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Antiviral Research 66 (2005) 103–110
Antiviral Research
www.elsevier.com/locate/antiviral

The antiviral activity of sulfated polysaccharides against dengue virus is dependent on virus serotype and host cell

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Received 8 September 2004; accepted 2 February 2005

Abstract

Two homogeneous sulfated polysaccharides obtained from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*, the kappa/iota/nu carrageenan *G3d* and the DL-galactan hybrid *C2S-3*, were assayed for their antiviral properties against the four serotypes of dengue virus (DENV) in different host cell types. Both seaweed derivatives were selective inhibitors of DENV-2 multiplication in Vero cells with inhibitory concentration 50% (IC_{50}) values around 1 μg/ml and selectivity indices >1000. The compounds had a lower antiviral effect against DENV-3 (IC_{50} values in the range 13.9–14.2 μg/ml), an even lower effect against DENV-4 (IC_{50} values in the range 29.3 to >50 μg/ml) and were totally inactive against DENV-1. With respect to the host cell, the polysulfates were inhibitors of DENV-2 and DENV-3 in the human hepatoma HepG2 and foreskin PH cells, with similar antiviral effectiveness as in Vero cells, but were totally inactive in mosquito C6/36 HT cells. Mechanistic studies demonstrated that *G3d* and *C2S-3* were active DENV-2 inhibitors only when added together with the virus or early after infection, and both initial processes of virus adsorption and internalization are the main targets of these compounds. Therefore, the variations in antiviral activity of the polysaccharides depending on the viral serotype and the host cell may be ascribed to differences in the virus-cell interaction leading to virus entry.

Keywords: Dengue virus; Flaviviruses; Antiviral activity; Sulfated polysaccharides; Viral entry

1. Introduction

Flavivirus is a genus of the family *Flaviviridae* composed by nearly 80 members. Many flaviviruses are arthropod-borne viruses that cause important human diseases, including yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV) and Japanese encephalitis virus (JEV) (Lindenbach and Rice, 2001). In particular, DENV has re-emerged in recent years as an increasingly important public health threat affecting more than 100 countries worldwide, with nearly 50 million infections each year and over 2.5 billion people at risk (Gubler, 2002).

DENV circulates in nature as four serotypes (DENV-1 to DENV-4), which are transmitted to humans by two species of mosquitoes, *Aedes aegypti* and *Aedes albopictus*. Infection with DENV produces a wide spectrum of clinical illness ranging from silent infection to either a mild febrile, self-limited acute syndrome known as dengue fever (DF) or the severe and often fatal dengue hemorrhagic disease (DHF) and dengue shock syndrome (DSS). Instead of the increasing global incidence of DF and DHF occurred in the last decades, there is neither specific chemotherapy nor vaccine for treatment and prevention of DENV infection. Supportive medical care and symptomatic treatment through hydration are the most important aids to patients and to improve survival in the severe forms of disease. Consequently, new

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0166-3542/$ – see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.antiviral.2005.02.001

approaches for the control of DENV infection are urgently needed.

The enveloped DENV virions contain a single-stranded, positive sense RNA that is translated as a single polyprotein and cleaved into three structural proteins, capsid (C), membrane (M) and envelope (E), and seven non-structural proteins. The viral replicative cycle starts with the attachment of virus to the surface of the host cell. The blockade of virus binding is a valuable antiviral strategy because it allows to establish a first barrier to suppress infection. Although the identity of the cellular receptor for DENV is at present controversial, several reports have shown that the glycosaminoglycan heparan sulfate (HS) is involved in the initial binding of glycoprotein E to host cells for DENV attachment (Chen et al., 1997; Hung et al., 1999; Hilgard and Stockert, 2000; Germi et al., 2002). Many diverse viruses have been shown to interact with HS for target cell binding (Spillmann, 2001), but the DENV interaction is unusual for its specificity for a highly sulfated form of HS (Chen et al., 1997).

Based on this putative receptor role of HS for DENV, other charged polyanions could be effective inhibitors of viral infectivity. We have previously reported that novel DL-galactan hybrids can inhibit DENV-2 replication in Vero cells (Pujol et al., 2002). In the present study, diverse natural sulfated polysaccharides isolated from the red seaweeds *Gymnogongrus griffithsiae* and *Cryptonemia crenulata* were assayed for their antiviral action against the four serotypes of DENV in Vero cells, and also their effectiveness in human and mosquito cells was comparatively evaluated.

2. Materials and methods

2.1. Compounds

The extraction and purification of the polysaccharides from *G. griffithsiae* and *C. crenulata*, two red seaweeds collected in Brazil, were previously described (Talarico et al., 2004). The crude polysaccharide extracts *G3* and *C2*, obtained from *G. griffithsiae* and *C. crenulata*, respectively, and two homogeneous compounds, the kappa/iota/nu carrageenan *G3d* and the DL-galactan hybrid *C2S-3*, obtained by KCl fractionation and DEAE-Sephacell chromatography from *G3* and *C2*, respectively, were assayed for anti-DENV activity.

Two commercial products, dextran sulfate with an average molecular weight of 8000 (DS8000) and heparin (Sigma–Aldrich Co.), were also tested for antiviral activity.

2.2. Cells and viruses

Vero (African green monkey kidney) (ATCC) 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%. The human diploid foreskin fibroblast cell line PH was obtained from Dr. G. Carballal (CEMIC, Buenos Aires, Argentina) and propagated in MEM supplemented with 10% fetal calf serum. The C6/36 HT mosquito cell line from *Aedes albopictus* (adapted to grow at 33 °C) was provided by the Instituto Nacional de Enfermedades Virales Humanas (INEVH) Dr. J. Maiztegui (Pergamino, Argentina) and 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 calf serum. The human hepatoma cell line HepG2 was obtained from Cátedra de Virología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (Buenos Aires, Argentina) and propagated in MEM containing 0.03% glutamine, 0.01% sodium pyruvate and 10% fetal calf serum.

DENV-1 strain Hawaii was obtained from INEVH. DENV-2 strain NGC, DENV-3 strain H87 and DENV-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 HT cells and titrated by plaque formation in Vero cells.

2.3. Cytotoxicity assay

Cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma–Aldrich) 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 assays

Antiviral activity was evaluated by two methods: plaque reduction and virus yield inhibition assays. In the plaque reduction test, 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 virus 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.

In virus yield reduction assays, Vero, HepG2, PH, and C6/36 HT cells grown in 24-well plates were infected with DENV-2 or DENV-3 at a multiplicity of infection (moi) of 0.1

in the presence of different concentrations of the compounds, two wells per concentration. After 48 h of incubation at 37 °C (for Vero, HepG2 and PH cells) or 33 °C (for C6/36 HT cells), cell supernatants were collected and the virus yields were determined by plaque formation in Vero cells. The IC_{50} values were calculated as indicated above.

2.5. Time of addition experiment

To test the effect of the time of addition of the compounds on their anti-DENV-2 activity, Vero cells grown in 24-well plates were infected with 100 PFU of DENV-2 in either MM containing 20 μg/ml of each compound (time 0) or MM without compound. After 1 h adsorption at 4 °C, medium containing unadsorbed virus was removed and cell cultures were washed twice with PBS. Then, MM with compound (20 μg/ml) was added immediately (time 0 and 1 h p.i.) or at various times post infection (from 2 to 10 h p.i.) and incubation was followed at 37 °C until 11 h p.i. At that time, supernatants were discarded, the monolayers were washed with PBS and overlaid with MM containing 1% methylcellulose. DENV-2 plaques were counted at 6 days after infection.

2.6. Assay for virus adsorption

Vero cells grown in 6-well plates were infected with 500 PFU of DENV-2 in the presence or absence of 20 μg/ml of the compounds and incubated for 0, 15, 30 or 60 min at 4 °C. Medium containing unadsorbed virus was then removed, cells were washed twice with PBS and covered with MM containing 1% methylcellulose. Virus plaques were counted after 6 days of incubation at 37 °C.

2.7. Assay for virus internalization

Vero cells grown in 6-well plates were infected with 500 PFU of DENV-2 at 4 °C. After 1 h of virus adsorption, unadsorbed virus was removed and cells were washed with PBS and incubated at 37 °C in the presence or absence of 20 μg/ml of the compounds. At different times post-adsorption (0, 30, 60 and 120 min) cells were washed with PBS and treated with 0.1 ml of citrate buffer (citric acid 40 mM, potassium chloride 10 mM, sodium chloride 135 mM, pH 3) for 1 min to inactivate adsorbed but not internalized virus. Cells were then washed with PBS and covered with MM containing 1% methylcellulose. Plaques were counted after 6 days of incubation at 37 °C.

3. Results

3.1. Spectrum of anti-DENV activity of the polysaccharides

To study the inhibitory properties against DENV of the polysaccharides from *G. griffithsiae* and *C. crenulata*, the crude extracts *G3* and *C2* obtained from both seaweeds were evaluated as inhibitors of DENV-2 by a plaque reduction assay in Vero cells. As they were found active (data not shown), we then screened the two homogeneous compounds extracted from *G3* and *C2*, the carrageenan *G3d* and the DL-galactan hybrid *C2S-3*, respectively, for antiviral activity against the four DENV serotypes in Vero cells. The purified compounds were chosen to perform the more extensive characterization of anti-DENV activity because they are homogeneous and chemically characterized polysaccharides (Talarico et al., 2004), and consequently, they are more suitable to analyze the interaction with the viral target. Heparin and DS8000, two commercial polysaccharides known for their antiviral properties against several enveloped viruses, were simultaneously tested as reference substances.

As seen in Table 1, the antiviral activity of the sulfated polysaccharides was dependent on the viral serotype. Both natural compounds *G3d* and *C2S-3* inhibited DENV-2 multiplication in Vero cells, with IC_{50} values in the range 0.9–1 μg/ml, whereas the IC_{50} values for the reference polysaccharides heparin and DS8000 were 1.9 and 0.9 μg/ml, respectively. No cytotoxicity was observed with any of these polysulfates when cell viability was studied in Vero cell monolayers by MTT method, at concentrations up to 1000 μg/ml, allowing us to estimate the CC_{50} for all compounds as >1000 μg/ml. Thus, the selectivity index (SI), defined as the CC_{50}/IC_{50} ratio, was at least >1000 for both algal compounds. Based on these results, *G3d* and *C2S-3* can be considered effective and selective inhibitors of DENV-2.

When the compounds were evaluated against DENV-3, the IC_{50}s against this serotype were approximately 5–20-fold higher than the values corresponding for DENV-2, indicating a lower susceptibility of DENV-3 to the polysulfates. However, the SI of *G3d* and *C2S-3* against DENV-3 multiplication in Vero cells was >70, indicating also a selective

Table 1
Spectrum of anti-DENV activity of sulfated polysaccharides in Vero cells

Compound	IC_{50}[a] (μg/ml)			
	DENV-1 HW	DENV-2 NGC	DENV-3 H87	DENV-4 8124
G3d	>50	0.9 ± 0.4	13.9 ± 0.3	>50
C2S-3	>50	1.0 ± 0.3	14.2 ± 3.1	29.3 ± 5.3
Heparin	>50	1.9 ± 0.2	10.8 ± 2.5	>50
DS8000	>50	0.9 ± 0.1	18.3 ± 1.2	31.2 ± 2.4

[a] 50% inhibitory concentration: concentration required to reduce plaque number in Vero cells by 50%. Each value represents the mean of duplicate assays ± S.D.

inhibition of this serotype by the compounds. An even lower antiviral effect was observed against DENV-4: the IC_{50} for *C2S-3* was 29.3 μg/ml (about 30-fold higher than the IC_{50} against DENV-2) and *G3d* inhibited DENV-4 replication only by 41% at the maximum concentration tested (50 μg/ml); thus, the IC_{50} was >50 μg/ml. The behavior of the two reference compounds was similar: DS8000 showed a weak activity (IC_{50} of 31.2 μg/ml, approximately 35-fold higher than the IC_{50} of DS8000 against DENV-2) and heparin was inactive. Finally, the compounds were inactive against DENV-1 at the highest concentration tested in the plaque reduction assay (IC_{50} > 50 μg/ml); only DS8000 inhibited DENV-1 infection by 46% at 50 μg/ml.

The variation in DENV serotype susceptibility to polysaccharides could be due to differences in their growth ability in Vero cells. To test this possibility, the multiplication curves of the four serotypes in Vero cells were studied during a period of 72 h after infection. As shown in Fig. 1, the four serotypes replicated with similar efficiency in Vero cells. Virus yields showed some differences among serotypes during the earliest times, but virus production at 72 h p.i. for the most susceptible serotype was similar to the yield of the polysulfate-resistant serotype, with titers of 3.7×10^6 and 4.0×10^6 PFU/ml for DENV-2 and DENV-1, respectively. Consequently, the differential susceptibility to sulfated polysaccharides could not be attributed to a differential growth ability in Vero cells.

3.2. Anti-DENV activity of polysaccharides in different host cells

It is known that DENV can infect a wide range of different cell types and cell tropism appears directly related to virus pathogenesis (McBride and Bielefeldt-Ohmann, 2000). Although Vero cells are widely used to propagate and detect DENV, they are not representative of the true target tissues in natural infection and consequently, are not totally appropriate for antiviral studies. Thus, the influence of the host cell on the inhibition of DENV infection by polysaccharides was analyzed by determining their antiviral action in two human cell lines, PH and HepG2, and in the mosquito cell line C6/36 HT. Due to the difficulties for DENV plaque assays in these cell lines, the antiviral activity was evaluated by a virus yield inhibition test. For these studies, the more susceptible serotypes DENV-2 and DENV-3 were assayed against *G3d* and *C2S-3* as well as against heparin and DS8000.

In the virus yield reduction assay on Vero cells, both serotypes were as sensitive to the antiviral action of the compounds as in the plaque reduction assay, showing IC_{50} values in the range 1.0–3.8 and 4.1–14.7 μg/ml against DENV-2 and DENV-3, respectively (Table 2). Given the higher multiplicity of infection employed in the virus yield reduction assay in comparison with the plaque reduction test (0.1 versus 0.0001), these results demonstrate that the antiviral activity of these polysulfates against DENV-2 and DENV-3 is independent of the input multiplicity of infection. The polysaccharides were effective inhibitors of DENV-2 and DENV-3 in the human HepG2 and PH cells, with an antiviral effect similar to that observed in Vero cells. Furthermore, no cytotoxicity was detected in both human cell lines after exposure to compound concentrations up to 1000 μg/ml. Thus, for the seaweed derivatives *G3d* and *C2S-3*, the values of SI in human cells against DENV-2 and DENV-3 were in the range >555 to >6250 and >70 to >277, respectively. These data are indicative of a selective inhibition of both serotypes in human cells with higher antiviral effectiveness against the serotype DENV-2, as described in Vero cells. In contrast, the compounds did not affect the multiplication of both DENV serotypes in mosquito C6/36 HT cells, at concentrations up to 50 μg/ml (Table 2). Again, we assessed that the differential susceptibility of vertebrate and invertebrate cells to the inhibitory action of polysulfates against DENV was not related to their ability to support virus growth, since DENV-2 and DENV-3 yields at 48 h p.i. were around 10^6 PFU/ml in Vero as well as in C6/36 cells (data not shown).

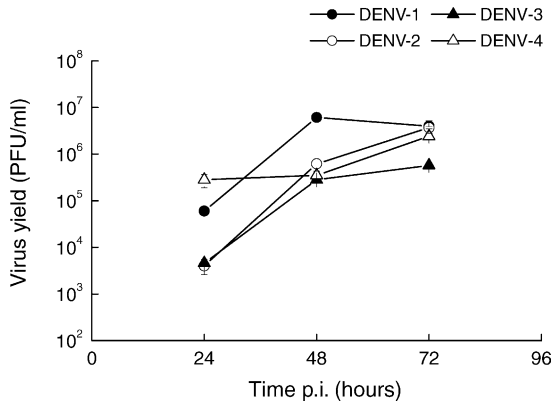

Fig. 1. Growth curves of DENV serotypes in Vero cells. Vero cells were infected with DENV serotypes at a moi of 0.1 and incubated at 37 °C. At different times after infection virus yields were determined. Each value represents the mean of duplicate assays ± S.D.

Table 2
Anti-DENV activity of sulfated polysaccharides in Vero, HepG2, PH and C6/36 HT cells

Compound	IC_{50}[a] (μg/ml)			
	Vero	HepG2	PH	C6/36 HT
DENV-2				
G3d	1.0 ± 0.1	1.8 ± 0.3	0.31 ± 0.01	>50
C2S-3	3.8 ± 0.1	1.4 ± 0.1	0.16 ± 0.01	>50
Heparin	1.6 ± 0.6	6.0 ± 0.4	2.1 ± 0.1	>50
DS8000	1.8 ± 0.2	3.6 ± 0.2	2.4 ± 0.2	>50
DENV-3				
G3d	5.2 ± 0.1	10.4 ± 1.2	9.5 ± 0.7	>50
C2S-3	14.7 ± 2.8	12.6 ± 4.0	3.6 ± 0.3	>50
Heparin	9.7 ± 1.2	6.2 ± 0.3	3.2 ± 0.5	>50
DS8000	4.1 ± 0.5	13.9 ± 1.2	6.3 ± 0.5	>50

[a] 50% inhibitory concentration, or concentration required to inhibit virus yield at 48 h p.i. by 50%. Each value represents the mean of duplicate assays ± S.D.

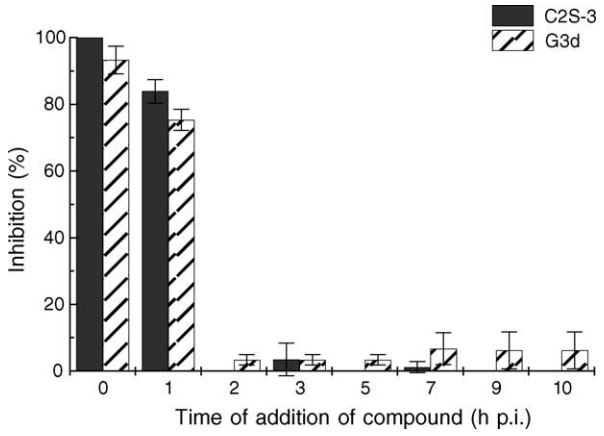

Fig. 2. Influence of time of addition of compounds on anti-DENV-2 activity. Vero cells were infected with 100 PFU of DENV-2 in the presence (0 h p.i.) or absence of 20 μg/ml of each compound. After 1 h of virus adsoption at 4 °C, cell monolayers were washed, refed with maintenance medium in the presence or absence of compounds and incubated at 37 °C. The polysaccharides were added at different times p.i. (1, 2, 3, 5, 7, 9 and 10 h). At 11 h p.i. medium was discarded and replaced with maintenance medium containing 1% methylcellulose. Each value represents the mean of duplicate assays ± S.D.

3.3. Influence of time of addition of polysaccharides on anti-DENV-2 infectivity

As an initial approach to understand the differential inhibition of polysaccharides on DENV, according to virus serotype and host cell, the mode of action of these compounds was studied in the system Vero cells-DENV-2. To locate the antiviral target during the virus multiplication cycle, the influence of the time of addition of the compounds *C2S-3* and *G3d* on DENV-2 plaque formation in Vero cells was determined. The polysaccharides were added simultaneously with virus or at 1-h intervals after exposure of cells to virus. As shown in Fig. 2, the highest inhibitory effect was observed when *C2S-3* and *G3d* were added to cells together with the virus (time 0). When added immediately after adsorption at 1 h p.i., polysulfates were also effective inhibitors, reducing virus plaques by more than 80%. By contrast, both compounds were ineffective to produce a significant reduction in DENV-2 plaque formation at later times. These results suggest that virus adsorption is the main target of the polysulfates against DENV-2 in Vero cells, but a very early stage of the virus cycle occurring immediately after adsorption during the first hour of infection, probably virus internalization, seems also to be affected.

3.4. Effect of polysaccharides on virus adsorption and internalization

To ascertain the antiviral action on the early steps of DENV-2 multiplication cycle, the kinetics of virus adsorption in the presence of *C2S-3* and *G3d* was evaluated. Vero cells were incubated with virus and compounds during different times at 4 °C, a condition to assess that virus attachment to the cell receptor is the only event of the virus cycle that occurs. Results shown in Fig. 3A confirmed that both compounds inhibited virus adsorption since the amount of cell-bound infectious virus was highly diminished in treated cells in comparison to non-treated infected cells.

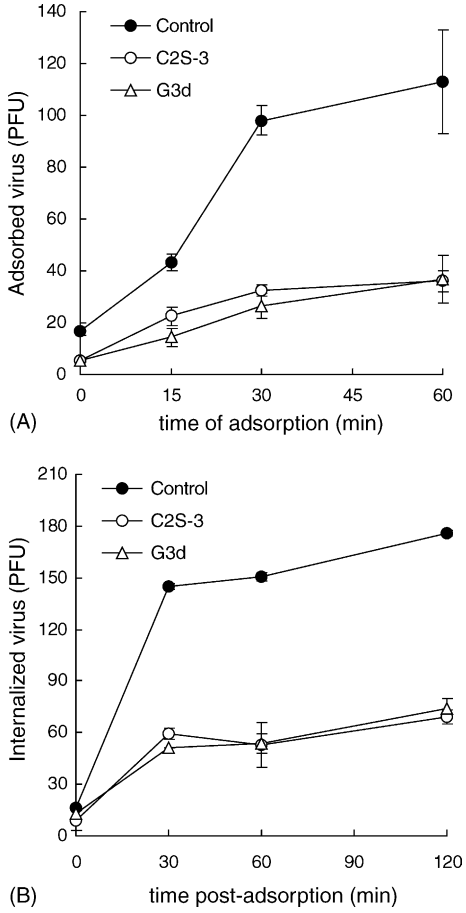

Fig. 3. Effect of compounds on DENV-2 adsorption and internalization kinetics. (A) Vero cells were incubated during 0, 15, 30 and 60 min at 4 °C with DENV-2 in the absence or presence of 20 μg/ml of each compound. Cells were then washed and refed with MM containing 1% methylcellulose. (B) Vero cells were infected with DENV-2 at 4 °C. After 1 h of virus adsorption, cells were incubated at 37 °C in the absence or presence of 20 μg/ml of each compound. At different times post-adsoption cells were washed, treated with citrate buffer and covered with MM containing 1% methylcellulose. Each value represents the mean of duplicate assays ± S.D.

The inhibitory action of the polysulfates on a subsequent post-adsorption step of the virus cycle was next analyzed, thereby examining the effect of *C2S-3* and *G3d* on the kinetics of virus internalization. To this end, Vero cells were infected with DENV-2 and incubated at 4 °C to allow virus adsorption. Then, the compounds were added to the cell cultures and temperature was raised to 37 °C to start virus penetration. Under these treatment conditions, the amount of internalized virus, measured by determining PFU number after inactivating the extracellular bound but non-penetrated virus with citrate buffer, was significantly reduced in the presence of the polysulfates (Fig. 3B).

Table 3
Influence of treatment period on the anti-DENV-2 activity of galactan *C2S-3* in different cells

Period of *C2S-3* treatment[a]	Inhibition[b] (%)		
	Vero	HepG2	PH
Virus adsorption	86.8 ± 0.6	82.7 ± 0.1	96.3 ± 1.7
Virus internalization	70.9 ± 4.1	81.8 ± 0.1	92.6 ± 0.1
Virus adsorption and internalization	95.2 ± 1.4	93.2 ± 1.1	100

[a] Cells were infected with DENV-2 (moi 0.1) in MM with or without compound (10 μg/ml for PH cells and 20 μg/ml for Vero and HepG2 cells). After adsorption at 4 °C cells were washed, overlaid with MM with or without compound and incubated at 37 °C for 2 h. Cells were then washed, treated with citrate buffer and covered with MM. After 48 h of incubation at 37 °C, virus yields were determined by a plaque assay in Vero cells.
[b] Results are expressed as the percent of inhibition in virus yield compared to the untreated control. Each value represents the mean of duplicate assays ± S.D.

The effect of the polysulfates on the events leading to entry of DENV into the host cell was also assessed in human cells. Both PH and HepG2 cells, as well as Vero cells as reference system, were infected with DENV-2 and treated with *C2S-3* under different treatment conditions: (a) during the virus adsorption period (1 h at 4 °C) only; (b) during virus internalization (1 h at 37 °C) only; (c) both during virus adsorption and internalization. After each treatment, cell supernatants were discarded and replaced by MM without compound. In all cases, extracellular virus yields were measured at 48 h after infection. In the three cell systems tested, significant antiviral efficacy was obtained if *C2S-3* was present either only during DENV-2 adsorption or internalization, and a slight increase of inhibition was observed when the treatment with *C2S-3* was extended during the whole period of adsorption and internalization (Table 3).

4. Discussion

Seaweed sulfated polysaccharides have been proved to inhibit the replication of several enveloped DNA and RNA viruses, including severe human pathogens such as retroviruses and herpesviruses among the most susceptible agents (Witvrouw and De Clercq, 1997; Damonte et al., 2004a). The studies reported here have shown that two sulfated polysaccharides extracted from South American red seaweeds, the DL-galactan hybrid *C2S-3* and the kappa/iota/nu carrageenan *G3d*, as well as the commercial compounds heparin and DS8000, were inhibitors of DENV replication, but their effectiveness was variable according to the viral serotype and the type of host cell.

The susceptibility of the four serotypes to the natural polysaccharides in Vero cells was in the order DENV-2 > DENV-3 > DENV-4 > DENV-1. DENV-2 was the most affected serotype, with SI values >1000 which are indicative of a highly selective virus inhibition by these compounds. It is interesting to remark that also a selective antiviral effect was also exerted by *G3d* and *C2S-3* against DENV-3, with SI values >70 (Table 1). In fact, this is the first report on a selective antiviral inhibition of DENV-3, a serotype which recently reappeared in the Americas, after a prolonged absence, particularly responsible of 2002–2003 dengue outbreaks in Brazil and Argentina (Miagostovich et al., 2002). In contrast, there was a very weak or lack of inhibition against the other two serotypes DENV-4 and DENV-1. In a recent brief communication, Lin et al. (2002) have reported a variable level of inhibition for DENV serotypes by heparin treatment in BHK-21 cells, and the authors suggested a correlation between the suppression effect of heparin on DENV and virus replication in the cells. However, we did not detect significant differences in virus production among DENV serotypes in Vero cells; thus, the variations observed in anti-DENV activity could not be attributed to differences in viral multiplication ability.

With respect to the type of host cell, the polysaccharides were active inhibitors of DENV-2 and DENV-3 multiplication in monkey Vero cells as well as in human hepatoma HepG2 and foreskin PH cells, but were inactive in mosquito C6/36 cells. There were no significant differences in the level of DENV multiplication in these vertebrate and invertebrate cell lines, confirming that differential susceptibility according to virus serotype or host cell is not due to virus growth ability.

Mechanistic studies performed in the three types of vertebrate cells indicated that the compounds were active DENV-2 inhibitors only during the first hour after infection, and the initial events of virus adsorption and internalization seemed to be the main target for these compounds during in vitro infection. When the inhibitory effect of *C2S-3* and *G3d* was separately assayed on both steps of the virus cycle, a comparable level of suppression was observed either for DENV-2 adsorption or internalization (Fig. 3; Table 3). This is a surprising result about the mode of action of polysulfates, since most studies on enveloped viruses have established that polysulfates act by preventing predominantly the first stage of virion binding to cell receptor (Witvrouw and De Clercq, 1997; Damonte et al., 2004a). In fact, our previous study on the anti-herpetic activity of *C2S-3* and *G3d* demonstrated that these compounds were inhibitors of HSV attachment and lacked any subsequent effect (Talarico et al., 2004). Probably, the dissimilar action of these compounds against DENV and HSV may be ascribed to differences in the internalization process between both viruses.

The mechanisms involved in DENV binding and internalization to the host cells are still not well understood. The E glycoprotein is certainly identified as the viral attachment protein (Anderson et al., 1992; Chen et al., 1996). However, the identity of the cellular receptor is at present controversial since both proteins and HS were implicated as candidate molecules. Studies reporting proteins as putative receptors in diverse types of vertebrate and invertabrate cells were described for DENV-2 (Daughaday et al., 1981; Ramos-Castañeda et al., 1997; Muñoz et al., 1998; Moreno-

Altamirano et al., 2002; Bielefeldt-Ohmann, 1998; Wei et al., 2003), DENV-1 (Marianneau et al., 1996) and DENV-4 (Salas-Benito and del Angel, 1997). In contrast, an involvement of HS was demonstrated for attachment of DENV-2 to human hepatocytes, Vero, BHK and CHO cells (Chen et al., 1997; Hung et al., 1999; Hilgard and Stockert, 2000; Germi et al., 2002). The mechanism of DENV internalization is also under discussion: electron-microscopic studies suggested that DENV-2 penetrated directly by fusion of the virion envelope with the plasma membrane (Hase et al., 1989; Lim and Ng, 1999), but it is most generally accepted that viral uptake for productive infection occurs through receptor mediated-endocytocis (Randolph and Stollar, 1990; Heinz et al., 1994; Lindenbach and Rice, 2001). Thus, a plausible mechanism emerging from these conflicting data is a multistep process for DENV entry consisting in the sequential interaction of the E protein with several target molecules on the cell membrane, where HS may be the primary receptor and then other molecules might participate as coreceptors for viral internalization, as suggested by Martínez-Barragán and del Angel (2001). In this context, the variations reported here in antiviral activity of the polysulfates *G3d* and *C2S-3* against DENV may be ascribed to differences in the virus-cell interaction leading to virus entry, as receptor usage or internalization process.

Although the life cycle of DENV presents a series of stages representing potential targets for antiviral drug discovery, the few attempts to find antiviral substances have been mainly focused on the screening of probable RNA synthesis inhibitors, such as ribavirin and other nucleoside analogues (Damonte et al., 2004b). But, the effectiveness of this type of compounds against DENV was very weak and non selective (Koff et al., 1982; Gabrielsen et al., 1992; Markland et al., 2000; Leyssen et al., 2000; Diamond et al., 2002; Crance et al., 2003). Only in recent years, a few studies about potential DENV attachment/entry inhibitors were reported. As mentioned above, heparin was the first polysaccharide shown to be effective to inhibit DENV-2 multiplication in Vero, BHK and human liver cells (Chen et al., 1997; Hung et al., 1999; Germi et al., 2002; Lin et al., 2002) and the minimum size required to occupy the E protein binding site was found to be a heparin-derived decasaccharide (Marks et al., 2001). Other polyanions such as sulfated galactans and polyoxometalates were recently described as inhibitors of DENV-2 multiplication in Vero cells (Pujol et al., 2002; Shigeta et al., 2003). The limitations of the available animal models of DENV infection have restricted the adequate in vivo evaluation of putative antiviral compounds (Charlier et al., 2004). However, two seed sulfated galactomannans with in vitro activity against DENV and YFV were able to protect mice against yellow fever by intraperitoneal inoculation of virus and compounds (Ono et al., 2003), demonstrating that polysulfates are an alternative for flavivirus chemotherapy.

Given their selectivity and antiviral effectiveness in human cells against DENV-2 and DENV-3, the seaweed polysulfates reported in this study deserve consideration as virus entry inhibitors. Due to their differential behavior, compounds like *G3d* and *C2S-3* may be useful tools to elucidate the mechanisms of binding and internalization of DENV serotypes to vertebrate and invertebrate cells, and to establish structure-activity relationships. Furthermore, our results illustrate the requirement to evaluate all viral serotypes when putative anti-DENV compounds are studied. DENV-2 is the better adapted virus to grow in vitro, and it is the most routinely used serotype in DENV studies, including antiviral tests.

The four serotypes cocirculate in epidemic regions and it is well known that the reinfection with a different serotype represents an important threat for human health due to the development of severe forms of DHF or DSS. For this reason, it should be required to assay the four serotypes for inhibitory activity, because responses similar to those here presented can be (re)produced with other antiviral substances.

Acknowledgements

This research was supported by grants from Agencia Nacional para la Promoción Científica and Tecnológica, Universidad de Buenos Aires (Argentina), and PRONEX-Carboidratos (CNPq, Brazil). LBT is fellow from CONICET (Argentina), EBD is a member of Research Career from CONICET, RGMZ is fellow from CAPES, PCSF from CNPq and MDN is Research Member from CNPq (Brazil).

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