as previously described.[29] Briefly, equal volumes (50 μl) of compounds (125 μM) and different dilutions of BSA or FCS solutions were mixed and incubated for 5 min at room temperature prior to the addition of 200 PFU of HSV-2 333 strain in serum-free medium. Additional incubation was then carried out for 15 min at 37 °C and the mixtures were then transferred to GMK AH1 cells. The complete procedure was conducted as described above for the viral plaque assay.

Statistical Analysis The mean values±standard deviations are representative of four determinations from two independent experiments. For the determination of CC_{50} and IC_{50} values, nonlinear regressions of concentration–response curves were used.

RESULTS

Our results demonstrated that GA and PG reduced HSV-2 replication in a concentration-dependent manner when either incubated with the virus prior to the addition of the mixture to cells, or added to and incubated with cells after their infection (Fig. 2). When the compounds were added during and after infection of cells with HSV-2, the IC_{50} values for GA were 33.56 and 64.35 μM, and for PG were 98.46 and 56.00 μM, respectively. Concentrations of compounds that reduced the GMK AH1 cells viability by 50% (CC_{50}) were $>$1000 and 275.54 μM for GA and PG, respectively, thus exceeding the IC_{50} values. In contrast, pre-treatment of cells with these compounds did not reduce HSV-2 infectivity (data not shown) suggesting that either the virus particle or the virus-infected cells were targeted by both compounds.

The effect of GA and PG on the cell-to-cell spread of HSV-2 was determined through the evaluation of the size of plaques developed in the presence of these compounds in the overlay medium. As shown in Fig. 3, PG reduced the cell-to-cell spread of HSV-2 more efficiently than GA, while the latter appeared to be a better inhibitor of viral infectivity.

In order to evaluate if the inhibitory activity of these compounds was related to impairment in the attachment step of infection, the binding of purified virions and isolated viral glycoproteins to cells in the presence of compounds was determined. Both GA and PG moderately interfered with the binding of virus particles to GMK AH1 cells (Fig. 4B). In relation to the binding of glycoproteins, only gB was affected, while the ability of gC to bind to cells in the presence of

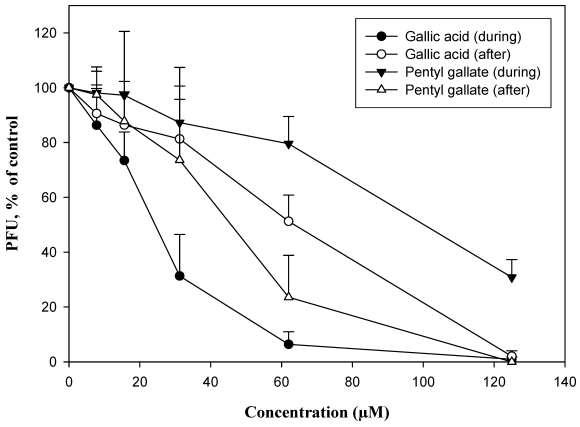

Fig. 2. Effects of Gallic Acid (GA) and Pentyl Gallate (PG) on HSV-2 Infectivity in GMK AH1 Cells Determined by Viral Plaque Number Reduction Assay

Mixtures of different concentrations of both compounds and 200 plaque forming units (PFU) of HSV-2 strain 333 were incubated for 10 min at room temperature prior to their addition to the cells during the infection period (2 h) or added to cells after the infection period. Values shown are the means of four determinations from two separate experiments.

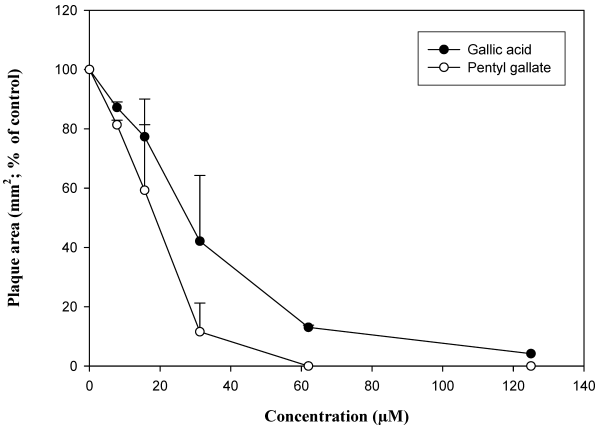

Fig. 3. Effects of Gallic Acid (GA) and Pentyl Gallate (PG) on HSV-2 Cell-to-Cell Spread in GMK AH1 Cells Determined by Viral Plaque Size Reduction Assay

Images of 20 viral plaques for each compound concentration were captured and subjected to area determination as described under Materials and Methods. Values shown are the means of four determinations from two separate experiments.

compounds remained basically similar to untreated controls (Fig. 4A).

The virus-inactivating activity of these compounds was also evaluated. The co-incubation of 4.0×10^4 PFU of the virus with compounds followed by the dilution of the mixture to non-inhibitory concentrations revealed that both GA and PG caused complete inactivation of HSV-2 infectivity (Fig. 5) at relatively low IC_{50} values. Given that microbicidal properties of different compounds are known to be diminished in protein-rich solutions, the virucidal activity of GA and PG in the presence of BSA and FCS solutions was investigated. Table 1 shows the residual infectivity of HSV-2 in the presence of BSA and FCS samples and 125 μM of each GA and PG.

DISCUSSION

In previous studies from our research group, the anti-HSV-

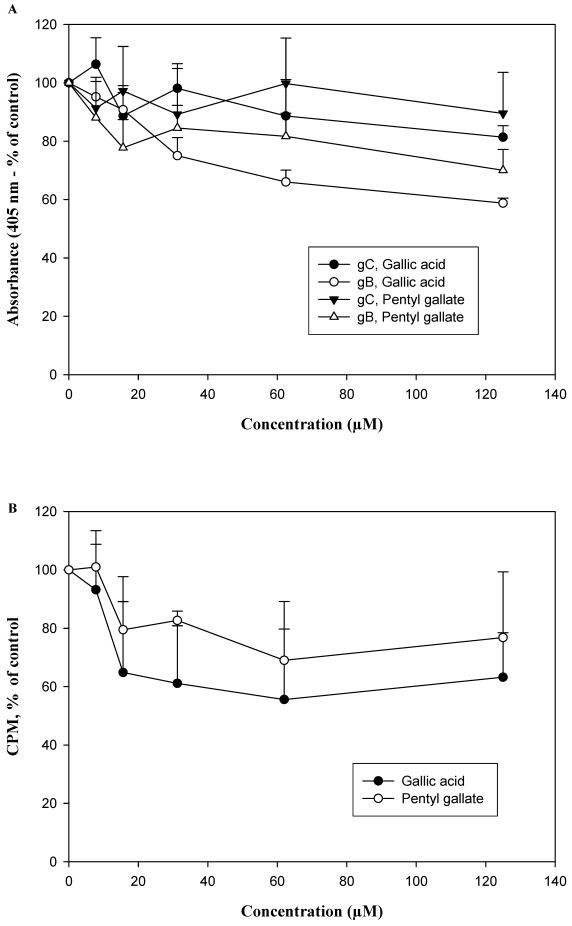

Fig. 4. Effects of Gallic Acid (GA) and Pentyl Gallate (PG) on the Binding of HSV-2 Virions and HSV-2 gB and gC Glycoproteins to GMK AH1 Cells

Different concentrations of both compounds were incubated with isolated HSV-2 gB and gC glycoproteins (A) or purified HSV-2 virions (B) for 15 min prior to and during 1 h period of virus adsorption to cells. The results are expressed as absorbance values of attached viral glycoproteins (A) and as percentages of attached viral cpm (B) in relation to the controls. Values shown are means of four determinations from two separate experiments.

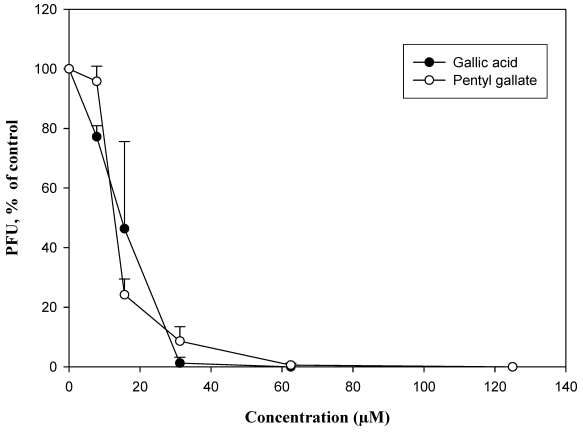

Fig. 5. Virus-Inactivating Activity of Gallic Acid (GA) and Pentyl Gallate (PG) Determined by Viral Plaque Number Reduction Assay

Different concentrations of both compounds were incubated with 4.0×10^4 PFU of HSV-2 for 20 min at 37 °C followed by the dilution of the mixtures to non-inhibitory concentrations prior to their addition to GMK AH1 cells. Values shown are means of four determinations from two separate experiments.

Table 1. Anti-HSV-2 Activity of Gallic Acid and Pentyl Gallate in the Presence of Protein-Rich Solutions

Sample	Gallic acid	Pentyl gallate
BSA (%)		
0.5	21.27±3.98	64.21±10.73
0.05	0.0	14.22±7.53
0.005	0.0	4.32±2.04
FCS (%)		
5	0.0	20.07±13.56
0.5	0.0	5.47±4.41
0.05	0.0	2.21±1.87

Gallic acid and pentyl gallate were co-incubated with specific protein-rich solutions before addition of 200 PFU of HSV-2. Results are expressed as percentages of the residual infectivity detected in virus incubated with the compounds and bovine serum albumin (BSA) or fetal calf serum (FCS) relative to the virus treated only with specific protein-rich samples. Values shown are means of four determinations from two separate experiments.

1 activity of GA and fifteen gallates was described[19] and the most promising compounds (GA and PG) were selected for investigation of their mode of anti-HSV-1 activity (Kratz J. M. *et al.*, unpublished data). In the present study we have characterized the anti-HSV-2 activity of GA and PG.

Initially, we investigated whether the compounds exerted their antiviral activity by targeting the virus particle or the cell. The pretreatment of uninfected GMK AH1 cells with GA and PG did not affect subsequent viral infection, however, preincubation of viral particles with compounds followed by the addition of the virus-compound mixture to cells interfered with viral infectivity. Likewise, addition of GA and PG to cells after the infection period of 2 h also inhibited viral replication (Fig. 2), indicating that the virus particles or the virus-infected cells are the sites of antiviral activity for both compounds. Nevertheless, GA presented better activity during than after infection while the opposite tendency was observed for PG.

These results suggested that impairment in the viral attachment could be implicated in the detected anti-HSV-2 activity. Thus, the binding of purified virions and isolated viral gB and gC glycoproteins to cells in the presence of the compounds was evaluated. As shown in Fig. 4B, GA and PG inhibited only 36.77% and 23.20% of attached virions, respectively. Likewise, the effect of both compounds on the binding of glycoproteins to cells was incomplete. The treatment with 125 μM of GA or PG inhibited the binding of gB by 41.21% and 29.98% (Fig. 4A), respectively, while the ability of gC to bind to cells remained basically comparable to untreated controls. This relationship could not be elucidated, but more experiments are in course to clarify this issue.

These results demonstrated that the antiviral activity of both compounds could be explained only in part by the interference with virus attachment or binding of isolated viral glycoproteins to cells.

Therefore, we also evaluated the effect of GA and PG on the cell-to-cell spread of HSV-2 (Fig. 3). Both compounds inhibited the lateral spread of infection in cultured cells, an effect manifested as a reduction in size of HSV-2 plaques formed in the presence of these compounds in the overlay medium. PG caused a greater reduction on the cell-to-cell spread of the virus than GA, an observation that may explain the lower IC_{50} value of this compound when added to the cells after infection in relation to its inhibitory activity during infection (Fig. 2).

The virus-inactivating property of both compounds was also evaluated. Both GA and PG caused complete inactivation of HSV-2 infectivity at lower IC_{50} values (13.16 μM for GA, and 14.35 μM for PG) than those obtained in the standard plaque assays (Fig. 5). Although the molecular basis of the virus inactivation requires further investigation, the relatively low inactivating concentrations of compounds indicate that their antiviral activities detected are mainly related to virucidal properties. The direct virucidal activity was also found in other gallate derivatives, such as epigallocatechin-3-gallate, prodelphinidin B-2 3′-*O*-gallate and octyl gallate.[20,21,30—32]

Given that virucidal activity of some compounds was decreased in the presence of human cervical secretions or protein-rich solutions,[33,34] the virucidal activity of GA and PG in the presence of BSA and FCS solutions was investigated. The BSA solution (5%) but not FCS neutralized to some extent the anti-HSV-2 activity of GA. The virucidal activity of PG was even more affected by both protein-rich solutions suggesting that higher concentrations of this compound would be needed to overcome the neutralizing effects detected *in vitro* (Table 1). Considering the potential topical application of these compounds, further investigations on the neutralizing effects of human cervical secretions, semen and saliva on their virucidal activity are merited.

During the course of our studies, another study[20] described the anti-HSV-1 activity of octyl gallate with a moderate cytotoxicity. In our study, the cytotoxicity presented a similar profile, although the antiviral activity was found only for the gallates with as much as seven carbons in the alkyl moiety. The discrepancies between the results of these studies may come from the different employed cell lines and used methodologies, as already supposed by the authors,[20] fact that corroborates the need for a standardization in this research area.

In summary, the anti-HSV-2 activity of GA and PG was ascribed to their virucidal effect on virus particles, a change that was likely accompanied by partial inhibition of the virus attachment to cells and its subsequent cell-to-cell spread activity. This suggests that these compounds can be regarded as promising candidates for development as topical anti-HSV-2 agents.

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