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Broad Antiviral Activity of Carbohydrate-Binding Agents Against Dengue Virus Infection

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1. Introduction

1.1. Origin and epidemiology

Dengue virus (DENV) is a member of the *Flavivirus* genus within the family of the *Flaviviridae* and is the most common mosquito-borne viral disease. *Flaviviruses* derived from a common viral ancestor 10,000 years ago. DENV has a relative recent revolutionary history originating 1000 years ago and establishes transmission in humans since a few hundred years. There is strong evidence that DENV was originally a monkey virus in non-human primates in Africa and Asia. Cross-species transmission to humans has occurred independently for all four DENV serotypes [1,2]. Each serotype shares around 65% of the genome and despite of the differences, each serotype causes nearly identical syndromes in humans and circulates in the same ecological niche [3]. First clinical symptoms of dengue infections date from the 10th century but it is not for sure that this was a dengue epidemic. The first large dengue epidemics were in 1779 in Asia, Africa and North America. The first reported epidemic of dengue hemorrhagic fever (DHF) was in Manilla, Philippines, in 1953 after World War II. It was suggested that the movement of the troops during World War II has led to the spread of the virus. It has been shown that in the 19th and 20th century, the virus was widespread in the tropics and subtropics where nowadays 3.6 billion people are at risk of getting infected with DENV (Figure 1). Every year, 50 million infections occur, including 500,000 hospitalizations for DHF, mainly among children, with a case fatality rate exceeding 15% in some areas [4,5]. In 40 years of time, DENV became endemic in more than 100 countries because of the increase in human population, international transport and the lack of vector control.



Figure 1. Global distribution of dengue virus infections in 2011. Contour lines represent the areas at risk (Source: WHO, 2012).

1.2. Transmission

The transmission of DENV can only occur by the bite of an infected female mosquito, the *Aedes aegypti* and the *Aedes albopictus*. *Aedes aegypti* originated, and is still present, in the rainforests of Africa feeding on non-human primates (sylvatic cycle, Figure 2). DENV infection in non-human primates occurs asymptotically. However, the mosquito became domesticated due to massive deforestation and breeds in artificial water holdings, like automobile tires, discarded bottles and buckets that collect rainwater [4]. On one hand *Aedes aegypti* is not an efficient vector because it has a low susceptibility for oral infection with virus in human blood. Since mosquitoes ingest 1 μl of blood, the virus titer in human blood has to be 10^5 - 10^7 per ml for transmission to be sustained. After 7-14 days the virus has passed the intestinal tract to the salivary glands and can be transmitted by the infected mosquito to a new host. On the other hand, *Aedes aegypti* is an efficient vector because it has adapted to humans and they repeatedly feed themselves in daylight on different hosts. After a blood meal, the oviposition can be stimulated and the virus can be passed transovarially to the next generation of mosquitoes (vertical transmission, Figure 2) [6].

The tiger mosquito *Aedes albopictus* is spreading his region from Asia to Europe and the United States of America (USA). In the 1980s, infected *Aedes Albopictus* larvae were transported in truck tires from Asia to the United States. Dengue viruses were introduced into port cities, resulting in major epidemics [6].

Because there is no vaccine available, the only efficient way to prevent DENV infection is eradication of the mosquito. In the 1950s and 1960s there was a successful vector control program in the USA organized by the Pan American Health Organization. They eradicated the mosquito from 19 countries. Unfortunately, the vector control program was stopped in 1972 because the government thought that DENV was not important anymore [2,4]. This resulted in a re-emergence of the mosquito and DENV infections in the USA. Both demographic and ecological changes contributed to the world wide spread of DENV infections.

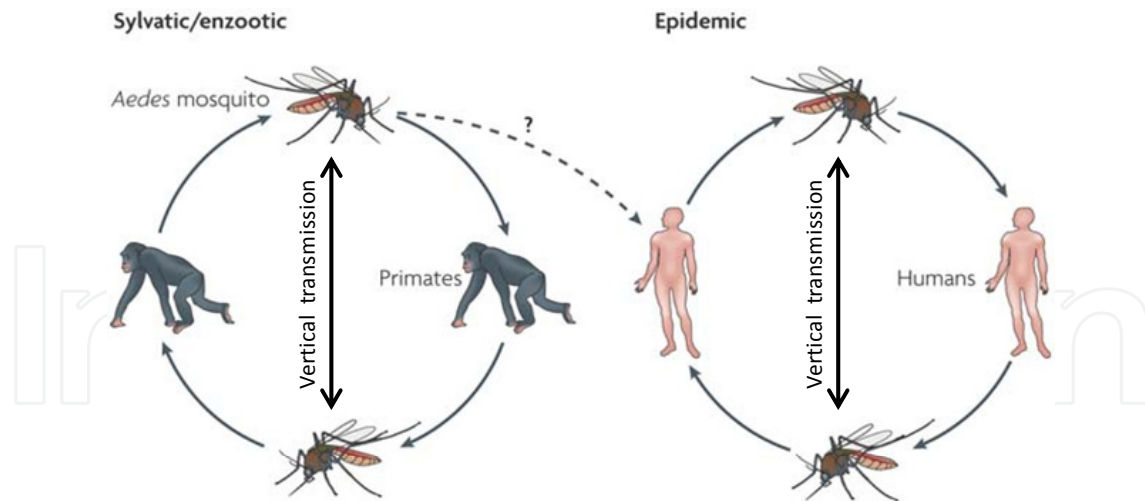


Figure 2. Transmission of DENV by *Aedes* mosquitoes. Modified from Whitehead et al. [7].

Very recently, another approach to attack the vector has been documented [8,9]. There was a mosquito made resistant to DENV infection after trans infection with the endosymbiont *Wolbachia* bacterium which can infect a lot of insects. A certain strain of the *Wolbachia* bacteria was trans infected in *Aedes* mosquitoes and was reported to inhibit the replication and dissemination of several RNA viruses, such as DENV. Embryos of a *Wolbachia* uninfected female die if the female has bred with a *Wolbachia* infected male. This means that *Wolbachia* infected mosquitoes can take over the natural population. This was recently tested in Australia, where dengue is endemic, and after 2 months the *Wolbachia* infected mosquitoes resistant to DENV had taken over the natural mosquito population. Thus, this indicates the beginning of a new area in vector control efforts with a high potential to succeed.

1.3. Pathogenesis

Although DENV infections have a high prevalence, the pathogenesis of the disease is not well understood. The disease spectrum can range from an asymptomatic or flu like illness to a lethal disease. After a bite of an infected mosquito, there is an incubation period of 3 to 8 days. Then there is an acute onset of fever ($\geq 39^{\circ}\text{C}$) accompanied by nonspecific symptoms like severe headache, nausea, vomiting, muscle and joint pain (dengue fever). Clinical findings alone are not helpful to distinguish dengue fever from other febrile illnesses such as malaria or measles. Half of the infected patients report a rash and is most commonly seen on the trunk and the insides of arms and thighs. Skin hemorrhages, including petechiae and purpura, are very common. Liver enzyme levels of alanine aminotransferase and aspartate aminotransferase can be elevated. Dengue fever is generally self-limiting and is rarely fatal [5,10,11].

The disease can escalate into dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). DHF is primarily a children's disease and is characterized as an acute febrile illness with thrombocytopenia ($\leq 100,000$ cells/mm³). This results in an increased vascular

permeability and plasma leakage from the blood vessels into the tissue. Plasma leakage has been documented by an increased hematocrit and a progressive decrease in platelet count. Petechiae and subcutaneous bleedings are very common [12].

DSS is defined when the plasma leakage becomes critical resulting in circulatory failure, weak pulse and hypotension. Plasma volume studies have shown a reduction of more than 20% in severe cases. A progressively decreasing platelet count, a rising hematocrit, sustained abdominal pain, persistent vomiting, restlessness and lethargy may be all signs for DSS. Prevention of shock can only be established after volume replacement with intravenous fluids [5,11]. When experienced clinicians and nursing staff are available in endemic areas, the case fatality rate is < 1%.

DHF and DSS occur during a secondary infection with a heterologous serotype. The first infection with one of the four serotypes provides lifelong immunity to the homologous virus. During a second infection with a heterologous serotype, non-neutralizing IgG antibodies can enhance disease severity. This phenomenon is called antibody-dependent enhancement (ADE). The pre-existing non-neutralizing heterotypic antibodies can form a complex with DENV and enhance the access to Fc-receptor bearing cells such as monocytes and macrophages [13,14] (Figure 3).

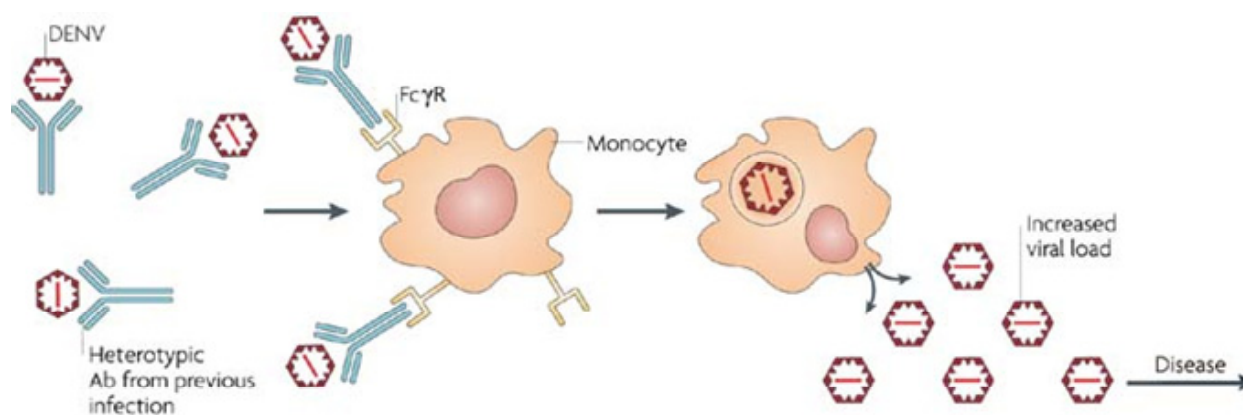


Figure 3. Mechanism of antibody-dependent enhancement (ADE). During a secondary infection caused by a heterologous virus, the pre-existing heterotypic antibodies can cross-react with the other DENV serotypes. The non-neutralizing antibody-virus complex can interact with the Fc-receptor on monocytes or macrophages. This will lead to an increased viral load and a more severe disease. Figure derived from Whitehead et al. [7].

This will lead to an increase in viral load and a more severe disease. These non-neutralizing antibodies can cross-react with all four virus serotypes, as well as with other flaviviruses. This phenomenon explains why young infants born to dengue immune mothers often experience a more severe disease due to transplacental transfer of DENV-specific antibodies [15]. Another approach to assist this phenomenon is the observation of increased viremia in non-human primates which received passive immunization with antibodies against DENV [16].

A second mechanism to explain ADE of flaviviruses is the involvement of the complement system. It has been shown that monoclonal antibodies against complement receptor 3 inhibit ADE of West Nile virus *in vitro* [14]. But Fc-receptor-dependent ADE is believed to be the most common mechanism of ADE.

2. DENV entry

2.1. Entry process

The infectious entry of DENV into its target cells, mainly dendritic cells [17], monocytes and macrophages, is mediated by the viral envelope glycoprotein E via receptor-mediated endocytosis [18]. The E-glycoprotein is the major component (53 kDa) of the virion surface and is arranged as 90 homodimers in mature virions [19]. Recent reports demonstrated also that DENV enters its host cell via clathrin-mediated endocytosis [20,21], as observed with other types of flaviviruses [22,23]. Evidence for flavivirus entry via this pathway is based on the use of inhibitors of clathrin-mediated uptake, such as chlorpromazine. However, DENV entry via a non-classical endocytic pathway independent from clathrin has also been described [24]. It seems that the entry pathway chosen by DENV is highly dependent on the cell type and viral strain. In case of the classical endocytic pathway, there is an uptake of the receptor-bound virus by clathrin coated vesicles. These vesicles fuse with early endosomes to deliver the viral RNA into the cytoplasm. The E-protein responds to the reduced pH of the endosome with a large conformational rearrangement [25,26]. The low pH triggers dissociation of the E-homodimer, which then leads to the insertion of the fusion peptide into the target cell membrane forming a bridge between the virus and the host. Next, a stable trimer of the E-protein is folded into a hairpin-like structure and forces the target membrane to bend towards the viral membrane and eventually fusion takes place [25,27,28]. The fusion results in the release of viral RNA into the cytoplasm for initiation of replication and translation (Figure 4).

2.2. The DENV envelope

The DENV E-glycoprotein induces protective immunity and flavivirus serological classification is based on its antigenic variation. During replication the virion assumes three conformational states: the immature, mature and fusion-activated form. In the immature state, the E-protein is arranged as a heterodimer and generates a "spiky" surface because the premembrane protein (prM) covers the fusion peptide. In the Golgi apparatus, the virion matures after a rearrangement of the E-protein. The E-heterodimer transforms to an E-homodimer and results in a "smoothy" virion surface. After a furin cleavage of the prM to pr and M, the virion is fully matured and can be released from the host cell. Upon fusion, the low endosomal pH triggers the rearrangement of the E-homodimer into a trimer [29].

The E-protein monomer is composed out of β -barrels organized in three structural domains (Figure 5).

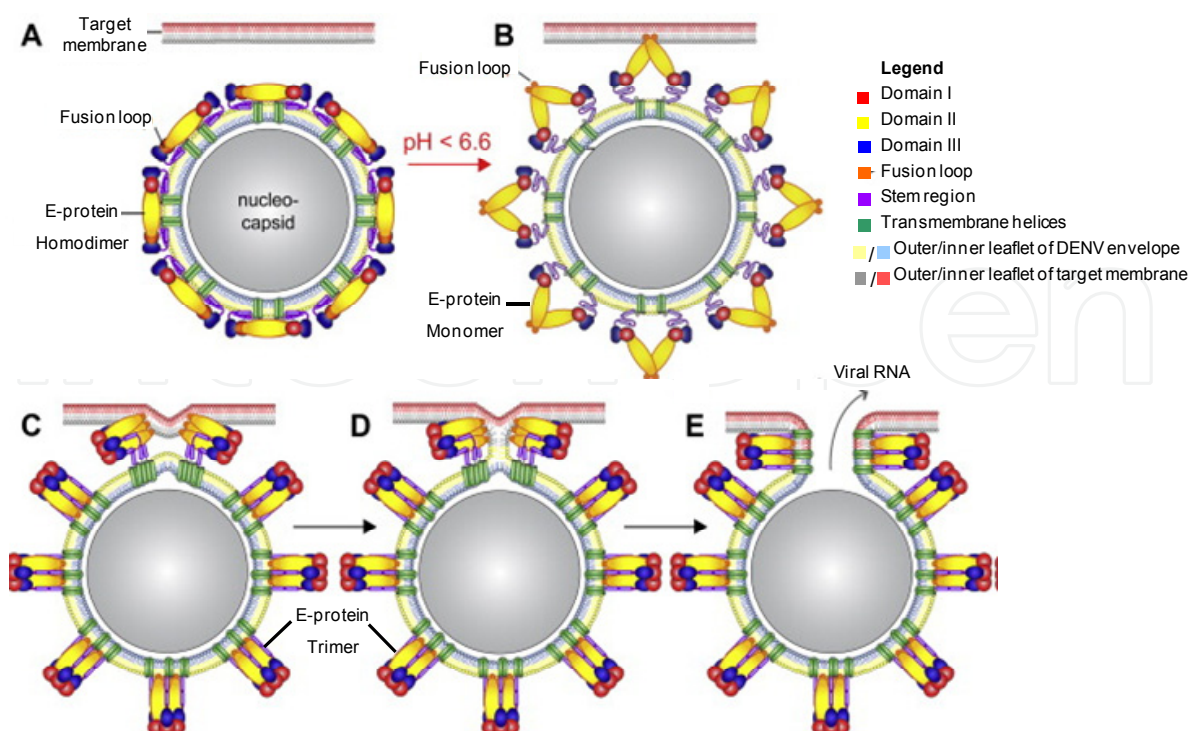


Figure 4. Schematic overview of the DENV membrane fusion process. (A) Pre-fusion conformation of the E-protein consists of homodimers on the virus surface. (B) Low endosomal pH triggers dissociation of the E-dimers into monomers which leads to the insertion of the fusion peptide with the endosomal target membrane. (C) A stable E-protein trimer is folded in a hairpin-like structure. (D) Hemifusion intermediate in which only the outer leaflets of viral and target cellular membranes have fused. (E) Formation of the post-fusion E-protein trimer and opening of the fusion pore allows the release of the viral RNA into the cytoplasm. Modified from Stiasny et al. [26].

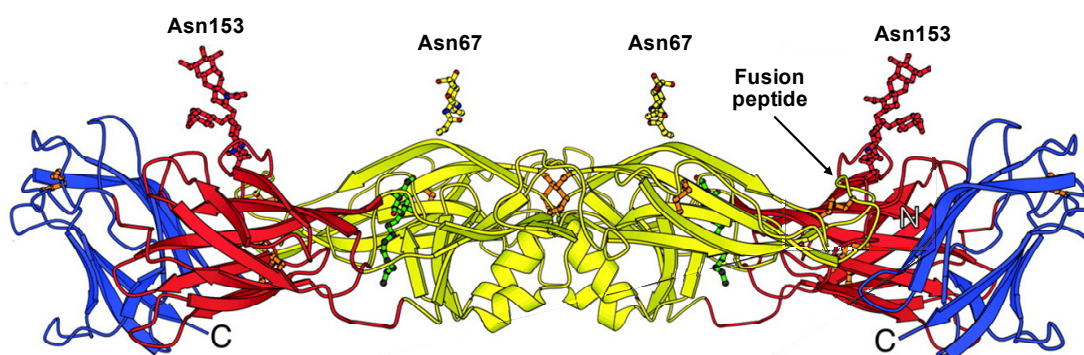


Figure 5. Location of the 2 N-glycans on the envelope protein of DENV. The DENV E-protein dimer carries 2 N-glycans on each monomer at Asn67 and Asn153. β -strands are shown as ribbons with arrows, α -helices are shown as coiled ribbons. Thin tubes represent connecting loops. Domain I is shown in red, domain II is shown in yellow and contains the fusion peptide near Asn153. Domain III is shown in blue. Disulfide bridges are shown in orange. In green, the ligand N-octyl-D-glucoside is shown, which interacts with the hydrophobic pocket between domain I and II. Modified from Modis et al. [30].

The central domain I contains the aminoterminal and contains two disulfide bridges. Domain II is an extended finger-like domain that bears the fusion peptide and stabilizes the dimer. This sequence contains three disulfide bridges and is rich in glycine. Between domain I and domain II is a binding pocket that can interact with a hydrophobic ligand, the detergent β -N-octyl-glucoside. This pocket is an important target for antiviral therapy because mutations in this region can alter virulence and the pH necessary for the induction of conformational changes. The immunoglobulin-like domain III contains the receptor binding motif, the C-terminal domain and one disulfide bond [30,31]. Monoclonal antibodies recognizing domain III are the most efficient of blocking DENV [32,33] and this domain is therefore an interesting target for antiviral therapy.

Because dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) (See 1.3.1) is identified as an important receptor for DENV in primary DC in the skin and DC-SIGN recognizes high-mannose sugars, carbohydrates present on the E-protein of DENV could be important for viral attachment. The E-protein has two potential glycosylation sites: asparagines 67 (Asn67) and Asn153. Glycosylation at Asn153 is conserved in flaviviruses, with the exception of Kunjin virus, a subtype of West Nile virus [34] and is located near the fusion peptide in domain II [30,31] (Figure 5). Glycosylation at Asn67 is unique for DENV [31].

3. Role of DC-SIGN in DENV infection

Prior to fusion, DENV needs to attach to specific cellular receptors. Because DENV can infect a variety of different cell types isolated from different hosts (human, insect, monkey and even hamster), the virus must interact with a wide variety of cellular receptors. In the last decade, several candidate attachment factor/receptors are identified. DC-SIGN is described as the most important human cellular receptor for DENV.

Since 1977, monocytes are considered to be permissive for DENV infection [35]. More recent, phenotyping of peripheral blood mononuclear cells (PBMCs) from pediatric DF and DHF cases resulted in the identification of monocytes as DENV target cells [36].

First, it was believed that monocytes are important during secondary DENV infections during the ADE process, because of their Fc-receptor expression. The complex formed between the non-neutralizing antibody and the virus can bind to Fc-receptors and enhance infection in neighboring susceptible cells [14,18,37]. However, *in vitro*, monocytes isolated from PBMCs, apparently have a very low susceptibility for DENV infection for reasons that remain to be elucidated.

More detailed observation of the natural DENV infection, changes the idea of monocytes being the first target cells. Following intradermal injection of DENV-2 in mice, representing the bite of an infected mosquito, DENV occurs to replicate in the skin [38]. The primary DENV target cells in the skin are believed to be immature dendritic cells (DC) or Langerhans cells [17,39-41]. Immature DC are very efficient in capturing pathogens whereas mature DC are relatively resistant to infection. The search for cellular receptors responsible

for DENV capture leads to the identification of cell-surface C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN; CD209) [42-45]. DC-SIGN is mainly expressed by immature DC, but also alveolar macrophages and interstitial DC in the lungs, intestine, placenta and in lymph nodes express DC-SIGN [46]. DC-SIGN is a tetrameric transmembrane receptor and is a member of the calcium-dependent C-type lectin family. The receptor is composed of four domains: a cytoplasmic domain responsible for signaling and internalization due to the presence of a dileucine motif, a transmembrane domain, seven to eight extracellular neck repeats implicated in the tetramerization of DC-SIGN and a carbohydrate recognition domain (CRD) (Figure 6) [47].

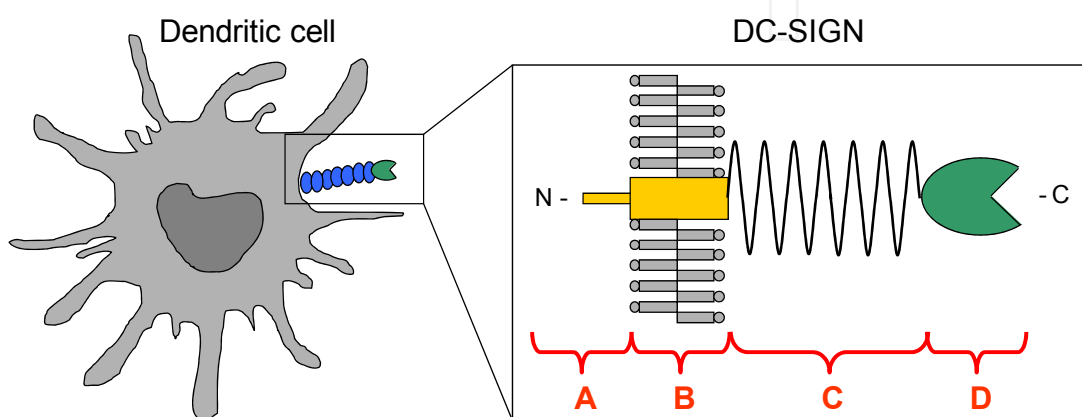


Figure 6. Structure of DC-SIGN. DC-SIGN, mainly expressed by human dendritic cells in the skin, is composed out of four domains: (A) cytoplasmic domain containing internalization signals, (B) transmembrane domain, (C) 7 or 8 extracellular neck repeats implicated in the oligomerization of DC-SIGN and (D) carbohydrate recognition domain which can interact calcium-dependent with a variety of pathogens.

Alen *et al.* [42] investigated the importance of DC-SIGN receptor in DENV infections using DC-SIGN transfected Raji cells versus Raji/0 cells. A strong contrast in DENV susceptibility was observed between Raji/DC-SIGN⁺ cells and Raji/0 cells. DC-SIGN expression renders cells susceptible for DENV infection. Also in other cell lines, the T-cell line CEM and the astrogloma cell line U87, expression of DC-SIGN renders the cells permissive for DENV infection. To evaluate the importance of DC-SIGN, Raji/DC-SIGN⁺ cells were incubated with a specific anti-DC-SIGN antibody prior to DENV infection. This resulted in an inhibition of the DENV replication by ~90%, indicating that DC-SIGN is indeed an important receptor for DENV. Also 2 mg/ml of mannan inhibited the DENV infection in Raji/DC-SIGN⁺ cells by more than 80%. This data indicate that the interaction between DC-SIGN and DENV is dependent on mannose-containing N-glycans present on the DENV envelope [42].

Thus, the CRD of DC-SIGN recognizes high-mannose N-glycans and also fucose-containing blood group antigens [48,49]. Importantly, DC-SIGN can bind a variety of pathogens like human immunodeficiency virus (HIV) [50], hepatitis C virus (HCV) [51], ebola virus [52] and several bacteria, parasites and yeasts [46]. Many of these pathogens have developed strategies to manipulate DC-SIGN signaling to escape from an immune response [46]. Following antigen capture in the periphery, DC mature by up regulation of the co-

stimulatory molecules and down regulation of DC-SIGN. By the interaction with ICAM-2 on the vascular endothelial cells, DC can migrate to secondary lymphoid organs [53]. Next, the activated DC interact with ICAM-3 on naïve T-cells. This results in the stimulation of the T-cells and subsequently in the production of cytokines and chemokines [54]. Inhibition of the initial interaction between DENV and DC could prevent an immune response. DC-SIGN could be considered as a target for antiviral therapy by interrupting the viral entry process. But caution must be taken into account as the DC-SIGN receptor has also an important role in the activation of protective immune responses instead of promoting the viral dissemination. However, several DC-SIGN antagonists have been developed such as small interfering RNAs (siRNA) silencing DC-SIGN expression [55], specific anti-DC-SIGN antibodies [56] and glycomimetics interacting with DC-SIGN [57]. The *in vivo* effects of DC-SIGN antagonists remain to be elucidated.

Besides DC, macrophages play a key role in the immune pathogenesis of DENV infection as a source of immune modulatory cytokines [58]. Recently, Miller *et al.* showed that the mannose receptor (MR; CD206) mediates DENV infection in macrophages by recognition of the glycoproteins on the viral envelope [59]. Monocyte-derived DC (MDDC) can be generated out of monocytes isolated from fresh donor blood incubated IL-4 and GM-CSF. After a differentiation process MDDC were generated highly expressing DC-SIGN (Figure 7A, B) and showing a significantly decrease in CD14 expression in contrast to monocytes [59,60]. Again, DC-SIGN expression on MDDC renders cells susceptible for DENV in contrast to monocytes (Figure 7A, B). MR is also present on monocyte-derived DC (MDDC) and anti-MR antibodies can inhibit DENV infection, although to a lesser extent than anti-DC-SIGN antibodies do (Figure 7C) [61]. Furthermore, the combination of anti-DC-SIGN and anti-MR antibodies was even more effective in inhibiting DENV infection. Yet, complete inhibition of DENV infection was not achieved, indicating that other entry pathways are potentially involved. Two other receptors on DC reported to be responsible for HIV attachment are syndecan-3 (a member of the heparan sulfate proteoglycan family) [62] and the DC immune receptor [63]. Since DENV interacts with heparan sulfate, syndecan-3 may be a possible (co)-receptor on DC. It has been hypothesized that DENV needs DC-SIGN for attachment and enhancing infection of DC *in cis* and needs MR for internalization [59]. In fact, cells expressing mutant DC-SIGN, lacking the internalization domain, are still susceptible for DENV infection because DC-SIGN can capture the pathogen [43].

Another C-type lectin, CLEC5A (C-type lectin domain family 5, member A) expressed by human macrophages can also interact with DENV and acts as a signaling receptor for the release of proinflammatory cytokines [64]. However, whereas the DC-SIGN-DENV interaction is calcium-dependent, CLEC5A binding to its ligand is not dependent on calcium. Mannan and fucose can inhibit the interaction between CLEC5A and DENV, indicating that the interaction is carbohydrate-dependent [64]. However, a glycan array demonstrated no binding signal between CLEC5A and N-glycans of mammals or insects [65]. The molecular interaction between CLEC5A and DENV remains to be elucidated.

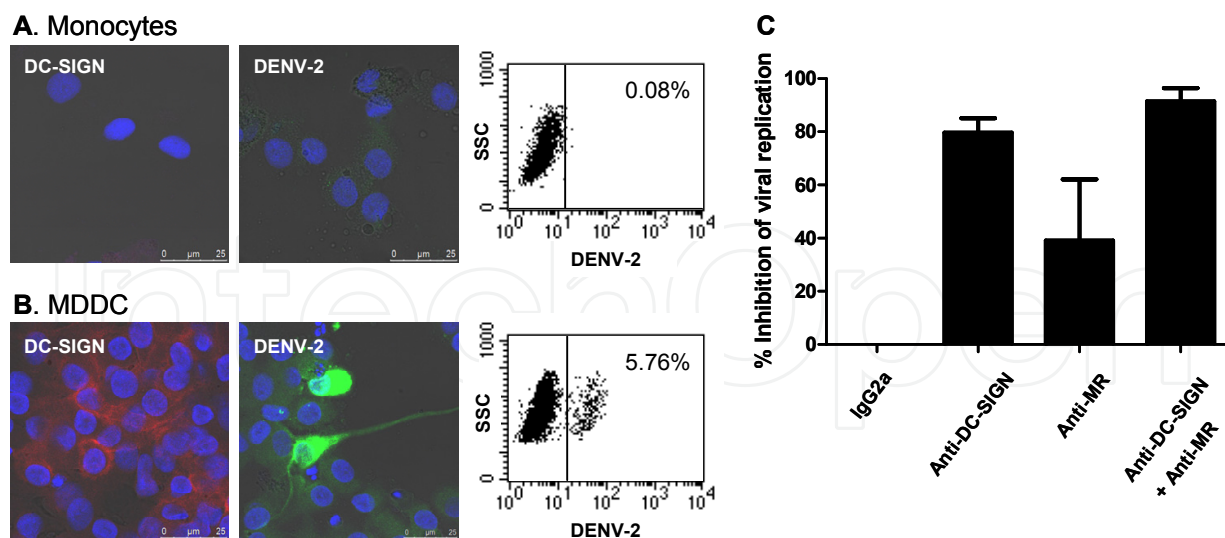


Figure 7. Infection of MDDC by DENV. Monocytes isolated from PBMCs were untreated (A) or treated with 25 ng/ml IL-4 and 50 ng/ml GM-CSF (B) for 5 days prior to DENV-2 infection. Two days after infection the cells were permeabilized and analyzed for DC-SIGN expression and DENV infection by confocal microscopy and flow cytometry. Uninfected cells were stained with a PE-labeled monoclonal DC-SIGN-antibody (red). Infected cells were stained with a mixture of antibodies recognizing DENV-2 E-protein and PrM protein (green). Nuclei were stained with DAPI (blue). Infected monocytes (A) and MDDC (B) were analyzed by flow cytometry to detect DENV-2 positive cells. The values indicated in each dot plot represent the % of DENV-2 positive cells. (C) MDDC were preincubated with 10 μ g/ml of isotype control IgG_{2a}, anti-DC-SIGN or anti-MR antibody for 30 minutes before DENV-2 infection. Viral replication was analyzed by flow cytometry. % Inhibition of viral replication \pm SEM of 4 different blood donors is shown.

Immune cells, in particular dendritic cells, are the most relevant cells in the discovery of specific antiviral drugs against dengue virus, but the isolation of these cells and the characterization is unfortunately labour intensive and time consuming.

Liver/lymph node-specific ICAM-3 grabbing non-integrin (L-SIGN) is a DC-SIGN related transmembrane C-type lectin expressed on endothelial cells in liver, lymph nodes and placenta [66,67]. Similar to DC-SIGN, L-SIGN is a calcium-dependent carbohydrate-binding protein and can interact with HIV [67], HCV [51], Ebola virus [52], West Nile virus [68] and DENV [45]. Zellweger *et al.* observed that during antibody-dependent enhancement in a mouse model that liver sinusoidal endothelial cells (LSEC) are highly permissive for antibody-dependent DENV infection [69]. Given the fact that LSEC express L-SIGN, it is interesting to focus on the role of L-SIGN in DENV infection. L-SIGN expression on LSEC has probably an important role in ADE *in vivo* and therefore it is interesting to find antiviral agents interrupting the DENV-L-SIGN interaction and subsequently prevent the progression to the more severe and lethal disease DHF/DSS. Although endothelial cells [70] and liver endothelial cells [71] are permissive for DENV and L-SIGN-expression renders cells susceptible for DENV infection, the *in vivo* role for L-SIGN in DENV entry remains to be established.

4. Antiviral therapy

At present, diagnosis of dengue virus infection is largely clinical, treatment is supportive through hydration and disease control is limited by eradication of the mosquito. Many efforts have been made in the search for an effective vaccine, but the lack of a suitable animal model, the need for a high immunogenicity vaccine and a low reactogenicity are posing huge challenges in the dengue vaccine development [7,72]. There are five conditions for a dengue vaccine to be effective: (i) the vaccine needs to be protective against all four serotypes without reactogenicity, because of the risk of ADE, (ii) it has to be safe for children, because severe dengue virus infections often affects young children, (iii) the vaccine has to be economical with minimal or no repeat immunizations, because dengue is endemic in many developing countries, (iv) the induction of a long-lasting protective immune response is necessary and finally (v) the vaccine may not infect mosquitoes by the oral route [7,73].

As there is no vaccine available until now, the search for antiviral products is imperative. The traditional antiviral approach often attacks viral enzymes, such as proteases and polymerases [74,75]. Because human cells lack RNA-dependent polymerase, this enzyme is very attractive as antiviral target without cytotoxicity issues. Nucleoside analogues and non-nucleoside compounds have previously been shown to be very effective in anti-HIV therapy and anti-hepatitis B virus therapy. The protease activity is required for polyprotein processing which is necessary for the assembly of the viral replication complex. Thereby, the protease is an interesting target for antiviral therapy. However, the host cellular system has similar protease activities thus cytotoxic effects form a major recurrent problem. Very recently, many efforts have been made in the development of polymerase and protease inhibitors of DENV, but until today, any antiviral product has reached clinical trials. This chapter is focusing on a different step in the virus replication cycle, namely, the viral entry process. In the past few years, progression has been made in unraveling the host cell pathways upon DENV infection. It is proposed that viral epitopes on the surface of DENV can trigger cellular immune responses and subsequently the development of a severe disease. Therefore, these epitopes are potential targets for the development of a new class of antiviral products, DENV entry inhibitors. Inhibition of virus attachment is a valuable antiviral strategy because it forms the first barrier to block infection. Several fusion inhibitors, glycosidase inhibitors and heparin mimetics have been described to inhibit DENV entry in the host cell. Here, specific molecules, the carbohydrate-binding agents (CBAs), preventing the interaction between the host and the N-glycans present on the DENV envelope are discussed.

4.1. Carbohydrate-binding agents (CBAs)

The CBAs form a large group of natural proteins, peptides and even synthetic agents that can interact with glycosylated proteins. CBAs can be isolated from different organisms: algae, prokaryotes, fungi, plants, invertebrates and vertebrates (such as DC-SIGN and L-SIGN) [76,77]. Each CBA will interact in a specific way with monosaccharides, such as mannose, fucose, glucose, N-acetylglucosamine, galactose, N-acetylgalactosamine or sialic acid residues present in the backbone of N-glycan structures. Because a lot of enveloped

viruses are glycosylated at the viral surface, such as HIV, HCV and DENV (Figure 5), CBAs could interact with the glycosylated envelope of the virus and subsequently prevent viral entry into the host cell [78,79]. Previously, antiviral activity against HIV and HCV [78-80] was demonstrated of several CBAs isolated from plants (plant lectins) and algae specifically binding mannose and N-acetylglucosamine residues.

Here, we focus on the antiviral activity of three plant lectins, *Hippeastrum* hybrid agglutinin (HHA), *Galanthus nivalis* agglutinin (GNA) and *Urtica dioica* (UDA) isolated from the amaryllis, the snow drop and the stinging nettle, respectively. In general, plant lectins form a large diverse group of proteins, exhibiting a wide variety of monosaccharide-binding properties which can be isolated from different sites within the plant, such as the bulbs, leaves or roots. HHA (50 kDa) and GNA (50 kDa) isolated from the bulbs are tetrameric proteins. For GNA, each monomer contains two carbohydrate-binding sites and a third site is created once if tetramerization had occurred, resulting in a total of 12 carbohydrate-binding sites (Figure 8). HHA specifically interacts with α 1-3 and α 1-6 mannose residues and GNA only recognizes α 1-3 mannose residues. UDA, isolated from the rhizomes of the nettle, is active as a monomer containing 2 carbohydrate-binding sites recognizing N-acetylglucosamine residues (Figure 8). In 1984, UDA was isolated for the first time and with its molecular weight of 8.7 kDa, UDA is the smallest plant lectin ever reported [81]. The plant lectins have been shown to possess both antifungal and insecticidal activities playing a role in plant defense mechanisms. Here, the antiviral activity of the plant lectins against DENV will be further highlighted and discussed in detail.

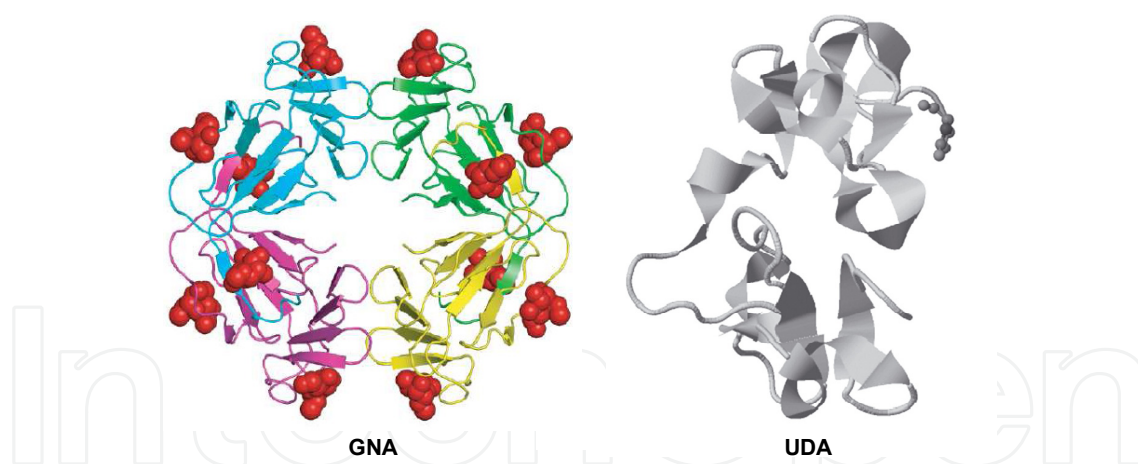


Figure 8. Structure of GNA and UDA. GNA is isolated from the snow drop and is a tetrameric protein. UDA is isolated from the stinging nettle and is a monomeric protein composed out of hevein domains.

Previously, concanavalin A (Con A), isolated from the Jack bean, binding to mannose residues and wheat germ agglutinin binding to N-acetylglucosamine (Glc-NAc) residues, were shown to reduce DENV infection *in vitro*. A competition assay, using mannose, proved that the inhibitory effect of Con A was due to binding α -mannose residues on the viral protein, because mannose successfully competed with Con A [82]. Together with the fact that HHA, GNA and UDA act inhibitory against HIV and HCV, we hypothesized that these plant lectins had antiviral activity against DENV, because DENV has two N-glycosylation

sites on the viral envelope and uses DC-SIGN as a cellular receptor to enter DC. The antiviral activity of the three plant lectins was investigated in DC-SIGN⁺ and L-SIGN⁺ cells and the infection was analyzed by flow cytometry, RT-qPCR and confocal microscopy.

4.2. Broad antiviral activity of CBAs against DENV

Because DC-SIGN interacts carbohydrate-dependent with DENV, the antiviral activity of the three plant lectins, HHA, GNA and UDA, recognizing monosaccharides present in the backbone of N-glycans on the DENV E-protein, was evaluated. A consistent dose-dependent antiviral activity was observed in DC-SIGN transfected Raji cells against DENV-2 analyzed by flow cytometry (detecting the presence of DENV Ag) and RT-qPCR (detecting viral RNA in the supernatants) [42].

Next, the antiviral potency of the three plant lectins was determined against all four serotypes of DENV, of which DENV-1 and DENV-4 are low-passage clinical virus isolates, in both Raji/DC-SIGN⁺ cells and in primary immature MDDC. The use of MDDC has much more clinical relevance than using a transfected cell line. MDDC resemble DC in the skin [83] and mimic an *in vivo* DENV infection after a mosquito bite. Moreover, cells of the hematopoietic origin, such as DC, have been shown to play a key role for DENV pathogenesis in a mouse model [84]. A dose-dependent and a DENV serotype-independent antiviral activity of HHA, GNA and UDA in MDDC was demonstrated as analyzed by flow cytometry (Figure 9). These CBAs proved about 100-fold more effective in inhibiting DENV infection in primary MDDC compared to the transfected Raji/DC-SIGN⁺ cell line.

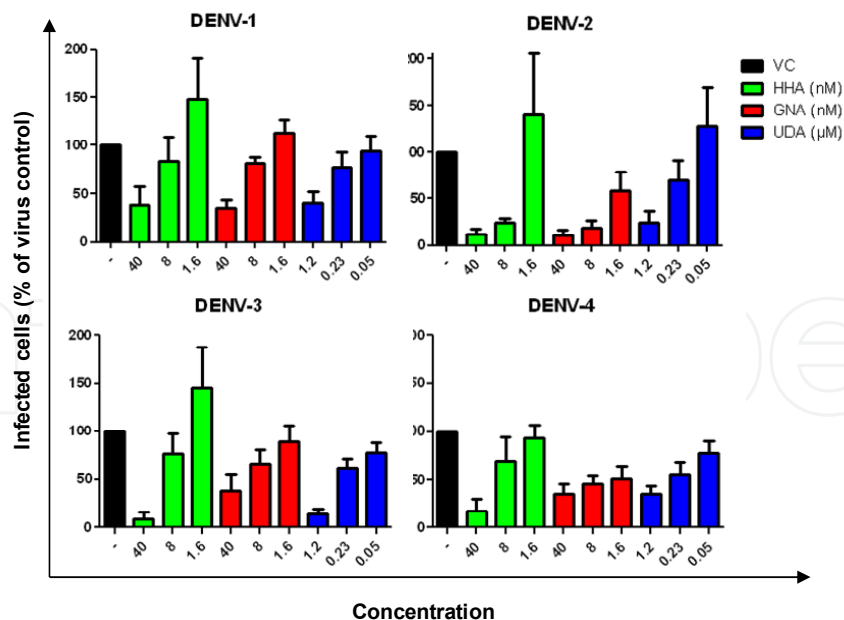


Figure 9. Dose-dependent antiviral activity of HHA, GNA and UDA in DENV-infected MDDC. MDDC were infected with the four serotypes of DENV in the presence or absence of various concentrations of HHA, GNA and UDA. DENV infection was analyzed by flow cytometry using an anti-PrM antibody recognizing all four DENV serotypes (clone 2H2). % of infected cells compared to the positive virus control (VC) \pm SEM of 4 to 12 different blood donors is shown. (Adapted from Alen et al. [61]).

When DENV is captured by DC, a maturation and activation process occurs. DC require downregulation of C-type lectin receptors [85], upregulation of costimulatory molecules, chemokine receptors and enhancement of their APC function to migrate to the nodal T-cell areas and to activate the immune system [86]. Cytokines implicated in vascular leakage are produced, the complement system becomes activated and virus-induced antibodies can cause DHF via binding to Fc-receptors. Several research groups demonstrated maturation of DC induced by DENV infection [87,88]. Some groups made segregation in the DC population after DENV infection, the infected DC and the uninfected bystander cells. They found that bystander DC, in contrast to infected DC, upregulate the cell surface expression of costimulatory molecules, HLA and maturation molecules. This activation is induced by TNF- α and IFN- α secreted by DENV-infected DC [40,89,90]. Instead, Alen *et al.* observed an upregulation of the costimulatory molecules CD80 and CD86 and a downregulation of DC-SIGN and MR on the total (uninfected and infected) DC population following DENV infection [61]. This could indicate that the DC are activated and can interact with naive T-cells and subsequently activate the immune system resulting in increased vascular permeability and fever. When the effect of the CBAs was examined on the expression level of the cell surface markers of the total DC population, it was shown that the CBAs are able to inhibit the activation of all DC caused by DENV and can keep the DC in an immature state. Furthermore, DC do not express costimulatory molecules and thus do not interact or significantly activate T-cells. An approach to inhibit DENV-induced activation of DC may prevent the immunopathological component of DENV disease.

However, since plant lectins are expensive to produce and not orally bioavailable, the search for non-peptidic small molecules is necessary. PRM-S, a highly soluble non-peptidic small-size carbohydrate-binding antibiotic is a potential new lead compound in HIV therapy, since PRM-S efficiently inhibits HIV replication and prevents capture of HIV to DC-SIGN⁺ cells [91]. PRM-S also inhibited dose-dependently DENV-2 replication in MDDC but had only a weak antiviral activity in Raji/DC-SIGN⁺ cells [61]. Actinohivin (AH), a small prokaryotic peptidic lectin containing 114 amino acids, exhibits also anti-HIV-1 activity by recognizing high-mannose-type glycans on the viral envelope [92]. Although DENV has high mannose-type glycans on the E-protein, there was no antiviral activity of AH against DENV infection. Other CBAs such as microvirin, griffithsin and Banlec have been shown to exhibit potent activity against HIV replication [93-95], but these CBAs did not show antiviral activity against DENV. Previously, it has been shown that the CBAs HHA, GNA and UDA also target the N-glycans of other viruses, such as HIV, HCV [79] and HCMV [80]. This indicates that the CBAs can be used as broad-spectrum antiviral agents against various classes of glycosylated enveloped viruses. Although, the three plant lectins did not act inhibitory against parainfluenza-3, vesicular stomatitis virus, respiratory syncytial virus or herpes simplex virus [79]. Together, these data indicate a unique carbohydrate-specificity, and thus also a specific profile of antiviral activity of the CBAs.

4.3. Molecular target of the CBAs on DENV

It was demonstrated in time of drug addition assays that the mannose binding lectin HHA prevents DENV-2 binding to the host cell and acts less efficiently when the virus had already attached to the host cell. It was shown that HHA interacts with DENV and not with cellular membrane proteins such as DC-SIGN on the target cell. The potency of HHA to inhibit attachment of DENV to Raji/DC-SIGN⁺ cells is comparable to its inhibitory activity of the capture of HIV and HCV to Raji/DC-SIGN⁺ cells [79]. CBAs could thus be considered as unique prophylactic agents of DENV infection.

To identify the molecular target of the CBAs on DENV, a resistant DENV to HHA was generated in the mosquito cell line C6/36 by Alen *et al.* (HHA^{res} DENV). Compared with the WT DENV, two highly prevalent mutations were found, namely N67D and T155I, present in 80% of all clones sequenced. Similar mutational patterns destroying both glycosylation motifs (T69I or T69A each in combination with T155I) were present in another 10% of the clones analyzed. The N-glycosylation motif ¹⁵³N-D-T₁₅₅ is conserved among the majority of all flaviviruses, while a second N-glycosylation motif ⁶⁷N-T-T₆₉ is unique among DENV [96]. In the HHA^{res} virus both N-glycosylation motifs were mutated either directly at the actual N-glycan accepting a residue of the first site (Asn67) or at the C-proximal Thr155 being an essential part of the second N-glycosylation site [97], thus both N-glycosylation sites on the viral envelope protein can be considered to be deleted. This indicates that HHA directly targets the N-glycans on the viral E-protein. In fact, all clones sequenced showed the deletion of the N-glycan at Asn153. However, 10% of the clones sequenced had no mutation at the glycosylation motif ⁶⁷N-T-T₆₉, indicating that this glycosylation motif [96] has a higher genetic barrier compared to ¹⁵³N-D-T₁₅₅. Though there are multiple escape pathways to become resistant to HHA, it seems not to be possible to fully escape the selective pressure of favoring a deglycosylation of the viral E-protein. In addition, there were no mutations found either apart from the N-glycosylation sites of the E-protein or in any of the five WT DENV-2 clones passaged in parallel. This is not fully unexpected as flaviviruses replicate with reasonable fidelity and DENV does not necessarily exist as a highly diverse quasispecies neither *in vitro* nor *in vivo* [98,99].

There are some contradictions in terms of necessity of glycosylation of Asn67 and Asn153 during DENV viral progeny. Johnson *et al.* postulated that DENV-1 and DENV-3 have both sites glycosylated and that DENV-2 and DENV-4 have only one N-glycan at Asn-67 [100]. In contrast, a study comparing the number of glycans in multiple isolates of DENV belonging to all four serotypes led to the consensus that all DENV strains have two N-glycans on the E-protein [101]. However, mutant DENV lacking the glycosylation at Asn153 can replicate in mammalian and insect cells, indicating that this glycosylation is not essential for viral replication [96,102]. There is a change in phenotype because ablation of glycosylation at Asn153 in DENV is associated with the induction of smaller plaques in comparison to the wild type virus [96]. Asn153 is proximal to the fusion peptide and therefore deglycosylation at Asn153 showed also an altered pH-dependent fusion activity and displays a lower stability [103,104]. In contrast, Alen *et al.* showed that the mutant virus, HHA^{res} lacking both

N-glycosylation sites, had a similar plaque phenotype in BHK cells (manuscript submitted). It has been shown that DENV lacking the glycosylation at Asn67 resulted in a replication-defective phenotype, because this virus infects mammalian cells weakly and there is a reduced secretion of DENV E-protein. Replication in mosquito cells was not affected, because the mosquito cells restore the N-glycosylation at Asn67 with a compensatory site-mutation (K64N) generating a new glycosylation site [96,105]. These data are in contrast with other published results, where was demonstrated that DENV lacking the Asn67-linked glycosylation can grow efficiently in mammalian cells, depending on the viral strain and the amino acid substitution abolishing the glycosylation process [102]. A compensatory mutation was detected (N124S) to repair the growth defect without creating a new glycosylation site. Thus, the glycan at Asn67 is not necessary for virus growth, but a critical role for this glycan in virion release from mosquito cells was demonstrated [102]. However, HHA resistant virus was found to replicate efficiently in mosquito and insect cells indicating an efficient carbohydrate-independent viral replication in these cell lines. A possible explanation for the differences between our data and data from previous studies could be that the mutant virus has been generated in mosquito C6/36 cells (during replication under antiviral drug pressure) and not in mammalian cells (after introducing the mutations by site-directed mutagenesis). In addition, in previous studies, other amino acid substitutions were generated, resulting in different virus genotypes and subsequently resulting in poorly to predict virus phenotypes.

The glycosylation at Asn67 is demonstrated to be essential for infection of monocyte-derived dendritic cells (MDDC), indicating an interaction between DC-SIGN and the glycan at Asn67 [96,106]. Also the HHA^{res} DENV was not able to infect efficiently DC-SIGN⁺ cells or cells that express the DC-SIGN-related liver-specific receptor L-SIGN. Interestingly, MDDC are also not susceptible for HHA^{res} DENV infection, indicating the importance of the DC-SIGN-mediated DENV infection in MDDC. Moreover, cells of the hematopoietic origin, such as DC, are described to be necessary for DENV pathogenesis [84]. If the CBA resistant DENV is not able to infect DC anymore, it can be stated that the CBAs interfere with a physiologically highly relevant target. DC-SIGN is postulated as the most important DENV entry receptor until now. The entry process of DENV in Vero, Huh-7, BHK-21 and C6/36 cell lines is DC-SIGN-independent and also carbohydrate-independent. Indeed, HHA^{res} DENV can efficiently enter and replicate in these cell lines. HHA^{res} DENV lacking both N-glycans on the envelope E-glycoprotein is able to replicate efficiently in mammalian cells, with the exception of DC-SIGN⁺ cells.

The HHA^{res} virus was used as a tool to identify the antiviral target of other classes of compounds as it could replicate in human liver Huh-7 cells. The use of Huh-7 cells has much more clinical relevance than using monkey (Vero) or hamster (BHK) kidney cells. The HHA^{res} DENV was found cross-resistant to GNA, that recognizes like HHA, α -1,3 mannose residues. UDA, which recognizes mainly the N-acetylglucosamine residues of the N-glycans, also lacked antiviral activity against HHA^{res} DENV in Huh-7 cells. This indicates that the entire backbone of the N-glycan is deleted. Likewise, pradimicin-S (PRM-S), a small-size α -1,2-mannose-specific CBA, was also unable to inhibit HHA^{res} DENV. This

demonstrates that PRM-S targets also the N-glycans on the DENV envelope. In contrast, ribavirin (RBV), a nucleoside analogue and inhibitor of cellular purine synthesis [74], retained as expected wild-type antiviral activity against HHA^{res} DENV. This argues against that there would be compensatory mutations in the non-structural proteins of DENV which are responsible for an overall enhanced replication of the viral genome [107,108]. SA-17, a novel doxorubicine analogue that inhibits the DENV entry process [109], was equipotent against WT and HHA^{res} DENV. The SA-17 compound is predicted to interact with the hydrophobic binding pocket of the E-glycoprotein which is independent from the N-glycosylation state of the E-glycoprotein [109]. These data confirm the molecular docking experiments of SA-17.

Generally, the function of glycosylation on surface proteins is proper folding of the protein, trafficking in the endoplasmic reticulum, interaction with receptors and influencing virus immunogenicity [110]. Virions produced in the mosquito vector and human host may have structurally different N-linked glycans, because the glycosylation patterns are fundamentally different [101,111]. N-glycosylation in mammalian cells is often of the complex-type because a lot of different processing enzymes could add a diversity of monosaccharides. Glycans produced in insect cells are far less complex, because of less diversity in processing enzymes and usually contain more high-mannose and pauci-mannose-type glycans. DC-SIGN can distinguish between mosquito- and mammalian cell-derived alphaviruses [112] and West Nile virus [68], resulting in a more efficient infection by a mosquito-derived virus, but this was not the case for DENV [101].

Although the CBAs HHA and GNA are not mitogenic and not toxic to mice when administered intravenously [113], caution must be taken in the development of the CBAs to use as antiviral drug in the clinic. First, the natural plant lectins are expensive to produce and hard to scale-up, but efforts have been made to express CBAs in commensal bacteria which provide an easy production process of this class of agents. Second, there can be a systemic reaction against the lectins such as in food allergies against peanut lectin or banana lectin [114,115]. Third, the CBAs can recognize aspecifically cellular glycans and could interfere with host cellular processes. But, DENV glycosylation is of the high-mannose or pauci-mannose type, which is only rare on mammalian proteins. The synthetic production of small non-peptidic molecules, such as PRM-S, with CBA-like activity, could overcome the pharmacological problems of the plant lectins. Therefore, PRM-S forms a potential lead candidate in the development of more potent and specific DENV entry inhibitors.

5. Conclusion

In conclusion, besides active vector control in tropical and subtropical regions, there is an urgent need for antiviral treatment to protect half of the world's population against severe DENV infections. DC-SIGN is thought to be the most important DENV receptor and that the DC-SIGN-DENV envelope protein interaction is an excellent target for viral entry inhibitors such as the CBAs. Resistance against HHA forces the virus to delete its N-glycans and subsequently this mutant virus is not able anymore to infect its most important target cells.

Thus the CBAs act in two different ways: prevention of viral entry by directly binding N-glycans on the viral envelope and indirectly forcing the virus to delete its N-glycans and lose the capability to infect DC. The plant lectins provided more insight into the entry pathway of the virus into the host cell. Hopefully some of these future derivatives with a comparable mode of action will reach clinical trials in the near future.

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