REVIEW

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Dengue virus entry and trafficking: perspectives as antiviral target for prevention and therapy

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ABSTRACT Dengue virus (DENV) is the etiological agent of the most important human viral infection transmitted by mosquitoes in the world. In spite of the serious health threat that dengue represents, at present there are no vaccine or antiviral agents available and treatment of patients consists of supportive therapy. This review will focus on the process of DENV entry into the host cell as a potential target for antiviral therapy. The recent advances in the knowledge of viral and cellular molecules and mechanisms involved in binding, internalization and trafficking of DENV into the host cell until virion uncoating are discussed, together with an overview of the strategies and compounds evaluated for development of antiviral agents targeted to DENV entry.

KEYWORDS
• antiviral agents • dengue virus • entry inhibitors • *Flavivirus* • internalization • receptors • viral binding • viral entry • virus trafficking

Dengue is currently the most widespread arbovirosis in the world, with a particularly high prevalence in diverse tropical and subtropical regions of America and Asia [1]. The WHO estimates an occurrence of 50–100 million annual infections, but more recent modelling evaluations increased the number of new possible infections to 390 million per year [2]. There are four serotypes of dengue viruses (DENV), designated DENV-1 to DENV-4, that cocirculate and are transmitted to humans by the bites of the mosquitoes *Aedes aegypti* and *Aedes albopictus*. Precisely, the failure in the programs for control of the mosquito vector is one of the reasons for the explosive re-emergence and global spread of dengue in the last few decades in >100 countries, resulting in a serious public health challenge.

All serotypes can produce either an inapparent infection or a wide spectrum of clinical outcomes ranging from a mild febrile illness known as dengue fever (DF) to the more severe and life-threatening forms of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [3]. The initial infection with one DENV serotype leads to lifelong protection against homologous reinfection, but only brief and partial protection against infection with other serotypes. In fact, the secondary infection with a heterologous serotype is considered a risk factor for developing DHF and DSS. These severe clinical manifestations have been associated to the phenomenon known as antibody-dependent enhancement (ADE) [3]. In this process, the antibodies elicited by the primary infection bind to the heterotypic virus without neutralization of viral infectivity, and these immune complexes are opsonized into Fc-receptor positive cells leading to an increase in DENV replication and pathogenesis [4,5].

DENV is a member of the genus *Flavivirus* in the family Flaviviridae. The virion is a small spherical particle, 40–50 nm in diameter, containing a single-stranded positive sense RNA included in an inner nucleocapsid and surrounded by a lipid envelope. The virus genome codes for a single polyprotein that is cleaved into three structural proteins (the capsid protein C,

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which links with the genome to form the nucelocapsid, a small membrane protein M, which matures from the precursor prM and the envelope glycoprotein E responsible for mediating attachment and fusion of viral and cellular membranes during virus entry) and seven nonstructural polypeptides.

At present, there are no vaccines or antiviral agents available and treatment of patients consists of supportive therapy. As it is well known for viruses in general, the best solution should be the obtention of a preventive vaccine. However, the failure to assess effective and equivalent protection against all DENV serotypes, a mandatory condition to avoid the risk of ADE for vaccinated persons, has been the main difficulty faced by the several vaccine candidates currently being evaluated in clinical or preclinical studies [6]. Therefore, the search for antiviral agents for specific chemotherapy is an urgent need. To this end, different strategies targeted to blocking a virus component or a host cell factor involved in DENV multiplication were intended for antiviral development [7,8]. Virus entry has become an attractive alternative for therapeutic intervention against enveloped viruses, as evidenced by the successful entry inhibitors approved for HIV [9], since it represents a barrier to block the beginning of infection and it is determinant of host range, cellular tropism and pathogenesis.

In this review, we present the knowledge about the mechanisms of the different steps of virus entry, from virion binding to uncoating and release of the nucleocapsid into the cytoplasm. The compounds identified as agents interfering with virus entry are also discussed.

Mechanism of DENV entry

There are two general strategies for entry of DENV into a host cell: the primary infection, corresponding to the initial infection of a cell in a culture or organism that has never been in contact with the virus and, consequently, occurs in absence of DENV antibodies, and the secondary infection or antibody-dependent infection, in the presence of nonneutralizing DENV antibodies elicited during a primary infection and related to the ADE process.

The primary or antibody-independent entry of DENV is driven by the envelope glycoprotein E. The adsorption of viral particles is started by the binding of E protein to the host cell receptor molecules distributed on the surface of the plasma membrane. After binding, virions are internalized into the cell by receptor-mediated endocytosis also guided by the E protein. Finally, the nucleocapsid leaves the endosome to release the viral genome into the cytoplasm. The molecular mechanisms involved in the sequential steps of primary DENV entry will be summarized in this section.

• Binding & receptors

Virion binding to the host cell surface is the first event leading to virus entry through the interaction between E glycoprotein and cellular receptors. The precise nature of the cell receptor is still unclear and diverse molecules, including proteins, carbohydrates and lipids, have been reported as putative DENV receptors in the last decades (summarized in **Table 1**). Dendritic cells in the skin, monocytes and macrophages are considered the initial target cells for DENV infection in humans, but virus can also infect lymphocytes, hepatocytes, endothelial cells, epithelial cells and fibroblasts. Additionally, virus maintenance in the natural environment involves the continuous occurrence of mosquito–vertebrate–mosquito cycles of transmission. Given this wide spectrum of susceptible cell types from different hosts, *in vivo* and *in vitro*, it appears that the virus can interact with different molecules acting as cell receptors depending on the cell as well as on virus serotype.

Receptors in mammalian cells

Two well-characterized molecules with proved relevance for DENV binding to mammalian cells are heparan sulfate (HS) and C-type lectins. HS is a member of the sulfated glycosaminoglycan family, composed of chains of repetitive disaccharides with uronic or L-iduronic acids and an O-sulfated glucosamine derivative and linked to core proteins forming the HS proteoglycans (HSPG). It is very abundant on the surface and in the extracellular matrix of most mammalian cells, and serves as initial receptor for various pathogens [10,11]. The participation of HS for DENV attachment was demonstrated in Vero, BHK and CHO cell lines [12–15], as well as in human hepatocytes [16] and endothelial cells [17]. The negative charges of the sulfate groups in HS contact with basic amino acids in the E glycoprotein and this interaction concentrates virions on the cell surface. It has been suggested that HS might serve as a primary

Table 1. Putative dengue virus receptors in mammalian and mosquito cells.

Cell	Receptor	Serotype	Ref.
Mammalian cells			
Dendritic cells	DC-SIGN	1–4	[29–32]
Macrophages	Mannose receptor	1–4	[33]
Monocytes/macrophages	CD14-associated protein	2	[34]
Monocytes, U937	Hsp70, Hsp90	2	[35]
HuH-7	Heparan sulfate	2	[16]
HepG2	Laminin receptor	1	[40]
	GRP78	2	[37–39]
K562	Glycosphingolipid	1–4	[44]
HUVEC	Heparan sulfate	4	[17]
ECV304	Unknown proteins 28-74 kD	2	[42,43]
Vero	Heparan sulfate	2, 4	[12,14,15]
	Unknown protein 74 kD	4	[15]
LLC-MK2	Glycosphingolipid	2	[45]
CHO	Heparan sulfate	2	[12,14]
BHK	Heparan sulfate	2	[13]
	Glycosphingolipid	1–4	[44]
HEK293, A549, CHO745, HuH75.1, epithelial cells, astrocytes	TIM, TAM	1–4	[36]
Mosquito cells			
C6/36 (*A. albopictus*)	Hsp-related protein 45 kD	4	[55]
	Tubulin-like protein 48 kD	2	[56]
	Laminin receptor	3, 4	[54]
C6/36, CCl-125 (*A. aegypti*), adult *A. aegypti*	Prohibitin	2	[57,58]
AP-61 *(A. pseudoculterallis)*	Glycosphingolipid	2	[45]
C6/36, midgut *A. aegypti*	Unknown protein 67–80 kD	1–4	[52]
Salivary glands *A. aegypti* and *A. polynesiensis*	Unknown protein 48–77 kD	1–4	[53]
Salivary glands, midgut, ovary *A. aegypti*	Unknown protein 45 kD	4	[51]

DC-SIGN: Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin; GRP-78: Glucose-regulated protein 78; Hsp: Heat-shock protein; TAM: Tyro3, Axl and Mer; TIM: T-cell immunoglobulin and mucin domain containing proteins.

receptor to facilitate the subsequent interaction with a secondary molecule or coreceptor of high affinity, probably a protein, for penetration and envelope-endosomal membrane fusion [15]. The specificity of the HS-E protein interaction is supported by the requirement of a highly sulfated form of HS for DENV attachment to the cell and the identification of putative HS-binding motifs at the carboxy terminus of E protein [12]. Furthermore, in a clone of the erythroleukemic K562 cells, the presence of a particular HSPG, syndecan-2, was found to be essential for DENV-2 entry [18]. The biological relevance of the participation of HS in virus entry was validated by different experimental approaches, such as the inhibition of virus adsorption/infection produced by HS-mimicking compounds [19–22], cell treatment with heparinases [12,14,16,17], site-directed mutagenesis of the HS-binding regions of E protein [23,24] or blockage with monoclonal antibodies (mAbs) that bind to these HS-reactive domains [25]. Interestingly, the experiments with HS-mimicking compounds have shown that HS residues of cellular proteoglycans appear to act not only as attachment factors, but also through its binding to the E protein serve as mediators for endosomal fusion and uncoating into the host cell [20,26]. However, the involvement of HS as initial receptor is not universal since there are variations depending on the host cell and the virus serotype in the usage of HS for entry (see below item 3).

The second type of relevant DENV receptor is represented by carbohydrate-binding molecules like lectins that are abundant on the surface of cells of the immune system involved in DENV infection. Among these lectins, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) has been identified as a DENV receptor in immature dendritic

cells, the primary target cell in human skin after the mosquito bite [27]. Like HS, DC-SIGN is also reported to bind a variety of pathogens, including viruses, bacteria, parasites and yeasts [28]. After taking the pathogen, dendritic cells maturate and present the corresponding antigen to T cells stimulating the production of cytokines. DC-SIGN is a calcium-dependent C-type lectin, which has a carbohydrate recognition domain mainly reactive with high-mannose N-glycans, oligosaccharide chains present in DENV E glycoprotein. Different experimental approaches have demonstrated that the four DENV serotypes use DC-SIGN for attachment to dendritic cells, but the molecule is dispensable for subsequent virus internalization [29–31]. Then, similar to the proposal for HS in certain cell types, it was suggested that DC-SIGN concentrates virions at the surface of dendritic cells to allow a posterior efficient interaction with another high affinity receptor responsible for penetration [31]. This model is in accordance with cryo-electron microscopy studies of the complex DENV E protein-carbohydrate recognition domain of DC-SIGN showing the existence of enough free space on the viral surface for an interaction with a secondary receptor [32].

Macrophages are also major targets of human infection with DENV and another lectin, the mannose receptor, was identified as a DENV receptor for the four DENV serotypes through binding and functional analyses [33]. A previous study proposed a CD14-associated protein as a DENV receptor in human monocytes/macrophages because bacterial lipopolysaccharide could inhibit DENV infection [34]. Other report postulated the chaperons heat shock protein (HSP) 70 and HSP90 as components of the receptor complex in monocytes [35], probably related to the CD14-associated proteins abovementioned, but without further confirmation.

A recent study identified members of the TIM (T-cell immunoglobulin and mucin domain containing proteins) and TAM (Tyro3, Axl and Mer) families of transmembrane receptors that bind to phosphatidylserine (PtdSer) as a new class of DENV entry factors [36]. These proteins appear to participate in binding of the four DENV serotypes to different types of human cells, but are not directly involved in virus internalization. Then, the authors propose that TIM/TAM receptors act primarily as attachment receptors to capture virions on the cell surface to facilitate the subsequent interaction with another molecule responsible for virus internalization, a mode of action similar to that proposed for DC-SIGN in dendritic cells [31]. These studies also revealed the exposed presence of PtdSer on the surface of DENV virions and its important role for TIM/TAM-mediated entry.

Besides the abovementioned molecules, a large list of cellular components have been reported as putative DENV receptors in diverse mammalian cells based on different experimental approaches such as viral overlay protein-binding assay, affinity or thin-layer chromatography, blockade by specific antibodies or siRNA, but in many cases a functional validation of the receptor molecule is lacking. The chaperone glucose-regulated protein 78 (GRP78) in hepatic cells [37–39], the laminin receptor also in hepatic HepG2 cells [40] as well as in a porcine kidney-derived cell line [41], the unidentified proteins of MW raging between 28 and 74 kD in endothelial cells [42,43] or Vero cells [15], and neutral glycosphingolipids in human K562 [44] or monkey LLC-MK2 cells [45] are included among potential DENV receptors. As can be seen in **Table 1**, it is not unusual that different receptors have been suggested for attachment in the same cell, a situation illustrative of the present lack of clear definition and controversial state in the issue of DENV receptors.

Receptors in mosquito cells

The study of DENV entry and the possible attachment/internalization receptors involved in mosquito cells **(Table 1)** is much less analyzed and more poorly understood than in mammalian cells. In contrast to the situation in vertebrate cells, the participation of HS is practically discarded by experimental studies with HS-mimicking inhibitors [46,47], a result also supported in the lower level of sulfation of HS in mosquito tissues with respect to human liver [48] and the low HS expression observed in *A. albopictus*-derived C6/36 cells [49]. Accordingly, the structural analysis of the HS-binding sites in DENV-2 E protein indicated that they are essential for binding to hamster cells, but are dispensable for attachment to mosquito cells [50].

In opposition, various proteins only designated by their molecular weight were reported as DENV receptors in the C6/36 cell line as well as in diverse tissues derived from *A. aegypti* and *Aedes polynesiensis* mosquitoes [51–53]. More recent

investigations have identified more accurately some cellular proteins with DENV-binding ability in mosquito cells, including a laminin-binding protein, a Hsp90-related protein, prohibitin and a tubulin-like protein [54–58]. Finally, as also shown in mammalian cells, glycosphingolipids specific for mosquito cells were found reactive with DENV-2 [45]. Clearly, further investigations are necessary to elucidate the nature of DENV receptor in mosquito cells.

• Internalization: endocytic routes

After the initial step of binding to the host cell surface, enveloped viruses can exploit two main pathways for internalization into the cytoplasm: fusion of the envelope with the plasma membrane at the cell surface or with the membrane of an intracellular vesicle after receptor-mediated endocytosis. As abovementioned for the receptor usage, the pathway for DENV internalization into the host cell is also a complex process and appears to be regulated by various cell- and virus-dependent factors. In fact, the possibility of binding and penetration into the host cell through different receptors may also conduct to alternative routes for internalization and trafficking inside the cell until the cellular location for genome expression and replication is attained.

The initial studies about DENV entry, based in electron microscopy observations, proposed a direct penetration of DENV particles by fusion between virus envelope and plasma membrane in human monocytes, C6/36 and BHK cells [59,60]. A related hypothesis of direct entry at the plasma membrane of mosquito cells by the formation of a pore between viral and cellular proteins leading to a fusion- and low pH-independent internalization of the DENV nucleocapsid was suggested in a very recent publication [61]. However, there is not any functional demonstration of fusion- or channel-mediated entry for DENV at the plasma membrane to support these proposals. By contrast, a sustained body of structural and functional evidence has led to the general acceptance that the infective entry of DENV occurs by receptor-mediated endocytosis dependent on acid pH as shown in **Figure 1**. The use of lysosomotropic agents that raise endosomal pH, like ammonium chloride or concanamycin A, inhibited the entry of intact virions in diverse vertebrate and invertebrate cells [62–65] as well as the internalization of retroviral reporter viruses pseudotyped with prM and E proteins in Huh7 cells [66]. In another experimental approach, the formation of syncytia was induced in DENV-infected cells by exposure to low pH [62]. Finally, a very elegant analysis of live cell imaging by real-time fluorescence microscopy of DENV-2-labeled particles in BSC-1 cells corroborated that fusion occurs exclusively within acidic endosomes that may be located at the cell periphery or at the perinuclear region [67]. Together with these functional studies and in support of an endocytic entry mechanism for DENV, structural analyses have demonstrated irreversible conformational changes in E protein that depend on acid pH and lead to membrane fusion. In mature infectious virions, the E protein is arranged as 90 homodimers that lay flat covering the envelope surface [68]. Acidic conditions similar to the endosomal environment trigger a rearrangement of E protein from the homodimeric array to homotrimers followed by the exposure of a hydrophobic domain, required for the fusion between virus envelope and endosomal membrane [69–71].

To proceed with endocytosis, the cell offers to viruses several options of internalization routes usually available for cellular uptake of different ligands. The best characterized endocytic pathways are clathrin-mediated endocytosis, caveolar/raft-dependent endocytosis and macropinocytosis, but also a set of less known pathways independent of clathrin and caveola have been more recently described for some cellular ligands, including viruses [72]. Clathrin-mediated endocytosis appears to be the classical and most commonly employed pathway for virus entry [73]. It involves the concerted action of clathrin and adaptor proteins like AP-2, AP180 and Eps15 as well as other factors implicated in cargo selection and the subsequent formation of clathrin-coated pits, seen by electron microscopy [73]. The charged coated pit undergoes a scission from the plasma membrane supported by the GTPase activity of dynamin originating the clathrin-coated vesicle. Finally, the clathrin cage is dissociated and the vesicle fuses with endosomes.

As reported for other viruses, the functional endocytic pathway for DENV entry into different cell types is dependent on clathrin. In mosquito C6/36 cells, the use of a clathrin-mediated route was demonstrated by using biochemical inhibitors such as chlorpromazine and dansylcadaverine as well as the overexpression of dominant negative mutants of the protein Eps15,

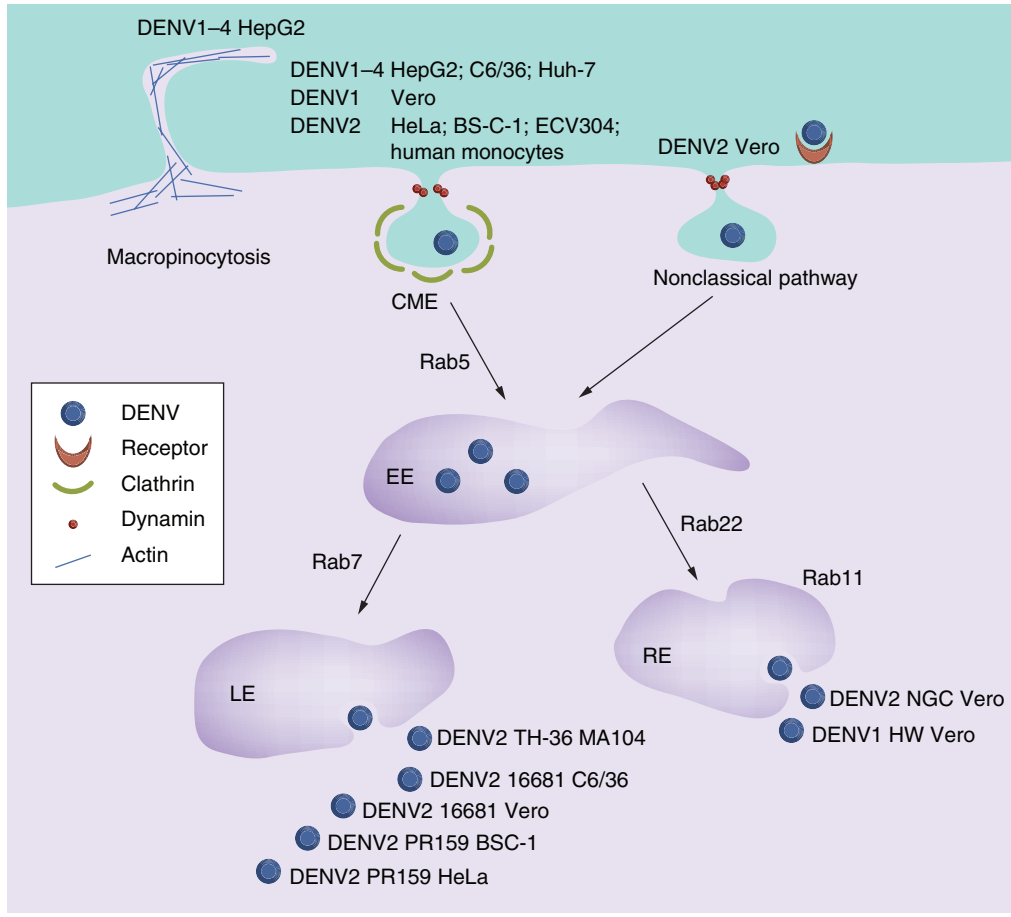

Figure 1. Internalization routes and intracellular trafficking for Dengue virus entry. Endocytic pathways exploited by different DENV serotype-host cell combinations.
CME: Clathrin-mediated endocytosis; DENV: Dengue virus; EE: Early endosome; LE: Late endosome; RE: Recycling endosome.

together with colocalization by confocal microscopy of DENV E glycoprotein with the marker protein transferrin and the observation of virions in clathrin-coated vesicles by transmission electron microscopy [63–65].

Regarding mammalian cells, the internalization of DENV-2, the most extensively studied serotype, was also found to be clathrin mediated in human cells like HeLa [62], HepG2 [39], A549 [74], ECV304 [75], human monocytes [76] and monkey BSC-1 cells [77]. By using RNA interference silencing methods, the infection of human hepatic Huh7 cells with the four serotypes DENV-1 to DENV-4 was also shown to be potently inhibited by siRNAs targeting genes associated with clathrin-mediated endocytosis [78].

However, the use of other clathrin-independent routes of entry may be hijacked by DENV similarly to that described for other viruses [73,79]. In Vero cells, a cell line usually employed for DENV quantification and for the development and production of DENV vaccines [80–82], alternative internalization routes can be used depending on the virus serotype. By treatment with biochemical inhibitors and overexpression of cellular proteins involved in the different endocytic routes, it was proved that the entry of DENV-1 for a productive infection requires clathrin-mediated endocytosis, whereas the infectious entry of DENV-2 into Vero cells occurs by a nonclassical endocytic pathway independent of clathrin, caveolae and lipid rafts, but dependent on dynamin [74]. Lastly, ultrastructural analysis showed the classical image of a clathrin-coated vesicle containing DENV-1 virions in Vero cells, whereas DENV-2 virions were contained in larger uncoated vesicles, with smooth borders **(Figure 2)**. It is noteworthy from these results that two DENV serotypes can use

a differential mechanism for entry into the same host cell, the Vero cell line. Furthermore, considering the above commented studies about clathrin-mediated entry, the serotype DENV-2 is able to exploit alternative routes for internalization into different cell types, from vertebrate or invertebrate origin. A study performed in the human HepG2 cells with the four DENV serotypes also proposed the occurrence of multiple entry pathways, including clathrin endocytosis and macropinocytosis, with a variable predominance of either one or another route depending on the virus serotype [83].

More recently, a similar phenomenon of alternative cellular pathways for virus entry was demonstrated for Japanese encephalitis virus (JEV), another mosquito-borne virus, suggesting that it may be a common possibility for flaviviruses. The JEV internalization into Vero cells occurs by clathrin-mediated endocytosis, whereas neuronal cells are infected via a clathrin-independent mechanism [84,85]. The utilization of optional entry routes by flaviviruses, as well as the usage of a range of putative receptors, may represent an advantage for these viruses that must infect in nature different classes of cells and tissues. This versatile ability of DENV to employ different cellular pathways may be beneficial to allow adaptation for virus infection of diverse host cells. In the opposite direction, it may be detrimental for viral infection if the virus is able to enter a particular cell by more than one endocytic route but only one of both pathways leads to a successful and productive infection. This hypothesis of productive and nonproductive infection has been suggested by the consistent increase in virus production observed in DENV-2-infected Vero cells when clathrin-mediated endocytosis is blocked by chlorpromazine and dansylcadaverine [74]. If the clathrin pathway would be a noninfective mode of entry for DENV-2 in Vero cells, when this pathway is blocked, the use of the infective nonclathrin route will be improved leading to a more efficient infection.

Besides receptors/coreceptors and cellular proteins participating in the endocytosis routes, lipids may also be involved in virus entry since viral and cellular lipid membranes interact during the process. In particular, the role of cholesterol as a component of lipid rafts has been extensively analyzed with several enveloped viruses. For DENV, the results about the influence of cellular cholesterol on virus entry are

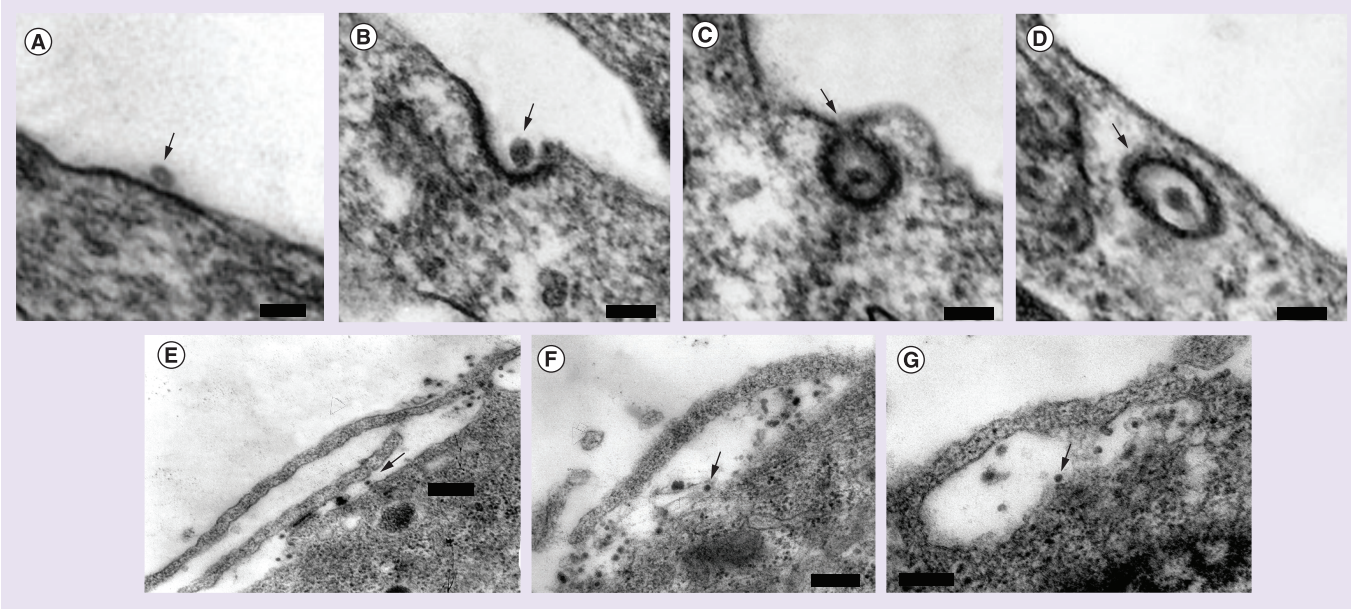

Figure 2. Dengue virus-1 and Dengue virus-2 entry into Vero cells. Vero cells were infected with DENV-1 strain Hawaii **(A–D)** or DENV-2 strain NGC **(E–G)** at a multiplicity of infection of 10 PFU/cell. After 60 min at 4°C and 25 min at 37°C, samples were processed for electron microscopy. **(A)** DENV-1 particle in the extracellular medium. **(B–C)** DENV-1 particle within invaginations of the plasma membrane. **(D)** DENV-1 particle within an endocytic coated vesicle. **(E & F)** DENV-2 particles in the extracellular medium surrounded by projections of the plasma membrane. **(G)** DENV-2 particles within smooth big-sized vesicles. Bar: 100 nm.
DENV: Dengue virus.

controversial. The entry of DENV was found independent of plasma membrane cholesterol in C6/36, Vero, A549, HepG2, ECV304, U937, K562 and Raji-DC-SIGN cells [63–65,74,75,83,86], but a cholesterol dependence was observed in human monocytes and mouse neuroblastoma N18 cells [35,87]. The main strategy used in the mentioned studies to deplete or alter membrane cholesterol was the cell treatment with cholesterol-reactive drugs like methyl-β-cyclodextrin, nystatin or filipin. The observed differences may be due either to variations in the characteristics of the endocytosis process or the requirement of cholesterol during virus internalization among different cells or virus strains. However, it must be also considered if the contradictory results may be ascribed to differences in the treatment conditions to achieve cholesterol depletion and the time of infection. About this point, it is important to take into account the virucidal properties of drugs affecting cholesterol. These agents not only affect cellular lipid membranes, but also the composition of the viral envelope. In fact, the strong virucidal activity of methyl-β cyclodextrin and nystatin against the four DENV serotypes was recently demonstrated [86]. Since the incubation of the virion suspension with the compound destroys infectivity, the cell treatment to evaluate its effect on virus entry must be done before virus infection to assess that inhibition is exerted during internalization and not by virus inactivation. When a different method for cholesterol depletion was employed such as serial passage of C6/36 cells in a medium with delipidated serum, DENV-1 and DENV-2 infection was not affected [88], corroborating the lack of cholesterol dependence observed with cholesterol-sequestering compounds. Notably, the addition of exogenous cholesterol to a suspension of DENV virions also exerted an inactivating effect on infectivity, indicating that a very narrow interval of cholesterol concentration in DENV membrane is optimal for complete infection [86,87]. In conclusion, the presence of cholesterol in the DENV envelope appears more crucial for infection than the content in the plasma membrane, but the requirement of cellular cholesterol in certain cell types cannot be discarded.

• Intracellular trafficking & viral uncoating

After internalization, viruses exploit vesicular membrane transport to reach the cell vesicle where virion uncoating takes place. The specific site where fusion between virion envelope and cell membrane occurs is essential to facilitate the genome access to the cell machinery needed for macromolecular biosynthesis.

The endosomal system is composed of different vesicular compartments, each one with a characteristic pH. It is well known that Rab GTPases serve as master regulators that contribute to the structural and functional identity of intracellular membranes. It was described that Rab4 and Rab5 are present in early endosomes (EE). Rab5 participates in the charge of clathrin-coated pits and the subsequent fusion with EE [89]. The EE are also called sorting endosomes because they can deliver the cargo molecules to different destinations. There are two recycling routes that start in Rab 4 microdomains within EE: one of them is a direct pathway to the plasma membrane, and the other one is called slow recycling route mediated by Rab22 and the Rab 11-enriched perinuclear recycling endosomes (RE). The third possible fate is the degradative pathway, which involves the Rab7 participation in the progression from EE to late endosomes (LE) and finally to lysosomes [90].

Lozach *et al.* have characterized the late virus penetration as that one with a time of fusion from 10–15 min up to a few hours and a pH threshold of 5.0–5.8 [91]. Although DENV has a pH threshold of 6.2, it is considered a late-penetrating virus because the fusion event is delayed. An elegant study demonstrated that E protein requires, besides an acidic pH, a specific interaction with anionic lipids present in LE membranes during the endosomal fusion process [92]. DENV utilizes anionic lipids such as bis(monoacylglycero)phosphate and phosphatidylserine to trigger the advance from the early hemifusion intermediates to the fusion pore opening with nucleocapsid release into the cytoplasm [92]. Then, virus–endosome fusion is mainly initiated in LE because anionic lipids are enriched in these compartments of mammalian cells. At present, DENV is the only flavivirus reported with this specific lipid requirement for membrane fusion. Accordingly with this location of DENV fusion in LE, several reports have proved that DENV virion uncoating occurs at 10–17 min postinfection using different methodological approaches, like single-virus tracking [67,92], kinetics of infectivity resistant to ammonium chloride, time course of C protein

escape from endosomes [93] and colocalization assays of EE and LE markers with DENV particles [64,78,94].

Krishnan *et al.* performed the first study of the intracellular pathway followed by DENV and West Nile virus (WNV). By using RNA interference and dominant negative mutants of Rab proteins, they determined that Rab5, but not Rab7, is required for entry of DENV-2 strain NGC into HeLa cells, concluding that transport to EE but not to LE is required [62]. Afterward, a single-virus tracking study done with DiD-labeled DENV-2 strain PR159 S1 in BSC-1 cells showed that 80% of virions penetrate in Rab7-positive endosomes [77]. To analyze if the discrepancy was due to the virus strain, the authors compared the infection of HeLa cells expressing dominant negative mutants of Rab5 and Rab7 with both viruses. In agreement with the above results, the strain S1 was severely inhibited by either mutant, whereas strain NGC was unaffected by the Rab7 mutant, suggesting that the intracellular trafficking may be dependent on the virus strain. This conclusion was confirmed when the endosomal intracellular trafficking for productive infection of Vero cells with DENV-1, strain Hawaii (HW), and DENV-2, strains NGC and 16681, was studied by employing dominant negative mutants of Rab5 and Rab7, as well as Rab22 and Rab11 mutants, cellular markers of RE and wortmannin, a pharmacological inhibitor that impairs the maturation from EE to LE. After transit of the three viruses by EE, DENV-2 16681 viral particles were sorted to LE in a Rab-7-dependent manner, whereas DENV-1 HW and DENV-2 NGC were transported to slow RE in a still unknown sorting event [93]. This study proved for the first time the involvement of the recycling pathway for productive DENV infection. Since DENV-1 and DENV-2 showed different clathrin dependence for internalization into Vero cells [74], the intracellular trafficking of DENV particles until membrane fusion appears to be independent of the route for initial virion uptake.

There are some experimental approaches that have suggested a relationship between vesicles involved in DENV intracellular trafficking and the induction of autophagy. Autophagy is a cellular response against stress wherein cytoplasmic components are sequestered and degraded in order to maintain cellular homeostasis. Induction of autophagy activates the formation of double-membraned autophagic vacuoles called autophagosomes. These vesicles can fuse with endosomes, to form amphisomes, and both autophagosomes and amphisomes finally fuse with lysosomes to form autophagolysosomes, which are the degradative vesicles. Autophagic machinery contributes to defense against viral infections; however, some viruses, in particular many positive-stranded RNA viruses, require autophagy for efficient replication. Several *in vitro* studies demonstrated that DENV-2 induces autophagy in different mammalian cell lines and these reports also revealed that autophagy inhibition significantly reduces viral replication [95–97]. Colocalization studies suggested that amphisomes would act as DENV-2 translation and replication sites [96]. Moreover, Khakpoor *et al.* reported that inhibiting autophagolysosome formation, by treatment with a lysosomal fusion inhibitor, increased DENV-2 multiplication indicating that amphisomes would be critical for productive DENV-2 infection [98]. However, this hypothesis is not consistent with data obtained by cryo-electron tomography, which revealed that viral RNA replication would take place in endoplasmic reticulum invaginations [99]. On the other hand, Heaton *et al.* found that induction of autophagy in DENV-infected cells would be implied in the alteration of lipid metabolism and a correlation between autophagy-dependent degradation of lipid droplets, which are cellular stores of triglycerides and cholesterol esters, and viral replication has been assessed [97,99,100]. Furthermore, it has been recently shown that antibody-enhanced DENV infection also induces autophagy in pre-basophil-like KU812 cells and immature mast cell-like HMC-1 cells [101]. Therefore, although a direct involvement of entry vesicles in the translation and replication of DENV genome has not been conclusively demonstrated, the entry of DENV particles through the endocytic pathway would trigger an autophagic response, which in turn would modulate cellular physiology promoting virus multiplication [99,102].

Very few studies were performed regarding the endosomal trafficking of DENV in mosquito cells, but it appears that there is also a late penetration mechanism. Fluorescent labeled particles of DENV-2, strain 16681, colocalize with EE antigen 1 marker at 5 min post infection, and

with Lyso Tracker™ (Life Technologies, CA, USA), an acidophilic dye that selectively stains low pH containing compartments, after 15–30 min of infection in C6/36 cells [64]. Furthermore, the inhibitory action against DENV infection of nocodazole, a drug that impairs the polymerization of microtubules affecting the movement of LE to perinuclear region, was also demonstrated as supporting evidence of a late penetrating event.

Viral entry as target of antiviral agents

DENV entry into the host cell is an attractive antiviral target since drug uptake is not always required. In addition, the blockage of virus entry will potentially limit the viremia and the hyperactivation of the immune system resulting in the prevention of severe dengue and reducing DENV transmission. Different approaches have been employed to identify inhibitors of DENV entry into the host cell, including screening of natural and synthetic molecules based in viral replication studies, structure-based rational design of molecules that interact either with E protein or cellular receptors and virtual screening of small molecules from different chemical databases [7,103]. Those antiviral molecules that target viral components are more specific and selective inhibitors, whereas targeting cellular factors reduces the risk of resistance development and would be useful for viruses that share the requirement of the same cellular process or factor for replication.

E protein is involved in receptor recognition and membrane fusion events that lead to the release of viral nucleocapsid into the cell cytoplasm; thus, most strategies to inhibit virus entry developed so far are focused on this protein. The N-terminal ectodomain of E protein has three domains (DI, DII and DIII), whereas the C-terminal comprises a membrane proximal stem region containing two α-helices (EH1 and EH2), connected by a stretch of conserved sequences. DIII would be involved in receptor binding and antibody neutralization, whereas DII contains the fusion peptide required for viral uncoating. In mature virions, E protein is arranged forming head-to-tail dimers that lie parallel on the virion surface. Low pH of the endosome environment causes the protonation of highly conserved histidine residues in E protein that triggers the reversible dissociation of E dimers and the exposure of the fusion peptide at the tip of DII by the movement of this domain around a hydrophobic pocket at the DI–DII junction. These conformational changes allow the insertion of the fusion peptide into the endosomal membrane followed by a re-association of E protein into trimers [69–71]. Several DENV entry inhibitors directed against the hydrophobic pocket or the stem region of E protein impair not only receptor recognition, but also E protein conformational rearrangements required for membrane fusion [104,105]. There are also molecules that exert an inhibitory action early during infection, but their mechanism of action remains unknown **(Figure 3 & Table 2)**. Here, we summarize the current knowledge about inhibitors that affect different stages of DENV entry into the host cell.

• Inhibitors of virus–receptor interaction

DIII, the putative receptor binding domain, competes with viral particles for cellular receptors acting as inhibitors of DENV-2 multiplication in mosquito and human cells [106]. Therefore, specific designed peptides, carbohydrate-binding agents (CBAs) and mAbs that interact with DIII, as well as molecules that interact with cell receptors, can be used to impair the onset of the infection.

Peptides

Antiviral peptides to target DIII of E protein were designed using a BioMoDroid algorithm [107]. Two of the synthesized peptides, DET2 and DET4, displayed minimal toxic effects and reduced DENV multiplication in monkey kidney cells. Transmission electron microscopy studies revealed that incubation of viral particles with these peptides causes changes in the surface of treated virions that correlate with the inhibition of virus–receptor interaction [107].

Monoclonal antibodies

Crill and Roehrig established that mAbs recognizing defined epitopes within DIII of E protein are the most effective blockers of DENV adsorption [25] and it was proved that binding of one of these mAbs, called 1A1D-2, induces changes in E structural arrangement [108]. In a further study, Shrestha *et al.* generated a panel of mAbs against DIII and found that two of these mAbs that neutralized all five DENV-1 genotypes showed therapeutic activity when administered to immunocompromised AG129

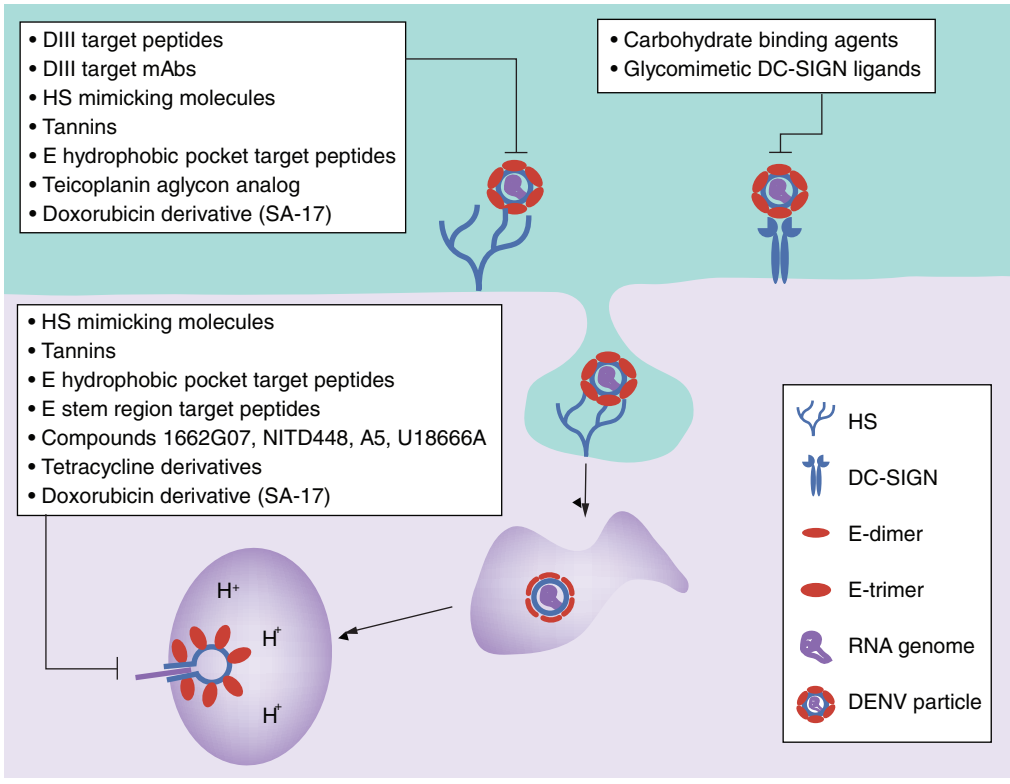

Figure 3. Mechanism of action of Dengue virus entry inhibitors. Schematic representation of DENV entry into the cell: mode of action of different inhibitors. Viral particles attachment to HS and DC-SIGN molecules as well as membrane fusion during viral uncoating are the main targets of the antiviral activity of the inhibitors commented in the text.
DC-SIGN: Dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin; DENV: Dengue virus; HS: Heparan sulfate.

mice after infection with a heterologous DENV-1 genotype [109]. The administration of neutralizing mAbs could affect the outcome of the infection due to the triggering of ADE and this has relevant implications for the design and construction of mAbs for clinical applications. In this regard, new strategies in the generation of mAbs with altered Fc structure should be further investigated to prevent upregulation of DENV infection by ADE [110].

Carbohydrate-binding agents

Cell surface C-type lectin DC-SIGN seems to be the most important cell receptor for DENV in dendritic cells. Inhibition of DENV entry into these cells could prevent cytokine release responsible for vascular leakage; thus, DC-SIGN is an interesting target of antiviral therapy. Since DC-SIGN interacts with E protein oligosaccharides, several CBAs such as the plant lectins *Hippeastrum hybrid* agglutinin, mannose-specific *Galanthus nivalis* agglutinin and N-acetylglucosamine-specific *Urtica dioica* agglutinin inhibit binding of DENV-2 to DC-SIGN-expressing Raji cells [111]. The lack of antiviral activity of CBAs in cells that do not express DC-SIGN contributes to the idea that the mechanism of DENV entry is cell type dependent. Interestingly, the lectins exert a strong inhibitory effect on the binding of all DENV serotypes to monocyte-derived dendritic cells [112].

Glycomimetic DC-SIGN ligands

A recent study reported the activity of a new class of inhibitors of DC-SIGN-dependent uptake [113]. These inhibitors consist of carbohydrates that mimic DC-SIGN ligands, which are presented on dendrimeric scaffolds. DC-SIGN are tetramers clustered in patches in the plasma membrane and interactions with viruses imply multipoint attachments. Therefore, multivalent oligodendrimers formed by a flexible polyester backbone conjugated to carbohydrate groups

that mimic E protein oligosaccharides would block virus–receptor interaction. One of these glycomimetic DC-SIGN ligands exhibited anti-DENV-2 activity in Raji cells and improvement of this inhibitory action might still be obtained as new dendrimers are analyzed [113].

Antibiotic derivatives

Certain synthetically modified glycopeptide antibiotics exhibit *in vitro* anti-DENV activity. In particular, the teicoplanin aglycon analog LCTA-949 inhibits the replication of several flaviviruses: hepatitis C virus [114], DENV-2, tick-borne encephalitis virus, WNV, JEV and the murine flavivirus Modoc virus. The inhibitory action of this compound on DENV-2 binding to the cell was demonstrated and an adverse effect of the analog on other stages of virus entry has not yet been ruled out. Moreover, LCTA-949 also interferes with antibody-mediated cell entry of DENV-2 particles [115].

• Inhibitors of virus adsorption & penetration

Sulfated polysaccharides

Charged polyanions such as heparin and other sulfated polysaccharides act as HS-mimicking molecules and interfere with E protein-HS

Table 2. Dengue virus entry inhibitors.

Inhibitor	Mode of action	Ref.
DIII target peptides	Impairment of virus cell recognition	[107]
DIII target mAbs	Impairment of virus cell recognition	[25,108,109]
CBAs: lectins HHA, GNA and UDA	Impairment of virus cell recognition	[111,112]
Glycomimetic DC-SIGN ligands	Impairment of virus cell recognition	[113]
Teicoplanin aglycon analog: LCTA-949	Impairment of virus cell recognition and probable of other entry stage	[115]
Sulfated galactans	Inhibition of virus adsorption and penetration	[46,117,118,120]
Fucoidans	Inhibition of virus adsorption and penetration	[119,120]
Carrageenans	Inhibition of virus adsorption and penetration	[20,46,49]
Sulfated xylomannans	Inhibition of virus adsorption and penetration	[120]
Heteropolysaccharide CrHWE	Inhibition of virus adsorption and penetration	[120]
Chondroitin sulfate E	Inhibition of virus adsorption and penetration	[21]
Curdlan sulfate	Inhibition of virus adsorption and penetration	[26]
Polysaccharide K5 from *Escherichia coli*	Inhibition of virus adsorption and penetration	[22]
Tannins: chebulagic acid and punicalagin	Inhibition of virus adsorption and penetration	[122]
E hydrophobic pocket target peptides	Inhibition of virus adsorption and penetration	[123]
Doxorubicin derivative: SA-17	Inhibition of virus binding and membrane fusion	[124,125]
E stem region target peptides	Blockade of membrane fusion	[126,127]
E stem region target peptide: DN59	Release of viral RNAs from viral particles	[128,129]
E stem region target small molecule:1662G07	Blockade of membrane fusion	[130]
E hydrophobic pocket target small molecule: NITD448	Blockade of membrane fusion	[131]
E hydrophobic pocket target small molecule: A5	Blockade of membrane fusion	[105]
Tetracycline derivatives: rolitetracycline and doxycycline	Probable blockade of membrane fusion	[132]
Inhibitor of cholesterol transport: U18666A	Blockade of virus uncoating	[135]
E hydrophobic pocket target peptides	Unknown	[136]
E hydrophobic pocket target small molecules	Unknown	[137,138]
Zosteric acid derivatives	Unknown	[139]

CBA: Carbohydrate-binding agent; E: Viral glycoprotein; GNA: Galanthus nivalis agglutinin; HHA: Hippeastrum hybrid agglutinin; mAb: Monoclonal antibody; UDA: Urtica dioica agglutinin.

receptor interaction [103,116]. A great variety of polysaccharides such as DL-galactan hybrids, fucoidans and carrageenans, obtained from marine algae, display anti-DENV-2 activity [20,46,117–119]. It has been demonstrated that whereas DENV-2 was the most susceptible serotype to carrageenan G3d and DL-galactan hybrid C2S-3, DENV-1 was resistant to both polysaccharides. Antiviral action of the polysaccharides is influenced by differences in virus–cell interactions among viral serotypes and cell types. These polysulfates display anti-DENV activity in mammalian cells, but were totally inactive in mosquito cells [46]. C2S-3 and carrageenans inhibit DENV-2 adsorption and penetration, and exhibit higher effectiveness than heparin [20,118].

The antiviral activity of carrageenans is influenced by their extent of sulfation, the position of sulfate groups, the sugar composition and the number of repeated units [46]. Among different chemical classes of carrageenans (ι, λ and κ), λ- and ι-carrageenans were the most active, being DENV-2 and DENV-3 the most susceptible serotypes. Interestingly, only ι-carrageenans exhibit anti-DENV activity in the C636 mosquito cell line, but levels of inhibition were lower than those obtained in mammalian cells [49].

In a more recent study, the anti-DENV activity of a diverse classes of polysaccharides obtained from red, brown and green seaweeds was reported. In accordance with previous reports, DENV-2 was the most susceptible serotype and polysaccharides exerted their antiviral effect during viral adsorption and internalization [120]. On the other hand, Kato *et al.* [21] described the *in vitro* antiviral effect of chondroitin sulfate E against all DENV serotypes, whereas chondroitin sulfate A, B, C or D did not affect DENV multiplication. In addition, chondroitin sulfate E and heparin competitively inhibited virus binding to mammalian cells and both polysaccharides would share common carbohydrate determinants for interaction with E protein as was demonstrated by surface plasmon resonance analysis. This study also indicated that a specific carbohydrate structure of the glycosaminoglycan molecule would be necessary for binding to E protein [21].

Recently, Ichiyama *et al.* [26] reported that curdlan sulfate, a sulfated 1→3-β-D glucan, which did not present serious side effects when evaluated in clinical trials against HIV infection and *Plasmodium falciparum* malaria, exhibits inhibitory action against all DENV serotypes. Consistent with the results obtained with marine alga derived polysaccharides [46,120], DENV-1 was the less susceptible serotype to curdlan sulfate antiviral action. Furthermore, curdlan sulfate inhibits virus binding to the cell and impairs E protein-mediated membrane fusion. It is noteworthy that this polysaccharide also prevents ADE of DENV infection and is able to inhibit DENV-2 multiplication in different mammalian cell lines, including DC-SIGN-expressing cells [26].

Interestingly, Vervaeke *et al.* [22] showed that two derivatives of the sulfated polysaccharide K5 from *Escherichia coli* exhibit antiviral activity against DENV-2 in human microvascular endothelial cells. Although DENV receptors in endothelial cells have not been surely identified, the inhibitory action of different sulfated polysaccharides on virus multiplication, as well as the fact that treatment of these cells with heparinase II interfered with viral infection, suggests that HSPGs would mediate DENV entry. Furthermore, K5 derivatives, which are structurally related with heparin and HS, did not display anticoagulant activity and inhibited virus replication even when they were present only during the internalization process [22].

It has been recently demonstrated that the cell type used to obtain DENV stocks can affect the antiviral activity of HS-mimicking compounds. After serial passaging of DENV-2 in Vero cells, virus became resistant to the inhibition of heparin and carrageenans and resistance was associated with a change in the mode of virus entry. By contrast, serial passages in mosquito cells did not alter DENV-2 susceptibility to sulfated polysaccharides. Given that Vero cells constitute a cellular system frequently used to obtain DENV stocks, these findings are highly relevant for the evaluation of the *in vitro* antiviral activity of entry inhibitors [121].

Tannins

Two hydrolyzable tannins, chebulagic acid and punicalagin, exhibit a broad spectrum antiviral activity against different viruses, including DENV-2, that employ HSPGs for cell entry. Adsorption and penetration studies performed in cell cultures indicated that both steps of viral replicative cycle are abolished in the presence of these compounds [122].

Peptides

Taking advantage of the available crystal structures of both prefusion and postfusion forms of DENV E protein, Costin *et al.* carried out a computational design of multiple peptides that showed DENV-2 entry inhibitory activity [123]. Two active peptides, which are directed against E hydrophobic pocket in the DI/DII hinge region, interfered with virus binding and penetration and caused structural changes at the virion surface. Since the membrane fusion process would depend on hinge region movements, the inhibitory action of these peptides could be ascribed to an uncoating blockade.

Antibiotic derivatives

SA-17, a derivative of the antineoplastic antibiotic doxorubicin, was predicted to dock in the E protein hydrophobic pocket and was proved to be a selective inhibitor of DENV-2 in Vero cells. Time of drug-addition experiments indicated that SA-17 acts at the very early steps of DENV infection. The compound also exhibited antiviral activity against DENV-1, DENV-3 and yellow fever virus, but was inactive against DENV-4 and other enveloped and nonenveloped viruses [124]. To further explore the SA-17 mechanism of action, the effect of the compound on binding and fusion of DENV-2 particles labeled with a lipophilic fluorescent probe was examined. The compound, which directly binds to DENV-2 particles, reduced not only virus binding to BS-C-1 cells, but also the fusion capacity of DENV-2 virions. Furthermore, SA-17 was active against ADE of both mature and immature DENV-2 particles when the compound was added before, during or after antibody opsonization and it has been proposed that ADE might be inhibited by a virion-bound compound at the membrane fusion step [125].

• Inhibitors of membrane fusion

Peptides

A set of peptides derived from the E protein stem region were shown to inhibit DENV-2 entry into BHK cells and fluorescence polarization measurements indicated that the extent of inhibition correlates with the affinity of peptides for the trimer postfusion conformation of E protein [126]. Furthermore, these authors also found that peptides derived from each of the four DENV serotypes inhibit the other serotypes [127]. A two-step model of peptide mode of antiviral action was proposed. An initial hydrophobic association of peptides and viral membrane would occur at neutral pH in the extracellular medium and virus–peptides complexes would be incorporated in endosomes where low pH-induced E rearrangements would enable a more specific association between peptides and E protein. This association might prevent fusion-pore formation, as was assessed using a liposome fusion assay [126].

On the other hand, Hrobowski *et al.* used the Wimley–White interfacial hydrophobicity scale, a physiochemical algorithm, together with known structural data to predict regions of DENV E protein that may play a role during conformational rearrangements implied in the fusion process [128]. Peptide DN59, corresponding to a pre-anchor domain sequence within the stem region of E protein not only displayed an inhibitory effect against DENV-2, but also exhibited cross-inhibition of WNV multiplication, suggesting that DN59 or similar peptides may act as broad spectrum flavivirus inhibitors [128]. Although sequences of inhibitory peptides abovementioned generated by Schmidt *et al.* [126,127] extensively overlap with DN59 sequence, a controversial different mechanism of action for DN59 has been proposed. Cryo-electron microscopy analysis revealed that DENV-2 particles previously incubated with DN59 had lost most of their RNA genomes. The release of viral RNAs from viral particles was also assessed by an RNAse digestion assay. Hence, these results suggest that DN59 induces the formation of holes in viral membrane; however, this peptide did not cause the genome release of other RNA enveloped viruses and did not exhibit adverse effect on cell membrane [129].

Small molecules

Schmidt *et al.* [130] adapted a fluorescence polarization assay, previously employed to identify stem-derived peptides that bind trimeric postfusion E protein [126], to perform a high-throughput screen for small molecules that inhibit viral entry. The screen allowed the characterization of the compound 1662G07 and its analogs as reversible inhibitors of DENV-2 infectivity. Certain analogs were active against all DENV serotypes and liposome fusion assays suggest that these compounds would block viral uncoating [130].

An *in silico* virtual screening allowed the finding of a small molecule, targeted toward

the hydrophobic pocket between DI and DII of E protein, which exhibited antifusion activity and anti-DENV-2 activity in cell cultures [131]. Another *in silico* docking screen based in compounds from Maybridge chemical database that bind the hydrophobic pocket of E protein revealed the antiviral activity of the compound A5 against DENV-2, WNV and yellow fever virus in Vero cells. In addition, A5 inhibited low-pH-induced cell fusion in mosquito C636 cells corroborating the importance of this hinge region in E protein fusion activity [105].

Antibiotic derivatives

Two derivatives of tetracycline, rolitetracycline and doxycycline, selected from a virtual screening based on molecular docking using structural databases of medical compounds, were recognized as inhibitors of DENV multiplication. These compounds would establish hydrophobic interactions between their tetracyclic rings and E protein hydrophobic pocket probably affecting membrane fusion during viral entry [132].

Inhibitors of cholesterol synthesis & transport

As first commented, although cholesterol seems to play a key role in DENV-2 replication and assembly [133,134], controversial results regarding the influence of cellular cholesterol levels on DENV entry have been reported [35,86,87]. U18666A, an amphipathic steroid that affects both cholesterol synthesis and cholesterol intracellular trafficking, inhibits DENV-2 entry to BHK cells when cultures were incubated with the compound prior to infection. While virus binding to the cell occurs normally, treatment with U18666A induced cholesterol accumulation in LE hampering the intracellular trafficking of internalized viral particles. The authors proposed that the high levels of cholesterol within the endocytic vesicles may impair proper membrane fusion or subsequent viral uncoating [135].

• Other entry inhibitors

Antiviral activity against DENV of other peptides [136] and small molecules [137,138] targeting the hydrophobic pocket of E protein has been reported and it was demonstrated that one of these small inhibitors arrests viral particles within endocytic vesicles [138]. Future studies are needed to unravel the mode of action of this new set of antiviral molecules.

On the other hand, derivatives of zosteric acid, an antiadhesive compound obtained from the marine eelgrass *Zostera marina*, display anti-DENV entry inhibitory effects in the monkey kidney epithelial cell line, LLCMK-2; however, their mechanism of action is still unknown [139].

• Clinical trials for antiviral compounds

Although several compounds display anti-DENV activity in studies performed in cell cultures, a small number of molecules have been evaluated in clinical trials. Among the assayed drugs, the only one that affects an early step of DENV multiplication is chloroquine, a lysosomotropic compound that raises endosomal pH preventing viral uncoating. Since the drug also affects the pH within the lumen of the trans-Golgi network, other stages of viral multiplication such as glycoprotein processing and transport would also be affected [140]. Two double-blind, randomized, placebo-controlled trials were performed with chloroquine, one in Vietnam and the other in Brazil. The first trial showed no significant impact on virological or immunological parameters of DENV infection in young adults [140], whereas the second study revealed that there was no significant difference in the duration of the disease or the degree and days of fever in DENV-infected patients treated with chloroquine compared with the control group [141]. Compounds with other mechanisms of action such as balapiravir, an inhibitor of viral polymerase and celgosivir, an iminosugar derivative that targets cellular endoplasmic reticulum α-glucosidases affecting viral glycoprotein processing, were also tested in clinical trials [142,143]. Both balapiravir and celgosivir were safe and well tolerated, but failed to reduce viral load or fever burden in adult patients with DF [142,143]. Besides the abovementioned studies, a trial to evaluate the efficacy of lovastatin, a cholesterol-reducing agent that affects viral assembly is currently underway [144].

Conclusion & future perspective

According to the present knowledge here summarized, it can be concluded that the process of DENV entry into the host cell is very complex and variable depending on the host cell and the virus serotype/strain. A considerable number of options are possible about receptor molecules, including apparently a ubiquitous molecule like

HS or DC-SIGN as a primary receptor to concentrate virions near the cell surface and a subsequent and more specific secondary receptor to trigger virus penetration. Similarly, DENV can also utilize different routes for uptake and intracellular trafficking until release of the viral nucleocapsid. In mammalian cells, virus internalization may occur by clathrin-mediated or other clathrin-independent endocytosis pathways. Independently of this entry route, virions may transit through EE to either LE or RE for membrane fusion. These alternative possibilities may contribute to the wide range of host cells that DENV is able to infect *in vitro* and *in vivo*. Given the advances in the knowledge of these events during the last decade, as well as the recent technologies like live imaging to follow the virus particle inside the cell, it can be expected that more precise information of DENV entry will be available in the near future. This is particularly mandatory in the human cells representative of the natural infection such as dendritic cells, macrophages and monocytes.

Given the continuous expansion of dengue re-emergence around the world and the high incidence of the severe forms of DHF or DSS, particularly in the pediatric population, the search for antiviral drugs for dengue chemotherapy is a priority in public health. In addition, decreasing viral loads by antiviral treatment will not only help to overcome disease, but will also reduce mosquito-mediated virus transmission playing a key role in the control of DENV epidemics.

Several inhibitors targeted to virus or host cell-related factors participating in virus entry have been identified and characterized in the last few years, with very different experimental approaches. A great advance has been made concerning the mechanism of action of HS-mimicking polysaccharides that affect both virus-receptor recognition and viral uncoating. However, physicochemical and pharmacological properties, including membrane permeability, binding to plasma proteins, anticoagulant activity and bioavailability, represent a serious drawback for the *in vivo* efficacy of this class of compounds. On the other hand, intensive research based on rational design of peptides using structural data as well as virtual screening of chemical compound libraries performed in the last few years have accelerated the identification of a diverse variety of novel antiviral molecules that block DENV entry into the host cell. Also promising is the efficacy shown in recent reports by a few inhibitors not only against primary DENV infection, but also against ADE models.

Since the four DENV serotypes cocirculate in most of the endemic areas, a promising antiviral for treatment as well as a preventive vaccine must be effective against all serotypes. Considering the present comprehension of variable molecules and mechanisms participating in DENV entry, above summarized, it appears more difficult to assess the effectiveness against the four serotypes of an agent targeted to any cellular component involved in virus recognition or penetration. On the other hand, the small compounds designed to interact specifically with an E protein domain crucial for entry may be a more successful tool for antiviral development in a few years. Since treatment in dengue or severe forms is limited in time, the problem of viral resistance always present in these viral targeted compounds may be overcome. However, the selection of virus-resistant variants cannot be totally discarded and, consequently, a combined therapy with a cellular related strategy may be advisable.

Most of the developed inhibitors await *in vivo* experimentation and certainly, the lack of a simple small animal model has been one of the major weaknesses for the rapid development of antiviral agents against DENV [103,145]. Chloroquine was the only entry-targeted compound that has attained clinical testing, but with disappointing results. Furthermore, it must be considered that rapid and precise diagnosis will be crucial for the efficacy of antiviral drugs against DENV. DF might be successfully treated with antiviral agents after an early diagnosis, but a lower efficacy is expected when disease has progressed to severe forms. Thus, the advance in diagnostic tests for DENV is required simultaneously with new therapeutic strategies.

Therefore, a better comprehension of the viral entry process and *in vivo* validation of results obtained in cell cultures with those molecules active against all DENV serotypes are the main challenges in the short term.

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EXECUTIVE SUMMARY

Binding and receptors

- DENV binding to the host cell takes place through the interaction between the envelope E glycoprotein and cellular receptors.
- Given the wide spectrum of cell types susceptible to DENV infection, *in vivo* and *in vitro*, it appears that the virus can interact with different molecules acting as cell receptors depending on the cell as well as on virus serotype.
- A multistep process was proposed with a low affinity and abundant primary receptor, heparan sulfate or DC-SIGN according to the cell type, for initial attachment and capture of virions in the cell surface. Then, a secondary interaction with another high affinity receptor is responsible of virion internalization.

DENV internalization

- DENV internalization occurs through receptor-mediated endocytosis dependent on low pH, but the endocytic pathway may be variable according to DENV serotype and host cell.
- The most commonly described route for virion uptake is clathrin-dependent endocytosis, but DENV can enter by other alternative non clathrin-mediated pathways.
- Different DENV serotypes can use distinct endocytic routes for entry into the same cell type.
- The same serotype is able to exploit alternative routes for internalization into different cell types, from vertebrate or invertebrate origin.
- The high variation in receptor molecule and endocytic pathway may represent an adaptative advantage to support the wide host range and tropism of DENV in cell culture and in nature.

Intracellular trafficking and virion uncoating

- Similarly to binding and internalization, the cell site for envelope-endosomal membrane fusion and virion uncoating may be variable.
- The time required for fusion and nucleocapsid release into the cytoplasm is in the range 10-17 min, indicative of a late-penetrating mechanism.
- All DENV viruses seem to transit through early endosomes, but subsequent intracellular trafficking may guide DENV particles either to late endosomes or to slow recycling endosomes at the perinuclear region.

DENV entry as antiviral target

- Screening of natural and synthetic molecules, structure-based rational design and virtual screening have contributed to the identification of novel anti-DENV entry inhibitors including sulfated polysaccharides, peptides, small molecules and antibiotic derivatives.
- Most entry inhibitors interact either with E glycoprotein or with cellular receptors.
- Sulfated polysaccharides exert their antiviral action at two stages of virus entry: binding to cell receptor and low pH activated membrane fusion. DENV serotypes exhibit differential susceptibility to these compounds, which are effective inhibitors in mammalian cells.
- Peptides targeting domain III of E protein interfere with viral adsorption whereas peptides and small molecules designed towards the hydrophobic pocket and the stem region of E protein mainly hinder membrane fusion during viral uncoating.
- There are few DENV entry inhibitors that have proved to be effective in ADE models: the polysaccharide curdlan sulfate and the antibiotic derivatives LCTA-949 (teicoplanin aglycon analog) and SA-17 (doxorubicin analog).

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