Differential inhibition of dengue virus infection in mammalian and mosquito cells by iota-carrageenan

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The antiviral activity against dengue virus-2 (DENV-2) of carrageenans reported here has shown a differential susceptibility of C6/36 HT and Vero cells, taken as models of mosquito and mammalian cells, depending on the structural class of polysaccharides: all polysaccharides blocked DENV-2 infection in monkey Vero cells, but only iota-carrageenans were virus inhibitors in mosquito cells. However, iota-carrageenans were less effective in mosquito cells in comparison with mammalian cells with effective concentration 50 % (EC50) values in C6/36 HT cells 4.9–17.5-fold higher than in Vero cells, as determined by virus yield reduction assay. The mode of action of iota-carrageenan in both cell types was strikingly different: in Vero cells the inhibitory activity was exerted only at the initiation of the cycle, affecting virion binding, whereas in mosquito cells DENV-2 adsorption was not affected and comparable levels of inhibition were obtained if the compound was added to cells together with the virus, after 8 h of infection or by cell pre-treatment before infection. Furthermore, iota-carrageenans induced a subtle alteration in mosquito cells, detected by cell proliferation and protein synthesis analyses, suggesting that a probable cellular target may be responsible for the refractory state of mosquito cells to DENV-2 infection produced by this class of polysulfates. The failure of iota-carrageenan to block DENV-2 adsorption to mosquito cells appeared to be related to the low presence of adequate heparan sulfate (HS) in C6/36 HT cell surface and is indicative of a differential participation of HS residues for DENV-2 entry in both types of cells.

INTRODUCTION

Dengue virus (DENV), a member of the genus Flavivirus in the family Flaviviridae, is the most widespread arbovirus and re-emerged as a global health problem in the last decades (Gubler, 2002). There are four serotypes (DENV-1–4) which can cause a mild illness known as dengue fever or the severe dengue haemorrhagic fever/dengue shock syndrome in human. In nature, DENV is transmitted to its vertebrate host through the bite of an infected mosquito from the species Aedes aegypti and Aedes albopictus, and the continuous occurrence of mosquito-vertebrate-mosquito transmission cycles allows virus maintenance in the environment.

The virion is an enveloped particle containing a positive-sense RNA that is translated as a polyprotein then cleaved into three structural proteins and seven non-structural proteins. DENV primary infection is initiated by the binding of the envelope E glycoprotein to a cell surface receptor. Although the precise nature of DENV receptor is still controversial, several investigators demonstrated that heparan sulfate (HS) is involved in the first interaction with E glycoprotein to initiate DENV multiplication cycle in different types of vertebrate cells (Chen et al., 1997; Germi et al., 2002; Hilgard & Stockert, 2000; Hung et al., 1999). HS is a member of highly sulfated glycosaminoglycans (GAGs) which is very abundant on the surface of most mammalian cells and serves as a receptor for many microbial agents including bacteria, parasites and viruses (Rostand & Esko, 1997; Spillmann, 2001). For DENV, the interaction with HS is unusual owing to its specificity for a highly sulfated form of HS (Chen et al., 1997). Based on the proposed role of HS for DENV entry, diverse HS-like molecules were evaluated as antiviral agents against flaviviruses. Sulfated polysaccharides including heparin, galactans, fucoidans, glucans, mannans and carrageenans were found to be very potent and selective inhibitors of DENV-2 multiplication in mammalian cells (Hidari et al., 2008; Lee et al., 2006; Lin et al., 2002; Marks et al., 2001;
 mode of inhibition in comparison to mammalian cells. Surprisingly, no inhibitory activity was observed when some polysulfates were evaluated in mosquito cells (Talarico et al., 2005; Thaisomboonsuk et al., 2005).

Here, we report the antiviral activity against DENV-2 in mosquito cells of a particular type of sulfated polysaccharide, the iota-carrageenans, that show a different behaviour respect to other classes of polysulfates and an unusual mode of inhibition in comparison to mammalian cells.

**RESULTS**

**Antiviral activity of carrageenans against DENV-2 multiplication in mosquito and Vero cells**

Three structural types of carrageenans and the reference polysaccharide heparin were assayed for anti-DENV-2 activity in Vero and C6/36 HT cells, as model systems of mammalian and mosquito cells, respectively, by a virus yield inhibition assay. As previously reported in plaque reduction antiviral test (Talarico et al., 2007), the three carrageenans were inhibitors of DENV-2 multiplication in Vero cells, with lambda- and iota-carrageenans as the most active compounds (Fig. 1a). By contrast, only the iota-carrageenan was an inhibitor of DENV-2 multiplication in mosquito cells (Fig. 1b). Heparin was also able to inhibit DENV-2 multiplication in Vero cells but was inactive in C6/36 HT cells. From data in Fig. 1(a, b) the values of effective concentration 50 % (EC50) calculated for iota-carrageenan in Vero and C6/36 HT cells were 0.4 ± 0.1 and 7.0 ± 0.7 μg ml−1, respectively.

Since the commercial iota-carrageenan was the only type of tested polysaccharide able to affect DENV-2 multiplication in C6/36 HT cells, we decided to assay another iota-carrageenan, obtained from a different source to evaluate if this property is related to the particular structural characteristics of this type of carrageenans. The iota-carrageenan provided by Sigma-Aldrich is isolated from *Eucheuma spinosa*. M3a is an homogeneous polysulfate isolated from *Meristielia gelidium* constituted predominantly by iota-carrageenan (88–90 %) and with minor amounts of nu- (6–8 %) and kappa- (4 %) disaccharide repeating units (de S.F-Tischer et al., 2006). The carrageenan M3a reduced DENV-2 yields in a dose-dependent manner (Fig. 1a, b), with EC50 values of 4.6 ± 0.6 and 0.93 ± 0.05 μg ml−1 in C6/36 HT and Vero cells, respectively. These results allow us to conclude that the ability to interfere with the multiplication of DENV-2 in mosquito cells seems to be a consistent property of the iota-carrageenans, independently of their origin.

The effect of iota-carrageenan against DENV-2 infection in C6/36 HT cells was also demonstrated by monitoring viral antigen expression in infected cells by immunofluorescence assay. C6/36 HT cells expressing viral antigen were not detected in cultures infected with DENV-2 in the presence of iota-carrageenan, whereas untreated cultures or cultures treated with heparin exhibited a similar amount of DENV-2 antigen-positive cells (Fig. 1c).

**Mode of antiviral action of iota-carrageenan against DENV-2 in mosquito and Vero cells**

To characterize the antiviral activity of iota-carrageenans in mosquito cells, the time-course of the inhibitory effect was analysed by a time-of-addition experiment. For comparative purposes, Vero cells were assayed simultaneously in the same type of assay. As previously reported for other sulfated polysaccharides (Talarico et al., 2005, 2007), the antiviral effectiveness in Vero cells of both iota-carrageenans was restricted to the first hour after infection. The highest inhibitory effect was observed when the compound was added to cells together with virus (time 0) or immediately after adsorption at 1 h post-infection (p.i.) (Fig. 2a, b). Surprisingly, the response of C6/36 HT cells was very different: a similar level of inhibition, greater than 90 %, was observed if the commercial iota-carrageenan was added either together with the virus or at any time after adsorption, up to 8 h after infection (Fig. 2a). For M3a, virus yields were also diminished more than 50–70 % even if the carrageenan was added between 5 and 8 h p.i.

Results presented in Fig. 2(a, b) suggested the possibility of an effect of the iota-carrageenan either producing the inactivation of virus particles released from infected cells (if compound remains cell-associated and has virucidal activity, it could interfere in viral spread from infected cells during infectious centre assay incubation) or inducing a cell refractory state to DENV-2 infection when added to the cell. To test these presumptions, the pre-treatment of virions or cells was performed separately before infection. DENV-2 infectivity was not affected by pre-incubation of virions with compound (Fig. 2c), demonstrating a lack of inactivating properties against DENV-2 of iota-carrageenan. When C6/36 HT cells were pre-treated with iota-carrageenan during 2 h at 33 °C and compound was eliminated before infection with DENV-2, virus yields at 48 h p.i. were reduced by about 1.5 log (Fig. 2c). In fact, the EC50 against DENV-2 by cell pre-treatment in C6/36 HT cells was 6.2 ± 0.3 μg ml−1, similar to the above-mentioned value of EC50 obtained when treatment was exerted during all the period of virus infection (data from Fig. 1b). The carrageenan M3a exhibited a similar behaviour, whereas heparin and lambda-carrageenan did not exert any inhibitory effect on DENV-2 infection of C6/36 HT cells by pre-treatment (data not shown). The pre-incubation of Vero cells with either the commercial iota-carrageenan or M3a did not induce any refractory state to the subsequent DENV-2 infection (Fig. 2c), indicating that the inhibitory activity of iota-carrageenans against DENV-2 by cell pre-treatment before infection is dependent on the type of host cell.

To further assess the differential target for DENV-2 inhibition by iota-carrageenan in Vero and C6/36 HT
cells, the effect of this compound on virus adsorption in both cells was analysed by measuring the number of cell-bound viral RNA molecules after 1 h of DENV-2 infection at 4 °C in the presence of carrageenan. A significant reduction in the amount of DENV-2 RNA attached to Vero cells was detected by real-time RT-PCR, whereas virus binding to C6/36 HT cells was not affected by the carrageenan (Fig. 3).

Results shown in Fig. 3 appeared to confirm the proposed role of HS for DENV-2 binding in Vero cells (Chen et al., 1997; Martínez-Barragan & del Angel 2001; Germi et al., 2002) and suggested the possibility of a different primary receptor for DENV-2 in C6/36 HT cells. To support this conclusion, virus adsorption was determined comparatively in both cells under two experimental approaches to affect HS in cell membrane. First, cells were treated with heparinase I to remove GAG-related molecules. After this treatment, DENV-2 adsorption to Vero cells was highly inhibited, whereas the attachment to C6/36 HT cells was not significantly affected (Fig. 4a). The enzyme specificity for GAG degradation was tested by immunostaining of cell surface with an anti-HS antibody. The abundant presence of HS on Vero cell surface was highly reduced after treatment with heparinase I, whereas HS was almost undetectable in untreated or treated C6/36 HT cells (Fig. 4b). The level of GAG sulfation was also reduced by growing cells in sulfate-free medium added of sodium chlorate (Baeuerle & Huttner, 1986). Again, under this condition DENV-2 adsorption to Vero cells was inhibited, but adsorption to mosquito cells was not affected (Fig. 4a). As control of the desulfation treatment, cells treated with chlorate were supplemented with sodium sulfate, which reversed the desulfation process and restored DENV-2 binding to Vero cells (Fig. 4a). Then, the failure of iota-carrageenan to affect DENV-2 adsorption to C6/36 HT cells, the effect of this compound on virus adsorption in both cells was analysed by measuring the number of cell-bound viral RNA molecules after 1 h of DENV-2 infection at 4 °C in the presence of carrageenan. A significant reduction in the amount of DENV-2 RNA attached to Vero cells was detected by real-time RT-PCR, whereas virus binding to C6/36 HT cells was not affected by the carrageenan (Fig. 3).

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Fig. 1. Antiviral activity of sulfated polysaccharides against DENV-2. (a, b) Vero (a) and C6/36 HT (b) cells were infected with DENV-2 (m.o.i. of 0.1) in the absence or presence of each compound. Virus yields were determined at 48 h p.i. Each value is the mean of duplicate assays ± so. (c) C6/36 HT cells were mock infected or infected with DENV-2 in the absence or presence of 50 μg ml⁻¹ iota-carrageenan or heparin. At 48 h p.i., immunofluorescence staining was carried out using anti-DENV E antibody. Magnification, ×100.
cells appears to be related to the low presence of adequate HS in surface of these cells and, consequently, virus is bound to another type of cell membrane component.

Finally, to corroborate the dissimilarities observed in the mechanism of antiviral activity in Vero and C6/36 HT cells, we intended to generate resistance to iota-carrageenan by serial passage of DENV-2 in a type of host cell, either mammalian or mosquito, in the presence of compound, and then to study comparatively the response to the variants in both cell types. We chose to select the variants by passage in Vero cells because they were more susceptible to the antiviral action of the iota-carrageenan than the mosquito cells (Fig. 1). After three passages of DENV-2 in the presence of iota-carrageenan, the virus designated DENV-2-iota 3 exhibited a high resistance to iota-carrageenan, whereas control virus passaged similarly in the absence of compound, named DENV-2-CV 3, maintained the antiviral susceptibility (Fig. 5a). By contrast, when the antiviral assay was carried out in C6/36 HT cells both variants exhibited the same profile in the dose-response curves (Fig. 5b). From data shown in Fig. 5(a, b), the values of EC50 for DENV-2-iota-3 were calculated as >50 and 1.3 μg ml⁻¹ in Vero and C6/36 HT cells, respectively, confirming the presence of a differential target for this polysaccharide in mosquito and mammalian cells.

**Fig. 2.** Influence of time of treatment with iota-carrageenans on anti-DENV-2 activity. (a, b) Vero and C6/36 HT cells were infected with DENV-2 and maintenance medium (MM) containing 20 or 50 μg ml⁻¹ of iota-carrageenan (a) or M3a (b), respectively, was added simultaneously with virus (time 0) or at the indicated times after infection. At 10 h p.i. medium was discarded and an infectious centre assay was performed in Vero cells. (c) Pre-treatment of cells: cells were pre-incubated with compound during 2 h; then, cells were washed and infected with DENV-2. Virus yields were determined at 48 h p.i. Pre-treatment of virus: DENV-2 suspensions were incubated with compound for 45 min and the remaining infectivity was determined by plaque assay. Each value is the mean of duplicate assays ± SD.
This conclusion was further assessed by evaluating the kinetics of virus adsorption of these viruses in both cells. The adsorption of the resistant variant DENV-2-iota 3 to Vero cells was highly reduced in comparison to control virus, indicating that the mechanism leading to resistance to iota-carrageenan in Vero cells is an alteration in virion ability to interact with HS in cell surface (Fig. 5c). By contrast, DENV-2-iota-3 adsorption to C6/36 HT cells was not impaired (Fig. 5d).

**Effect of iota-carrageenan on cell proliferation and protein synthesis**

Data from Figs 1(b) and 2(c) suggested that the antiviral activity of iota-carrageenan against DENV-2 in mosquito cells may be mainly due to a long lasting effect exerted by any cellular factor triggered as response of the cell to the presence of the compound. The effects of treatment with these compounds on the host cell were analysed by monitoring proliferation of actively growing cells in the presence of carrageenans at concentrations close to the range used in antiviral determinations. When cytotoxicity of carrageenan was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method in uninfected confluent cultures of Vero and C6/36 HT cells after 48 h of treatment, as in the antiviral assay, no significant reduction in cell viability was detected (Fig. 6a). It must be noted that all DENV infections in the experimental protocols presented in this study were carried out in confluent cell monolayers. When iota-carrageenan was added at 2.5 h after seeding the cells, the increase in Vero cell number after 48 h of exposure to the compound was not significantly altered in comparison with compound treatment on confluent cells (Fig. 6a). By contrast, the proliferation ability of C6/36 HT cells was affected by iota-carrageenan with a reduction in the viability percentage of growing cells treated with compound respect to untreated cells of around 30–38 % in the range of assayed concentrations (Fig. 6a).

The activity of iota-carrageenan on cell metabolism was further studied by analysing cell protein synthesis after compound treatment. As seen in Fig. 6(b), no inhibition of protein synthesis in iota-carrageenan-treated Vero cells was
observed. In C6/36 HT cells, a reduction in the general labelling pattern of cellular polypeptides was detected, confirming a direct effect of this compound on mosquito cells in accordance with the results obtained in the cell proliferation assay. Similar results were obtained with M3a, whereas the lambda-carrageenan did not exert any inhibition in mosquito cell proliferation and protein synthesis (data not shown).

To further corroborate the effect of iota-carrageenan on mosquito cells, the viability of actively growing and confluent C6/36 HT cells after compound treatment was also evaluated by other methods such as neutral red (NR) and crystal violet (CV) assays. Results were comparable to MTT data with a reduction in the number of proliferative cells proportional to the compound dose (Fig. 6c).

In conclusion, a subtle but reiterative alteration was induced in mosquito cells after treatment with iota-carrageenan, which may be involved in the inhibition observed in DENV-2 multiplication in C6/36 HT cells.

**DISCUSSION**

Mosquitoes and mammals are the natural hosts of DENV which is successfully transmitted through alternate cycles
of virus replication in each host forcing the virus to maintain its ability to infect both types of cells. The analysis of the antiviral activity against DENV-2 of carrageenans reported here has shown a differential susceptibility of C6/36 HT and Vero cells, taken as models of mosquito and mammalian cells, depending on the structural class of polysaccharides: all polysaccharides blocked DENV-2 infection in Vero cells, but only iota-carrageenans were virus inhibitors in mosquito cells.

The lack of antiviral activity against DENV-2 of heparin, lambda- and kappa-carrageenan in mosquito cells was in accordance with previous reports of polysulfates, which were effective against DENV infection in diverse mammalian cells, but were totally inactive in mosquito cell lines C6/36 HT and AP61 (Talarico et al., 2005; Thaisomboonsuk et al., 2005). By contrast, the two evaluated iota-carrageenans showed an exceptional ability to interfere with DENV-2 multiplication in both cells, but with distinctive differences in their inhibitory action. First, mosquito cells were less susceptible in comparison to mammalian cells. Second, the mechanism of inhibition was strikingly different: in Vero cells the activity of iota-carrageenan was exerted only at the initiation of the cycle, affecting virion binding, whereas in mosquito cells comparable levels of inhibition were obtained if compound was added to cells together with the virus, after 8 h of infection or by cell pre-treatment. The response to iota-carrageenan in mosquito cells is totally different to that usually described for polysulfates not only against DENV but also against other enveloped viruses (Damonte et al., 2004).

From these results it can be concluded that the inhibition of DENV-2 multiplication in Vero cells appeared to be exerted by interference with the initial interaction of glycoprotein E with HS but the inhibition in C6/36 HT cells is independent of HS. The expression level of GAG in Vero cells is high as we could detect here and was reported by other investigators (Avirutnan et al., 2007), whereas HS in C6/36 HT membrane appeared sparsely distributed with a very weak staining. Not only the level of GAG expression in a cell may influence the virus–cell interaction, but also its structural characteristics since certain ligands require specific HS structures for binding. For instance, a highly sulfated form of HS is the reported target for DENV-2 and other flaviviruses to initiate infection in diverse mammalian cells (Barth et al., 2006; Chen et al., 1997). HS has been extensively studied in a wide variety of animals, including mammals, birds and flies (Bishop et al., 2007), but very little information is known about it in insects of medical importance. Sinnis et al. (2007) reported the first isolation of HS in Anopheles stephensi, the vector for Plasmodium parasites causing malaria, finding a lower degree of sulfation with respect to human liver HS. Although no information on Aedes HS composition is presently available, if the sulfated state of HS in Aedes is similar to Anopheles and considering the low HS expression observed in C6/36 HT cell surface reported here, the participation of HS for DENV entry appears to be minimal in comparison to mammalian cells, providing an explanation for our results. Accordingly, structural analyses indicated that HS-binding sites of the domain III of DENV-2 E glycoprotein are essential for binding to BHK-21 cells but are not involved in attachment to mosquito cells (Hung et al., 2004). Also, recent investigations have reported the identification of diverse proteins with virus-binding ability as receptors for DENV-2 in C6/36 cells (Chee & AbuBakar, 2004; Kudakitan et al., 2010; Paingankar et al., 2010; Salas-Benito et al., 2007) without apparent involvement of HS, whereas in Vero and other epithelial mammalian cells HS was proposed as primary attachment receptor to concentrate virions on the cell surface and trigger the interaction with a second receptor of protein nature for virus internalization (Martínez-Barragán & del Angel, 2001).

Like heparin, lambda- and iota-carrageenans are more heavily sulfated than most tissue-derived HS (Esko & Selleck, 2002), a characteristic that usually supported the great antiviral potency of these compounds in mammalian cells. Carrageenans consist of linear chains of alternating (1-3)-b-D-galactopyranoses and (1-4)-a-L-galactopyranosyl (or 3,6-anhydrogalactopyranosyl) (Damonte et al., 2004). They differ in the amount and position of sulfate groups: iota-carrageenan contains two sulfates per disaccharide repeating unit at axial positions, lambda- has close to three equatorial sulfates and kappa-only one. Other difference is related to the monosaccharide composition: lamba-carrageenans are constituted by galactose units whereas iota- and kappa- contain equal amounts of galactose and 3,6 anhydrogalactose, with higher hydrophobic character. Therefore, iota-carrageenans show a combination of ionic and hydrophobic zones in the same macromolecule, not found in lambda- and kappa-carrageenans. This particular structure may allow specific interactions that probably account for the differential effects among carrageenans observed in mosquito cells.

Polysaccharide samples may contain contaminating substances from the natural source that remain after the
extraction and purification processes. To assess that the effect detected in C6/36 HT cells was effectively caused by the polysaccharide, the commercial iota-carrageenan was extensively dialysed against water in a membrane with a cut-off size of 250 kDa. The antiviral activity against DENV-2 of this dialysed sample was similar to that obtained with original carrageenan (data not shown), indicating that the inhibition is not due to an additional effect produced by any small molecule associated to the polysaccharide. Furthermore, a similar behaviour to that here observed for DENV-2 in C6/36 HT cells has been reported in vertebrate cells for human papillomavirus (HPV), a DNA virus also requiring cellular HS for infection (Buck et al., 2006). In addition to blocking the initial interaction of capsid HPV protein with cells, iota-carrageenan also exerts a post-attachment inhibitory effect on HPV infectivity up to 12 h after infection that does not involve HS.

In conclusion, results presented here reveal the ability of iota-carrageenan to attack different targets of DENV-2 infection depending on the type of host cell: virus adsorption in Vero cells or apparently a still unidentified cellular pathway in C6/36 cells. This interesting observation of cell-dependent mode of action is a signal to be considered when any antiviral agent is evaluated in a particular cell system. In addition, new evidence is provided here about the failure for HS involvement in the initial interaction of DENV-2 in mosquito cells contrasting with mammalian cell infection. Further research is required to fully understand the HS-independent mechanism of inhibition of iota-carrageenan in mosquito cells. A recent genome RNA interference screen in insect cells identified 116 candidate host factors required for infection, several of them predicted to be membrane-associated in consistency with the remodelling of cellular membranes observed in DENV-infected cells (Sessions et al., 2009). Although the possibility of a post-entry viral target in the antiviral response to iota-carrageenan observed in C6/36 HT cells cannot be totally discarded, our results point out to an interference with any of the cellular products found associated to DENV-2 infection.

METHODS

Compounds and antibodies. Commercial iota-, lambda- and kappa-carrageenans, and heparin were purchased from Sigma-Aldrich. M3a is a homogeneous carrageenan fraction obtained from M. gelidium as described previously (de S.F-Tischer et al., 2006). Stock solutions of compounds were prepared in distilled water at 2 mg ml⁻¹.

The mouse IgG mAb against DENV E glycoprotein was from Abcam, the anti-HS IgM mAb was from Seikagaku Corporation, goat anti-mouse IgG conjugated to FITC was from Sigma-Aldrich and FITC-conjugated goat polyclonal anti-mouse IgM was from Chemicon International Inc.

Cells and virus. Vero (African green monkey kidney) cells were grown in Eagle’s minimum essential medium (MEM) (Gibco) supplemented with 5 % FBS. For maintenance medium (MM), the serum concentration was reduced to 1.5 %. The C6/36 HT mosquito cell line from A. 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 % FBS.

The stocks of DENV-2 strain NGC were prepared in C6/36 HT cells and titrated by plaque formation in Vero cells.

Antiviral assay. Antiviral activity was evaluated by a virus yield inhibition assay. C6/36 HT and Vero cells grown in 24-well microplates (3.0×10⁵ cells per well) were infected with DENV-2 at an m.o.i. of 0.1 in the presence of different concentrations of the compounds, two wells per concentration. After 48 h of incubation at 33 °C (for C6/36 HT cells) or 37 °C (for Vero cells), cell supernatants were collected and the virus yields were determined by plaque formation in Vero cells. The EC₅₀ values were calculated as the compound concentration able to reduce virus plaques by 50 %. All determinations were performed twice and each in duplicate.

Indirect immunofluorescence assay. Infection of 1×10⁵ C6/36 HT cells grown in coverslips with DENV-2 (m.o.i. of 0.1) was performed in the presence or absence of iota-carrageenan or heparin (50 μg ml⁻¹). At 48 h post-infection, cell monolayers were washed with cold PBS and fixed in methanol for 15 min at −20 °C for cytoplasmic immunofluorescence. Indirect staining was carried out by using anti-DENV mouse mAb and FITC-labelled goat anti-mouse IgG. 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) and visualized on a fluorescence microscope Olympus BX51.

Time of addition experiment. C6/36 HT and Vero cells (3.0×10⁵ cells per well) were infected with DENV-2 (m.o.i. of 1) in either MM containing 50 or 20 μg ml⁻¹ of M3a or iota-carrageenan, respectively, (time 0) or MM without compound. After 1 h adsorption at 4 °C, unadsorbed virus was removed, cultures were washed with PBS, and MM with compound was added immediately (time 0 and 1 h p.i.) or at 2, 3, 5 and 8 h p.i. An infected culture without compound treatment was performed simultaneously as virus control. At 10 h after infection, cell supernatants were withdrawn, the monolayers were washed with PBS and trypsinized with 0.25 % trypsin-EDTA to disperse cells. Protease treatment was stopped by adding 2 mM PMSF in PBS containing 3 % BSA (PBS-3 % BSA), then cells were resuspended in MM, and the final cell suspensions were serially 10-fold diluted and plated onto confluent monolayers of Vero cells. After 1.5 h incubation at 37 °C, monolayers were overlaid with MM containing 1 % methylcellulose. Infectious centres were counted after 6 days of incubation.

Effect of pre-treatment of cells or virus with compound prior to infection. Pre-treatment of cells: cells (3.0×10⁵ cells per well) were pre-incubated with MM containing different compound concentrations for 2 h at 33 or 37 °C. Then, cells were thoroughly washed with PBS, and infected with DENV-2 (m.o.i. of 0.1) in the absence of compound. Virus yields were determined at 48 h p.i.

Pre-treatment of virus: a DENV-2 suspension (1×10⁶ p.f.u. ml⁻¹) was incubated in MM containing or not different compound concentrations at 37 °C during 45 min, and then the remaining infectivity was titrated by p.f.u.

Cell viability assays. Cell viability was measured by three different assays in resting and actively growing cells. For resting cells, confluent cultures in 96-well plates (5×10⁴ cells per well) were exposed for 48 h to serial twofold compound dilutions, three wells for each concentration, and then viability was tested. To monitor proliferation...
of actively growing cells, 1 × 10^6 cells were seeded in 96-well plates and allowed to adhere during a 2.5 h incubation period at 33 or 37 °C for C6/36 HT and Vero cells, respectively. Thereafter, compound was added and incubation followed during 48 h before viability determination.

In the MTT method, 10 µl of MM containing MTT (final concentration 0.5 mg ml^-1) was added to each well. After 2 h of incubation at 33°C (C6/36 HT cells) or 37°C (Vero cells), the supernatant was removed, ethanol was added to each well and absorbance was measured at 595 nm.

In NR assay, cells were incubated for 2 h with NR (100 µg ml^-1) dissolved in MEM without serum in darkness. Then the supernatant was removed, cells were washed with PBS, elution medium (EtOH/AcCOOH, 50/1%) was added and absorbance was recorded at 540 nm.

For CV assay, cells were fixed with 10% formaldehyde during 15 min. Then, cells were washed with water and stained with CV (0.003% CV in 10% EtOH) during 30 min. After washing, colourant was eluted with EtOH/AcCOOH, 50/0.1% and absorbance was recorded at 590 nm.

**Cell protein synthesis.** C6/36 HT and Vero cells (3.0 × 10^5 cells per well) were incubated in the presence or absence of compound during 11 h at 33 or 37 °C, respectively. Then, cell monolayers were washed with PBS and incubated in methionine-cysteine-free medium in the presence or absence of compound for 1 h, and then labelled by the addition of 100 µCi EXPRE³²S (NEN Dupont) ml^-1 for 4 h. After labelling, cells were washed with PBS and lysed in sample electrophoresis buffer [5% SDS, 2% 2-mercaptoethanol, 10% glycerol (v/v) and 0.005% bromophenol blue in 0.0625 M Tris/HCl, pH 6.8]. Cell lysates were sonicated for 1 min, boiled during 2 min and loaded for electrophoresis on 15% SDS-polyacrylamide slab gels (lysates corresponding to1.0 × 10^6 cells per well). Protein bands were visualized by fluorography.

**Virus-binding assay.** C6/36 HT and Vero cells grown in six-well microplates (1.2 × 10^5 cells per well) were infected with DENV-2 (m.o.i. of 1) in the absence or presence of 50 or 20 µg ml^-1 of iota-carrageenan, respectively. After 1 h incubation at 4 °C, cells were washed extensively with cold PBS to remove unadsorbed virus and total RNA was extracted from cells with TRIzol (Invitrogen) according to the manufacturer’s instructions. The amount of cell bound viral RNA was quantified by real-time RT-PCR as described previously (Talarico & Damonte, 2007).

**Heparinase treatment.** C6/36 HT and Vero cells in six-well microplates (1.2 × 10^5 cells per well) were incubated for 1 h at 33 or 37 °C, respectively, in the absence or presence of 3 U heparinase I (Sigma-Aldrich) ml^-1. Then, cells were washed with PBS and infected with DENV-2 (m.o.i. of 1). After 1 h adsorption at 4 °C, cells were washed with cold PBS and disrupted by freezing and thawing. The amount of infectious bound virus was then measured by plaque formation.

To assess the effect of heparinase I on cell surface-exposed HS residues, C6/36 HT and Vero cells grown in coverslips were treated with the enzyme as above and then fixed with 4% paraformaldehyde followed by permeabilization with 0.2% Triton X-100. After fixation, cells were washed with PBS containing 0.5% BSA (PBS-0.5% BSA), and immunolabelled with monoclonal anti-HS and FITC-conjugated goat polyclonal anti-mouse IgM. After a final washing with PBS-0.5% BSA, cells were incubated in a glycerol solution containing 2.5% Dabco.

**Inhibition of cellular sulfation.** C6/36 HT and Vero cells (1.2 × 10^6 cells per well) were grown in standard MEM or sulfate-free MEM added with either 30 mM of the sulfation inhibitor sodium chlorate or 30 mM sodium chloride plus 20 mM sodium sulfate for reversal of sulfation inhibition. After 48 h incubation at 33 or 37 °C, according to cell type, all cultures were washed with MEM without serum, infected with DENV-2 and adsorbed virus was then measured as for heparinase treatment.

**Isolation of drug-resistant variant viruses.** Vero cells grown in six-well microplates (1.2 × 10^6 cells per well) were infected with DENV-2 (m.o.i. of 0.1) in the presence of iota-carrageenan during virus adsorption and throughout the period of incubation. Supernatants were collected at 4–7 days p.i. and titrated by plaque formation. The supernatant with the highest virus titre was selected to perform the next passage and serial passages were continued in this manner. The compound concentration was 1 µg ml^-1 for the initial two passages and 2 µg ml^-1 for passage 3. The EC50 of each passage against iota-carrageenan was determined in Vero cells to monitor the appearance of resistant variant viruses. Simultaneously, control passages of DENV-2 in Vero cells without carrageenan were also performed.

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