The Effects of the Diterpenes Isolated from the Brazilian Brown Algae *Dictyota pfaffii* and *Dictyota menstrualis* against the Herpes Simplex Type-1 Replicative Cycle

**Authors**

Juliana L. Abrantes¹, Jussara Barbosa¹, Diana Cavalcanti², Renato C. Pereira², Carlos Frederico L. Fontes¹, Valeria L. Teixeira², Thiago Moreno L. Souza³*, Izabel C. P. Paixão³*

**Affiliations**

The affiliations are listed at the end of the article

**Abstract**

We describe in this paper that the diterpenes 8,10,18-trihydroxy-2,6-dolabelladiene (1) and (6R)-6-hydroxydichotoma-4,14-diene-1,17-dial (2), isolated from the marine algae *Dictyota pfaffii* and *D. menstrualis*, respectively, inhibited HSV-1 infection in Vero cells. We initially observed that compounds 1 and 2 inhibited HSV-1 replication in a dose-dependent manner, resulting in EC₅₀ values of 5.10 and 5.90 µM, respectively, for a multiplicity of infection (MOI) of 5. Moreover, the concentration required to inhibit HSV-1 replication was not cytotoxic, resulting in good selective index (SI) values. Next, we found that compounds 1 sustained its anti-herpetic activity even when added to HSV-1-infected cells at 6 h after infection, while compound 2 sustained its activity for up to 3 h after infection, suggesting that these compounds inhibit initial events during HSV-1 replication. We also observed that both compounds were incapable of impairing HSV-1 adsorption and penetration. In addition, the tested molecules could decrease the contents of some HSV-1 early proteins, such as UL-8, RI-1, UL-12, UL-30 and UL-9. Our results suggest that the structures of compounds 1 and 2, Brazilian brown algae diterpenes, might be promising for future antiviral design.

**Introduction**

Herpes viruses are a large and diverse family of enveloped viruses composed of three subfamilies: alpha-, beta-, and gamma-herpes viruses. Herpes simplex virus type 1 (HSV-1) is a ubiquitous alpha-herpes virus, whose virions consist of a large, double-stranded DNA genome packaged within an icosahedral capsid encased by a proteinaceous tegument and a lipidic envelope composed of various glycoproteins [1]. The HSV-1 replication cycle has been well characterized [2]. HSV-1 entry into cells is a complex process that involves an initial attachment of virus glycoproteins to cell surface receptors [3]. Once inside the cell, the HSV-1 replication cycle can be divided into three phases. First, HSV-1 immediate-early (IE; α) gene expression depends on the binding of the α-trans-induction factor (αTIF)/ C1/Oct-1 multiprotein complex to the TAAGARAT (R, purine; GARAT) sequences of the cis-acting site in the HSV-1 genome [4]. Consequently, viral IE proteins, such as infected-cell protein 0 (ICP0), ICP27 and ICP4, regulate subsequent steps of viral replication. Second, early (E; β) genes, such as viral helicase (UL-8), DNA polymerase (UL-30) and thymidine kinase, are expressed and viral DNA is synthesized. Last, late (L; γ) genes encoding for structural proteins such as gB, gC and ICP5 are expressed [2]. It has been proposed that inhibition of any of these steps may lead to the blockage of HSV-1 replication [5,6]. After primary infection of mucosal tissues, HSV-1 establishes latency and can be reactivated under several conditions, causing a variety of infections, principally in individuals immunocompromised by AIDS, cancer, or organ transplantation [7]; this reactivation is considered to be an important event associated with increased HIV transmission [8]. Several anti-herpetic agents have been extensively investigated and used in the treatment of HSV-1-infected individuals, including acyclovir and penciclovir, and their prodrugs valacyclovir and famiclovir [9]. However, long-term administration of these drugs to immunocompromised patients may result in the emergence of drug-resistant strains of HSV-1.

* Both authors contributed equally to this paper.

---

**Key words**

- Dictyotaceae
- *Dictyota menstrualis*
- *Dictyota pfaffii*
- marine algae
- diterpenes
- antiviral
- HSV-1

**Correspondence**

Juliana Lourenço Abrantes, BSc, MSc
Instituto de Bioquímica Médica
Cidade Universitária
Ilha do Fundão
Centro de Ciências da Saúde (CCS)
Bloco H, segundo andar, sala 26
Rua Cesar Pernetta 400
CEP 21941–590, Rio de Janeiro
Brasil
Phone: +55 21 25 62 67 84
Fax: +55 21 25 62 67 84
abrantes@bioqmed.ufrj.br

**Bibliography**

Published online September 17, 2009
Planta Med 2010; 76: 339–344
© Georg Thieme Verlag KG Stuttgart · New York
ISSN 0032–0943

**received** April 10, 2009
**revised** August 9, 2009
**accepted** August 19, 2009
sistant HSV-1 isolates. Therefore, the search for new antiviral agents, especially with different mechanisms of action, is a crucial goal [10].

Polysaccharides, alkaloids, phenolic compounds, terpenoids, steroids and peptides are examples of non-nucleoside natural products reported as inhibitors of HSV-1 replication [12, 13]. We have previously described the anti-HSV-1 effects of alkaloids isolated from a marine sponge [14] and of diterpenes isolated from the Brazilian brown alga D. pfaffii [15, 16]. The anti-HIV-1 activities of products derived from this alga have also been reported, together with the effects of molecules from another brown alga, D. menstrualis [15–20]. In our previous work, we demonstrated the inhibition of HSV-1 cytopathic effects by diterpenes isolated from D. pfaffii, as well as their cytotoxicity toward Vero cells [15]. However, the mechanism of action by which HSV-1 replication was inhibited remained to be elucidated. Thus, in order to understand the mode of action of the main active product from D. pfaffii and to investigate the anti-HSV-1 activity of another diterpene isolated from D. menstrualis, we performed in vitro studies to determine the EC50 and CC50 of these natural products, as well as their effects on HSV-1 adsorption, penetration and the virus replication cycle. We found that the studied compounds were potent inhibitors of HSV-1 replication endowed with the ability to diminish the content of important early viral proteins.

Materials and Methods

Compounds

The diterpenes 8,10,18-trihydroxy-2,6-dolabelladiene (1) and (6R)-6-hydroxydichotoma-4,14-diene-1,17-dial (2) (Fig. 1) were obtained as described previously [15, 17]. These molecules were isolated from the Brazilian brown algae D. pfaffii and D. menstrualis, respectively, and tested for purity as described elsewhere [16, 21]. Both algae were collected at the Brazilian shore; D. pfaffii was collected at Atol das Rocas, Rio Grande do Norte, BR, and D. menstrualis at Buzios, Rio de Janeiro, BR, and they were identified by V. L. Teixeira, one of the authors of this paper. The algae were deposited at the Herbarium of the Universidade do Rio de Janeiro, Brazil (HBR), with the voucher numbers HBR[10017 and HBR[9117 for Dictyota menstrualis and Dictyota pfaffii, respectively. The diterpenes, at over 99% purity, were diluted in 100% dimethyl sulfoxide (DMSO) and stored at −20°C. The resulting DMSO concentrations during the assays were below 0.1%, a level that is not significantly cytotoxic.

Cells and virus

African green monkey kidney cells (Vero cells; ATCC) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U/mL penicillin, and 100 µg/mL streptomycin and incubated at 37°C in 5% CO2. In order to prepare virus stocks, Vero cells were infected with HSV-1 (AR-29 strain) [22] at a multiplicity of infection (MOI) of 0.1. At 24 h postinfection (p.i.), the cells were lysed by three cycles of freezing and thawing, centrifuged at 1500 × g at 4°C for 20 min, and the supernatant was collected and stored at −70°C for further studies [23].

Cytotoxicity assay

Monolayers of Vero cells in 96-multiwell plates (1 × 104/well) were treated with either compound 1 or 2 for 72 h, and 50 µL of a 1 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) diluted in DMEM without serum was then added to the cells. MTT was removed after 3 h, 50 µL of acid-isopropyl alcohol (0.04 N HCl in isopropyl alcohol) was added and the optical density (OD) was read using an automatic plate reader with a 570 nm test wavelength and a 690 nm reference wavelength [24]. The cytotoxic concentration in 50% (CC50) was calculated by linear regression analysis of the dose-response curves generated from the data using Excel for Windows software.

Plaque-forming assay

Monolayers of Vero cells in 6-well plates (3 × 104/well) were exposed to supernatant from the yield reduction assay for 1 h at 37°C. Next, residual viruses were removed, and DMEM containing 5% PBS and 1% methylcellulose (Fluka) (overlay medium) was added to cells. After 72 h at 37°C, the monolayers were fixed with 10% formaldehyde in PBS and stained with a 0.1% solution of crystal violet in 70% methanol, and the virus titers were calculated by scoring the plaque-forming units (PFU) [25].

Yield reduction assay

Monolayers of Vero cells in 24-well plates (1 × 105/well) were infected with HSV-1 at a MOI of 1 or 5 for 1 h at 37°C. Cells were washed and the compounds were added at various concentrations in DMEM with 5% FBS. After 20 h, the cells were lysed, cellular debris were cleared by centrifugation, and virus titers in the supernatant were determined by a plaque-forming assay using Vero cells. In every assay, we included acyclovir (ACV; Sigma, > 99% pure) as a control. The antiviral activity at 50% (EC50) was measured by the linear regression analysis of the dose-response curve mentioned above, using Excel for Windows software.

Adsorption and penetration inhibition assays

In order to evaluate whether compound 1 or 2 could inhibit virus adsorption, Vero cells in 6-well plates were exposed to 100 PFU of HSV-1/well and treated with the EC50 of these compounds at 4°C. Next, cells were washed and covered with overlay medium, and the temperature was raised to 37°C for 72 h [26], after which plaques were analyzed as described in the plaque-forming assay. For this kind of assay, heparin (Sigma; > 99% pure) was used as a control.

In order to measure HSV-1 penetration, we used the protocol mentioned above with some modifications. In brief, the same number of Vero cells was incubated with 100 PFU/well of HSV-1 at 4°C for 1 h. Next, the temperature was raised to 37°C in the presence of compound 1 or 2 and, 1 h after that, cells were washed with PBS containing glucose at pH 2.2. Cells were then

Fig. 1 The chemical structures of compounds 1 and 2 [16, 21].
covered with overlay medium and plaques were counted after 72 h of infection [14].

**Time-of-addition experiments**

In order to analyze whether addition of compound 1 or 2 could be delayed without losing their ability to block HSV-1 replication, and to better understand at what moment of the HSV-1 replication cycle these compounds might be acting, we performed a time-of-addition assay. Vero cells in 6-well plates were infected with 100 PFU/well for 1 h and washed, and the compounds were added at 20 µM (2 × EC50) at different times post-infection (p.i.) [26]. At the same time that compound 1 or 2 was added, cells were also covered with overlay medium and, after 72 h p.i., plaques were counted. As a control, we also performed additional assays with the reference compound acyclovir.

**HSV-1 protein synthesis assay**

In order to understand whether compounds 1 and 2 were able to interfere in HSV-1 protein synthesis, we performed pulse-and-chase experiments. Vero cells in 25 cm² bottles (2 × 10⁶ cells) were infected with HSV-1 at a MOI of 5 or 20 for 1 h at 37°C. Next, residual viruses were removed and the cells were treated with compound 1 or 2 at 10 µM for 4 h. After this period, the cells were replenished with methionine-free DMEM in the presence of the compounds, supplemented with [35S]-methionine (50 µCi/mL) for 2 h. Then, the cells were lysed with buffer A (1 M Tris-HCl, pH 6.8; 0.02% bromophenol blue; 5% β-mercaptoethanol; 10% SDS and 10% glycerol). These cell lysates were counted by liquid scintillation (Packard; model TRI-CARB 2100 TR) and 20 µg of the crude cell extracts, derived from assays in which we used a MOI of 20, were analyzed by SDS-PAGE electrophoresis, as described previously [27]. The protein electrophoresis was performed at least four times and the results are displayed as a representative gel. In every assay performed, an identical protein profile was seen; thus, p values for the representative proteins that had their content impaired were < 0.001.

**Statistical analysis**

All data presented in this paper represent the means ± standard error of the means (SEM) of at least three independent experiments. All statistical analyses were performed using Excel for Windows and p values equal to or less than 0.05 were considered statistically significant by Student’s t-test.

**Results and Discussion**

Since macro- and microalgae represent a fruitful source of natural products [11, 28], and considering that we have previously described the antiviral activity of the diterpenes derived from two distinct Brazilian brown algae, *D. pfaffii* and *D. menstrualis* [15–20], we further explored the mode of inhibition of the main compounds of these algae, compounds 1 and 2, against HSV-1 replication. Initially, we analyzed the effects of compound 1 toward this virus’s replication and found a potent dose- and MOI-dependent effect of this molecule, resulting in an EC50 equal to 1.2 µM with respect to an MOI of 1 and 5.1 µM when tested at an MOI of 5, with low cytotoxicity (CC50 = 184; Table 1). Based on the ratio of CC50 and EC50, we calculated the selective index (SI) values, which were equal to 153 and 36 at MOIs of 1 and 5, respectively. Moreover, we also showed that another brown alga natural product, the diterpene compound 2 derived from *D. menstrualis*, was also able to inhibit HSV-1 replication in Vero cells (Table 1). One more, we observed a pronounced dose- and MOI-dependent inhibition. The resulting EC50 was equal to 1.6 µM and 5.9 µM at MOIs of 1 and 5, respectively (Table 1). As a result of this difference between the CC50 and EC50 for compound 2, a high SI was seen, with values of 625 and 169.4 for MOIs of 1 and 5, respectively.

For comparison, the effect of the reference compound ACV was also studied under our assay conditions. Importantly, the EC50 values for acyclovir were 1.2 µM and 6.5 µM for the MOIs of 1 and 5, respectively (Table 1). As a result of this difference between the CC50 and EC50 for compound 2, a high SI was seen, with values of 625 and 169.4 for MOIs of 1 and 5, respectively.

An additional advantage of our compounds is that both compounds 1 and 2 are inhibitors of HIV replication [15–20]. Therefore, since HSV-1 is an opportunistic pathogen found in HIV-infected individuals [8], our compound formulations could be potentially interesting as leads to the synthesis and/or the search for novel molecules able to simultaneously target the replication of both HIV-1 and HSV-1. This, in the future, from a more pragmatic point of view, might also reduce the number of drugs taken by HIV-infected persons.

Next, in order to gain insight into the stage at which compounds 1 and 2 were acting, we performed time-of-addition assays. Thus, HSV-1–infected Vero cells were treated for different times with the compounds at twofold their EC50 values, with respect to an MOI of 5. Such a dose was used to reach maximum inhibition while preventing nonspecific effects. In this assay, we chose the

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CC50</th>
<th>EC50</th>
<th>SI*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MOI = 1</td>
<td>MOI = 5</td>
<td>MOI = 1</td>
</tr>
<tr>
<td>1</td>
<td>184 ± 21 **</td>
<td>1.20 ± 0.04</td>
<td>5.10 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>1000 ± 83</td>
<td>1.60 ± 0.08</td>
<td>5.90 ± 0.20</td>
</tr>
<tr>
<td>ACV</td>
<td>860 ± 32</td>
<td>1.20 ± 0.10</td>
<td>6.50 ± 0.06</td>
</tr>
</tbody>
</table>

* The selective index (SI) was calculated based on the ratio between CC50 and EC50. ** This value was obtained from our previous work [15].

Table 1 Cytotoxicity and antiviral activity of the natural products derived from the algae *Dictyota pfaffii* and *Dictyota menstrualis*. 
periods of drug addition based on the kinetics of the HSV-1 replicative cycle. For each chosen time, there is a peak of HSV-1 protein synthesis, characteristic of each step of HSV-1 replication [2]. In addition, cells were treated from 0 to 20 h p.i., because one complete HSV-1 replicative cycle lasts for this period [2]. We found that the anti-herpetic activities of compounds 1 and 2 were preserved when these drugs were added to HSV-1-infected cells up to 6 h and 3 h after infection, respectively, declining thereafter (Fig. 2). This result suggests that these diterpenes inhibit initial events during HSV-1 replication. For comparison, addition of acyclovir, which inhibits the early phase of HSV-1 replication, could be delayed up to 6 h after infection. This result suggests that compound 1 and acyclovir might share similar kinetics of inhibition, while compound 2 and the reference compound have different mechanisms of action. Nevertheless, these observations, together with other data in the literature [23,26], raise at least three possible targets for our compounds: viral adsorption, viral penetration and the initial steps of the HSV-1 replication process.

In order to further investigate our natural products’ mechanisms of action, we performed adsorption and penetration assays. Initially, we analyzed the effects of compounds 1 and 2 on viral adsorption. For this purpose, Vero cells were infected and treated at 4°C, since at this temperature, viruses bind but they do not enter the cells. When the temperature was raised to 37°C, only bound viruses could enter; thus, plaque formation after 72 h p.i. was directly proportional to HSV-1 adsorption. We noticed that the natural compounds did not affect viral adsorption (Fig. 3A). For comparison, we used heparin as a positive control at 1 µg/mL; this concentration could inhibit ~80% of HSV-1 adsorption, as has been shown previously [30]. Second, we investigated the effects of compounds 1 and 2 on HSV-1 penetration. Vero cells were infected with HSV-1 at 4°C, the temperature was raised to 37°C, and the test compounds were immediately added to the infected cells. At 4°C, viruses would only bind but they could not enter; when the temperature was raised, viruses would penetrate in the presence of the compound. Therefore, the plaque formation after 72 h was directly proportional to virus penetration. We failed to observe (Fig. 3B) HSV-1 penetration inhibition by the natural compounds.

Since our natural products inhibit HSV-1 at an initial event during its replication, but after viral adsorption/penetration, and considering that it has been described that HSV-1 replication leads to a shut-off of cellular protein synthesis [2], we took advantage of this viral feature to get insight into which viral proteins would have their concentration impaired upon treatment with compound 1 or 2. Vero cells were infected for 1 h at 37°C and were then treated with the compounds at two times their EC50 values. We observed (Fig. 4A) that HSV-1 infection could reduce Vero cell protein content. This event was prevented when the HSV-1-infected Vero cells were treated with either compound 1 or 2. We also noticed that mock-infected cells treated with the natural products had a transient reduction of their protein content; this is probably a transient event because the drug concentration used in this assay is not cytoxic (Fig. 4A). Additionally, to further qualitatively analyze the proteins in HSV-1-infected cells, we performed this same assay using an MOI of 20. This MOI was chosen since it produces a strong inhibition of cellular protein synthesis and, thus, might be useful to evaluate the main targets of our compounds on HSV-1 replication. As shown in Fig. 4B and C, the compounds did not change the protein content of mock-infected cells (lanes 1 and 2 of both panels).
inhibited by this molecule (RL-1 (a modulator of cell cycle and viral gene expression; 66.19 KDa) and UL-9 (ori-binding protein; 94.26 KDa)) (Fig. 4; lanes 3 and 4). This effect was shared, in part, with compound 2, since the concentrations of the proteins UL-8 (viral helicase; 79.92 KDa), RL-1 (a modulator of cell cycle and viral gene expression; 66.19 KDa) and UL-12 (a viral deoxyribonuclease; 67.50 KDa) were also inhibited by this molecule (Fig. 4C, lanes 3 and 4). These targets were identified based on their molecular weights using the proteomic database for HSV-1 strain 17 [31].

Taken together, our results suggest that the natural products used in this study are endowed with a mechanism of inhibition different from classical anti-HSV-1 agents in clinical use, such as ACV and foscarnet [32]. In addition, the results described in the paragraph above lead us to some possible mechanisms by which compounds 1 and 2 are inhibiting HSV-1 replication, since we observed that the compounds diminished viral proteins belonging to the early (E) phase of HSV-1 replication. We believe that the compounds could be directly acting on this phase or that they might be acting at a previous step of HSV-1 replication, such as the immediate-early (IE) phase. In this last case, considering the role of IE proteins as regulatory molecules [33], we think that the viral target for our natural products would be active even during later events of HSV-1 replication, such as early phase. An alternative explanation for our results might be that compounds 1 and 2 could be targeting, and thus impairing, the formation of the pre-initiation complex, as has been shown for other natural compounds [34]. These possibilities are under investigation in our laboratories through several cellular and molecular approaches. Finally, we consider that the continuous study on natural compounds may provide future bases for therapeutic molecules endowed with novel mechanisms of action. Along these lines, we propose that the structures of compounds 1 and 2, brown algae diterpenes obtained from D. pfaaffii and D. menstrualis, respectively, might be encouraging ones for future broad-spectrum antiviral design [15–20].

**Affiliations**

1 Laboratório de Estrutura e Regulação de Proteínas, Programa de Pós-Graduação em Química Biológica, Instituto de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil
2 Departamento de Biologia Marinha, Instituto de Biologia, Universidade Federal Fluminense, Niterói, RJ, Brasil
3 Laboratório de Virologia Molecular, Departamento de Biologia Celular e Molecular, Programa de Pós-Graduação em Neuroimunologia, Instituto de Biologia, Universidade Federal Fluminense, Niterói, RJ, Brasil
4 Laboratório de Vírus Respiratórios e do Sarampo, Laboratório de Referência para Influenza e Vírus Exantemáticos, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil

**References**

1 Heldwein EE, Krummenacher C. Entry of herpesviruses into mammalian cells. Cell Mol Life Sci 2008; 65: 1–16
3 Spear PG. Herpes simplex virus: receptors and ligands for cell entry. Cell Microbiol 2004; 6: 401–410

![Fig. 4](image-url) Effects of the diterpenes compounds 1 and 2 on some viral proteins.

Vero cells were infected with HSV-1 at an MOI of 5 and treated with compound 1 or 2 at 10 µM for 4 h. The cells were then replenished with DMEM containing [³⁵S]-methionine (50 µCi/mL) for 2 h. Cells lysates were counted by liquid phase scintillation (A). Lysates from HSV-1-infected Vero cells (MOI of 20) were also analyzed by SDS-PAGE electrophoresis and the gels were revealed by autoradiography (B and C). In panel A, data represent means ± SEM of four independent experiments and the asterisks represent p < 0.05. Panels B and C display representative gels with the following lanes: 1 – mock-infected Vero cells, 2 – mock-infected Vero cells treated with compound 1 (B) or compound 2 (C), 3 – HSV-1-infected Vero cells and 4 – HSV-1-infected Vero cells treated with compound 1 (B) or compound 2 (C). The molecular weights of the diminished proteins UL-30, UL-9, UL-8, UL-12 and RL-1 are 115.32, 94.26, 79.92, 67.50 and 66.19 KDa, respectively.
Abrantes JL et al. The Effects of ... Planta Med 2010; 76: 339–344