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Focus on flaviviruses: current and future drug targets

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Abstract

Background—Infection by mosquito-borne flaviviruses (family *Flaviviridae*) is increasing in prevalence worldwide. The vast global, social and economic impact due to the morbidity and mortality associated with the diseases caused by these viruses necessitates therapeutic intervention. There is currently no effective clinical treatment for any flaviviral infection. Therefore, there is a great need for the identification of novel inhibitors to target the virus lifecycle.

Discussion—In this article, we discuss structural and nonstructural viral proteins that are the focus of current target validation and drug discovery efforts. Both inhibition of essential enzymatic activities and disruption of necessary protein–protein interactions are considered. In addition, we address promising new targets for future research.

Conclusion—As our molecular and biochemical understanding of the flavivirus life cycle increases, the number of targets for antiviral therapeutic discovery grows and the possibility for novel drug discovery continues to strengthen.

Members of the *Flaviviridae* family belong to one of three genera: *Flavivirus*, *Pestivirus* and *Hepacivirus*. **West Nile virus** (WNV), **dengue virus** (DEN) and **yellow fever virus** (YF) are among the more than 70 members of the genera *Flavivirus*, which also includes Japanese encephalitis (JEV) and tick-borne encephalitis. These viruses are transmitted by arthropods, and infection results in symptoms that range from mild fever to severe disease and mortality. WNV differs from both DEN and YF in that it is maintained in a mosquito–bird transmission cycle with humans as the dead-end host rather than in a mosquito–human transmission cycle. DEN is transmitted by the *Aedes* genus of mosquito, YF by *Aedes* and *Haemogogus* and WNV by *Culex*.

Increasing health concern

While many cases of WNV are asymptomatic, clinical symptoms of infection range from headache, fever and rash associated with West Nile fever to seizures, myelitis, polyradiculitis and meningitis, encephalitis and even flaccid paralysis associated with more severe forms of the disease. WNV was first isolated in Uganda in 1937 [1]. Prior to 1999, WNV was found only in the Eastern hemisphere and is perhaps historically the least virulent of the flaviviruses

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examined here. Within the last decade, however, outbreaks of WNV have increased in both frequency and severity. In addition to Africa and West and Central Asia, WNV has now become endemic in Europe and North America [201]. According to the US CDC, in 2008, there were 1370 cases of WNV in the USA, resulting in 37 deaths. More than 45% of the reported cases exhibited the severe symptoms associated with encephalitis and meningitis [201].

Initial symptoms of urban YF include headache, fever, chills and fatigue. These symptoms escalate, generally following a short period of remission, to include hemorrhagic symptoms, jaundice, seizures and coma. YF was first described in the Yucatan in 1648 and was first isolated in Ghana in 1927 [1]. Today, the disease is restricted to sub-Saharan Africa and Central and South America, where the death rate following infection can range from 15 to 50% in unvaccinated populations [202].

Asia, Africa and North America concurrently experienced the first reported epidemics of dengue in 1779–1780 [202], and the first isolation of the virus from humans occurred in 1944 (Hawaii: DEN-1 and New Guinea: DEN-2) and 1957 (Philippines: DEN-3 and DEN-4) [1]. There are four antigenically distinct serotypes of the virus (DEN-1, DEN-2, DEN-3 and DEN-4) and immunity is serotype specific. Symptoms of dengue fever include fever, rash and arthralgia. Dengue hemorrhagic fever is the more severe form of the disease and occurs following reinfection with a second serotype of the virus. Hemorrhagic disease is symptomatic of dengue hemorrhagic fever often giving rise to edema. Dengue shock syndrome occurs when leakage and/or bleeding is sufficient to induce shock. Dengue shock syndrome is often fatal without well-managed fluid replacement.

The re-emergence of the Flaviviruses as significant human pathogens has raised concerns worldwide [2]. Globally, there are an estimated 50–100 million cases of DEN and 200,000 cases of YF reported each year, which result respectively in approximately 20,000 and approximately 30,000 deaths annually. More than 2 billion people are at risk of infection by DEN with 600 million and 1 billion at risk by YF and WNV, respectively [203]. Following the 1999 outbreak of WNV in New York, human disease or infected mosquitoes are now found in all but two US states. [201]. Transmission is not restricted to the poor in tropical areas, and instances of both transmission and of outbreaks are predicted to rise as urbanization and worldwide travel increase and public health infrastructures around the world continue to deteriorate. Indeed, the flavivirus mosquito vector *Aedes aegypti* is now found in southern states of the USA, setting the stage for an outbreak [204]. Despite the morbidity and mortality caused by flavivirus infection, to date, vaccines are only available for YF, tick-borne encephalitis and JEV, and there is currently no effective chemotherapeutic treatment for infection by any member of the *Flavivirus* family. The combined global socioeconomic impact of the flavivirus pathogens necessitates the identification and validation of viral targets (Figure 1) and the rational design of novel antiviral compounds.

Flavivirus replication cycle

The flavivirus genome consists of positive-sense single-stranded (ss)RNA. The approximately 11,000-nucleotide ssRNA bears a 5'-type-1 methyl-guanosine cap structure required for translation initiation, but no 3'-poly-A tail. Acting much like a cellular mRNA, the genome encodes a single open reading frame bordered by 5'- and 3'-untranslated regions (Figure 2). The open reading frame is translated in a single precursor polypeptide that is cleaved by viral and host proteases to produce three structural proteins (C, prM and E) and eight nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, 2K, NS4B and NS5) [3]. The nonstructural proteins are responsible for replicating the viral genome and altering the host cell environment such that replication is efficient and that the host innate immune response does not interfere with replication [3]. Replication takes place in modified membrane structures derived from the

endoplasmic reticulum (ER). A noncapped negative-strand copy of the genome is initially generated by the NS5 RdRp. Large numbers of capped genomic viral RNAs are synthesized to provide a template for translation and production of the precursor polypeptide and for packaging into virions.

The structural proteins C, prM and E and a single copy of positive-strand genomic RNA make up the flavivirus virion, organized into an icosahedral glycoprotein shell with a lipid bilayer surrounding a viral RNA containing nucleocapsid. Viral assembly to produce the noninfectious immature particle occurs on the ER [4] and the endomembrane system and exocytosis are utilized for export of virions from the infected cell. The prM of the immature virion is cleaved by furin, a host cell protease in the low pH of the trans-Golgi. Additional structural rearrangements of the glycoprotein shell [5] result in the production of mature, infectious particles that are released via exocytosis. Viral entry requires E-mediated attachment of the virion to the cell surface and receptor-mediated endocytosis. Internalization is assisted by clathrin-containing rafts within the bilayer of naive cells [6]. Fusion of the viral and cell membranes triggers a pH-mediated and irreversible trimerization of the E protein, which results in particle disassembly and the release of viral RNA into the cytoplasm of the host cell [7,8], initiating a replication cycle.

Current targets

NS3

The NS3 protein is a multifunctional enzyme that contains protease, helicase and RNA triphosphatase (RTPase) activities. The N-terminal 184 amino acids of NS3 enable protease activity and create the first domain of this three-domain protein structure. The remaining two domains consist of 450 amino acids that allow for RNA helicase activity [9–11] and RTPase activity.

NS3Pro activity

An important target within NS3 is the trypsin-like serine protease domain (NS3Pro) [12,13]. For NS3Pro to be active it must be in a complex with its cofactor NS2B. This protease (NS2B/NS3Pro) plays an essential role in the cleavage of the viral precursor polyprotein and disruption of this function is lethal to viral replication [14]. There are two primary avenues for antiviral inhibition of NS3Pro: the first is to target the enzymatic activity of an existing NS2B/NS3Pro complex and the second is to block association of NS2B with NS3Pro.

Initial efforts to optimize existing substrate-like inhibitors of protease activity were unsuccessful [15], with compounds such as benzamidine, PMSF, leupeptin and tosyl-L-lysine chloromethyl ketone failing to inhibit the WNV protease at 100 μ M concentrations [16]. X-ray crystallography has since provided a wealth of information for identifying and optimizing inhibitors of NS3 enzymatic function (details of available structures are provided in Table 1). Most recently, the crystal structure of the full length NS3Pro domain from DEN-4 was reported, and this structure provides tremendous insight into the structural complexity of NS3 [17]. Structural information is also available for substrate-free and inhibitor-bound WNV NS2B/NS3Pro [18]. Collectively, these data have advanced our knowledge of critical interactions between NS2B and NS3, and provide the foundation for the rational design of inhibitors that mimic substrate association. Computational docking of virtual compound libraries into the substrate-binding cleft and high-throughput screening (HTS) of millions of compounds have yet to produce a significant number of new NS2B/NS3Pro inhibitor candidates [19]. Bioassay systems have been more successful in identifying compounds active against **NS3 protease** function. Using luciferase-expressing WNV replicons, substrate-based aldehyde analogs have been identified as potential inhibitors with EC₅₀ values of approximately 1.4 μ M and selectivity

indexes of approximately 100 for WNV [20]. In another study, a cationic tripeptide with a non-peptidic cap at the N-terminus and an aldehyde at the C-terminus was shown to have a K_i of 9 nM against WNV protease activity, to be stable in serum (>90% intact after 3 h at 37°C), cell permeable, and exhibit an EC_{50} of 1.6 μ M with low cytotoxicity (CC_{50} > 400 μ M) [21].

A second avenue for antiviral development targeting NS3 protease is the identification of compounds able to block NS2B/NS3Pro association. Molecular dynamic simulations, principal-component analysis, molecular docking, mutagenesis and bioassays revealed the importance of the NS2B/NS3 complex and of the conformational changes resulting from the association for protease activation [22]. Targeting complex formation has yet to be vigorously tested as a strategy for inhibition, but it has significant potential. The interface between NS2B and NS3Pro has been resolved following crystallography [18,23]. These structural studies and a number of biochemical and virologic experiments have defined the specific interactions between NS2B and NS3 that are important to NS2B/NS3Pro activity [19,24–27], and have led to the preliminary identification of a 5'-amino-1-(phenyl)sulfonyl-pyrazol-3-yl class of compounds suggested by *in silico* docking to interfere with NS2B/NS3Pro association [28].

An increased understanding of the interactions between NS2B/NS3Pro may be gained from structural investigations of the hepatitis C virus NS3 and NS4A complex, and similarities may be exploited for flavivirus antiviral discovery. Historically, poor bioavailability and rapid degradation of peptidomimetic inhibitors identified for inhibition of hepatitis C virus (HCV) has compromised their effectiveness as antivirals. However, recent evaluation of the peptidomimetic inhibitor compound 3 and the tripeptide ketoamide compound, SCH 503034, suggest that the use of peptidomimetic inhibitors may indeed be viable [21,29]. Macrocyclic compounds are also performing well in both enzymatic assays and in cell-based replicon systems, and are yielding promising results in clinical trials. BILN 2061 provides confirmation for the application of macrocyclic compounds: after patients infected with HCV were treated with the macro-cyclic BILN 2061, there was an impressive reduction in HCV RNA plasmid levels 2 days after treatment [30]. BILN 2061 has also undergone numerous pharmacological tests to investigate its preclinical safety, and it was found to be well tolerated in various *in vitro* and *in vivo* assays with good metabolic properties in a variety of species [30]. The positive results obtained for HCV protease inhibitors are encouraging and reinforces the potential for developing protease inhibitors targeting the flavivirus NS2B/NS3Pro.

NS3 helicase activity

Helicase activity is required for the separation of dsRNA formed during viral replication and for the removal of proteins bound to the viral RNA during replication. For either activity, ATP is required and the two C-terminal domains of the NS3 protein comprise the ATPase activity [20]. Mutational analysis has shown that alteration of helicase, RTPase or ATPase activities results in the reduction or elimination of viral replication [10,31,32].

Recently, several high-resolution structures of the flaviviral NS3 have been solved (details provided in Table 1) which illustrate the expected three protein domains. However, the protease and helicase domains build an unexpected elongated structure with an RNA-binding tunnel that transverse the length of the protein. The structures are important because they represent a divergence from the hitherto accepted (HCV) model for helicase structure/conformation in mosquito-borne flaviviruses [33,34].

The **NS3 helicase** is a challenging target for drug design because its enzymology is not clearly understood. The most likely target of the NS3 helicase is the ATPase-binding site, however, this target is potentially problematic because of selectivity issues [20,35]. Currently, halogenated benzotrioles have been shown to inhibit WNV helicase activity by targeting ATPase activity and ring-expanded nucleosides have also been reported to inhibit JEV and WNV

helicases [35,36]. Sampath *et al.* demonstrated that an alanine substitution of Lys 396 drastically reduces helicase activity [10]. Interestingly, the amino acid substitution decreased activity to a greater degree than current small-molecule inhibitors [10,20]. The location of Lys 396 on the surface of domain II suggests that rather than targeting the ATP-binding sites for inhibition, perhaps the nucleoside-binding pockets surrounding the RNA-binding tunnel should be considered (see 'Future perspective' section).

RNA-dependent RNA polymerase

The **RNA-dependent RNA polymerase** (RdRp) is a strong candidate target for ant flaviviral drug development. The RdRp is located within residues 273–900 of NS5, and is responsible for synthesis of both the negative-strand RNA and positive-strand genomic RNA. Because the RdRp is essential for viral replication and a similar enzyme is not found within host cells, it is considered of great value as a target for the development of potent and specific antivirals.

Flavivirus negative- and positive-strand RNA synthesis is a complex and dynamic process, and an excellent review of the replication process and RdRp function was recently published [37]. Briefly, viral RNA released into the cytoplasm from infecting virions is first used for translation of the viral polyprotein, and the newly translated NS5 RdRp enzyme initiates synthesis of a negative-sense copy of the genomic viral RNA. Once bound to the 3'-untranslated region, the RdRp (in conjunction with the helicase activity of NS3) will polymerize a full-length negative-strand copy of the genomic RNA. The function of the negative-strand RNA is to serve as a template for the synthesis of a large number of positive-strand genomic RNAs. The RdRp recognizes and binds to the double-stranded, positive-negative RNA duplex and, in combination with NS3 helicase activity, synthesizes a nascent positive-strand RNA, displacing the original positive strand from the RNA duplex in the process. In a coupled process, the newly synthesized positive-strand RNA is capped by the combination of the NS3 RTPase, NS5 MTase and an as yet unidentified GTase.

The X-ray crystallographic structures for WNV and DEN-3 RdRp domains were recently solved [38,39] and both structures have the same basic structural components that characterize all known RdRp enzymes (i.e., palm, finger and thumb domains) [40]. When comparing the WNV and DEN-3 RdRp structures, most of the variability is observed within the finger domains, with the palm domain being the most conserved. DEN-3 RdRp assumed an open conformation and the WNV structure showed the finger subdomain rotated towards the thumb by an angle of approximately 8° [39]. The individual domains are similar to those of a DNA polymerase, however, the flavivirus RdRp does appear to have some distinctive characteristics. For example, the DEN-3 RdRp domain has fingertips that connect the thumb and the finger domains, allowing for a more compact, spherical shape [39].

Currently, HTS against DEN-2 RdRp using chemical libraries from both academic and commercial sources is being performed to identify both nucleoside inhibitors (NI) and non-NIs (NNIs) in order to find a suitable antiviral drug [37]. NIs are usually converted to nucleotide analogs by kinases present in the host cells and target the active site of the polymerase [41]. If the NI binds to the RdRp domain, the elongation processes that the polymerase performs is terminated. NNIs tend to bind to allosteric surface cavities that are located throughout the target polymerase, do not directly compete with NTPs and generally inhibit the polymerase at a stage prior to the elongation reaction [41]. NNIs have been shown to inhibit the replication of bovine virus diarrhea virus with high efficiency, and these allosteric inhibitors may also have an effect on WNV, DEN and YF. Targeting NNI-binding sites in HCV is a current focus for drug development [37], and covalent inhibitors with submicromolar biochemical affinity have been observed [42]. To date, two cavities predicted to be potential target locations for NNIs have been identified within the DEN-3 RdRp domain and five cavities identified within the WNV RdRp domain [37].

NS5 N-terminal methyltransferase adomet binding

The N-terminal, approximately 260 amino acids, of the NS5 protein contains the **methyltransferase** enzyme necessary for the formation of the mature RNA cap structure present at the 5'-- end of the viral genomic RNA. S-adenosyl methionine (AdoMet) is used by methyltransferases as a methyl group donor for substrate modification. Mutation of residues involved in NS5 AdoMet binding disrupt viral replication [43,44]; however, identifying compounds that specifically inhibit flavivirus MTase AdoMet binding, but not cellular methyltransferase activity, may be problematic due to the conserved nature of the AdoMet binding motif. Indeed, the conserved KDKE tetrad motif was initially used to classify the N-terminus of NS5 as a methyltransferase [45]. Additionally, the postreaction methyltransfer product AdoHyc is copurified and co crystallized in the AdoMet-binding site of all flavivirus MTase proteins crystallized to date [44,46,47], indicating that the site is likely to be occupied at high frequency *in vivo*. The K_d of AdoMet binding to the flavivirus MTase has not been empirically determined, but the presence of AdoHyc in the MTase crystal structures indicates that at least AdoHyc is relatively tightly bound (Figure 3) [44,47–50]. Furthermore, published methyltransferase assays detect post-reaction products rather than directly detecting ligand displacement, making *in vitro* HTS problematic [51]. However, the tight association of AdoMet to the MTase and the well-resolved crystal structures have allowed for virtual screening of compounds that could displace AdoMet. Luzhkov *et al.* identified one compound from *in silico* screening (compound 7) that could displace AdoMet from the MTase protein in an *in vitro* assay [52]. No toxicity or selectivity data has been reported for compound 7, but as a novel scaffold, it may be a valuable starting point for subsequent medicinal chemistry efforts to identify potent and selective AdoMet analogs that possess suitable toxicity and bioavailability profiles for further drug development.

NS5 N-terminal methyltransferase GTP binding

The GTP-binding domain of the NS5 N-terminal MTase domain is involved in the 2'-O methylation reaction [43,53,54]. Mutation of amino acids in the DEN MTase RNA cap-binding domain abolishes viral replication [43,55,56], indicating that GTP and RNA cap binding are essential to viral replication and may represent a novel drug target site. Comparison of the GTP-binding site structure from the various guanine-binding proteins in the PDB database with the flavivirus MTase GTP-binding site suggests that the flavivirus MTase proteins utilize a novel architecture for binding guanosine [49]. Most GTP-binding proteins with known structures interact with the guanine base via the guanine C-6 carbonyl group and two aromatic or charged residues that sandwich the guanine ring [57–59]. By contrast, the flavivirus MTases bind the cap on only one face of the guanine base, leaving the other highly exposed (Figure 3). The MTase Leu 16 and Leu 19 peptide backbone carbonyl groups interact with the guanine N-1 atom (via a conserved water bridge) and C-2 amino group. There is also a strong, directed stabilizing effect observed between the guanine ring and Phe24 [47,49,50]. These data suggest that the N-1 and C-2 positions represent the major determinants for specificity. This novel guanosine-binding configuration, including the lack of interaction with the C-6 carbonyl and the presence of the N-1 water bridge coupled with the open guanine-binding architecture, represents a significant difference as compared with known endogenous GTP-binding proteins, and may allow for virus-specific compounds to be identified. In addition, the GTP-binding site is highly amenable to *in vitro* HTS due to the availability of stable fluorescent GTP analogs [47]. Finally, all flavivirus NS5 MTase proteins appear to adopt the same mechanism to bind GTP, indicating that broadly active antivirals can be developed toward the GTP-binding site. Collectively, these factors advocate the flavivirus MTase RNA-cap-binding site as an ideal target for antiviral drug development. Our group has performed *in vitro* HTS for compounds that displace GTP from the MTase cap-binding pocket, and has identified a number of molecules with K_i values of less than 10 μ M (Unpublished Data). Additional *ex vivo* and structure-based drug-design testing is underway to further optimize potential inhibitors. While

little has been published to date on antiviral development targeting this domain, the conservation of the GTP-binding site amongst flaviviruses, ease of HTS assay development, and critical nature of GTP binding for viral replication makes the MTase an exciting new target for novel drug development [60].

E protein

The envelope (E) protein, which forms the glycoprotein shell of the virion consists of three domains: D1 (N-terminal), DII (includes a hydrophobic fusion peptide) and DIII (containing an immunoglobulin-like domain). Domain DII is involved in the dimerization of the E protein, and DIII is thought to play a role in receptor binding and antibody neutralization.

Receptor binding is a crucial step for virion internalization into host cells, and the interaction between the E protein and the host cell via a specific receptor would provide an important avenue for therapeutic intervention. To date, however, flavivirus receptor molecules have not been conclusively identified. Numerous of cellular proteins shown to be capable of mediating virus attachment including integrins [61], C-type lectins [62–65] and non-Fc receptor proteins [66,67]. Glycoproteins have also been identified as part of the receptor complex in the *Aedes albopictus* C6/36 cell line [68]. The C-type lectin, dendritic cell-specific intercellular adhesion molecule (ICAM)3-grabbing nonintegrin (DC-SIGN) is essential for DEN infection. Both soluble DC-SIGN and anti-DC-SIGN antibodies inhibit infection [64,65]. However, recent studies suggest that DC-SIGN is not the actual receptor and may be acting as an attachment molecule [63]. Cryo-EM reconstruction of DC-SIGN in complex with DEN-2 E protein propose Asn 67 as to the primary site for interaction with the DEN-2 E protein [69]. WNV E protein has also been shown to interact with a C-type lectin (DC-SIGNR) [70], however, YF lacks E protein glycan modifications. Whether the essential receptor interaction can be blocked directly preventing viral entry will be dependent on the identification of the specific receptor.

As the flavivirus virion progresses from the immature to the fusion-activated state, the glycoprotein shell undergoes three essential structural rearrangements: the prM and E heterodimers first rearrange into E homodimers prior to virion release, and the acidified endosomes subsequently reorganize the E homodimers into the fusion-competent E homotrimeric form. Each structural rearrangement is essential for viral maturation, and as such, each provides a potential target point for therapeutic intervention. Mounting knowledge of each structural rearrangement is the result of analyses of the growing number of atomic resolution structures for the E protein available in both pre- and postfusion conformations. Available E structures are included in Table 1. However, as the focus of this review is the current approaches and targets for drug design, details of the protein–protein interactions and structural rearrangements are not included here, and have been recently reviewed elsewhere [5]. In addition to providing insights into protein–protein interactions and rearrangements, the structural information has provided insights in to the actions of recombinant therapeutic antibodies [71–73].

Inhibition of essential protein–protein interactions is a path towards intervention that is currently being explored. Expression of recombinant forms of DIII have successfully (and specifically) prevented viral membrane fusion and blocked entry [74–77]. Fusion inhibition by a soluble DEN-2 DIII is explained mechanistically by the association of recombinant DIII with homotrimer fusion intermediates [76].

In 2003, the β -OG (*N*-octyl- β -D-glucoside)-binding pocket was identified as a potential small molecule target site: as a result of crystallographic studies of the E protein [78], the small hydrophobic channel was identified located between the DI and DII domains of E protein monomers. Mutational analysis of the hydrophobic residues that line the channel revealed the influence of these residues on the threshold pH necessary for fusion [78]. Access to the open-

ended channel is governed by the movement of the E protein kl loop, and targeting this pocket during virus assembly is predicted to inhibit subsequent virus entry. Recently, a family of thiazole molecules able to inhibit viral replication in cell-based assays was identified by a virtual screening and optimization paradigm [79]. A docking and scoring approach was also implemented to identify a compound observed to compete for the β -OG pocket [80] and block replicon amplification. The highest affinity compounds resulting from both studies were effectual at low micromolar concentrations.

Future perspective

As more becomes known about the molecular mechanisms governing flaviviral entry, replication and virogenesis, a host of new and exciting targets for chemotherapeutic intervention are identified for drug development. Research is expected to increasingly focus on these targets over the next 5–10 years. In this section, we touch on some of the most promising new targets and speculate on the possible ways that each may be utilized in *in silico*, *in vitro* and *ex vivo* drug-screening campaigns to identify potent and specific inhibitors of viral replication.

Inhibitors of virion assembly

Flavivirus virions are relatively simple structures composed of a large number of repeating protein units. The flavivirus virion is composed of an inner shell formed by interacting subunits of highly basic capsid (C) protein that interact with genomic RNA, an intermediate shell composed of membrane derived from the host ER, and an outer shell containing the viral glycoproteins M and E proteins. The nucleocapsid (RNA + C) is assembled in direct association with the RNA replication complex on the cytoplasmic face of the ER, and the viral glycoproteins (E and prM) drive virion budding [81–83]. The RNA-containing nucleocapsid associates with the budding glycoproteins, and the immature virion is released into the lumen of the ER. Immature virions are trafficked through the Golgi apparatus, where both the prM and E proteins are *N*-glycosylated and prM is cleaved to M by the furin protease. Maintenance of the immature virion until release from the cell is dependent on the interaction of E with prM, as prM inhibits the rearrangement of the E protein to a fusogenic conformation until the mature virion is released thus preventing reinfection of the cell. It is apparent that virion assembly is a highly orchestrated process, and chemotherapeutic disruption of any step of the process will severely affect viral viability.

Capsid dimerization

Several points along the virion assembly pathway provide intriguing targets for antiviral drug development. Nucleocapsid assembly is one such target. As mentioned earlier, the nucleocapsid is composed of one copy of the genomic viral RNA and an indeterminate number of the 11-kDa C protein. The flavivirus C protein is separated from prM on the viral polypeptide via NS3-mediated cleavage, and mature C associates with the cytoplasmic surface of the ER. The positive charge in the amino acid region 75–97 of C is thought to interact with genomic viral RNA [84]. The solution structure of the DEN-2 C protein indicates that the C protein forms dimers, with helix $\alpha 2$ and $\alpha 4$ forming the dimerization domain, and helix $\alpha 4$ presenting a highly basic surface that likely interacts with the phosphate backbone of the viral genomic RNA. Both the helical association and charged surface interaction present possible avenues of therapeutic intervention. Disruption of the basic charge on helix $\alpha 4$ may inhibit RNA binding to the capsid dimer and disrupt virion formation. However, it is unlikely that a small-molecule inhibitor could shield sufficient basic charges along the $\alpha 4/\alpha 4'$ dimer to significantly disrupt the protein–RNA interaction at a clinically available concentration. A perhaps more promising approach is to either disrupt or destabilize C–C dimerization via the interaction of helix $\alpha 2/\alpha 2'$ or $\alpha 4/\alpha 4'$. The geometry of C–C dimer interaction is very precise based on the solution

structure of the DEN C protein, and chemicals that bind between the homologous or heterologous helices may interfere with nucleocapsid formation and virion assembly, or could stabilize the nucleocapsid such that virions cannot efficiently release their genomic RNA into cells. Viral capsid disruption has been utilized against viruses such as hepatitis B virus [85, 86], Sindbis virus [87,88] and HIV-1 [89–91]. Several approaches for identifying capsid-disrupting compounds are possible. Since the NMR structures of the DEN capsid dimer are available, rational drug design methodologies can be used to identify both potential sites of disruption and chemical entities that may bind between the dimers. Alternatively, approaches that monitor capsid dimerization may be utilized. These may include *in vitro* fluorescence energy transfer (FRET)-style screens with purified and chemically labeled protein, or live cell screening using new fluorescence techniques such as bimolecular fluorescence complementation (BiFC) to identify compounds that disrupt C–C dimerization (Figure 4).

Heterologous structural protein interactions

Disrupting the interactions between viral structural proteins (C, M and E) is another potential area for antiviral development. However, these interactions may represent difficult drug development targets for several reasons. Cryoelectron microscopic reconstruction of WNV and DEN virions indicate that the N-terminal receptor binding and fusion domains are on the exterior of the virion and the carboxyl termini of the proteins do not extend significantly into the virion interior [92,93]. The lack of a significant cytoplasmic tail for E or prM makes it difficult to determine how C may specifically interact with the proteins, although the presence of a hydrophobic region on the C dimer (helix $\alpha 1$, $\alpha 2$, $\alpha 1'$ and $\alpha 2'$) may provide some interaction with the minimal E endodomain that is not appreciated in the cryo-EM structures. Alternatively, C may not form direct interactions with E or prM, but rather may interact directly with membranes. If this is the case, compounds that bind to the hydrophobic region in the C dimer and extend past the surface of the helices may hinder capsid dimers interaction with the membrane. In either case, targeting the hydrophobic pocket of the capsid dimer may be a viable option for disrupting the C-C, C-E or C-M interaction and viral budding.

Likewise, disruption of the interaction between M and E may prove to be difficult. Based on the cryo-EM reconstructions described earlier, there are no conserved contacts visible between the E or M coiled coil transmembrane helices that could account for specific interaction. While this does not eliminate the helix–helix interaction between M and E as a potential drug target, it does limit the usefulness of rational design methodologies in identifying compounds that block this interaction. A more promising target for interfering with M:E interactions is within the ectodomains of both proteins. During virion maturation, prM inhibits the membrane fusion activity of E during egress of the immature virion through the trans-Golgi network [94]. Cleavage of prM by the cellular furin protease [95] enables the E protein to homodimerize and the virions to become infectious [96,97]. The crystal structure of the ectodomain of prM complexed with the E ectodomain at low pH has been solved [98], a development that appears to provide a number of interesting targets. Inhibition of the interaction between the ectodomains of prM and E could be addressed by several mechanisms, including *in silico* modeling of compounds that specifically bind to the interaction interface or through *in vitro* HTS campaigns similar to those described earlier to identify candidates that interfere with C-C homodimer formation. Alternatively, compounds could be identified that bind to the furin protease cleavage site on prM, which would not block the release of virion particles but would make the particles unable to enter naive cells and essentially non-infectious.

Disruption of viral replication

Viral genomic replication is a highly orchestrated process that is essential to the production of progeny virus, and as such is a rich environment for the discovery of new antiviral targets and compounds. Currently, two viral nonstructural proteins, NS3 and NS5, are considered prime

targets for antiviral development due to their essential roles for viral RNA replication. The currently targeted enzymatic functions of NS3 helicase/protease and NS5 are described earlier. There are several additional antiviral development targets within both the NS3 and NS5 proteins, as well as within the other viral nonstructural proteins, that have yet to be exploited.

NS3 RTPase domain

The known RNA capping machinery for flaviviruses is encoded within two flaviviral enzymes, NS3 (RTPase) and NS5 (MTase). The NS3 RTPase binds to the 5'-triphosphate of the nascent positive-strand viral RNA and removes the γ -phosphate [99], resulting in a 5'-diphosphorylated RNA, which is the substrate for the guanosine monophosphate moiety transferred from the 5'-RNA guanylyltransferase (GTase). A low-resolution crystal structure of the Kunjin NS3 helicase/NTPase/RTPase domain was recently solved [33], but is not currently of sufficient resolution to be useful in rational drug design efforts. However, with greater resolution, rational design efforts can be utilized to find compounds that bind to and inhibit the ATP or RNA-binding site. In the meantime, an HTS assay to monitor the release of the γ -phosphate based on scintillation proximity assays could be developed, although this would require a greater understanding of the biochemistry of phosphate removal and RNA binding to the NS3 protein to discriminate between inhibitors of helicase and RTPase activity.

NS5 RNA binding

The NS5 MTase domain binds to viral RNA and methylates both the guanine cap and the ribose 2'-OH on the viral RNA. Disrupting the essential step of RNA association to the methyltransferase would impair its function and halt viral replication. The exact mechanism for RNA binding to the methyltransferase domain has not yet been definitively demonstrated, but there is growing evidence that the RNA could interact with a basic channel formed by helix $\alpha 3$ and the random coil region between β -sheets $\beta 2$ and $\beta 3$ [49]. The highly basic nature and apparent conservation of charge structure suggest that this region may bind nonspecifically to RNA. Strategies that can monitor displacement of RNA from a protein, such as fluorescence polarization assays or high-throughput scintillation proximity assays, could be used in HTS to identify compounds that interfere with RNA binding. Alternatively, as the structure of the putative RNA-binding domain is known (Figure 3), structure-based drug design could be used to develop compounds that fit tightly and selectively into the RNA-binding channel of the NS5 enzyme. An exciting concept is chemically linking compounds that individually block GTP binding and RNA binding such that both sites are occluded simultaneously (Figure 4). The conjugation strategy has been shown effective using pegylated interferon linked to ribavirin [100]. Such an approach would be advantageous for limiting antiviral resistance, as the likelihood of multiple resistance mutations arising within one generation of viral replication is extremely low.

Nonstructural protein interactions

Viral RNA replication is a complex process that involves each of the nonstructural proteins in various capacities. The role of each nonstructural protein in replication has been reviewed previously [3]. While the basic function of each viral protein in RNA replication is known, the overall organization of the viral replication complex is incompletely understood. However, it is clear that multiple protein-protein interactions are required for proper RNA replication and packaging. Some previously described nonstructural protein interactions have been the subject of intense investigation. The interaction between NS3 and the linker region of NS5 has been shown to stimulate the NTPase activity of NS3 [101]. Furthermore, the RdRp domain of NS5 is known to interact with the NS3 helicase [102]. The polymerase is predicted to require the helicase activity during replication of dsRNA and for effective capping, and as such the NS3-NS5 interaction provides an additional target for intervention. Unfortunately, molecular details

of the interaction are currently unavailable. While what is known of viral nonstructural protein interactions provides valuable information about some aspects of the viral replication complex, a more systematic analysis of nonstructural protein interactions needs to be performed. Live cell-imaging techniques, such as BiFC or FRET, should be adapted to determine relevant interactions between nonstructural proteins, and advanced techniques, such as BiFC-FRET and targeted peptide disruption, can be used to define ternary interactions or interaction interfaces, respectively (Figure 4). A valuable offshoot of these studies would be that the BiFC or FRET systems may be useful in downstream HTS drug screening assays to identify novel inhibitors that disrupt relevant protein–protein interactions.

Numerous cellular proteins are also known to be involved in the replication of viral RNA as determined by biochemical and genetic studies [103–110]. Elucidation of the complement of viral nonstructural–cellular protein interactions that form the replication complex will provide a rich target environment for future drug development. However, the number of experiential interactions described in the literature is likely far smaller than the actual number of interactions that take place *in vivo* and, in addition, interactions between the nonstructural proteins and cellular proteins may be cell-type specific. Some steps are currently being taken to overcome this dearth of interaction information. Recent RNAi screening studies have begun to clarify the cellular proteins that are important for viral replication [107], and proteomic approaches are beginning to reveal the complement of cellular proteins that are induced during flavivirus infection [111]. These approaches do not provide direct information about interactions occurring during infection, and to date a comprehensive analysis of the infection interactome has not yet been reported. Such a study could utilize techniques such as reporter complementation screens (e.g., yeast-two-hybrid, BiFC and FRET) using various human tissues libraries to identify new and important interactions that may be downstream targets for antiviral drug development.

Evasion of the host innate immune response

Viral replication within the host cell is generally detrimental to the viability of the host cell and the host organism. As such, hosts have developed molecular mechanisms to limit or cease viral replication both at the innate and adaptive immune level. Flaviviruses have, however, developed their own mechanisms for interfering with or evading the host's innate immune response. These evasion mechanisms generally work by inactivating the IFN- α/β signal transduction pathway. Targeted disruption of the viral immune evasion mechanisms using either novel small-molecule inhibitors or peptide therapeutics may allow the host's innate immune response to act against viral replication and rapidly eliminate the infection.

Interferon antagonists

Several lines of evidence indicate that flaviviruses specifically inhibit interferon signaling in infected cells. These interferon antagonistic activities predominantly reside within several of the nonstructural proteins, including NS1, NS2A, NS4A, NS4B and NS5. The known functions of these proteins have been reviewed elsewhere [3]. We will briefly touch on NS1 and NS2A as examples of the potential of interferon antagonists, although the anti-interferon activity of NS4A, NS4B and NS5 may also be exploitable as novel antiviral targets.

NS1 from WNV has recently been shown to interfere with Toll-like receptor (TLR)-3 activity [112]. In this study, NS1 was shown to block TLR-3-mediated nuclear translocation of IRF-3 and NF- $\kappa\beta$, blocking activation of IFN- α and NF- $\kappa\beta$ promoters. NS1 is localized in the ER lumen and secreted from infected mammalian cells, and while the mechanism of NS1-mediated TLR-3 inhibition is currently unclear, it is feasible that NS1 interacts and inhibits TLR-3 function on the extracellular surface of the plasma membrane or in endosomes. As biochemical data concerning the possible interaction between NS1 and TLR-3 become available, techniques

to monitor disruption of the NS1–TLR-3 interaction could be used in HTS campaigns to identify compounds that block this interaction and allow TLR-3 signaling to occur normally. The structure of TLR-3 ectodomain is known [113], and elucidation of the contact points between NS1 and TLR-3, whether biochemically or structurally, would provide an excellent starting point for the development of antivirals that disrupt this interaction.

NS2A is a small (~22 kDa) hydrophobic protein embedded within the ER membrane of infected cells. NS2A is important in virion assembly, but accumulating evidence suggests that NS2A is also disrupts activation of IFN- α/β via an as yet unknown mechanism [114–116]. A mutation in NS2A (Ala30Pro) abrogates NS2A-mediated interference with IFN- β signaling, but does not disrupt its function in viral replication and virion assembly [116]. The defect in IFN- β antagonist activity with the Ala30Pro mutant provides an excellent starting point for determining the interactions necessary for interferon repression via functional genomics or biochemical purification approaches. Obtaining X-ray crystallographic information for transmembrane proteins such as NS2A is technically difficult and, as such, *de novo* rational drug design methods may not be immediately useful for anti-NS2A drug development. However, bioassays in stable NS2A-expressing A549 cell lines that monitor de-repression of IFN- β -linked reporter genes in the presence of small-molecule libraries may be useful for identifying compounds that interfere with the anti-interferon activity of NS2A.

Conclusion

While the need for antiviral compounds able to halt flaviviral infections is critical in light of the significant worldwide mortality and morbidity associated with WNV, DEN and YF infections, there is currently no available treatment for a flavivirus infection. In this review, we provide insights into the current state of flavivirus drug discovery and provide our analysis for future directions. There is a plethora of enzymatic targets and protein–protein interactions that are either currently under exploration or are possible future avenues of inquiry. While the identification of candidate molecules as new therapeutic agents to target one or multiple flaviviruses remains in its infancy, there is exciting potential for future success.

Executive summary

Increasing health concern

- Insect-borne flaviviruses (e.g., West Nile virus, dengue virus and yellow fever virus) are responsible for significant worldwide morbidity and mortality, yet no effective therapeutics are currently available to treat infected individuals.
- The identification and validation of novel flaviviral drug targets and the development of safe and cost-effective therapeutic compounds is essential for combating these diseases, especially in developing countries.

Targets currently under consideration

- NS3 protease, NS3 helicase, RNA-dependent RNA polymerase, NS5 methyltransferase (AdoMet and GTP binding sites) and E protein are structural and nonstructural targets of current efforts to identify novel inhibitory chemical entities.
- Current efforts are largely focused on inhibiting enzyme ligand binding, disrupting enzymatic function or blocking protein conformational changes.

Future targets

- New insights into protein structure, rational drug-design methodologies and high-throughput screening techniques have identified valuable new targets for antiviral drug development.
- Promising new targets include interfering with the association of viral structural proteins involved in virion assembly, disrupting interactions between nonstructural proteins and cellular proteins that mediate viral replication, and attenuation of the viral mechanisms used to evade the host innate immune response.

Conclusion

- Our understanding of the molecular biology and biochemistry of flavivirus replication and virogenesis has matured over the last 2 decades.
- There exists a plethora of current and future targets that may be exploited for therapeutic intervention.
- Although the process of identifying novel antiviral drugs remains in its infancy, there is exciting potential for future success.

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Glossary

Flavivirus	A genus of small, enveloped, positive-sense single-strand viruses commonly transmitted to humans or animals by insect vectors
West Nile virus	A member of the genus that is maintained in a bird–mosquito transmission cycle and that can cause encephalitis in humans
Dengue virus	A member of the genus. It has four immunologically distinct serotypes (DEN-1–4) that can cause severe hemorrhagic fever in humans
Yellow fever virus	A member of the flavivirus genus that can cause acute liver dysfunction in humans
NS3 protease	Nonstructural protein 3 protease. The enzyme responsible for post-translational cleavage of the viral polyprotein
RNA-dependent RNA polymerase	The enzyme responsible for replicating the viral RNA genome
NS3 helicase	Nonstructural protein 3 helicase. The enzyme involved in unwinding double-stranded RNA intermediates formed during viral RNA replication
Methyltransferase	Located at the N-terminus of NS5, this enzyme is responsible for methylating the viral RNA cap structure

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- of considerable interest

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Figure 1. Stages in the flavivirus lifecycle that could be targeted for therapeutic intervention
ER: Endoplasmic reticulum.

Figure 2. Polyprotein precursor: structural proteins (brown) and nonstructural protein (green)
UTR: Untranslated region.

Figure 3.

(A) Surface representation of the yellow fever NS5 N-terminal methyltransferase domain indicating locations of GTP cap-binding domain, AdoMet-binding domain and RNA/phosphate-binding site. (B) Relative location of the three binding sites. (C) Yellow fever NS5 N-terminal methyltransferase residues associated with GTP binding to the GTP cap-binding site. (D) Yellow fever NS5 N-terminal methyltransferase residues associated with AdoMet binding to the AdoMet-binding site [47].

Figure 4. BiFC and FRET are two cell-based techniques to monitor protein–protein interactions
(A) In BiFC, the targeted proteins are linked to the N-terminal and C-terminal halves of a fluorescent protein (YFP). If the targeted proteins are within 15 nm of each other and in the correct spatial orientation, the N- and C-terminal halves of YFP can re-fold and form an active fluorophore that produces a detectable emission signal upon excitation. If the targeted proteins are farther apart than 15 nm, the N- and C-terminal halves of YFP are not able to re-associate and will not produce an emission signal upon excitation. In FRET, the two proteins whose interactions are being monitored are linked to two different fluorescent proteins whose emission (CFP) and excitation (YFP) wavelengths overlap. If the targeted proteins (and their fluorescent partners) are within 1–10 nm of each other, the emission of CFP is capable of exciting YFP and producing a YFP emission signal. If the proteins are farther apart than 10

nm, the emission from CFP is not able to excite YFP, and no YFP emission is observed. BiFC: Bimolecular fluorescence complementation; FRET: Fluorescence resonance energy transfer.

Table 1

Available structural information for West Nile virus, dengue virus and yellow fever virus proteins.

PDB-ID	Virus	Resolution (Å)	Notes	Ref.
<i>NS3 protease</i>				
2QID	DEN	2.1	Complex with mung-bean Bowman-Birk inhibitor	[117]
1DF9	DEN-2	2.1	Complex with mung-bean Bowman-Birk inhibitor	[117]
1BEF	DEN-2	2.1		[118]
<i>NS3 helicase</i>				
1YMF	YF	2.6	Complex with ADP	[119]
1YKS	YF	1.8		[119]
2JLZ	DEN-4	2.2	Complex with ssRNA, ADP and manganese glycerol	[9]
2JLY	DEN-4	2.4	Complex with ssRNA, ADP phosphate, chloride ion and manganese ion	[9]
2JLX	DEN-4	2.2	Complex with ssRNA, ADP vanadate, phosphate ion and glycerol	[9]
2JLW	DEN-4	2.6	Complex with ssRNA2, chloride ion, manganese ion and glycerol	[9]
2JLV	DEN-4	1.9	Complex with ssRNA and AMPPNP	[9]
2JLU	DEN-4	2.0	Complex with ssRNA and glycerol	[9]
2JLS	DEN-4	2.2	Complex with ADP, β-mercaptoethanol, chloride ion, manganese ion and glycerol	[9]
2JLR	DEN-4	2.0	Complex with AMPPNP	[9]
2JLQ	DEN-4	1.7	Apo enzyme with chloride ion and glycerol	[9]
2BMF	DEN-2	2.4	Residues 1646-2093	[120]
2BHR	DEN-2	2.8	Residues 1646-2093, complexed with sulfate ion	[120]
<i>NS5 methyltransferase</i>				
2OY0	WNV	2.8	Complex with S-adenosyl-L-homocysteine	[44]
2P41	DEN-2	1.8	Complex with 7MeGpppG2'OMe and S-adenosyl-L-homocysteine	[50]
2P40	DEN-2	2.7	Complex with 7MeGpppG	[50]
2P3Q	DEN-2	2.8	Complex with GpppG and S-adenosyl-L-homocysteine	[50]
2P3O	DEN-2	2.8	Complex with 7MeGpppA and S-adenosyl-L-homocysteine	[50]
2P3L	DEN-2	2.2	Complex with GpppA and S-adenosyl-L-homocysteine	[50]
2P1D	DEN-2	2.9	Complex with GTP and S-adenosyl-L-homocysteine	[50]
1R6A	DEN-2	2.6	Complex with S-adenosyl-L-homocysteine, ribavirin 5' triphosphate and sulfur ion	[46]
1L9K	DEN-2	2.4	Complex with S-adenosyl-L-homocysteine and sulfate ion	[49]
3EVA	YF	1.5	Complex with S-adenosyl-L-homocysteine	[47]
3EVB	YF	1.9	Complex with S-adenosyl-L-homocysteine	[47]
3EVC	YF	1.6	Complex with S-adenosyl-L-homocysteine and GTP	[47]
3EVD	YF	1.5	Complex with S-adenosyl-L-homocysteine and GTP	[47]
3EVE	YF	1.7	Complex with S-adenosyl-L-homocysteine and GpppA	[47]
3EVF	YF	1.5	Complex with S-adenosyl-L-homocysteine and me7-GpppA	[47]
3EVG	DEN-2	2.2	Complex with S-adenosyl-L-homocysteine	[47]

PDB-ID	Virus	Resolution (Å)	Notes	Ref.
<i>NS5 RNA-dependent RNA polymerase</i>				
2HFZ	WNV	3.0	Complexed with magnesium	[38]
2HCS	WNV	2.3	Residues 317-905, complexed with zinc	[38]
2HCN	WNV	2.4	Residues 317-905, complexed with calcium	[38]
2J7U	DEN-2	1.9	complexed with chloride, magnesium, zinc and glycerol	[39]
2J7W	DEN	2.6	Complexed with 3'DGTP and zinc	[39]
<i>Envelope protein</i>				
2I69	WNV	3.1		[71]
2HG0	WNV	3.0		[121]
2R6P	DEN-2	C-EM	Complex with Fab 1A1D-2	[122]
1UZG	DEN-3	3.5	Envelope protein	[123]
1TG8	DEN-2	2.6	E glycoprotein	[124]
1OK8	DEN-2	2.0	In postfusion conformation, complex with <i>N</i> -acetyl-D-glucosamine and chloride ion	[8]
1OKE	DEN-2	2.4	Complex with <i>N</i> -octyl-β-D-glucoside and <i>N</i> -acetyl-D-glucosamine	[78]
1OAN	DEN-2	2.8	Complexed with <i>N</i> -acetyl-D-glucosamine and sodium ion	[78]
1S6N	WNV	NMR	Domain III, strain 385-99	[125]
2P5P	WNV	2.8	Domain III	[126]
1ZTX	WNV	2.5	Domain III in complex with neutralizing E16 antibody fab	[121]
2JV6	YF	NMR	Domain III	[127]
2JQM	YF	NMR	Domain III (S288-K398)	[127]
3EGP	DEN-1	2.4	Domain III	[128]
2JSF	DEN-2	NMR	Domain III	[129]
2R69	DEN-2	3.8	Domain III with Fab 1A1D-2	[122]
2H0P	DEN-4	NMR	Envelope protein domain III	[130]
<i>Immature precursor membrane protein</i>				
1N6G	DEN-2	C-EM		[131]
<i>NS3 protease helicase</i>				
2VBC	DEN-4	3.2		[17]
<i>Core particle</i>				
1SFK	WNV	3.2		[132]
<i>Capsid</i>				
1R6R	DEN-2	NMR		[84]
<i>NS2B-NS3 complex</i>				
2IJO	WNV	2.3	Complex with bovine pancreatic trypsin	[18]
2GGV	WNV	1.8	His51Ala mutant	[18]
2FP7	WNV	1.7	Complex with Bz-Nle-Lys-Arg-Arg-H	[23]
2FOM	DEN-2	1.5	Complex with chloride ion and glycerol	[23]
<i>Precursor membrane protein and E protein complex</i>				
3C6E	DEN-2	2.6	Heterodimer at neutral pH	[98]

PDB-ID	Virus	Resolution (Å)	Notes	Ref.
3C5X	DEN-2	2.2	Heterodimer at low pH	[98]
<i>Immature virus</i>				
2OF6	WNV	C-EM	Immature virus	[133]
1NA4	YF	C-EM	Immature virus	[131]
3C6R	DEN-2	C-EM	Immature virus, low pH	[134]
3C6D	DEN-2		Immature virus	[98]
1TGE	DEN-2	C-EM	Immature virus at 12.5 Å	[124]
<i>Other</i>				
2R29	DEN-2	3.0	With serotype crossreactive antibody	[122]
2B6B	DEN	C-EM	Complex with CRD of DC-SIGN	[69]
1P58	DEN-2	C-EM	Complex organization, 9.5 Å	[135]
1THD	DEN-2	C-EM	Complex organization, 9.5 Å	[124]

C-EM: Cryo-electron microscopy; DEN: Dengue virus; NMR: Nuclear magnetic resonance spectroscopy; WNV: West Nile virus; YF: Yellow fever.