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An Algal-Derived DL-Galactan Hybrid is an Efficient Preventing Agent for *in vitro* Dengue Virus Infection

Abstract

The DL-galactan hybrid **C2S-3**, isolated from the red seaweed *Cryptonemia crenulata* (Halymeniaceae, Halymeniales), is a potent and selective inhibitor of the multiplication of diverse strains of DENV-2 in Vero cells with higher effectiveness than the reference polysaccharide heparin. The presence of the compound either only at virus adsorption or at virus internalization exerted a significant and dose-dependent inhibition in DENV-2 plaque number. The compound failed to inactivate DENV-2 directly by incubation of virus before cell infection as well as to induce a refractory state by cell pretreatment. Thus, the inhibitory effect was exclusively exerted through a blockade in virus multiplication during the infectious process. When the entry of DENV-2 particles into the cell is bypassed, as occurs in virus RNA transfection, the polysaccharide **C2S-3** failed to block the completion of the multiplication cycle. Furthermore, the antiviral properties of **C2S-3** are not correlated with anticoagulant activity.

Key words
Antiviral agent · dengue virus · sulfated galactan · marine alga · *Cryptonemia crenulata* · Halymeniaceae

Introduction

The potential of sulfated polysaccharides and polysulfates in general to inhibit infectivity of heparan sulfate (HS)-binding viruses has long been known [1]. These compounds are targeted to inhibit virus binding and/or entry into the host cell by mimicking the structural characteristics of HS, a glycosaminoglycan component of cell surface proteoglycans that functions as the initial receptor for several enveloped viruses. The inhibition of virus entry is a new and very effective way of preventing viral infection. One of the more recent antiviral agents approved for the therapy of human immunodeficiency virus (HIV) infections is a fusion inhibitor named enfuvirtide whereas several compounds that block HIV entry are currently in development [2].

Dengue virus (DENV) is an arthropode-borne flavivirus that causes a wide range of clinical illnesses in human [3]. DENV exists as four serotypes, DENV-1 to DENV-4, which cocirculate in the population. Even though DENV has a major health impact in tropical and subtropical regions around the world, there is no specific therapy for patient treatment. Since the initial report of HS as a putative receptor for DENV [4], several studies tested the ability of HS-mimetic compounds to inhibit DENV infectivity [5], [6], [7], [8].

In a previous report the sulfated galactans extracted from two red seaweeds, *Gymnogongrus griffithsiae* and *Cryptonemia crenulata*, were evaluated for their antiviral properties against the four serotypes of DENV in different cells [9]. DENV-2 was the most susceptible serotype, with values of effective concentration 50%

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(EC_{50}) around 1 μg/mL in mammalian cells, but the polysaccharides were inactive in mosquito cells. This differential susceptibility appeared to be related to the presently unknown mode of entry of DENV into diverse cells. Here, we analyze the mechanism of interference of the DL-galactan hybrid **C2S-3**, isolated from *C. crenulata*, in the multiplication of DENV-2 in Vero cells concluding that the compound affects the initial steps of virus adsorption and internalization into the host cell.

Materials and Methods

Compounds

The procedures for extraction and purification of the polysaccharides from *Cryptonemia crenulata* (Halymeniaceae, Halymeniales), a red seaweed collected in Brazil, were previously described [10]. Briefly, the DL-galactan hybrid **C2S-3** (Fig. **1**) was obtained by aqueous extraction followed by KCl fractionation of the crude extract **C2** and DEAE-Sephacel chromatography. Chemical and spectroscopic analyses showed that **C2S-3** (MW 236 kDa) is constituted of 3-linked β-D-galactose 2- and 2,6-disulfated and 4-linked α-D- and α-L-galactose 6- and 2,6-disulfated besides 3,6-anhydro-α-D- and α-L-galactose. The last unit is C-2 substituted either by sulfate or methoxy groups [11]. Heparin (Sigma-Aldrich Co.; St. Louis, MO, USA) was used as reference substance.

Cells and viruses

Vero monkey cells (American Type Culture Collection; Manassas, VA, USA) were grown in Eagle's minimum essential medium (MEM) (GIBCO; Grand Island, NY, USA) supplemented with 5% calf serum. For maintenance medium (MM), the serum concentration was reduced to 1.5%. The C6/36 HT *Aedes albopictus* cell line (provided by Dr. D. Enría, INEVH, Pergamino, Argentina) was cultured at 33 °C in L-15 Medium (Leibovitz, GIBCO; Grand Island, NY, USA) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% MEM non-essential amino acids solution and 5% fetal bovine serum.

Stocks of the DENV-2 reference strains NGC (provided by Dr. A. Mitschenko, Hospital R. Gutiérrez, Buenos Aires, Argentina) and 16 681 (provided by Dr. A. Gamarnik, FIL, Buenos Aires, Argentina), and the clinical isolate 67 655 (provided by Dr. D. Enría, INEVH, Pergamino, Argentina) were prepared in C6/36 HT cells and titrated by plaque formation in Vero cells.

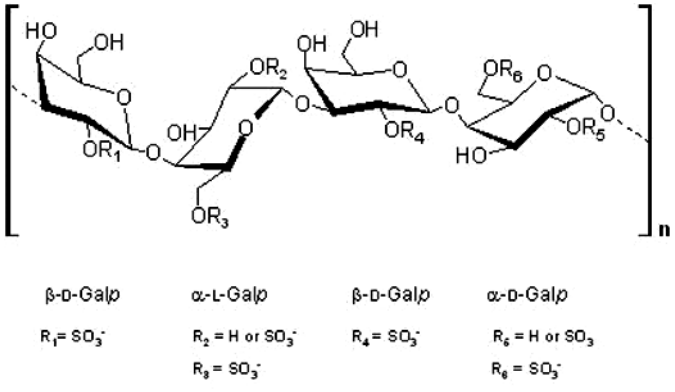

Fig. **1** Structural units of the DL-galactan hybrid **C2S-3**.

Antiviral assay

Antiviral activity was evaluated by a plaque reduction assay as previously described [9]. Briefly, Vero cells were infected with 50 PFU/well of DENV-2 in the absence or presence of serial compound dilutions. After 1 h at 37 °C, MM containing 1% methylcellulose and the corresponding dose of compound was added. Plaques were counted at 6 days post-infection (p.i.). The EC_{50} was calculated as the compound concentration required to reduce virus plaques by 50%. All determinations were performed twice and each in duplicate.

Inhibitory action of C2S-3 during DENV-2 adsorption and internalization

The effect of **C2S-3** on DENV-2 adsorption and internalization was studied by different experimental approaches, as follows:

a) Plaque reduction assay under different treatment conditions: Virus adsorption: cells were infected with 50 PFU of DENV-2 in MM containing serial compound dilutions and, after 1 h adsorption at 4 °C, cells were washed with PBS and overlaid with compound-free MM containing methylcellulose. Virus internalization: Vero 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 different **C2S-3** concentrations. 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. Virus adsorption and internalization: cells were infected with DENV-2 and maintained in MM containing **C2S-3** during both adsorption and internalization periods, and processed as above. Virus infection cycle: cells were infected and maintained in the presence of compound during adsorption and the whole period required for plaque formation.

b) Adsorption assay of DENV-2: Adsorption assay of DENV-2 to Vero cells was performed as previously described [8]. Briefly, cells were infected with DENV-2 in the presence or absence of 20 μg/mL of **C2S-3**. After 1 h adsorption at 4 °C, cells were washed with PBS and disrupted by freezing and thawing. Then, cell-bound virus was determined by PFU.

c) Internalization assay of DENV-2: For the internalization assay [8], Vero cells were infected with DENV-2 and after 1 h adsorption at 4 °C, cells were incubated at 37 °C for 1 h in MM containing or not 20 μg/mL of **C2S-3**. Thereafter, cells were treated with proteinase K for 45 min at 4 °C, proteinase K was inactivated with 2 mM phenylmethanesulfonyl fluoride (PMSF), and cells were pelleted by low speed centrifugation. Serial dilutions of the cell suspension were plated onto Vero cells to quantify the amount of internalized virus by infectious center formation.

Effect of pretreatment of cells or virus with C2S-3 prior to infection

Pretreatment of cells: Vero cells were pre-incubated with MM containing different **C2S-3** concentrations during 2 h at 37 °C. Then, cells were washed with PBS, infected with DENV-2 in the absence of compound, and incubated 1 h at 37 °C. Virus yields were determined at 48 h p.i.

Pretreatment of virus: A DENV-2 suspension was incubated in MM containing or not different **C2S-3** concentrations at 37 °C during 45 min, and then the remaining infectivity was titrated by PFU.

Treatment during virus infection: Vero cells were infected with DENV-2 in the presence of different **C2S-3** concentrations. Then, cultures were incubated 1 h at 37 °C and virus yields were determined at 48 h p.i.

RNA transfection

RNA was extracted from a DENV-2 virion suspension using the commercial kit TOTALLY RNA (Ambion; Austin, TX, USA) and quantitated by UV absorption at 260 nm. Vero cells grown in coverslips were transfected with 16 μg of DENV-2 RNA using Lipofectamine 2000 (Invitrogen; Carlsbad, CA, USA) and Opti-MEM I (GIBCO). After 2 h at 37 °C, monolayers were washed with PBS and refed with MM containing or not 20 μg/mL of **C2S-3**. After 18 h incubation at 37 °C, cells were washed with PBS and overlaid with MM in the absence of compound. At 96 h post-transfection, supernatants were harvested for plaque assay and cells were fixed with methanol for immunofluorescence staining.

Indirect immunofluorescence assay

DENV-2 infected and RNA DENV-2 transfected Vero cells were processed for immunofluorescence staining at 48 h p.i. and 96 h post-transfection, respectively, by using anti-DENV-2 mouse hyperimmune ascitic fluid (provided by Dr. A.V. Gamarnik, FIL, Buenos Aires, Argentina) and fluorescein-labeled goat anti-mouse IgG (Sigma-Aldrich Co.; St. Louis, MO, USA). The incubation time for the RNA transfection assay was longer than the time in the virus infection assay due to the lower amount of viral RNA that could be transfected in comparison to inoculated PFU. After a final washing with PBS, cells were stained with Evans blue and mounted in a glycerol solution containing 1,4-diazabicyclo [2.2.2]octane (DABCO).

Anticoagulant activity

The activated partial thromboplastin time (APTT) was determined incubating 100 μL of pooled human plasma with different concentrations of test sample (30 μL) and 100 μL of APTT reagent (Wiener Lab; Rosario, Argentina) for 1 min at 37 °C. Then, 70 μL of 0.025 M $CaCl_2$ were added and the time to clot formation was recorded.

Results and Discussion

We first tested the activity of **C2S-3** against different strains of DENV-2 to evaluate if the susceptibility to polysulfates shown by this serotype is independent of the virus isolation. Both NGC and 16681 laboratory reference strains of DENV-2 were highly susceptible to inhibition by **C2S-3** with EC_{50} values about 1 μg/mL (Table **1**). As this compound lacked any cytotoxicity for Vero cells up to 1000 μg/mL [10], the antiviral activity of **C2S-3** against DENV-2 was very selective. As seen in Table **1**, the EC_{50} for the clinical isolate 67655 was higher than the values found for the other strains. However, the higher inhibitory effectiveness against the three DENV-2 strains exhibited by the natural galactan in comparison with heparin, assayed as a reference substance, is noticeable (Table **1**).

Table **1** Antiviral activity of galactan **C2S-3** against diverse strains of DENV-2

DENV-2 strain	*EC_{50} (μg/mL)[a]*	
	C2S-3	*Heparin*
NGC	1.0 ± 0.3	3.0 ± 0.2
16681	0.8 ± 0.1	4.8 ± 0.5
67655	16.0 ± 0.3	> 100

[a] Compound concentration required to reduce DENV-2 plaque number in Vero cells by 50%. Each value is the mean of duplicate assays ± standard deviation.

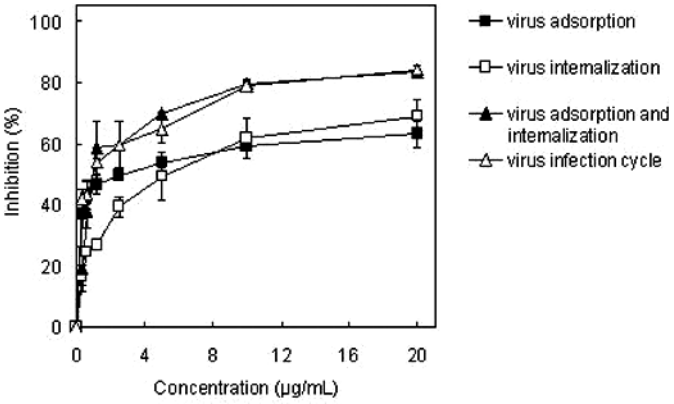

Fig. **2** Inhibitory action of **C2S-3** during DENV-2 adsorption and internalization. Vero cells were infected with DENV-2 under four different treatment conditions. *Virus adsorption:* cells were infected with DENV-2 in MM containing compound and, after 1 h adsorption at 4 °C, were overlaid with compound-free MM containing methylcellulose. *Virus internalization:* DENV-2 was adsorbed to Vero 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 MM containing methylcellulose. *Virus adsorption and internalization:* cells were infected and maintained in the presence of compound during adsorption and internalization and then processed as above. *Virus infection cycle:* cells were infected and maintained in the presence of compound during adsorption and the 6 day period for plaque formation. Results are expressed as percent of inhibition in compound-treated cultures compared to untreated ones. Each value is the mean of duplicate assays ± standard deviation.

The mechanism of inhibition of **C2S-3** was further studied using the NGC strain as model system. Our preliminary studies about the time course of anti-DENV activity of *C. crenulata* and *G. griffithsiae* polysaccharides have shown that the compounds were effective only when added either simultaneously with virus or at least until 1 h after infection [9]. To precisely locate the effect of **C2S-3** in the events occurring during the first hour of infection, a plaque reduction assay under different treatment conditions was performed. The presence of the compound either only at virus adsorption at 4 °C or at virus internalization at 37 °C exerted a significant and dose-dependent inhibition in plaque number (Fig. **2**). Comparatively, the inhibition of **C2S-3** against DENV-2 was slightly higher when treatment was during adsorption (EC_{50} = 2.5 ± 0.1 μg/mL, from data in Fig. **2**) with respect to treatment only during internalization (EC_{50} = 5.5 ± 0.7 μg/mL). The inhibitory effect was increased when **C2S-3** was included during both stages of adsorption and internalization,

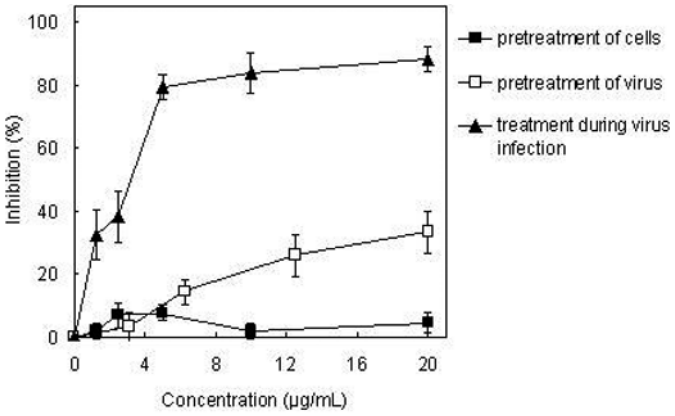

Fig. **3** Effect of pretreatment of cells or virus with **C2S-3** prior to infection. *Pretreatment of cells:* Vero cells were pre-incubated with **C2S-3**, then compound was removed and cells were infected with DENV-2. Virus yields were determined at 48 h p. i. *Pretreatment of virus:* DENV-2 suspensions were incubated with **C2S-3** and then the remaining infectivity was determined by plaque assay. *Treatment during virus infection:* compound was added to Vero cells simultaneously with virus. Virus yields were determined at 48 h p. i. Results are expressed as percent of inhibition in compound-treated cultures compared to untreated ones. Each value is the mean of duplicate assays ± standard deviation.

but a similar level of inhibition was observed when the compound was present at adsorption and maintained during the whole period of 6 days required for plaque formation. In fact, the EC_{50} obtained from data in Fig. **2** when treatment was restricted to adsorption and internalization was 1.0 ± 0.3 µg/mL whereas the EC_{50} for treatment during all infection cycle was 1.0 ± 0.2 µg/mL, suggesting that the antiviral activity of **C2S-3** is exerted exclusively at virus entry.

The effect of **C2S-3** on DENV-2 adsorption and internalization was also analyzed by determining the amount of bound and internalized DENV-2 particles in the presence of test compound. When adsorption was performed at 4 °C in the presence of **C2S-3** the amount of DENV-2 infectious particles bound to Vero cells was reduced by 82%. If DENV-2 was adsorbed at 4 °C in the absence of compound, and then virus was internalized during 1 h at 37 °C in the presence of **C2S-3**, the amount of internalized virus was reduced by 68%, corroborating the data from the experiments shown in Fig. **2**.

Next, the possibility that the drug acted directly either on the virions or on the cells to be infected was investigated by performing each separate treatment. When cell monolayers were pre-incubated with **C2S-3,** and then the compound was removed before infection, no reduction in virus yield was observed (Fig. **3**). Similarly, **C2S-3** was not effective in reducing virus titer following pre-incubation with virions for 45 min at 37 °C before cell infection. By contrast, when the compound was added to cells simultaneously with virus inoculum, virus yields were significantly reduced in a dose dependent-manner (Fig. **3**). Thus, **C2S-3** failed to neutralize DENV-2 directly as well as to induce a refractory state by cell pretreatment, and the inhibitory effect was exclusively exerted through a blockade in virus multiplication during the infectious process.

Following virus entry to the host cell, the positive stranded RNA genome of DENV is translated to viral proteins. To discard any inhibitory action of **C2S-3** after virus entry into the cell, the effect of this polysaccharide in the expression of viral RNA introduced to Vero cells by transfection was evaluated. After immunofluorescence staining with anti-DENV-2 antibody, the number of viral antigen-expressing cells in compound treated and untreated transfected cells was similar (Figs. **4A** and **B**), demonstrating that the compound did not affect the expression of the viral genome. As a positive control, Vero cells infected with DENV-2 in the presence and absence of the compound were monitored by immunostaining. The number of DENV-2 antigen expressing cells was highly reduced by **C2S-3** in comparison with untreated infected cells (Fig. **4D** and **E**). Furthermore, the titer of DENV-2 infectious particles released from untreated transfected cells at 4 days post-transfection was 5.0×10^2 PFU/mL whereas in **C2S-3** treated transfected cells virus production raised to 5.1×10^2 PFU/mL. Thus, when the entry of DENV-2 particles into the cell is bypassed, as occurs in virus RNA transfection, the polysaccharide **C2S-3** failed to block the completion of the multiplication cycle.

Since sulfated polysaccharides are generally endowed with anticoagulant properties which hamper their usefulness as antiviral agents, the APTT was measured to evaluate the anticoagulant activity of **C2S-3**. The value of 34.5 sec, corresponding to APTT for the control blood treated with PBS, showed a very small increment when **C2S-3** was added at a concentration near the antiviral EC_{50} (2 µg/mL, Table **2**). APTT increased only about 2-fold at a **C2S-3** concentration of 20 µg/mL, a dose exceeding the EC_{50}. He-

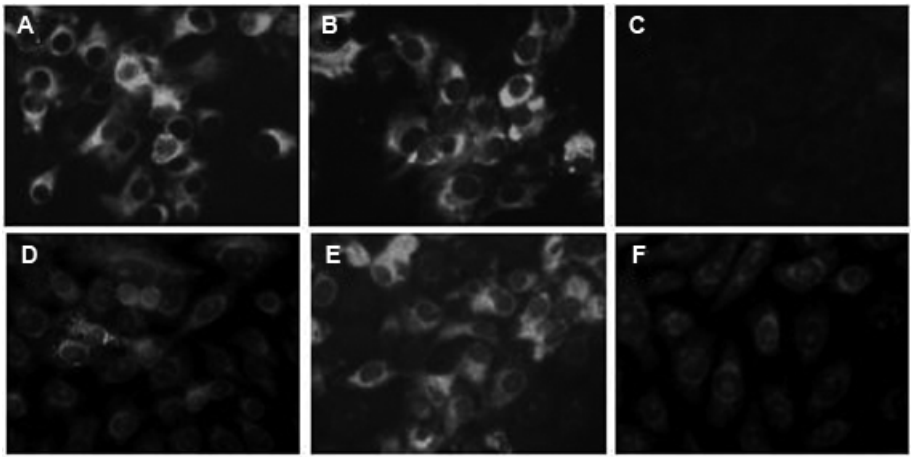

Fig. **4** Effect of **C2S-3** in DENV-2 RNA transfected and DENV-2 infected Vero cells. **A**, **B**, and **C** Vero cells grown in coverslips were mock transfected (**C**) or transfected with DENV-2 RNA, and 2 h later monolayers were incubated in MM containing (**A**) or not (**B**) **C2S-3**. At 96 h post-transfection, cells were immunostained. **D**, **E**, and **F** Vero cells were mock infected (**F**) or infected with DENV-2 in the presence (**D**) or absence (**E**) of **C2S-3**. At 48 h p. i., immunofluorescence staining was carried out. Magnification: 400×.

Table **2** Anticoagulant activity of **C2S-3**

Compound	*Concentration (μg/mL)*	*APTT (sec)*[a]
C2S-3	2	45.5 ± 4.9
	20	93.0 ± 2.8
	200	> 180
Heparin	2	> 180
	20	> 180
	200	> 180
PBS	–	34.5 ± 2.1

[a] Activated partial thromboplastin time. Each value is the mean of duplicate assays ± standard deviation.

parin was assayed as a reference anticoagulant substance. The APTT of **C2S-3** was of the same order of those of heparin only at concentrations 100 times higher (200 and 2 μg/mL for **C2S-3** and heparin, respectively). Thus, the antiviral properties of **C2S-3** are not correlated with anticoagulant activity.

In conclusion, the results reported here demonstrate that the algal DL-galactan hybrid **C2S-3** is a very selective inhibitor of *in vitro* DENV-2 infection. In spite of the close structural similarities between HS and heparin, the antiviral action of **C2S-3** is exerted with higher effectiveness than heparin against diverse strains of DENV-2. As previously described [11] **C2S-3** presents the diads [→3)-β-D-($^4C_1$)-Gal*p*-2-OSO_3-(1→4)-α-L-($^1C_4$)-Gal*p*-6′-OSO_3-(1→] and [→3)-β-D-($^4C_1$)-Gal*p*-2-OSO_3-(1→4)-α-D-($^4C_1$)-Gal*p*-6′-OSO_3-(1→]. Both disaccharide units present in *C. crenulata* polysaccharide are similar to the repeating structures of the minimal sequence of HS necessary to interact with glycoprotein C of herpes simplex virus (HSV) [12]. Therefore these diads could also contribute for the inhibition of the interaction of DENV with HS. Furthermore, an additional advantage for **C2S-3** with respect to heparin is the lack of significant anticoagulant properties.

Adsorption-internalization studies together with the RNA transfection assays indicate that **C2S-3** blocked the initial binding of the virus to the cell and its subsequent penetration, preventing the delivery of DENV-2 RNA into the cytoplasm to start with viral macromolecule biosynthesis. All these characteristics distinguish **C2S-3** as a promising DENV-2 entry inhibitor. At present, the limitations of the available animal models of DENV infection have restricted the *in vivo* evaluation of compounds with *in vitro* proved antiviral activity. However, algal polysaccharides have shown *in vivo* antiviral activity against other enveloped viruses like murine leukemia virus [13], HSV [10], [14], cytomegalovirus [15] and yellow fever virus [16], warranting the future perspectives of these natural compounds for prevention against dengue.

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