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Caulerpin as a potential antiviral drug against herpes simplex virus type 1

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Abstract: About 80% of the human adult population is infected with HSV-1. Although there are many anti-HSV-1 drugs available (acyclovir, ganciclovir, valaciclovir, foscarnet), their continuous use promotes the selection of resistant strains, mainly in ACV patients. In addition to resistance, the drugs also have toxicity, particularly when administration is prolonged. The study of new molecules isolated from green algae with potential antiviral activity represents a good opportunity for the development of antiviral drugs. Caulerpin, the major product from the marine algae *Caulerpa* Lamouroux (Caulerpales), is known for its biological activities such as antioxidant, antifungal, acetylcholinesterase inhibitor (AChE) and antibacterial activity. In this work, we show that caulerpin could be an alternative to acyclovir as an anti-HSV-1 drug that inhibits the alpha and beta phases of the replication cycle.

Article

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Introduction

The type 1 herpes simplex viruses (HSV-1) are enveloped viruses containing double-stranded linear DNA belonging to the subfamily Alphaherpesvirinae (Gilbert et al., 2002). They are the primary cause of oral and facial lesions in humans, establishing lytic infection in mucocutaneous epithelial cells and latent infection in sensory ganglia (Placek & Berger, 2010).

The lytic cycle of HSV-1 involves the expression of three viral gene sets: an immediate early (IE) or α phase, an initial (I) or β-phase and a late (L) or γ phase (Hancock et al., 2006). In the alpha and beta phase are synthesized factors responsible for regulation of viral replication, antigenic presentation and genome replication, respectively, and in the gamma phase, the proteins that form the capsid (Whitley & Roizman, 2001). After lytic infection and local replication in the mucosa, HSV-1 can reach nerve terminals and infect peripheral sensory neurons. The nucleocapsid moves through axonal transport to the trigemeal ganglia, where the viral genome is released and rapidly associated with histones to form episomal DNA (Knipe & Cliffe, 2008). During latency, the viral transcription is restricted to Latency Associated Transcripts (LAT) and some authors have suggested that LAT produce anti-apoptotic effects (Allen et al., 2011; Perng et al., 2000). In neuronal cells, LAT were shown to reduce viral gene expression and replication during productive infection (Mador et al., 1998).

About 80% of the human adult population is now infected, but mostly may not show clinical manifestations, which contributes for their its greater spread (Kelly et al., 2009). Diseases caused by HSV-1 infection appear frequently (Rozenberg et al., 2011). In children about 3 to 5 years old, clinical manifestations last 5 to 12 days (López Garcia et al., 2002). Bioinformatic analyses revealed a number of putative TH responsive elements (TRE) located in the critical regulatory regions of HSV-1 genes such as thymidine kinase (TK) latency associated transcript (LAT) (Hsia et al., 2011). In addition, infections caused by HSV-1 may be responsible for complications, especially in immunocompromised patients, leading in some cases to permanent injury, encephalitis, and even death death, depending on the severity of the case (Jones & Isaacs, 2004).

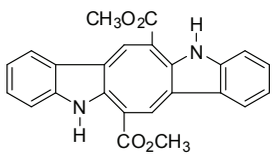
Caulerpa Lamouroux is an exclusively marine and benthic genus of green algae (Caulerpales, Caulerpaceae). *Caulerpa* species are characterized

by a stem divided into an upright portion (fronds) of various sizes and shapes and a prostrate portion with the rhizoids for attachment to the substrate. Due to this external differentiation with the formation of stolons, algae of the genus *Caulerpa* can colonize rocky and sandy substrates (Reviers, 2006). The species *Caulerpa racemosa* (Forsskål) J.Agardh is considered to be an alga with great invasive potential; in just seventeen years after the first report, it colonized twelve countries, as well as several islands in the Mediterranean Sea and Atlantic Ocean (Verlaque et al., 2000, 2004). In addition to the impacted areas, the genera is also found in native form in the southeast of Perth and Hopetown and was introduced in Adelaide in South Australia, where the waters are less impacted (Klein & Verlaque, 2008).

Extracts of the alga *C. racemosa* obtained using solvents of different polarities are reported to exhibit various biological activities such as antioxidant (Cavas & Yurdakoc, 2005, Chew et al. 2008, Matanjun et al., 2008), antifungal, acetylcholinesterase (AChE) inhibitor, antibacterial (Radwan et al., 2007), and antinociceptive activities (Souza et al., 2009). Moreover, they can reduce blood pressure and treat rheumatism (Novaczek, 2001).

Although there are few studies of this species, pharmacology has shown great interest in algae in recent years, mainly because of the secondary metabolites that are potentially useful for the development of new drugs for the treatment for human diseases caused by viruses, bacteria and fungi, as well as certain types of cancer and Alzheimer's (Smit, 2004, Wijesekara et al., 2011).

According to Rocha et al. (2007), caulerpin (**1**) is the major substance in the acetone extract of Brazilian *C. racemosa.* Caulerpin (**1**), a non-toxic red pigment, has a unique bisindole structure found in 80% of the species of *Caulerpa* (Vidal et al., 1984). However, this substance is not unique to *Caulerpa*, having also been isolated from the red alga *Chondrus armatus* (Harvey) Okamura. According to Rocha (2004), this finding reinforces the idea of a consortium of *Caulerpa* and bacterial biofilms, since the production of caulerpin requires the intervention of bacteria capable of making nitrogen available in the water column for the metabolism of these algae.



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Because the drugs currently used to control infection by HSV-1 can cause side effects and strains resistant to these drugs have emerged, this work reports the antiviral activity of caulerpin. Earlier studies with *Caulerpa* had shown anti-HSV activity in sulphated polysaccharides from *Caulerpa brachypus* Harvey, *C. okamurae* Weber-van Bosse and *C. scapelliformis* (R. Brown ex Turner) C. Agardh (Lee et al., 2004). In this work, we analyze the anti-HSV-1 activity of caulerpin isolated from *C. racemosa* collected in the archipelago of São Pedro and São Paulo, a group of rocky islands located about 1000 km off the coast of Rio Grande do Norte.

Materials and Methods

The archipelago of São Pedro and São Paulo is situated at latitude 00° 55'N and longitude 029° 21'W, lying about 600 km from the archipelago of Fernando de Noronha and about 1000 km from Cabo Calcanhar in Rio Grande do Norte, the nearest point on the continental Brazilian coast. Specimens of *Caulerpa racemosa* were collected in May of 2007 and 2008 by scuba diving in the sublittoral zone on the western side of the archipelago in the region called the "Caulerpa zone", at depths ranging from 50 to 10 m. The seaweeds were washed with local sea water and separated from sediments, epiphytes and other associated organisms. The algae were collected and identified by one by us (RCV).

All solvents were HPLC grade. Analytical thin-layer chromatography (TLC) separations were carried out on Merck silica gel 60 F-254 (0.2 mm) precoated aluminum plates. Once developed, the plates were visualized by spraying with 2% ceric sulphate in sulfuric acid, followed by gentle heating. Silica gel 60 (Merck, 70-230 and 230-400 mesh) was used for column chromatography. Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ (100%, Aldrich). After extraction with CH_2Cl_2 at room temperature, the material was filtered and the solvent was removed by using a rotary evaporator under reduced pressure. The extract was stored under refrigeration. The extract was subjected to thin layer chromatography with eluents of different polarities. Analyses by analytical thin layer chromatography (TLC) were performed on silica gel 60 F254 aluminum plates. The revelations of the chromatographic spots were made by inspection under UV light (at 254 and 366 nm) and spraying with a solution of 2% ceric sulfate in sulfuric acid, followed by heating. The column chromatography was performed on silica gel 60 (70-230 mesh) and RP-18 (70-230 mesh).

Air-dried specimens (70 g) were extracted successively with CH_2Cl_2. Evaporation of the crude extract yielded 2.4 g of residue (about 3.3% of the dry mass) of which 1 g was subjected to purification by silica gel-column chromatography (elution with *n*-hexane, CH_2Cl_2 and EtOAc). The fractions eluted with CH_2Cl_2/ EtOAc (1:1) contained 1, obtained in pure form as orange-red needle-shaped crystals after re-crystallization from n-hexane. The identification of 1 as caulerpin was

based on comparison of physical and spectroscopic data with literature data

Cells and viruses

Vero cells (African green monkey *Cercopithecus aethiops* kidney cells; ATCC, Hanassas, VA, USA) were cultured in Dulbecco´s modified medium (DMEM) supplemented with 5% of fetal bovine serum (FBS; HyClone, Logan, UT, USA), 0.1 µM HEPES and 2.5 µg/mL gentamycin at 37ºC in 5% CO_2. Vero cells were subconfluent in all assays and were used prior to passage 20. The stock of HSV-1 was obtained with the strain HSV-1 (AR-29) (Lagrota et al., 2004; Andrighetti-Fröhner et al., 2005) at a multiplicity of infection (MOI) equal to 0.1 for 1 h at 37 ºC. Briefly, after the incubation, the monolayer was washed with phosphate-buffered saline (PBS) and cells were cultured for an additional 48 h. After this period, the cells were lysed by three cycles of freezing, centrifuged at 1500×*g* at 4 ºC for 20 min to remove cellular debris, and the supernatants collected, titered by plaque assay and stored at -70 ºC for further studies.

Cytotoxicity assay and selectivity index (SI)

The HSV-1 KOS strain, susceptible to acyclovir (ACV), was diluted in DMEM without serum and multiplied in VERO cells, using a multiplicity of infection (MOI) of 0.1 (Lagrota et al., 1994; Esquenazi et al., 2002). After 24 h of infection, the cells were lysed by freezing and centrifuged at 400×*g* for 20 min at 4 °C. The HSV-1 titer was determined by viral plaque reduction assay (Kuo et. al., 2001). Virus stocks were stored at -70 ºC until use.

Cytotoxicity assays

To evaluate the cytotoxicity of caulerpin (**1**), we used the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma), a water-soluble compound that has a yellow color in aqueous solution and is readily incorporated by viable cells, where it is reduced by dehydrogenases present in mitochondria. When reduced, MTT is converted into formazan, a purple-colored water-insoluble substance stored in the cytoplasm of the cell. The MTT cytotoxic assays was performed with Vero cells in 96-multiwell plates (10^5 cells/well) treated with different concentrations (50, 250,500 and 1000 µM) of caulerpin (**1**) at 37 ºC in an atmosphere of 5% CO_2 for 72 h. After this, 50 µL of a 1mg/mL stock solution of MTT was added to each well. After incubation for 3 h, each well received 50 µL of acidic isopropanol (0.04N HCl in isopropanol). The concentrations were determined in an automatic plate reader at 570 nm as the measurement wavelength and 690 nm as the reference wavelength (Mosmann, 1983; Denizot & Lang, 1986). The 50% cytotoxic concentration (CC50) was calculated from the experimental dose-response curves by linear regression analysis.

Plaque reduction assay

For the plaque assay, we infected monolayers of Vero cells (10^5) in 24-well plates with HSV-1 (KOS strain) at an MOI equal to 1 for 1 h at 37 ºC. Cells were washed with PBS to remove residual virus and the test substance was added diluted in DMEM with 2.5% FBS. After 20 h, cells were lysed and cellular debris was cleared by centrifugation. The virus titer was determined in Vero cells maintained in six well plates (3 x 10^5 cells/well) and infected with different dilutions of HSV-1 for 1 h at 37 °C in an atmosphere of 5% CO_2. After this period, residual virus was removed and the monolayers were covered with DMEM with 5% fetal bovine serum and 1% methylcellulose. After 72 h, cell monolayers were fixed with 10% formaldehyde and stained with 0.1% crystal violet. Then the viral titer was determined by the number of viral plaques (PFU/mL). For comparison, linear regression of the dose response curves for acyclovir (ACV) was also performed to calculate EC50 values.

Time course infection

In order to determine the inhibition of the replicative cycle of HSV-1 by caulerpin (**1**), Vero cells were cultured in six well plates (3 x 10^5 cells/well) and separated into five groups (Gong et al., 2002). *Adsorption*: Vero cells were treated with 50 µM of the compound for 2 h at 4 ºC. After washing, cells were infected with 100 PFU/mL for 1 h at 37 °C in an atmosphere of 5% CO_2. *Penetration*: Vero cells were infected with 100 PFU/mL at 4 °C for 1 h, washed with glycine solution in PBS, pH 2.2, and the temperature raised to 37 °C for 1 h. *0-3 h p.i.*: Vero cells were infected with 100 PFU/ml HSV-1 for 1 h at 37 °C in an atmosphere of 5% CO_2. After this period (time 0), the viral inoculum was removed and the cells were cultured with the test substance (50 µM) for 3 h. *3-6 h p.i.*: Vero cells were infected with 100 PFU/ml HSV-1 for 1 h at 37 °C in an atmosphere of 5% CO_2. After 3 h p.i., the cells were treated with the substance, 6-20h p.i.: Vero cells were infected with 100 PFU/ml HSV-1 for 1 h at 37 ° C in an atmosphere of 5% CO_2. At 6 *h p.i.* the cells were treated with 10 µM test substance, which remained in contact with the cell. After each of these procedures, the monolayers were covered with DMEM with 5% fetal bovine serum and 1% methylcellulose for 72 h, fixed with 10% formaldehyde and stained with 0.1% crystal violet to determine the number of viral plaques.

Results and Discussion

Tests of cell viability are important to determine the cytotoxicity of new substances in the early stages of drug development (Putnam et al., 2002). The equilibrium between the pharmacological and toxicological effects of a substance is an important requirement for its applicability as a future drug (Melo et al., 2000). In this experiment, we determined the concentrations of the substance that are able to kill 50% of Vero cells (CC50) and compared these values with those obtained with acyclovir, used as the positive control. The viability test performed in Vero cells showed that caulerpin is very promising as a potencial drug for human cells. The CC50 value of caulerpin (**1**) was even better than that of acyclovir (Table 1), reaching almost 18% of the reference value. These results encouraged us to continue the examine its anti-HSV-1 activity.

By definition, cytopathic effects (CPE) are irreversible modifications caused by viral replication in the host cells. For Vero cells submitted to HSV-1 replication, there is a loss of adherence and the cells acquire a round shape and bright appearance and are often linked to each other by cytoplasmic processes (Figure 1). Our results showed an EC50 value very similar to that of acyclovir. We cannot say at this time whether these substances act in the same stage of the replicative cycle. According to Rang et al. (2001) there are numerous mechanisms of action of antiviral drugs and those that are most employed in clinical use during the lytic cycle act on the viral polymerase in the adsorption and penetration phases (Rang et al., 2001, De Clercq, 2001, 2004). The discovery of acyclovir (Zovirax®) as a selective anti-herpes agent by Schaeffer and colleagues (Elion et al., 1977, Schaeffer et al., 1978) began a new era in antiviral chemotherapy in 1978. ACV is a nucleoside analogue with an incomplete guanine ribosyl lacking the hydroxyl groups at carbons 2' and 3'. In its mechanism of action, it is phosphorylated by a viral thymidine kinase and subsequently is di-and tri-phosphorylated by celular enzymes. Finally, when it is captured by the viral DNA polymerase (complex UL30/UL42), it results in disruption of the formation of the DNA strand due to the absence of the 3'-OH (Cheng et al., 1983, Coen, 1996; De Clercq, 2004). Our next step will be to study more thoroughly the action with the viral polymerase.

Based on the results obtained for cell viability and the antiviral inhibitory effect, it is possible to calculate the selectivity index (SI), which represents the degree of safety for the use of a compound. This pharmacological parameter was calculated from the ratio between the CC50 and the EC50 of the substance. For caulerpin (**1**), the S.I. value was higher than that of acyclovir (Table 1), showing that it is somewhat safer as an antiviral. Caulerpin (**1**) thus represents a possible candidate for further esting for anti-HSV-1 therapy in pre-clinical studies.

Table 1. Cell viability (CC50), antiviral effect (EC50) and selectivity index (S.I.) for caulerpin (**1**) in pre-clinical tests compared to acyclovir.

Substance	CC50 (μM)*	EC50 (μM)*	S.I.*
caulerpin	1167	1,29	904
acyclovir	960	1,09	880

*All assays were performed in triplicate with Vero cells according to methods described in the literature.

Another approach that permits a more detailed analysis of viral inhibition is accomplished through successive stages of disruption of the genes involved in the replicative cycle of HSV-1. The replicative cycle of herpes simplex virus results in the activation and regulation of three consecutive groups of genes: immediate, early and late, which express the alpha, beta and gamma proteins, respectively. Through changes in the temperature of incubation and different periods in the presence of the infected cells, we could verify the stage where caulerpin stops the viral replication, *i.e.*, at the alpha, beta or gamma genes.

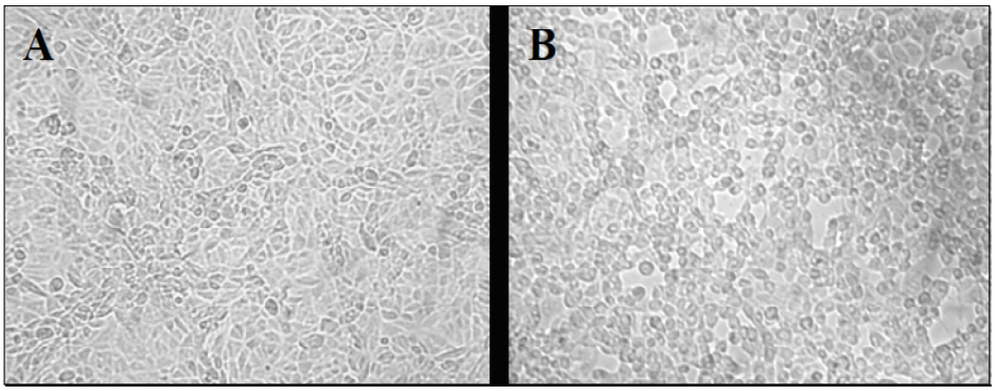

Figure 1. Representative photomicrographs of Vero cells with cytopathic effects after 72 h of infection with HSV-1. A. Control without treatment; B. Infected without treatment.

The anti-HSV-1 activities of several compounds derived from brown algae have been reported. Abrantes et al. (2010) showed that 8,10,18-trihydroxy-2,6-dolabelladiene and 6-hydroxydichotoma-4,14-diene-1,17-dial inhibited HSV-1 replication in a dose-dependent manner, resulting in EC50 values of 5.10 and 5.90 μM, respectively. Our results showed that caulerpin (**1**) could partially inhibit the expression of the early and late genes of HSV-1 (Figure 2). The β-phase proteins include enzymes that are necessary for replication of the viral genome: DNA polymerase (complex UL30/UL42); thymidine kinase (TK); binding protein of the single-stranded DNA (SSB), also known as ICP8; DNA helicase-primase; origin binding protein (UL9 protein); and those involved in nucleotide metabolism. The viral DNA replication stimulates the transcription of the late genes encoding structural proteins of the virion (Cliffe & Knipe, 2008). The kinetics of gene expression end with the appearance of the proteins of the γ phase, which are the structural proteins of the virion, including tegument and envelope and viral particle assembly proteins (Boehmer & Lehman, 1997).

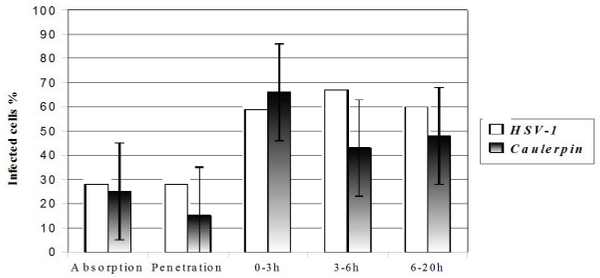

Figure 2. Time course of the infection of HSV-1 in the presence of caulerpin (50 μM).

The reduction of 34% in the replication means that this inhibition is not complete, but we believe that the concentration of the substance may have been underestimated. Caulerpin (**1**) has no effect as a virucidal substance (data not shown), even at 4 h of incubation in systems without the presence of cells.

In conclusion, the study of new molecules isolated from green algae with potential antiviral activity appears to be a good opportunity for the development of antiviral drugs, especially for the α and γ phases of the replication of HSV-1, which acyclovir is not able to inhibit. The development of new substances against these phases of the replication of HSV-1 is of particular interest since there are currently no drugs available at the clinic stage that target these steps. Moreover, these steps are different from those inhibited by ACV. The current study clearly demonstrates that caulerpin (**1**) is a promising substance for anti-HSV-1 therapy. As a result, we are currently conducting additional toxicity and activity studies to determine the effectiveness of caulerpin *in vivo.*

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