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Antiviral activity against dengue virus of diverse classes of algal sulfated polysaccharides

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ABSTRACT

Diverse classes of sulfated polysaccharides obtained from the red seaweeds (Rhodophyta) *Grateloupia indica*, *Scinaia hatei* and *Gracilaria corticata*, the brown seaweeds (Phaeophyta) *Stoechospermum marginatum* and *Cystoseira indica* and the green seaweed (Chlorophyta) *Caulerpa racemosa* were assayed for antiviral activity against the four serotypes of dengue virus (DENV). DENV-2 was the most susceptible serotype to all polysulfates, with inhibitory concentration 50% values in the range 0.12–20 μg/mL. The antiviral potency of the sulfated polysaccharides depended on the sulfate content, the position of sulfate group, the sugar composition, and the molar mass. Independently of the sugar composition, the antiviral effect was mainly exerted during DENV-2 adsorption and internalization.

1. Introduction

Dengue virus (DENV) is a member of the family Flaviviridae transmitted to human by two species of mosquitoes, *Aedes aegypti* and *Aedes albopictus*. The virion is an enveloped particle containing a single positive-stranded RNA genome and three structural proteins. Human DENV infection can be asymptomatic or present a range of clinical manifestations from the self-limited febrile illness called dengue fever to the more severe forms of dengue hemorrhagic fever and dengue shock syndrome, with a high degree of lethality [1]. There are four serotypes (DENV-1 to DENV-4) which co-circulate in tropical and subtropical regions. Currently, dengue is endemic in more than 100 countries in Southeast Asia, the Western Pacific, America, Africa and the Middle East and is considered the most prevalent arthropod-borne disease worldwide [2,3].

Despite this threat for human health, no specific chemotherapy or safe vaccination for DENV infection is currently available [4,5]. The only treatment for patients is supportive therapy. Therefore, there is a requirement for effective antiviral agents and therapeutic strategies for DENV infection. Since the first report about the role of heparan sulfate (HS) in the initial interaction for DENV attachment to vertebrate cells, diverse HS-like glycosaminoglycans were evaluated as antiviral agents against DENV [6–11]. Within this field, seaweeds represent a natural source rich in sulfated polysaccharides, compounds mimicking HS produced at low cost and with few adverse effects. Different types of hybrid DL-galactans and carrageenans obtained from red seaweeds are the most extensively studied class of algal polysaccharides analyzed against DENV infections and were found to be very potent and selective inhibitors of DENV-2 multiplication in mammalian cells [12–16]. A few studies have evaluated other type of natural polysaccharides with variable results [17–20].

The aim of the present study was to evaluate comparatively the antiviral activity against DENV of different structural classes of sulfated polysaccharides isolated from red, brown and green seaweeds collected in the Arabian Sea. These polysulfates proved previously to be potent and selective inhibitors of herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) interfering with virus binding to the host cell [21–26]. Here we report their in vitro effectiveness against all DENV serotypes and the mode of action to block the infection of DENV-2 in Vero cells.

2. Materials and methods

2.1. Sulfated polysaccharides

The extraction and fractionation of the sulfated polysaccharides from the Rhodophyta *Grateloupia indica*, *Gracilaria corticata* and *Scinaia hatei*, the Phaeophyta *Cystoseira indica* and *Stoechospermun marginatum*, and the Chlorophyta *Caulerpa racemosa*, all collected

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from the Gujarat coast of the Arabian Sea in India, were previously described [21–26]. Briefly, each depigmented algal powder was extracted with cold (25–35 °C) or hot water (80 °C) to obtain the crude water extracts GiWE, GcWE, ShWE, SmWE, CiWE and CrHWE from *G. indica*, *G. corticata*, *S. hatei*, *C. indica*, *S. marginatum*, and *C. racemosa*, respectively. Then, the purified fractions GiF3, GcF3, SmF3 and CiF3 were obtained by anion exchange chromatography from GiWE, GcWE, SmWE and CiWE, respectively, whereas the fraction ShF1 was obtained by size exclusion chromatography from ShWE.

Two commercial products, dextran sulfate with an average molecular weight of 8000 (DS8000) and heparin (Sigma–Aldrich Co., U.S.A.) were also tested as control compounds.

2.2. Cells and viruses

Vero (African green monkey kidney) cells were grown in Eagle's minimum essential medium (MEM) (GIBCO) supplemented with 5% calf serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%. For plaquing medium (PM), methylcellulose was added to a final concentration of 1%. The C6/36 mosquito cell line from *Aedes albopictus*, adapted to grow at 33 °C, was cultured in L-15 Medium (Leibovitz) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% MEM non-essential amino acids solution and 5% fetal bovine serum.

DENV-1 strain Hawaii was obtained from Instituto Nacional de Enfermedades Virales Humanas (INEVH) Dr. J. Maiztegui (Pergamino, Argentina). DENV-2 strain NGC, DENV-3 strain H87 andDENV-4 strain 8124 were provided by Dr. A.S. Mistchenko (Hospital de Niños Dr. Ricardo Gutiérrez, Buenos Aires, Argentina). Virus stocks were prepared in C6/36 cells and titrated by plaque formation on Vero cells.

2.3. Cytotoxicity assay

Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma–Aldrich Co., U.S.A.) method. Confluent cultures in 96-well plates were exposed to different concentrations of the polysaccharides, with three wells for each concentration, using incubation conditions equivalent to those used in the antiviral assays. Then 10 μL of MM containing MTT (final concentration 0.5 mg/mL) was added to each well. After 2 h of incubation at 37 °C, the supernatant was removed and 200 μL of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm. The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

2.4. Antiviral assay

Antiviral activity was evaluated by a virus plaque reduction assay. Vero cell monolayers grown in 24-well plates were infected with about 50 PFU/well in the absence or presence of various concentrations of the compounds. After 1 h of adsorption at 37 °C, residual inoculum was replaced by MM containing 1% methylcellulose and the corresponding dose of each compound. Plaques were counted after 6–12 days of incubation at 37 °C, according to virus serotype. The inhibitory concentration 50% (IC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50%. All determinations were performed twice and each in duplicate.

2.5. Virucidal activity

A DENV-2 suspension containing 6×10^5 PFU/mL was incubated with an equal volume of MM with or without different concentrations of GiWE, CrHWE and SmWE, for 1 h at 37 °C. Then, the samples were diluted in MM and the remaining infectivity was titrated by plaque formation. The sample dilution effectively reduced the drug concentration to be incubated with the cells at least 100-fold to assess that titer reduction was only due to cell-free virion inactivation. The virucidal concentration 50% (VC_{50}), defined as the concentration required to inactivate virions by 50%, was then calculated.

2.6. Effect on virus adsorption and internalization

Vero cells grown in 24-well plates were infected with 500 PFU of DENV-2 following different treatment conditions. Adsorption: cells were exposed to DENV-2 in the presence of 1, 3 or 30 μg/mL of GiWE, CrHWE or SmWE, respectively. After 1 h at 4 °C, both compounds and unadsorbed virus were removed. The cells were washed with cold phosphate-buffered saline (PBS) and overlaid with plaquing medium. Internalization: cells were infected in compound-free MM and after 1 h adsorption at 4 °C, cells were washed and further incubated at 37 °C during 1 h in MM containing 1, 3 or 30 μg/mL of GiWE, CrHWE or SmWE, respectively. Thereafter, cells were washed with PBS and treated with citrate buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3) for 1 min to inactivate adsorbed but not internalized virus. Then, cells were washed with PBS and covered with MM containing methylcellulose. Always: the compounds were present during adsorption at 4 °C and in the medium added after adsorption. For all treatments, virus plaques were counted after 6 days of incubation at 37 °C and results were expressed as % inhibition of each treatment with respect to untreated cell control.

3. Results and discussion

3.1. Chemical composition of sulfated polysaccharides

Many viruses display affinity for cell surface HS proteoglycans with biological relevance to virus entry. This raises the possibility of the application of sulfated polysaccharides in antiviral therapy. In this study, we have analyzed the activity of sulfated galactan, fucan and xylomannan-containing fractions isolated from different seaweeds against DENV.

The sugar composition, the molecular mass and the degree of sulfation of the crude and purified fractions were previously reported in detail [21–26]. A brief summary of the chemical properties of the different polysaccharides here evaluated for anti-DENV activity is presented in Table 1.

According to their sugar composition, the sulfated polysaccharides GiWE, GiF3, GcWE and GcF3 were classified as sulfated galactans, the products from *S. hatei* ShWE and ShF1 were sulfated xylomannans, and the polymers SmWE, SmF3, CiWE and CiF3 were sulfated fucans. The hot water-extracted fraction from *C. racemosa* CrHWE showed a very heterogeneous sugar composition with the presence of similar amounts of galactose, glucose, arabinose and xylose, together with smaller amounts of mannose and rhamnose as minor components and hence it was named as heteropolysaccharide [21].

3.2. Antiviral activity against DENV-2

The antiviral activity against DENV-2 of the crude and purified fractions isolated from different seaweeds was evaluated in Vero cells by a virus plaque reduction assay. Previously, the cytotoxicity