Parthenolide Influences *Herpes simplex virus 1* Replication in vitro

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Keywords

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Abstract

Background/Aims: Parthenolide is a sesquiterpene lactone that is present in plants of the *Tanacetum* genus, for which many biological effects have already been reported, including antiherpetic activity. Although the effectiveness of parthenolide against *Herpes simplex virus 1* (HSV-1) has already been demonstrated, such findings are still controversial. The objective of this study was to investigate the ways in which parthenolide exerts anti-HSV-1 activity. **Methods:** The cytotoxicity and antiviral activity of parthenolide were determined by the MTT method and plaque reduction assay, respectively. The expression of cell and viral proteins during the treatment of infected cells was investigated by Western blot. **Results:** Both strains of HSV-1 were sensitive to parthenolide, and parthenolide was active only after penetration of the virus into the host cell. The expression of p65 protein decreased, the expression of caspases 8 and 9 increased, and the expression of c-Jun N-terminal kinase (JNK) and p38 pro-

tein was altered in infected cells after parthenolide treatment, resulting in lower cell survival. The low expression of viral proteins gB, gD, and ICP0 confirmed the reduction of HSV-1 particle production. **Conclusion:** Parthenolide exerts anti-HSV-1 activity by impairing cell viability, which consequently interferes with the efficient infection and production of new viral particles.

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Introduction

Herpes simplex virus 1 (HSV-1) is a DNA virus that is protected by an enveloped icosahedral capsid that causes latent infection. An estimated 60–80% of the world's population carry the virus, regardless of whether they manifest recurring symptoms [1]. The treatment of choice for HSV-1 is the use of nucleoside analogs that modulate the enzymatic activity necessary for the multiplication of viral genetic material. Mutations, insertions, or deletions of the HSV-1 genome can lead to resistance, which can be problematic for immunocompromised patients, the elderly, and patients with recurrent infection [2]. The

search for compounds with anti-HSV-1 activity that act via an alternative mechanism would be valuable for overcoming the emergence of resistant strains.

The sesquiterpene lactone parthenolide is present in plants of the *Tanacetum* genus, and it is the most important secondary metabolite of *Tanacetum parthenium*. Parthenolide is a key molecule in the synthesis of guaianolide, a sesquiterpene lactone that is also biologically active [3]. Parthenolide has antiparasitic [4, 5], antiviral, and anti-inflammatory effects. It also has antineoplastic activity in vitro and induces apoptosis in neoplastic cells, thus interfering with signaling pathways [6–9].

The anti-HSV-1 activity of parthenolide was demonstrated in a preliminary study [10]. However, another study [11] did not confirm its anti-HSV-1 activity. This study sought to clarify the anti-HSV-1 activity of parthenolide as an important compound of extracts from the aerial parts of plants of the *Tanacetum* genus.

Materials and Methods

Cells and Viruses

Vero cells (American Type Culture Collection CCL-81) were cultured in DMEM (Gibco) supplemented with 10% FBS (Gibco) and gentamycin (50 µg/ml). Samples of the HSV-1 strains KOS and AR-29 were provided by Dr. Marcelo Alves Pinto (Fiocruz, RJ, Brazil).

Determination of Cytotoxicity and Antiviral Activity

Ninety-six-well plates were prepared with Vero cells, and different concentrations of parthenolide (Sigma-Aldrich, 99% purity) were added, followed by incubation at 37 °C for 72 h in an animal cell culture CO₂ incubator. To determine antiviral activity, the cells were previously infected with the tissue culture infectious dose 50 (TCID₅₀) of HSV-1 (KOS strain), and incubated for 1 h at 37 °C until viral adsorption and penetration occurred [12]. Different concentrations of parthenolide were then added, followed by incubation at 37 °C for 72 h. In both cases, cell viability was determined by the MTT method, in which 25 µL of MTT solution (2 mg/mL) was added to each well and incubated at 37 °C for 4 h. Subsequently, the solution was removed, and the purple-colored formazan that formed was solubilized with 15 µL of dimethylsulfoxide and read at 570 nm in a microplate reader. The cytotoxic concentration for 50% of the cells (CC₅₀) and effective concentration for 50% of the cells (EC₅₀) were calculated by linear regression analysis. Acyclovir (Sigma-Aldrich, 99% purity) was used as a positive control for anti-HSV-1 activity.

Determination of Antiviral Activity by the Plaque Reduction Assay

Cell monolayers in 24-well plates were infected with roughly 60 PFU/well of HSV-1 (KOS or AR-29 strain) [13]. To determine at which stage of viral infection parthenolide was active, it was added at different times. First, viruses were treated with parthenolide for 1 h at 37 °C (virucidal assay) before infecting the cell monolayer.

The viruses were then diluted to 60 PFU, layered in contact with the host cell for 1 h at 37 °C, and covered with DMEM that contained 0.5% carboxymethylcellulose. Second, different concentrations of parthenolide were placed in contact with the cell and the virus at the same time during incubation for 1 h at 4 °C (adsorption). The wells were then washed with PBS and covered with DMEM that contained 0.5% carboxymethylcellulose. Third, to evaluate adsorption/penetration interference, the assay was quite similar to the second treatment regimen above, with the exception of the incubation temperature, which was 37 °C. Fourth, the cells were infected with HSV-1, washed with PBS, and coated with different concentrations of parthenolide in DMEM that contained 0.5% carboxymethylcellulose (after infection). Fifth, host cells were treated with parthenolide prior to viral infection (prophylactic assay) and then infected for 1 h at 37 °C, washed with PBS, and covered with DMEM that contained 0.5% carboxymethylcellulose. In all cases, after 72 h of incubation, the plates were fixed with 10% formaldehyde and stained with crystal violet for plaque counting. Acyclovir was used as the reference drug.

Effect of Parthenolide on Caspase-8 and Caspase-9 Expression and on DNA Damage

The evaluation of caspase expression was performed as described previously [9], with minor modifications. Six-well plates were prepared with Vero cells, infected with HSV-1 (KOS strain), and treated for 24 h with 2.5 µg/mL parthenolide (EC₅₀). After this period, the cells were lysed with lysis buffer and centrifuged. The protein sample was then separated by SDS-PAGE. The proteins were transferred to a nitrocellulose membrane, blocked with 5% egg albumin, labeled overnight with caspase-8, caspase-9, gD, and β-actin antibodies (Santa Cruz Biotechnology), and stained with anti-mouse horseradish peroxidase secondary antibody. Detection was performed with chemiluminescence reagent (Santa Cruz Biotechnology). Readings were performed with the ChemiDoc MP Imaging System (BioRad) and ImageLab software. The optical density and relative band expression were normalized to β-actin and determined using the same software. To determine differences between treatments, one-way ANOVA was performed, followed by the Dunnett test. Values of $p < 0.05$ were considered statistically significant.

To evaluate the DNA damage induced by parthenolide treatment, Vero cells were plated under round glass coverslips inserted into 24-well plates. After confluence, cells were infected and treated for 24 h with 2.5 µg/mL parthenolide (EC₅₀). As control for apoptosis, uninfected cells were treated for 24 h with 40 µM camptothecin (Sigma-Aldrich, ≥90% purity). As a control for necrosis, cells were treated for 10 min with 98 µM digitonin (Sigma-Aldrich). Cell monolayers were stained with Hoechst (Thermo-Fisher; excitation/emission: 350/461 nm for 15 min, concomitant with propidium iodide labeling (Sigma-Aldrich; excitation/emission: 535/617 nm for 10 min). The coverslips were examined in a fluorescence microscope (Olympus BX51), coupled with an image acquisition system (Olympus U-TV0.63XC). The images were acquired with a ×40 magnification.

Effect of Parthenolide on Cellular Signaling Pathways

To evaluate the effects of parthenolide on NF-κB and mitogen-activated protein kinase (MAPK) pathways, 6-well plates were prepared with Vero cells, infected with HSV-1 (KOS strain), and treated with 2.5 µg/mL parthenolide, 3.3 µg/mL curcumin (Acros Or-

Fig. 1. Interference of parthenolide at different stages of viral multiplication in vitro by plaque reduction assay.

ganics, 98% purity; control for anti-NF- κ B activity; [14]), and 20 μ M anisomycin (Sigma-Aldrich, 98% purity; positive control for anti-MAPK activity). The cytoplasmic and nuclear material of the cells was separated with a commercial kit (nuclear extraction kit, Cayman Chemical) for the NF- κ B p65 subunit and viral protein evaluation. To determine MAPK protein expression, the total cell lysate was used. Included in the lysis buffer were inhibitors of both protease (Sigma-Aldrich) and phosphatase (Thermo Fisher Scientific). After the separation of polyacrylamide gels, the proteins were transferred to a nitrocellulose membrane, blocked with egg albumin, and labeled with p65, ICP0, β -laminin, gD, gB, p38, phosphorylated p38 (p-p38), c-Jun N-terminal kinase (JNK), p-JNK, extracellular signal-regulated kinase (ERK), p-ERK, and β -actin antibodies (Santa Cruz Biotechnology). Labeling with peroxidase-conjugated secondary antibody was performed and revealed as described above, and is presented as the relative expression normalized to β -actin. Readings, measurements, and statistical analyses of the optical density were performed as described above.

Results and Discussion

Cytotoxicity and Antiviral Activity of Parthenolide Determined by the MTT Method

Confirming previous results [10], parthenolide presented anti-HSV-1 activity, with an EC_{50} of 0.3 μ g/ml. Cell viability was determined by the MTT method (Table 1). The anti-HSV-1 effects of a crude extract of *Tanacetum vulgare* were previously confirmed [10, 11], but the anti-HSV-1 activity of parthenolide has been contested [11], with the anti-HSV-1 activity of the fractions being attributed to other compounds isolated from *T. vulgare* leaves. Further inspection of these studies suggests that the different results might be attributable to the different strategies used to investigate the antiviral activity of the fractions and parthenolide. In the present study, the EC_{50} values were based on the percentage of cell protection (i.e., cell viability, detected by the MTT colorimetric method). In contrast, the previous study determined the

Table 1. Cytotoxicity, antiviral activity against HSV-1 (KOS strain) and selectivity index of parthenolide by the MTT method

Compounds	CC_{50} , μ g/mL	KOS	
		EC_{50} , μ g/mL	SI
Parthenolide	3.4 $\pm$ 0.04	0.3 $\pm$ 0.01	11.9
Acyclovir	817.5 $\pm$ 24.75	0.07 $\pm$ 0.002	11.679

Data are expressed as mean $\pm$ SD from 3 independent experiments. CC_{50} , toxic concentration for 50% of the cells; EC_{50} , concentration which inhibited 50% of viral destruction; SI, selectivity index (CC_{50}/EC_{50}).

Table 2. Antiviral activity of parthenolide against KOS and AR-29 strains by plaque reduction method

	EC_{50} , μ g/mL	
	KOS	AR-29
Parthenolide	2.3 $\pm$ 0.02	1.8 $\pm$ 0.07
Acyclovir	0.06 $\pm$ 0.01	12.5 $\pm$ 2.12

EC_{50} , concentration which inhibited 50% of viral destruction.

EC_{50} based on the percentage of wells that presented a cytopathic effect (CPE) which was analyzed by light microscopy [11]. Considering the purity that was employed in this study (99%), parthenolide was able to inhibit the production of HSV-1 particles in Vero cell cultures at a low concentration. Nonetheless, we cannot necessarily attribute anti-HSV-1 activity solely to parthenolide present in the crude extract of *Tanacetum* sp., because parthenolide is not the only biologically active substance that is found in the extract [15].

Fig. 2. a Western blot analysis. Expression of caspases 8 and 9 in Vero cells infected with HSV-1: lane 1, Vero cells (cell control); lane 2, Vero cells infected with KOS strain (virus control); lane 3, Vero cells treated with parthenolide; lane 4, Vero cells infected with KOS strain and treated with 2.5 µg/mL parthenolide. **b** Relative quantification of Western blot analysis in bar graphs. * $p < 0.05$ when compared with lane 2.

Fig. 3. a Western blot analysis. Effect of parthenolide on the expression of viral proteins in the cytoplasm of cells infected with HSV-1: lane 1, Vero cells (cell control); lane 2, Vero cells infected with KOS strain and treated with 2.5 µg/mL parthenolide; lane 3, Vero cells treated with parthenolide; lane 4, Vero cells infected

with KOS strain and treated with curcumin; lane 5, Vero cells infected with KOS strain (virus control). **b** Relative quantification of Western blot analysis in bar graphs. **** $p < 0.0001$ when compared with lane 5.

Fig. 4. Fluorescence microscopy analysis of Vero cells infected with HSV-1 treated with parthenolide. Vero cells stained with Hoechst (**a**) and propidium iodide (**b**): (1) treated with digitonine (necrosis control); (2) treated with camptothecin (apoptosis control); (3) nontreated (cell control); (4) infected with KOS strain (virus control); (5) treated with parthenolide; (6) infected with KOS strain and treated with 2.5 µg/mL parthenolide. Asterisks indicate cells with DNA condensation and nuclear fragmentation.

Anti-HSV-1 Activity of Parthenolide Determined by the Plaque Reduction Assay

After evaluating the antiviral activity of parthenolide against infection with the KOS strain, we investigated whether parthenolide also has activity against the AR-29 strain, which is resistant to acyclovir. Table 2 shows that parthenolide maintained its activity, regardless of the tested strain.

We performed experiments to determine the step at which parthenolide acts on the viral multiplication cycle, and found that it acts mainly on the viral postentry stage (Fig. 1). These results confirm those of a previous study [10], indicating that parthenolide does not act on the initial phase of infection (i.e., before or during viral penetration), but appears to be able to prevent viral replication after viral particle internalization.

Parthenolide acts on cell signaling pathways, independent of the presence of pathogens, and these pathways are required for cell viability [7]. Considering the wide range of parthenolide's biological activity that has been described in the literature, we investigated whether it influences the death and survival of HSV-1-infected cells.

Effect of Parthenolide on Caspase-8 and Caspase-9 Expression in HSV-1-Infected Cells

Herpes viruses generally use strategies to prevent apoptosis (i.e., a natural defense mechanism of the cell to avoid viral infection). Some *HSV-1* genes regulate cell death and allow cell survival. This balance is important because, if the virus leads to the death of the host cell, this can be harmful to its replicative cycle [16, 17]. To evaluate the effects of parthenolide on cell death in HSV-1-infected cells, we investigated caspase-8 and caspase-9 expression. Figure 2 shows that untreated HSV-1-infected cells and parthenolide-treated noninfected cells exhibit an increase in the expression of caspase-9 compared with controls. HSV-1 appeared to be able to maintain control over the expression of caspase-9 to avoid apoptosis (Fig. 2b, column 2). Parthenolide increased the expression of both caspase-8 and caspase-9 (Fig. 2b, columns 3 and 4), which can trigger apoptosis and led to the impairment of viral production, reflected by a decrease in viral protein expression (Fig. 3a).

A previous study found that parthenolide altered the expression of the intrinsic (caspase-9) and extrinsic (caspase-8) pathways [9]. Both pathways converge inside the cell. Activation of the extrinsic pathway can promote interactions with intermediates, leading to mitochondrial stress and resulting in the activation of the intrinsic cell death pathway [16]. Parthenolide also induced apoptosis

Fig. 5. a Western blot analysis. Effect of parthenolide on the expression of cellular protein p65 and viral protein ICP0 in the nucleus of cells infected with HSV-1: lane 1, cell control; lane 2, KOS strain (virus control); lane 3, parthenolide; lane 4, infected cells treated with curcumin; lane 5, infected cells treated with 2.5 μg/mL of parthenolide. **b** Relative quantification of Western blot analysis in bar graphs. ** $p < 0.005$; *** $p < 0.0001$ when compared with lane 2.

in neoplastic cells by upregulating Bax and caspase-3 expression [18], thus corroborating our findings.

Death by apoptosis can also be evidenced by damage of cellular DNA, which becomes fragmented and condensed, as well as by morphological changes in the cellular nucleus [19]. Parthenolide treatment of HSV-1-infected cells showed that there was an increase in the cell population with DNA damage, similar to that found with the camptothecin treatment (Fig. 4).

Effect of Parthenolide on the NF-κB Pathway and Viral Proteins

The transcription factor NF-κB in mammalian cells regulates the expression of genes that are related to growth, programmed cell death, cytokine production, and inflammation. When the NF-κB pathway is not activated, the p65 subunit remains blocked in the cytoplasm by IκB protein. When the pathway is stimulated, IKK protein phosphorylates IκB protein, which is then degraded and allows the migration and accumulation of the p65 subunit in the cell nucleus. This results in the regulation of the expression of various genes. For successful infection by HSV-1, the NF-κB pathway needs to be activated to ensure the viability of cells that are infected by the virus and allow the efficient replication of viral genetic material [20, 21]. To evaluate cellular and viral protein expression in cytoplasmic and nuclear portions of cells that were infected by HSV-1 and treated with parthenolide, we analyzed the expression of the p65 subunit protein and viral ICP0 protein (Fig. 5).

Parthenolide reduced NF-κB p65 subunit accumulation in the cell nucleus in infected and treated cells compared with infected and untreated cells. The lower expression of viral ICP0 protein confirmed the relationship between the NF-κB pathway and antiviral activity. Treatment of the infected cells with parthenolide reduced ICP0 expression in both the nucleus and cytoplasm and decreased the expression of other HSV-1 surface glycoproteins (Fig. 3).

ICP0 is considered the primary viral protein that is responsible for successful infection. It can act on in 2 stages: (1) in the first hours, it accumulates in the nucleus of the cell, increasing the efficiency of replication of viral genetic material, and (2) between 5 and 9 h after HSV-1 infects the host cell, ICP0 protein migrates to the cytoplasm, ensuring the assembly of viral particles and inhibiting the activation of immune responses [22]. When viral ICP0 protein is present in the cytoplasm, it can interact with IκB protein, triggering a cascade of reactions that allows migration of the p65 subunit from the cytoplasm to the nucleus [20]. In this study, parthenolide decreased the expression of ICP0 protein in the nucleus of infected cells. In the cytoplasm, ICP0 expression significantly decreased. Thus, parthenolide retained ICP0 protein in the nucleus. Interactions with IκB protein did not occur, and the p65 subunit remained in the cytoplasm. Blockade of the translocation of ICP0 protein to the cytoplasm appears to be harmful to the virus because the host immune system is activated [23, 24].

Fig. 6. a Effect of parthenolide in the expression of MAPK proteins in Vero cells: lane 1, treated with anisomycin (positive control); lane 2, untreated (cell control); lane 3, infected with KOS strain (virus control); lane 4, treated with parthenolide; lane 5, infected

with KOS strain posttreated with 2.5 µg/mL of parthenolide. **b** Relative quantification of Western blot analysis in bar graphs. ** $p < 0.005$ when compared with lane 3.

Lower expression of the viral surface glycoproteins gB and gD was observed in the cytoplasm in infected and treated cells compared with infected and untreated cells. This may be explained by the fact that parthenolide exerts its actions after penetration of the viral particle in the host cell by impairing ICP0 expression, which in turn influences the expression of gD and gB proteins and the assembly of new viral particles. Such proteins are produced in the late phase of the HSV-1 replication cycle, constituting components of the viral envelope that are necessary for the viral adsorption and penetration process.

Treatment with parthenolide appears to control HSV-1 infection without requiring specific interactions with

the viral particle. Blocking the NF-κB pathway and triggering apoptosis would make HSV-1 infection less efficient. This can be advantageous because parthenolide does not act through cellular pathways that are unique to viral replication. Therefore, genetic mutation of the viral particle does not affect the activity of parthenolide, as in the case of acyclovir-resistant viral strains.

Epstein-Barr-infected cells that were treated with parthenolide exhibited an increase in the expression of caspase-8 and caspase-9 and decrease in the accumulation of p65 subunit in the nucleus [9]. *Houttuynia cordata* extract blocked activation of the NF-κB pathway in HSV-1-infected cells and in cells that were stimulated with TNF-α [14].

The NF- κ B pathway also influences inflammatory responses in the host. When we consider in vivo application, a compound with both antiviral and anti-inflammatory activity could be beneficial because the inflamed lesion can cause pain and discomfort to the patient. Furthermore, attenuating infection-induced inflammation may improve the appearance (redness and edema) of the injured area.

Effect of Parthenolide on Cellular Pathways Mediated by Kinases

Viruses generally appropriate the cellular machinery of infected cells and modify their metabolism for the efficient replication of viral genetic material. Thus, the virus maintains the viability of the host cell and prevents apoptosis until its genetic material has been replicated. Beyond the NF- κ B pathway, HSV-1 influences other cellular signaling pathways, such as those of the MAPKs, responsible for cellular maintenance, growth, and death. MAPKs are proteins that regulate the cell cycle, adhesion, growth, differentiation, and apoptosis. Depending on the stimulus that is received, some phosphorylation cascades are activated, resulting in the transcriptional regulation of certain genes [25, 26]. Previous studies showed that HSV-1 also interferes with the MAPK pathway by increasing p38 and JNK expression, reducing ERK expression, and manipulating the cell cycle, thus ensuring efficient replication of the virus and preventing cell death [27].

In this study, as expected, infected and untreated cells exhibited an increase in p-JNK and p-p38 expression and a decrease in p-ERK expression (Fig. 6). However, treatment with parthenolide appeared to reverse these changes by reducing the expression of p-JNK and p38 and increasing the expression of p-ERK. These findings confirm that parthenolide exerts antiviral effects by interfering with cellular pathways that support efficient virus replica-

tion. Thus, these pathways are necessary for HSV-1 replication, and parthenolide at low concentrations may be useful as a complementary therapy for the treatment of HSV-1 infection.

Viral ICP0 protein plays a fundamental role in the HSV-1 replication cycle. Other studies suggested that the expression of ICP0 is related to activation of the NF- κ B pathway and the expression of JNK [20, 28]. We found that interfering with the migration of ICP0 protein from the nucleus to the cytoplasm altered the expression of JNK/p-JNK proteins and the NF- κ B p65 subunit. The cascade of MAPK activation interferes with the expression of JNK and p38 and ERK proteins [29]. Parthenolide does not appear to act directly on HSV-1, but it modulates several pathways that are necessary for cellular maintenance and viral replication, leading the infected cell to collapse and preventing the progression of infection.

Parthenolide is an important bioactive compound of *Tanacetum* sp. It represents one of the constituents that are responsible for anti-HSV-1 activity against both acyclovir-resistant and acyclovir-sensitive strains. Significant cellular protection was observed when HSV-1-infected cells were treated in vitro with parthenolide. Although parthenolide did not act directly on viral particles, it retained viral ICP0 protein in the nucleus, modulating the NF- κ B and MAPK pathways and regulating the expression of caspases that are responsible for cell death. Therefore, in addition to other compounds that are present in *Tanacetum* sp., parthenolide appears to contribute to anti-HSV-1 activity by modulating the defense mechanisms of the host cell.

Disclosure Statement

The authors declare no conflicts of interest.

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