Cell entry of dengue virus

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Dengue virus is an expanding public health problem in tropical and subtropical regions of the world, mainly owing to failure in the maintenance of control programs for the mosquito vector *Aedes aegypti* and increasing and unplanned urbanization. It has been estimated that over 50 million dengue virus infections of varying severity occur globally each year, making this virus the most significant mosquito-borne human pathogen. However, there is no specific antiviral therapy or vaccine for treatment or prevention. This review focuses on recent data describing the putative molecules and mechanisms involved in the complex process of dengue virus binding and entry into mosquito and mammalian cells in primary infections. Furthermore, the perspectives of these early events in the virus life cycle as a target for antidengue therapeutic strategies are also considered.

Keywords: antiviral therapy, dengue virus, envelope E glycoprotein, flavivirus, receptor, virus attachment, virus entry

Dengue virus (DENV) is a mosquito-borne member of the genus *Flavivirus*, family *Flaviviridae*, which includes many important human pathogenic viruses such as yellow fever virus, tick-borne encephalitis virus and West Nile virus. The virion is an enveloped particle containing a single positive-stranded RNA genome and three structural proteins (the capsid [C] protein, a small membrane [M] protein and the envelope [E] glycoprotein). There are four antigenically related serotypes (DENV-1–4), which cocirculate in tropical and subtropical regions around the world between their vectors, the mosquitoes *Aedes aegypti* and *Aedes albopictus*, and the vertebrate hosts.

DENV human infection results in a wide clinical spectrum ranging from either an asymptomatic infection or a benign self-limited febrile illness called dengue fever (DF), to a severe disease such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) [1]. Primary infection provides immunity against the infecting serotype, but the secondary infection with another serotype appears to enhance the risk of developing DHF/DSS through an immunopathological process termed antibody-dependent enhancement (ADE), with formation of immune complexes between virus and nonneutralizing heterotypic antibodies that infect Fc-receptor positive cells via the Fc portion of the immunoglobulin [2,3].

The failure to maintain programs for controlling the mosquito vector has caused the global re-emergence of this infection, turning DENV into a public health problem. Currently, it is estimated that the virus is endemic in more than 100 countries, resulting in approximately 50 million cases of DF per year and 250,000–500,000 cases of life-threatening DHF/DSS [4].

Despite the importance and increasing incidence of DENV as a human pathogen, there are no antiviral agents or vaccines available for treatment or prevention, and little is known about the cell biology or the life cycle of DENV in mosquitos or mammalian cells. The development of a successful chemotherapy for DENV infection requires a better understanding of the viral life cycle to elucidate potential targets and, thus, to obtain key information for the rational design of antiviral drugs. In this review we present the recent data on the molecules and mechanisms involved in DENV entry in primary infection. Furthermore, the perspectives of this early process as a target for anti-DENV strategies are considered.

DENV attachment: multiple cell receptors?

The events involved in virus entry comprise virion binding to the cell surface followed by penetration into the cytoplasm. The success of both sequential steps is determined by host range and tissue tropism, key factors for viral pathogenesis. For DENV infection, this early interaction seems to be particularly complex. Although the precise mechanisms of DENV entry are not still totally elucidated, a probable reason for this complexity may be the great diversity in DENV cellular tropism *in vivo* and *in vitro* and the putative possibility of a different receptor or entry pathway dependent on the class of the infected host cell.

In primary infection, the E glycoprotein has been identified as the DENV protein involved in both attachment and internalization into the cell, playing a central role in the control of infection and tropism. In contrast to the certain involvement of the E glycoprotein as a viral attachment protein, the identity of the cellular receptor for DENV is, at present, controversial. The ability of DENV to replicate *in vitro* in a wide spectrum of primary and continuous cell cultures derived from many mammalian and arthropod tissues suggests the presence of either a single ubiquitous receptor or divergent receptors according to the type of host cell. In support of this last hypothesis, several recent reports, reviewed in Table 1, have implicated a long list of receptor candidate molecules.

For mosquito cells, two glycoproteins of 40–45 kDa were reported as putative receptors in C6/36 cells for binding of DENV-4 [5,6], two proteins of 67–80 kDa and a 48 kDa tubulin-like protein were described for binding of DENV-2 [7,8], whereas a laminin-binding protein was identified as a DENV-3 and -4 receptor in the same cells [9]. For mammalian cells, different proteins have been proposed in human, mouse and monkey cells, including monocytes, macrophages, dendritic cells, B and T leukocytes, endothelial cells, and bone marrow-, hepatoma-, neuroblastoma- and kidney-derived cells. The putative protein DENV receptors include heat shock protein (Hsp)70 and Hsp90 [10], GRP78 [11], laminin receptor [12], mannose receptor [13], CD14-associated protein [14,15], DC-SIGN [16,17] and diverse, not fully characterized, polypeptides [18–25]. Among the candidate protein receptors, the best characterized molecule is DC-SIGN, which can mediate infection with the four serotypes of DENV. In contrast with the aforementioned reports, involvement of heparan sulfate (HS) was demonstrated for DENV attachment to cells of human, monkey and hamster origin [26–29]. HS is a very ubiquitous glycosaminoglycan present on the cell surface and in the extracellular matrix, and is used by many pathogens as an initial receptor [30]. However, the DENV interaction with HS is unique owing to its specificity for a highly sulfated form [26]. In addition to the identification of diverse molecules for DENV binding to different cells, the initial virus–cell interaction in the same host cell was also shown to be serotype dependent (Table 1) [12,31,32].

Collectively, these conflicting data lead to the proposal of a multistep process consisting of the sequential interaction of the E glycoprotein with at least two target molecules on the cell membrane. First, an abundant and low affinity attachment receptor, which may be HS or DC-SIGN according to the cell type, would serve primarily to concentrate virus particles on the cell surface, and then the interaction with a high affinity or second receptor of protein nature mediates virion internalization [23,33]. The serotype and cell dependence of these processes may be ascribed to variations in the corresponding domains of the E glycoprotein among serotypes and their interaction with the cell to be infected.

DENV internalization

After binding to the cellular receptor(s), viruses depend on their ability to penetrate and release the viral genome into the cell cytoplasm. For enveloped viruses, penetration involves membrane fusion and this process may take place either at the cell surface (pH-independent penetration) or within intracellular vesicles (pH-dependent penetration). The majority of viruses need endocytic internalization for productive infection, probably because endocytosis offers the advantage of guiding the virion to an adequate site for replication, bypassing many cytoplasmatic barriers [34]. As a result, viruses have evolved to hijack the multiple portals of entry that are available in the cells (Figure 1) [35–37]. There are four well-defined routes:

- Phagocytosis
- Macropinocytosis
- Clathrin-mediated endocytosis
- Caveolae-mediated endocytosis.

In addition, other nonclathrin- and noncaveolar-mediated pathways are less characterized:

- Lipid raft-mediated endocytosis dependent on dynamin;
- Lipid raft-mediated endocytosis independent of dynamin;
- The nonclathrin, noncaveolar-mediated pathway independent of lipid rafts, in which dynamin participation has been proposed but has not been determined.

Initial electron microscopy studies indicated that DENV-2 penetrated directly into the cytoplasm of human monocytes, mosquito and BHK cells by fusion of the virion envelope with the plasma membrane at physiological pH [38,39]. However, at present it is generally accepted that for productive infection DENV uptake occurs through receptor-mediated endocytosis, which is

Table 1. Putative dengue virus receptors.

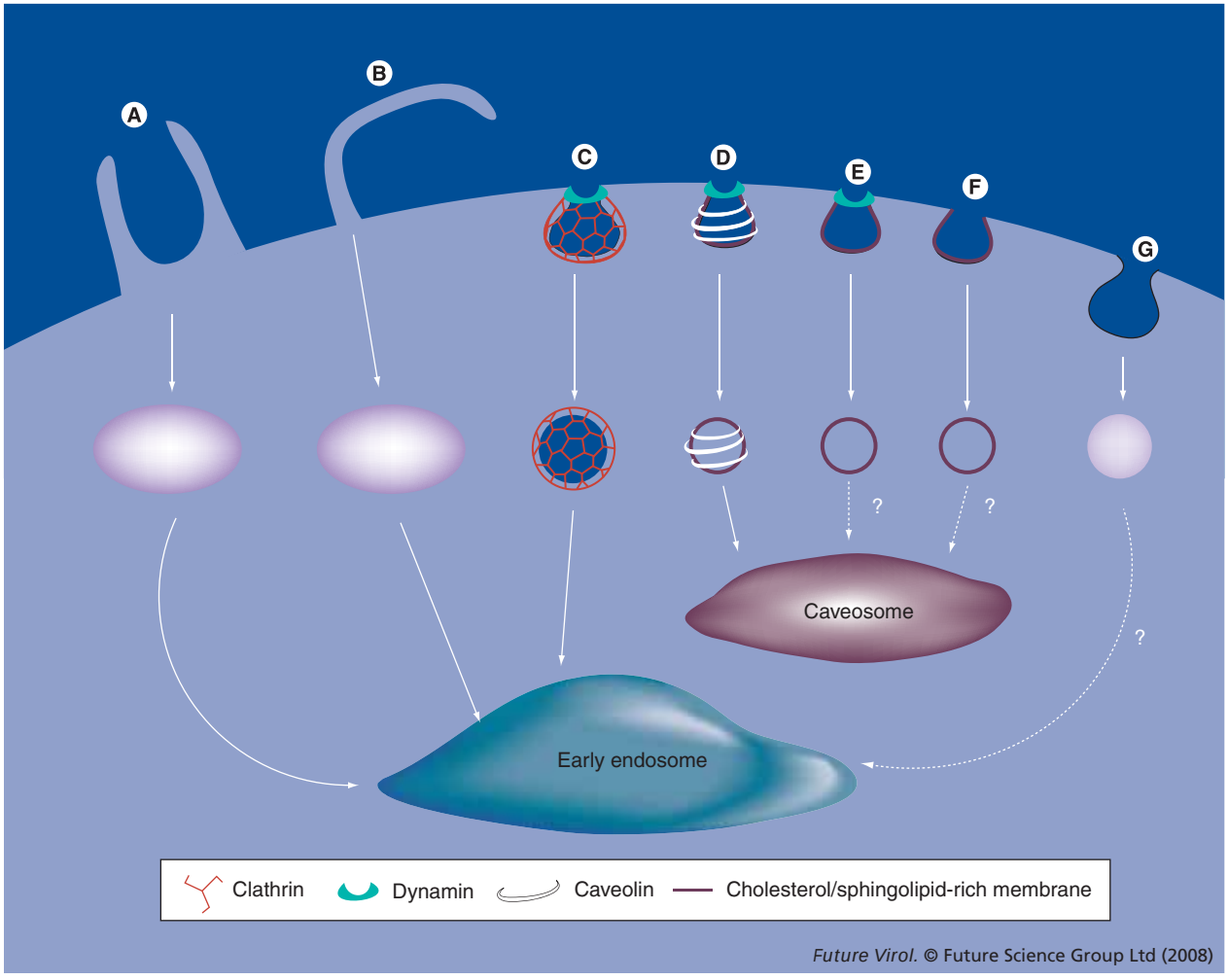
DENV serotype	Cell type	Receptor characteristics	Ref.
Proteins			
DENV-1	Human hepatoma (HepG2), monkey kidney (Vero)	Protein	[18]
	Human hepatoma (HepG2)	Laminin receptor	[12]
DENV-2	Mosquito cell line (C6/36)	Proteins (67, 80 kDa)	[7]
	Mosquito cell line (C6/36)	Tubulin-like protein (48 kDa)	[8]
	Human hepatoma (HepG2)	GRP78	[11]
	Human endothelial (ECV304)	Proteins (28, 34, 43 kDa)	[25]
	Human bone marrow (K562)	Protein (100 kDa)	[19]
	Human and mouse neuroblastoma (SK-N-SH, N1E-115)	Protein (65 kDa)	[20]
	Human monocytic (U937) and neuroblastoma (SK-SY-5Y)	Hsp 90, Hsp 70	[10]
	Human monocytes	Protein (CD14-associated molecule)	[14,15]
	Human macrophages	Proteins (27,45, 67, 87 kDa)	[24]
DENV-2,-3	Human myelomonocytic (HL60) and B-cell line (BM13674)	Proteins (40, 70 kDa)	[21]
	Human B- and T-leukocyte cell lines (Raji, Molt4, LK63)	Proteins	[22]
DENV-3,-4	Mosquito cell line (C6/36)	Laminin receptor	[9]
DENV-4	Mosquito cell line (C6/36)	Glycoproteins (40, 45 kDa)	[5,6]
	Monkey kidney (Vero)	Protein (74 kDa)	[23]
DENV-1–4	Human macrophages	Mannose receptor	[13]
	Human dendritic cells	DC-SIGN	[16,17]
Glycosaminoglycans			
DENV-2	Monkey kidney (Vero), hamster ovary (CHO)	HS	[26,29]
	Hamster kidney (BHK)	HS	[27]
	Human hepatocytes (HuH-7)	HS	[28]

DENV: Dengue virus; HS: Heparan sulfate; Hsp: Heat shock protein.

dependant on exposure of the virus to low pH for membrane fusion activity. Evidence for this pH requirement is provided by diverse experimental approaches, such as entry inhibition using lysosomotropic agents in cells infected with DENV-2 virions [40,41] or retroviral reporter viruses pseudotyped with pre-membrane and envelope (prM/E) proteins of DENV-2 [42], low pH-induced formation of syncytia in DENV-infected cells [40,43] and silencing of the vacuolar ATPase gene by siRNA [40]. Very recently, pH-dependent penetration was also demonstrated by single virus tracking [44]. The authors demonstrated that the internalized particles have different types of transport behavior, leading to membrane fusion in endosomal compartments located in distinct cellular localizations, either at the periphery or at perinuclear regions of the cell. As a counterpart to these functional studies, structural analyses have shown that under acidic conditions, as those encountered in endosomes, the E glycoprotein undergoes irreversible conformational changes to expose hidden fusion domains that trigger the membrane fusion process required to release viral RNA into the cytoplasm [45–48].

Despite the general consensus regarding a pH requirement for viral fusion, the information available about the mode of DENV entry into cells is scanty and controversial. Very recently the use of dominant-negative mutants, including Eps15, a cellular protein necessary for clathrin-dependent endocytosis, underlined that the functional entry pathway of DENV-2 in mosquito and HeLa cells is clathrin dependent [40,41]. In both cases the evidence also suggests that DENV requires transport to early, but not late, endosomes for viral infection. Even though it was demonstrated that mosquito cell DENV entry is independent of lipid raft integrity [41], some reports indicate a strong dependence on membrane cholesterol for DENV-2 infection in human monocytes [10] and mouse neuroblastoma cells [49], suggesting that cholesterol-rich membrane fractions are important for DENV entry in these cell systems. These contrasting

Figure 1. Different mechanisms of endocytosis in the cells.



(A) Phagocytosis, **(B)** macropinocytosis, **(C)** clathrin-mediated endocytosis, **(D)** caveolae-mediated endocytosis, **(E)** lipid raft-mediated endocytosis devoid of caveolin and clathrin but dependent on dynamin, **(F)** lipid raft-mediated endocytosis devoid of caveolin, clathrin and dynamin and **(G)** nonclathrin noncaveolar pathway independent of lipid rafts.

results suggest that DENV particles may be internalized into cells by different transport routes. In this respect, unpublished data from our laboratory suggests that DENV-2 entry may occur through a nonclathrin, noncaveolar pathway in certain cell systems.

Further studies are required to elucidate if viral particles are targeted to different transport routes in different cell systems or, more intriguingly, if different infectious pathways may be undertaken within the same cell. Additionally, there are no reports addressing the functional entry mechanism utilized by serotypes other than DENV-2. The fact that distinct receptors are proposed for different serotypes within a same host cell (Table 1) raises the question whether alternative entry routes are taken by different serotypes.

DENV entry as an antiviral target

The blockade of DENV entry into the host cell is an interesting antiviral strategy because it represents a barrier to suppress the beginning of infection [50]. The antiviral target is the viral glycoprotein E in its interaction with components of the cell membrane that allow virus binding and internalization. The different types of inhibitors reported to be active against DENV entry are summarized in Table 2.

Polyanionic substances

Since the initial finding of highly sulfated HS as a putative primary receptor for DENV in certain types of mammalian cells [26], the antiviral efficacy of polyanionic compounds of diverse structures has been demonstrated *in vitro*. Heparin, a

close structural homolog of HS, has shown ability to inhibit DENV-2 binding to Vero [26,29,51], LLC-MK_2 [51] and BHK [27] cells, as well as diverse types of human hepatic cells [31], in support of the hypotesis of HS as a DENV receptor. In addition, it was determined that heparin oligosaccharides smaller than a decasaccharide failed to inhibit E glycoprotein binding [26]. A structure–activity relationship study carried out to examine the E glycoprotein-binding ability of different heparin-like polyanions, including suramin (a pharmaceutical polysulfonate), glycosaminoglycans and hyaluronic acid oligosaccharides, confirmed the need for a minimum chain size equivalent to the heparin decasaccharide as well as high levels of charge density and structural flexibility for optimal interaction between the polyanion and E glycoprotein [52]. Aside from the described compounds, other types of polyanions, such as pentosan sulfate, the sulfated phosphomanno-oligosaccharide PI-88 [53], sulfated DL-galactan hybrids extracted from seaweeds [32,54,55], sulfated derivatives of natural α-D-glucans [56], and carrageenans of natural and commercial origin [32,57–59], also demonstrated anti-DENV-2 activity in mammalian cells, whereas sulfated galactomannans exhibited anti-DENV-1 activity in mosquito cells [60].

All these polyanionic compounds acted as HS-mimetic substances, interfering with the interaction of E glycoprotein with the cellular HS receptor. The mode of action of sulfated polysaccharides on DENV-2 multiplication indicated that the blockade of the interaction of DENV-2 with HS not only affected binding but also virus internalization into the host cell [27,32,55,59], since both initial events of the DENV infective cycle were equally inhibited with high efficacy. The post-adsorption inhibition of DENV infection obtained when the polysulfate is added after virus binding suggests that the interaction of the compound with the virion bound to the cell surface would avoid the normal transit of the viral genome into the cytoplasm. The experimental approaches utilized to analyze the mechanism of action of polysulfates indicated that virions enter the cell but the fusion event leading to uncoating of the nucleocapsid and escape from the endosome is blocked as a consequence of the association of the polysaccharide with the DENV E glycoprotein [59].

A differential susceptibility of DENV serotypes to sulfated polysaccharides has also been shown in Vero and BHK-21 cells and was in the order DENV-2>DENV-3>DENV-4>DENV-1, with DENV-2 being by far the most affected serotype [31,32,59]. The variations observed in anti-DENV activity of polysulfates in Vero cells according to virus serotype may be ascribed to the aforementioned differences in virus–cell interactions leading to virus entry. In fact, as shown in Table 1, HS was reported as the receptor molecule for DENV-2 in Vero cells

Table 2. *In vitro* antiviral activity of dengue virus entry inhibitors.

Compound	Virus serotype	Ref.
Polyanionic substances		
Heparin	DENV-2	[26,27,29,31,51]
Suramin	DENV-2	[26,53]
Pentosan sulfate	DENV-2	[53]
PI-88	DENV-2	[53]
DL-galactan hybrids	DENV-2	[32,54,55]
Sulfated α-D-glucans	DENV-2	[56]
ι-, κ-, λ-, κ/ι/ν-carrageenans	DENV-2 and -3	[32,57,58,59]
Sulfated galactomannans	DENV-1	[60]
Polyoxotungstates	DENV-2	[61]
Lectins		
Concanavalin A	DENV-2	[27]
Inhibitors of viral fusion		
Amantadine hydrochloride, rimantadine hydrochloride	DENV-1, -2, -3 and -4	[63,64]
Chlorpromazine	DENV-2	[41]
Synthetic peptides	DENV-2	[70]
Tetracycline derivatives	DENV-2	[71]

[26,29], whereas for DENV-1 and -4 a proteinaceous receptor was identified in the same cell line [18,23].

Inorganic polyanionic substances such as polyoxotungstates substituted with vanadium or titanium were also reported to be inhibitors of DENV-2 multiplication in Vero cells, probably through interference with DENV binding to host cells [61].

The limitations of the available animal models of DENV infection have restricted the adequate *in vivo* evaluation of those compounds with proved *in vitro* antiviral activity. In addition, at present there are very few reports of compounds exhibiting effective antiviral action *in vivo*. Among entry inhibitors, the oligosaccharide PI-88 was assayed in a murine model of type I and II interferon receptor-deficient mice inoculated with DENV-2 by intravenous route [53]. The therapeutic effect of PI-88 was very weak since treatment ameliorated disease in infected animals without eliciting a significant increase in survival rate. Several attempts to establish small animal models for DENV infection by peripheral routes with a simple management for antiviral testing are in progress [62].

Lectins

Another group of molecule that inhibit virus entry are lectins – sugar-binding proteins that block specific residues in E glycoprotein involved in the interaction with cellular receptors. Concanavalin A, a lectin that binds to α-linked terminal mannose residues, blocked DENV-2 binding and penetration into BHK cells [27], and the soluble domain of the lectin DC-SIGN also inhibited virus infection [16].

Inhibitors of viral fusion

This class of early inhibitors of the DENV multiplication cycle includes amantadine hydrochloride and rimantadine hydrochloride, known blockers of influenza virus uncoating. However, both compounds showed very weak *in vitro* anti-DENV activity [63,64]. More recent studies demonstrated that chlorpromazine, a pharmacologic drug, which inhibits clathrin-dependent endocytosis, affected the entry of another flavivirus, Japanese encephalitis virus, into Vero cells without decreasing cell viability [65]. This drug also inhibited DENV-2 multiplication in mosquito C6/36 cells [41].

Executive summary

Introduction

- Dengue virus (DENV) is the most significant mosquito-borne human pathogen, responsible for over 50 million infections of variable severity each year.
- There is no specific antiviral therapy or vaccine for treatment or prevention.

DENV attachment: multiple cell receptors?

- The envelope (E) glycoprotein is involved in DENV attachment and internalization, but the nature of the cellular receptor is, at present, controversial.
- A multistep process was proposed consisting of the initial interaction of the E protein with a low affinity attachment receptor (heparan sulfate or DC-SIGN, according to the cell type) to concentrate virus particles on the cell surface – a high affinity receptor of protein nature then triggers virus internalization.

DENV internalization

- DENV internalization occurs by receptor-mediated endocytosis, which is dependent on low pH, required for conformational rearrangement of the E glycoprotein and membrane fusion.
- Clathrin-mediated endocytosis was addressed for DENV-2 internalization, but alternative transport pathways may be undertaken by this and other serotypes in certain cells.

DENV entry as an antiviral target

- Selective inhibition of *in vitro* DENV-2 multiplication was achieved with diverse entry inhibitors such as polysulfates, polyoxotungstates, lectins, peptides and tetracycline derivatives.

Future perspective

- The elucidation of the complex aspects related to DENV cell entry is starting to be obtained through different experimental approaches, and it will guide the development of rational strategies to establish a successful therapy for DENV infections in the near future.

Recent progress in the structural characteristics of DENV E glycoprotein [45,66–69] has enabled the use of physico–chemical algorithms for the rational design of peptide inhibitors of DENV entry that could interfere with the virus–cell membrane fusion process. These synthetic peptides function through a sequence-specific mechanism to inhibit DENV-2 infectivity in LLC-MK_2 cells and thus could serve as lead compounds for the development of optimized peptide drugs [70]. Another design-oriented approach used virtual screening based on the recent finding of a hydrophobic detergent-binding pocket in the E glycoprotein of DENV-2 involved in the pH-induced conformational rearrangement that is essential for virus entry [67]. In this study, two novel tetracycline derivatives that target this pocket displayed significant inhibitory effects on DENV-2 propagation in cell culture [71].

Future perspective

Currently, there are several experimental approaches in progress aiming to elucidate the molecules and mechanisms involved in DENV entry into a wide spectrum of host cells. This knowledge will be essential for the discovery of DENV inhibitors targeted to the initial steps of the virus multiplication cycle. The studies reviewed here show that many aspects of DENV entry, such as the involvement of different receptors dependent on the virus serotype and the class of host cell, the route and mechanism of virus internalization to the cytoplasm and the understanding of conformational changes in E glycoprotein during viral fusion, can guide the development of new strategies to establish an effective anti-DENV therapy with potential medical application in the near future.

Financial & competing interests disclosure

Research in the authors' laboratory was supported by Agencia Nacional de Promoción Científica y Tecnológica, Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and Universidad de Buenos Aires, Argentina. EB Damonte is a member of Research Career from CONICET and EG Acosta is a fellow from the same institution. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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