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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

Table 1
Chemical composition of the sulfated galactans (GiWE and GiF3 from *G. indica*, and GcWE and GcF3 from *G. corticata*), the fucans (CiWE and CiF3 from *C. indica*, and SmWE and SmF3 from *S. marginatum*), the xylomannans (ShWE and ShF1 from *S. hatei*) and the heteropolysaccharide (CrHWE from *C. racemosa*).

	GiWE	GiF3	GcWe	GcF3	CiWE	CiF3	SmWE	SmF3	ShWE	ShF1	CrHWE
Total sugar[a]	43	43	34	27	40	40	43	37	39	40	37
Uronic acid[a]	3	2	Tr[d]	Tr	4	2	4	Tr	–[e]	–	4
Sulfate[a]	11	16	11.5	10	8	9	10	13	9	8	9
Mol. mass (kDa)	Nd[c]	60	Nd	30	Nd	35	Nd	40	Nd	160	70–130
Rha[b]	–	–	Tr	Tr	Tr	–	Tr	–	–	–	1
Fuc[b]	1	–	Tr	Tr	75	87	91	96	–	–	Tr
Ara[b]	–	–	–	Tr	Tr	Tr	–	–	–	–	19
Xyl[b]	2	–	Tr	Tr	14	7	3	2	38	23	15
Man[b]	–	–	–	–	Tr	1	1	–	62	77	5
6-Me-Gal[b]	–	–	33	32	–	–	–	–	–	–	–
Anhy-Gal[b]	–	–	20	19	–	–	–	–	–	–	–
Gal[b]	94	100	47	49	11	5	3	2	Tr	–	31
Glc[b]	3	–	Tr	Tr	Tr	Tr	2	–	Tr	–	30

[a] Values expressed as percent weight of fraction dry weight.
[b] Values expressed as mol.% of anhydro sugar.
[c] Nd: not determined.
[d] Tr: trace.
[e] –: not detected.

of these products was evaluated in the same cell system and no alterations were detected in cell viability as determined by MTT assay at concentrations up to 1000 μg/mL (data not shown). Then the CC_{50} values were considered greater than 1000 μg/mL for all crude and purified fractions.

As shown in Table 2, all the compounds tested were active against DENV-2 with IC_{50} in the range 0.12–20 μg/mL. The most active compounds were the sulfated galactans GiWE and GiF3 derived from *G. indica*, with IC_{50} values around 0.1 μg/mL, followed by the sulfated xylomannans from *S. hatei* and the heterogeneous sulfated polysaccharides from *C. racemosa*, with IC_{50} values in the range 0.6–1.1 μg/mL. Interestingly, although all the sulfated galactans showed selectivity toward DENV-2, the polysaccharides GcWE and GcF3 obtained from the red seaweed *G. corticata* exhibited an anti-DENV-2 activity very lower in comparison to the galactans GiWE and GiF3 from *G. indica* with IC_{50} values about 100–200-fold higher. The antiviral potency of a sulfated polysaccharide appears to depend, *inter alia*, on the position of the sulfate group, the sugar composition and the molecular mass [27,28]. Fraction GcF3, with a molecular mass of 30 kDa, consisted of a backbone of 3-linked β-D- and 4-linked α-D-galactopyranosyl residues. This linear galactan contained Gal_2Xyl_1, Gal_2AnGal_2, Gal_4 and Me-Gal_3AnGal_2 as oligomeric building subunits and sulfate group when present was located at C-4 of 3-linked galactopyranosyl residues of this polymer [23]. In contrast, the galactan GiF3 from *G. indica* had higher molecular mass (60 kDa) and the positions of the sulfate groups were also different [24]. Here, sulfate groups, if present, were located mostly at C-2/6 of 4-linked and C-4/6 of 3-linked galactopyranosyl units. Moreover, the presence of anhydro-galactose residue was not detected. Therefore, these chemical features justify the higher antiviral potency of GiF3 in comparison to GcF3.

Table 2
Antiviral activity of sulfated polysaccharides from different seaweeds against DENV-2.

Seaweed	Fraction	IC_{50} (μg/mL)[a]	SI [b]
Rhodophyta			
G. indica	GiWE	0.18 ± 0.06	>5555
	GiF3	0.12 ± 0.07	>8333
S. hatei	ShWE	1.1 ± 0.2	>909
	ShF1	0.6 ± 0.1	>1667
G. corticata	GcWE	10.0 ± 0.9	>100
	GcF3	20.0 ± 2.5	>50
Phaeophyta			
S. marginatum	SmWE	6.3 ± 1.0	>158
	SmF3	4.1 ± 1.3	>246
C. indica	CiWE	8.1 ± 2.7	>124
	CiF3	4.0 ± 1.4	>250
Chlorophyta			
C. racemosa	CrHWE	0.6 ± 0.1	>1667
Reference compounds			
	Heparin	1.9 ± 0.2	>526
	DS8000	0.9 ± 0.1	>1111

[a] IC_{50} (inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Mean of two determinations ± SD.
[b] SI (selectivity index): CC_{50}/IC_{50}. CC_{50} (cytotoxic concentration 50%): concentration required to reduce 50% the number of viable Vero cells after 6 days of incubation with the compounds. This concentration was >1000 μg/mL for all the compounds tested.

With respect to the fucans, both fractions derived from *S. marginatum* and *C. indica* showed moderate activity against DENV-2, with IC_{50} values between 4.0 and 8.1 μg/mL. The crude water-extracted fraction SmWE from *S. marginatum* and the purified fraction SmF3 obtained by anion exchange chromatography consisted of a backbone of 4- and 3-linked-α-L-fucopyranosyl residues sulfated mostly at C-2 and/or C-4 position [22]. On the other hand, the purified fucan CiF3 isolated from *C. indica* contained a backbone of 3-linked α-L-fucopyranosyl residues substituted at C-2 with fucopyranosyl and xylopyranosyl residues [25]. This sulfated fucan, considered the active principle of the *C. indica* water extract (CiWE), also contained variously linked xylose and galactose units and glucuronic acid residues. Sulfate groups, if present, were located mostly at C-4 of 3-linked α-L-fucopyranosyl units. Perhaps the relatively similar average molecular mass of SmF3 (40 kDa) compared to CiF3 (35 kDa) and their similar sulfate contents (8–13%, weight/weight) and sugar compositions (L-fucose as the major sugar) are responsible for exhibiting similar potency toward DENV-2.

Heparin and DS 8000, two commercial polysaccharides known for their antiviral properties against several enveloped viruses, were simultaneously tested as reference substances showing an intermediate behavior in relation to the algal extracts.

Given the lack of cytotoxicity of these polymers, high values of selectivity index (SI), defined as the CC_{50}/IC_{50} ratio, were obtained for all tested fractions (Table 2). Particularly, the products derived from *G. indica* presented SI higher than 5000 and could be considered very effective and selective inhibitors of DENV-2.

Therefore, the anti-DENV-2 activities of the sulfated polysaccharides of present study are not merely a function of high charge density, but have distinct structural specificities such as position of the sulfate groups, the molar mass and constituent sugars which are undoubtedly important.

Table 3
Spectrum of antiviral activity of sulfated polysaccharides against DENV serotypes.

Compound	IC_{50} (μg/mL)[a]		
	DENV-1	DENV-3	DENV-4
GiWE	25.0 ± 2.5	6.25 ± 0.7	>200
ShWE	25.0 ± 2.3	6.25 ± 1.0	69.8 ± 6.5
SmWE	>100	100.0 ± 2.2	>100
CrWE	>100	16.5 ± 2.3	>100
Heparin	>100	10.8 ± 2.5	>100
DS8000	>100	18.3 ± 1.2	31.2 ± 2.4

[a] IC_{50} (inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Mean of two determinations ± SD.

3.3. Spectrum of antiviral activity against DENV serotypes

Next, the spectrum of antiviral activity against the four serotypes of DENV was analyzed. To this end, an active sample representative of each class of algal sulfated polysaccharide was tested, together with the reference substances. Interestingly, the sulfated polysaccharides of present study showed selectivity against different serotypes of DENV. As shown in Table 3, DENV-3 was inhibited by all the sulfated polysaccharides, but the level of antiviral susceptibility exhibited by this serotype was low in comparison to DENV-2. In fact, the IC_{50} values against DENV-3 were 5–35-fold higher than those corresponding to DENV-2. The reactivity of the polysulfates with DENV-1 and DENV-4 was still weaker, with either a very low or a total lack of virus inhibition even at high concentrations of the algal extracts.

The differential susceptibility of DENV serotypes to diverse classes of sulfated polysaccharides here shown is in agreement with previous studies about the antiviral activity of heparin, carrageenans and natural galactans against DENV serotypes in mammalian cells, reporting the high susceptibility of DENV-2 and the resistance of DENV-1 [7,14,16]. The structural differences among sulfated polysaccharides here tested did not appear to be the sole factor that influences their variable effectiveness against DENV serotypes. These variations may be probably ascribed to the differences in virus–cell interactions during entry of DENV-1 and DENV-2 into Vero cells [29], and may represent a disadvantage for the future possibilities of these compounds to be used as anti-DENV therapeutic agents, due to the co-circulation of the four serotypes in endemic regions [2,3].

3.4. Mode of inhibition of DENV-2

The virucidal activity of GiWE, SmWE and CrHWE against DENV-2 was first analyzed to elucidate the possibility that these sulfated polysaccharides may act directly on the virus particles. Although sulfated polysaccharides/oligosaccharides usually lack virus inactivating properties, there are some reports on this type of substances as virucidal agents [12,27,30,31]. After direct incubation of a DENV-2 suspension with the compounds, the remaining infectivity in the mixture was determined by plaque formation in Vero cells. No inactivating effect was observed with GiWE and SmWE at concentrations 5-fold exceeding the antiviral IC_{50}, whereas for CrHWE the VC_{50} was 2.5 μg/mL, a value nearly 4-fold higher than the IC_{50} (0.6 μg/mL). Consequently, even in this last case, the inhibitory action of these sulfated polysaccharides against DENV-2 may be mainly ascribed to an interference with any step of the virus multiplication cycle and not to an interaction with cell-free virions.

Given the probable interference of these algal polysaccharides with the initial interaction between DENV-2 and the host cell membrane during virus entry [6,14,16], the effect of GiWE, SmWE and CrWE on the initial steps of virus adsorption and internalization was studied performing a plaque reduction assay under different

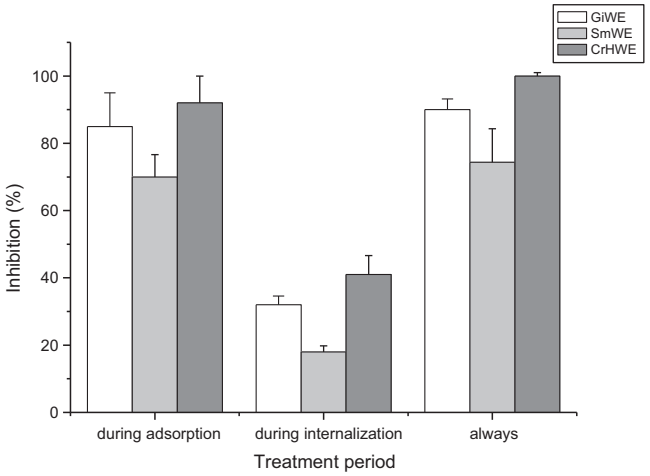

Fig. 1. Effect of sulfated polysaccharides on DENV-2 adsorption and internalization. Vero cells were infected with 500 PFU of DENV-2 in a plaque assay under different treatment conditions with GiWE (1 μg/mL), CrHWE (3 μg/mL) and SmWE (30 μg/mL). Adsorption: cells were infected with DENV-2 in MM containing compound and, after 1 h adsorption at 4 °C, were overlaid with compound-free plaquing medium. Internalization: DENV-2 was adsorbed to cells at 4 °C, then MM containing compound was added and incubation continued at 37 °C for 1 h. Then, cells were treated with citrate buffer and covered with plaquing medium. Always: cells were infected and maintained with compound during adsorption and throughout all the incubation period for plaque formation. In all cases, plaques were counted after 6 days of incubation at 37 °C. Results are expressed as % inhibition respect to untreated infected controls. Each value is the mean of duplicate determinations ± SD.

treatment conditions. Vero cells were infected with DENV-2 and the compounds were exposed to the cells during different periods: (a) only during virus adsorption (1 h at 4 °C); (b) only during virus internalization (1 h at 37 °C, after virus adsorption at 4 °C in compound free-medium); and (c) both during adsorption and throughout all the incubation period after adsorption. As can be concluded from data presented in Fig. 1, a strong inhibitory effect was observed when the three sulfated polysaccharides were present only during adsorption. Under these treatment conditions, the level of inhibition was similar to that observed when the compounds were added during adsorption and maintained throughout the whole incubation period, indicating that DENV-2 adsorption is the main antiviral target for the different algal polysaccharides. However, a lower but significant activity of GiWE, SmWE and CrWE when absent during DENV-2 adsorption and present only during the subsequent step of virus internalization (1 h at 37 °C after adsorption) was also observed. The amount of virus internalized to the cells in presence of the polysulfates was reduced in comparison to untreated cells, showing an inhibition of 18, 32 and 41% for SmWE, GiWE and CrHWE, respectively (Fig. 1).

Interference with viral adsorption agrees with our previous studies about the antiviral activity of these polysulfates against HSV and consist in blocking virus binding to residues of HS proteoglycans present in the cell [22,24,25]. A similar mode of action as virus attachment inhibitors was reported for the antiviral activity of other algal sulfated polysaccharides against HSV and other enveloped viruses. In contrast, no post-adsorption inhibitory action against HSV was observed in the above mentioned studies as here reported for DENV-2 internalization, a disparity probably due to differences in the internalization process into Vero cells between both viruses: endocytocis for DENV and fusion at plasma membrane for HSV.

4. Conclusions

In conclusion, the results reported here demonstrate that diverse classes of sulfated polysaccharides isolated from red, brown

or green seaweeds are potent and selective inhibitors of DENV-2. The reactivity of the most active compounds was variable against the other three DENV serotypes, with an effective inhibitory action against DENV-3 and a weak effect or lack of inhibition against DENV-1 and DENV-4. Independently of the sugar composition, these polysaccharides were found to be competitors of host cell membrane components involved in virus entry since the anti-DENV activity here described might be attributed mainly to a blockade in virus adsorption and also a minor inhibitory effect in virus internalization. Furthermore, this novel work opens new avenues for related studies on sulfated polysaccharides from other seaweeds, also increasing the library of knowledge and lead compounds for antiviral studies.

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