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Short communication

Changes in antiviral susceptibility to entry inhibitors and endocytic uptake of dengue-2 virus serially passaged in Vero or C6/36 cells

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ABSTRACT

The aim of the present study was to analyze the influence of virus origin, mammalian or mosquito cell-derived, on antiviral susceptibility of DENV-2 to entry inhibitors and the association of this effect with any alteration in the mode of entry into the cell. To this end, ten serial passages of DENV-2 were performed in mosquito C6/36 cells or monkey Vero cells and the antiviral susceptibility of each virus passage to sulfated polysaccharides (SPs), like heparin and carrageenans, was evaluated by a virus plaque reduction assay. After serial passaging in Vero cells, DENV-2 became increasingly resistant to SP inhibition whereas the antiviral susceptibility was not altered in virus propagated in C6/36 cells. The change in antiviral susceptibility was associated to a differential mode of entry into the host cell. The route of endocytic entry for productive Vero cell infection was altered from a non-classical clathrin independent pathway for C6/36-grown virus to a clathrin-mediated endocytosis when the virus was serially propagated in Vero cells. Our results show the impact of the cellular system used for successive propagation of DENV on the initial interaction between the host cell and the virion in the next round of infection and the relevant consequences it might have during the in vitro evaluation of entry inhibitors.

Dengue virus (DENV), a member of the genus *Flavivirus* in the family *Flaviviridae*, is at present the most widespread arbovirus in the world (Guzmán et al., 2010). The World Health Organization estimates that 50–100 million infections occur each year, though new appraisals indicate that apparent and inapparent infections would reach the number of 350 million per year (Bhatt et al., 2013). Four serotypes of DENV, DENV-1 to DENV-4, co-circulate in tropical and subtropical regions of Asia, Africa and America by human transmission through the bite of an infected mosquito from the species *Aedes aegypti* and *Aedes albopictus*. All serotypes may cause an inapparent infection, a mild illness known as dengue fever or the severe dengue hemorrhagic fever/dengue shock syndrome (Halstead, 2007). However, no specific chemotherapy neither vaccine for dengue is currently available and treatment is based only in supportive care.

Different types of sulfated polysaccharides (SPs) were found to be potent and selective inhibitors of DENV infection. The antiviral activity of SP against DENV is dependent on virus serotype and the host cell used for the antiviral assay. For virus plaque, focus or yield reduction tests performed in Vero (Pujol et al., 2012; Talarico et al., 2005), BHK-21 (Hidari et al., 2008; Lin et al., 2002) HepG2 (Talarico and Damonte, 2007) or LLC-MK2 cells (Ichiyama et al., 2013) it has been reported that DENV-2 is the most susceptible serotype to SP inhibition, whereas DENV-3, DENV-4 and DENV-1, in this order, require higher compound concentration for an effective action. It was demonstrated that the antiviral action of SP against DENV-2 is exerted by a blockade of virus adsorption and penetration (Ichiyama et al., 2013; Talarico and Damonte, 2007), due to the structural similarities with the heparan sulfate (HS) residues of membrane proteoglycans, proposed as putative initial cell receptor for DENV-2 (Chen et al., 1997; Dalrymple and Mackow, 2011; Germi et al., 2002; Hilgard and Stockert, 2000). With respect to the host cell, the efficacy of SPs against DENV-2 was similar in the cited mammalian cells, but they were almost inactive in mosquito C6/36 cells (Talarico et al., 2005).

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In the above-mentioned studies, the working viral stocks used to analyze antiviral activity were propagated in mosquito C6/36 cells. In nature, the first round of infection in a human being is with virus derived from mosquito cells, and subsequent infections in the same host occur with human-derived virus as well as the next round of transmission from the human host to a new mosquito vector. Differences in the source of virus may affect the interaction between the virus and the host cell and, likely, the antiviral susceptibility. The effect of viral source has been examined for some particular DENV–host interactions such as virulence/attenuation, replication ability and receptor affinity (Añez et al., 2009; Hacker et al., 2009; Lee et al., 2006, 2011; Prestwood et al., 2008). The aim of the present study was to evaluate the impact of virus origin, mammalian or mosquito cell-derived, on antiviral susceptibility of DENV-2 to SP and the association of this effect with any alteration in the mode of entry into the cell.

The C6/36 mosquito cell line was cultured at 33 °C in L-15 medium (Leibovitz) (GIBCO) supplemented with 0.3% tryptose phosphate broth, 0.02% glutamine, 1% minimum essential medium (MEM) non-essential amino acids solution and 5% fetal calf serum whereas Vero monkey cells were grown at 37 °C in Eagle's MEM (GIBCO) supplemented with 5% calf serum. Maintenance medium (MM) consisted of MEM containing 1.5% calf serum. For cellular virus adaptation, ten serial passages of the original stock of DENV-2 strain NGC (provided by Dr. A.S. Mistchenko, Hospital de Niños Dr. Ricardo Gutiérrez, Buenos Aires, Argentina), usually produced in C6/36 cells and designed DENV-2 V0, were carried out either solely in Vero (named V for Vero cells and the corresponding passage number) or C6/36 cells (named M for mosquito cells and passage number). To this end, Vero or C6/36 cell monolayers grown in 6-well microplates were infected with DENV-2 at an m.o.i. of 0.1 PFU/cell. Supernatants were collected at 4–7 days post-infection (p.i.) and titrated by plaque formation in Vero cells. The supernatant with the highest virus titer was selected to perform the next passage and serial passages were continued in this manner. Two series of independent sequential cell passages were performed and analyzed separately.

First, the antiviral activity of heparin (Sigma–Aldrich) against C6/36- or Vero-adapted DENV-2 was evaluated by a virus plaque reduction assay in the presence of serial two-fold compound concentration as previously described (Talarico et al., 2005). A dose dependent inhibitory response was observed for the original virus and the four initial passages in Vero cells, as shown for DENV-2 V0 and DENV-4 V4 in Fig. 1. However, for the virus serially propagated in Vero cells, the susceptibility to heparin decreased dramatically between passages 4 and 5, and no inhibition in plaque number was detected for DENV-2 V10 (Fig. 1). By contrast, the virus continuously propagated in mosquito cells DENV-2 M10 presented a susceptibility to heparin very similar to DENV-2 V0 (Fig. 1).

The variable resistance to heparin after DENV-2 passage in Vero or C6/36 cells was clearly evidenced when the values of effective concentration 50% (EC_{50}), the compound concentration required to reduce plaque number by 50%, were calculated from data shown in Fig. 1. No inhibition of DENV-2 V10 multiplication was observed up to the highest concentration tested of heparin (50 μg/ml) whereas EC_{50} value for the original DENV-2 V0 was 2.6 μM (Table 1). A similar trend of change from DENV-2 V0 to DENV-2 V10 was observed when the antiviral susceptibility was evaluated against other types of SP exhibiting more potent activity against DENV-2 such as λ- or ι-carrageenan (Sigma–Aldrich). The values of EC_{50} against carrageenans corresponding to DENV-2 continuously replicated in Vero cells were always higher than the EC_{50} of the original DENV-2 V0, especially in the case of ι-carrageenan (Table 1). Differently from DENV-2 V10, DENV-2 M10 did not show significant increased resistance to any SP treatment (Table 1).

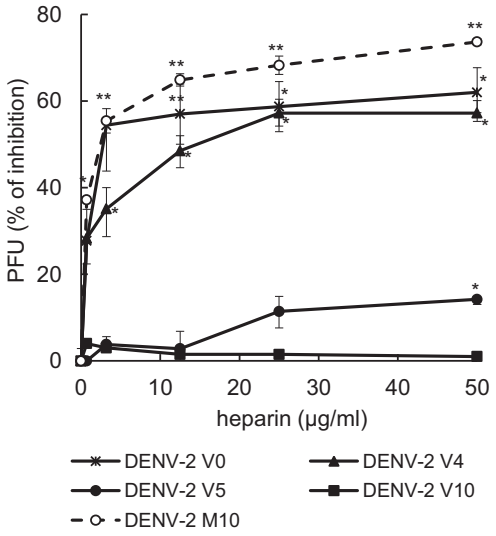

Fig. 1. Effect of heparin on DENV-2 infection. Vero cells were treated with serial twofold concentrations of heparin during 1 h and then infected with about 50 PFU/well of each DENV-2 passage in the presence of heparin. After adsorption, residual inoculum was replaced by MM containing methylcellulose. At 6 days p.i. cells were fixed with 10% formaldehyde, stained with 1% crystal violet and plaques were counted. Results are expressed as % inhibition of plaque number in compound-treated cultures compared to untreated ones. Each value is the mean of duplicate assays ± SD. Student's *t*-test was used to determine statistical significance between treated and control infected cells for each passage. Asterisks indicate *P* values in each dose–response curve (*$P \leq 0.05$; **$P \leq 0.001$).

A noticeable differential susceptibility to heparin and carrageenans was also reported among DENV serotypes (Talarico and Damonte, 2007). Interestingly, DENV-2 and DENV-1, the most susceptible and resistant serotypes to SP, respectively (Ichiyama et al., 2013; Talarico et al., 2005) use a different pathway for infectious entry into Vero cells: DENV-1 utilizes a clathrin-dependent pathway of endocytosis whereas the entry of DENV-2 occurs by a non-classical endocytic route independent of clathrin and caveolae, but dependent on dynamin (Acosta et al., 2009). Thus, we decided to assay the route of entry into Vero cells, regarding to clathrin-dependence, of the DENV-2 suspensions serially passaged in Vero or C6/36 cells to find the basis of their differential susceptibility to SP.

The involvement of clathrin-mediated pathway in the entry of DENV-2 after each passage was addressed by determining the effect of chlorpromazine (CPZ), a pharmacological inhibitor of this endocytic route (Wang et al., 1993). Vero cells were treated with 50 μM CPZ, the maximum noncytotoxic concentration, only for 2 h before infection and during the first hour of infection with each DENV-2 passage when entry takes place. Under these treatment conditions the clathrin-mediated endocytic pathway is effectively blocked, as assessed by transferrin internalization assays (data not shown). Extracellular virus yields were determined at 48 h p.i. by plaque formation in Vero cells. As previously reported for the original DENV-2 V0 (Acosta et al., 2009), CPZ treatment did not produce any inhibitory effect on infection of Vero cells with initial passages of DENV-2 V0 in Vero cells (DENV-2 V1 to V3), but surprisingly, after 4 passages a 50% reduction in virus yield was observed after CPZ treatment as compared to untreated control (Fig. 2A). The inhibitory activity of CPZ against DENV-2 increased with Vero cell passage number and after 7 passages the inhibition in virus yield remained over 90% with respect to control non-CPZ treated cells. The parallel 10 serial passages of DENV-2 V0 in mosquito C6/36 cells resulted in the generation of virus populations without increased susceptibility to CPZ treatment in Vero cells. Conversely, a tendency to produce higher amounts of

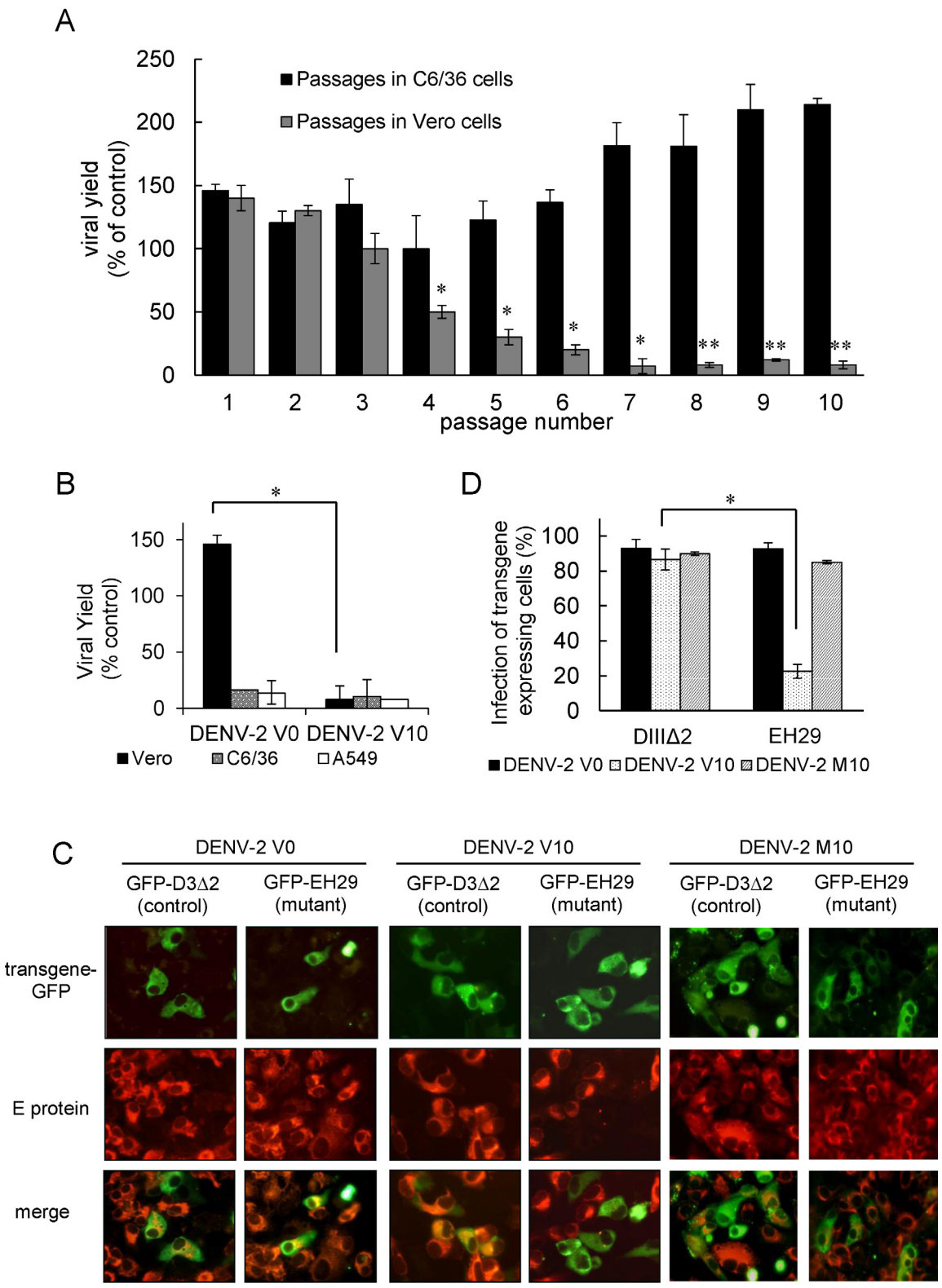

Fig. 2. Effect of inhibition of chlatrin-mediated endocytosis on DENV-2 infection. (A) Vero cells were pretreated with 50 μM CPZ for 2 h at 37 °C, and then infected with DENV-2 stocks serially propagated in Vero cells or C6/36 cells, at an m.o.i. of 0.1 PFU/cell in the presence of drug. After 1 h of infection at 37 °C, virus inocula were removed, cultures were washed three times with PBS and further incubated at 37 °C in MM without compound. Virus yields were quantified at 48 h p.i. by plaque assay in Vero cells. Results are expressed as % of virus yield in compound-treated cultures compared to untreated ones. (B) Vero, A549 and C6/36 cells were pretreated with 50 μM CPZ for 2 h. Then, cells were infected with DENV-2 V0 or DENV-2 V10 and virus yields were determined at 48 h p.i. as in (A). (C) To determine the effect of Eps 15 dominant negative mutant on DENV-2 infection, cells transiently transfected with the constructs GFP-DIIIΔ2 (control) or GFP-EH29 (mutant) of Eps15 were infected with DENV-2 V0, DENV-2 V10 or DENV-2 M10 (m.o.i. 1 PFU/cell). At 24 h p.i. cells were fixed and viral antigen expression was visualized by immunofluorescence staining using mouse anti-glycoprotein E monoclonal antibody (Abcam) and rhodamine-labeled anti-mouse IgG (Sigma–Aldrich). (D) For quantification of samples shown in (C), 250 transfected cells with similar levels of GFP expression were screened and cells positive for DENV-2 antigen were scored. In (A), (B) and (D) values represent the mean ± SD of two independent experiments. Student's *t*-test was used to determine statistical significance between control infected/transfected cells and treated infected/mutant transfected cells. *P* values statistically significant are indicated by asterisks: $^{*}P \leq 0.05$; $^{**}P \leq 0.001$.

Table 1
Antiviral activity of sulfated polysaccharides against DENV-2 passaged in Vero or C6/36 cells.

Virus	$EC_{50} \pm SD$ (μg/ml)[a]			RR[b]		
	Heparin	λ-Carr	ι-Carr	Heparin	λ-Carr	ι-Carr
DENV-2 V0	2.6 ± 0.1	0.08 ± 0.02	0.39 ± 0.06	1	1	1
DENV-2 V10	>50*	0.5 ± 0.2	22.5 ± 3.9**	>19.2	6.3	57.6
DENV-2 M10	2.4 ± 0.3	0.21 ± 0.09	0.64 ± 0.04	0.9	2.6	1.6

[a] Effective concentration 50%: compound concentration required to reduce virus plaque number in Vero cells by 50%. Each value is the mean of duplicate assays ± standard deviation. Student's t-test was used to determine statistical significance. Asterisks indicate statistically significant differences in EC_{50} from serially passaged DENV-2 in comparison to DENV-2 V0.
[b] Relative resistance: EC_{50} of each DENV-2 passage/EC_{50} of DENV-2 V0.
* $P \leq 0.05$.
** $P \leq 0.001$.

infectious virions from CPZ-treated cells was detected as just described for the original DENV-2 stock (Acosta et al., 2009). Then, these results suggest there is a shift from a clathrin-independent route of entry to a clathrin-mediated endocytic uptake in Vero cells as DENV-2 is adapted to grow in this cell system, while clathrin-independence for infectious entry in Vero cells is sustained as the virus is propagated in mosquito cells. This alteration in virus entry route between DENV-2 V0 and V10 was a unique property for Vero cell infection. When both virus populations were tested for CPZ susceptibility in mosquito C6/36 cells or human A549 cells, cellular systems in which mosquito-grown DENV-2 exploits the clathrin-mediated pathway for infectious entry (Acosta et al., 2008, 2009; Mosso et al., 2008), DENV-2 V0 and DENV-2 V10 were similarly inhibited (Fig. 2B).

To confirm the differential behavior for Vero cell entry between DENV-2 continuously passaged in C6/36 or Vero cells, the EH29 dominant negative mutant of Eps15, a cellular protein required for clathrin-mediated endocytosis, was used (Benmerah et al., 1999). As a negative control we included in the assay the DIIIΔ2 construct, which encodes for a truncated version of Eps15 that does not affect the clathrin pathway (Benmerah et al., 1998). Vero cells were transfected with plasmids encoding the GFP-tagged versions of these proteins using Lipofectamine 2000 reagent (Invitrogen, USA) as previously described (Acosta et al., 2009) and 24 h post-transfection, cells were infected and then processed to detect GFP auto-fluorescence and viral antigen expression by indirect immunofluorescence staining. Infection rates were determined at 24 h p.i. for each virus population by scoring cells positive for viral antigen in 250 cells with comparable levels of transgene expression. As expected, the cell fraction positive for DENV-2 V0 represented around 90% of transgene-expressing cells in both GFP-EH29 and GFP-DIIIΔ2 transfected cells whereas for DENV-2 V10 the percentage of virus positive cells diminished from 86.6% to 22.5% in GFP-DIIIΔ2 control transfected cells respect to GFP-EH29 mutant expressing cells, respectively (Fig. 2C and D). By contrast, the continuous growth of DENV-2 V0 in mosquito cells, illustrated by DENV-2 M10, maintained the level of infected transgene expressing cells over 85% in both control and mutant transfected cultures (Fig. 2C and D). In conclusion, both pharmacological and molecular inhibitors of clathrin-mediated endocytosis confirmed that DENV-2 is capable of entering and causing a productive infection in Vero cells lacking a functional clathrin-mediated endocytosis when virus is grown in mosquito cells, but DENV-2 propagation in Vero cells produces a shift in the entry route into Vero cells to a clathrin-dependent endocytic pathway. This change in entry route was not related to a differential ability to propagate in Vero cells since the three virus populations grow at the same rate in Vero cell cultures with high titers of virus production in the range 10^6–10^7 PFU/ml at 3–4 days p.i.

The envelope E glycoprotein is mainly involved in the sequential steps leading to DENV entry into the host cell (binding, internalization and membrane fusion) and as antiviral target in the activity of SP that block virus adsorption and penetration. Given the alterations in both the route of virus entry and the susceptibility to SP detected in DENV-2 V10, the sequences of E gene corresponding to DENV-2 V0, V10 and M10 were obtained to compare possible alterations in this protein among these viral populations. The GenBank/EMBL/DDBJ accession numbers for the E gene sequences of DENV-2 M10, V0 and V10 are KF752594, KF752595 and KF752596, respectively. In comparison to DENV-2 V0, the DENV-2 variant serially transferred for 10 passages in Vero cells did not present any alteration whereas only one change in the position 599 was found in DENV-2 M10 in the E region of the nucleotide sequence (Fig. S1), resulting in a change in the amino acid sequence of E from a neutral amino acid to a positive charged-one(Q200R) but this substitution was not related to a change in the endocytic route (Fig. 2). Thus, the molecular mechanism responsible of the changes in antiviral susceptibility and endocytic uptake remains to be fully elucidated.

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Other reports showed that adaptation of DENV to cell culture may lead to enhance virus affinity for HS, probably linked to mutations that increase the positive charges in the E envelope glycoprotein (Añez et al., 2009; Lee et al., 2006; Prestwood et al., 2008). Apparently, the interaction of DENV-2 with Vero cells presents several unique properties. First, our previous studies demonstrated that Vero is the host cell where alternative entry routes and intracellular trafficking pathways have been shown for mosquito-grown virus depending on DENV serotype and/or strain (Acosta et al., 2009, 2012). By contrast, a classical clathrin-mediated pathway appears to be the only route for infective DENV-2 penetration in mosquito cells (Acosta et al., 2008; Mosso et al., 2008) and in other mammalian cells, such as HeLa (Krishnan et al., 2007), BSC-1 (van der Schaar et al., 2008), Huh7 (Ang et al., 2010) and HepG2 (Alhoot et al., 2012). Second, DENV-2 propagation in Vero cells selects for viral populations with reduced affinity for HS, as clearly evidenciated here by the increased resistance to HS-mimicking inhibitors, like heparin and carrageenans, in comparison to mosquito C6/36 cell-derived virus. In addition, this study establishes for the first time a clear association between the alteration in response to SP and a change in the route of endocytic entry to Vero cells from a non classical clathrin-independent pathway to a classical clathrin-mediated endocytosis when the host cell for DENV-2 propagation was shifted from mosquito to Vero cells.

The Vero cell line is a system usually employed to propagate and assay DENV, to evaluate compounds in anti-DENV assays and also is a representative cell line to produce virus in different approaches for the development of DENV vaccines (Huang et al., 2013; Lee et al., 2011; Sanchez et al., 2006). Then, it must be taken into consideration the impact of the cellular system used for successive propagation of DENV on the initial interaction between the host cell and the virion in the next round of infection and the relevant consequences it might have during the in vitro evaluation of

entry inhibitors. Not only the activity of HS-related compounds on DENV multiplication may be affected but also other drugs, like CPZ, targeted to cellular components involved in the endocytic process.

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