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Review

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Flavivirus Entry Inhibitors

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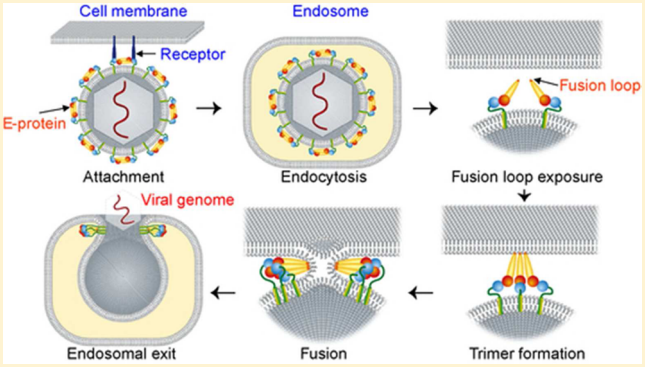
ABSTRACT: Many flaviviruses are significant human pathogens that are transmitted by mosquitoes and ticks. Although effective vaccines are available for yellow fever virus, Japanese encephalitic virus, and tick-borne encephalitis virus, these and other flaviviruses still cause thousands of human deaths and millions of illnesses each year. No clinically approved antiviral therapy is available for flavivirus treatment. To meet this unmet medical need, industry and academia have taken multiple approaches to develop antiflavivirus therapy, among which targeting viral entry has been actively pursued in the past decade. Here we review the current knowledge of flavivirus entry and its use for small molecule drug discovery. Inhibitors of two major steps of flaviviral entry have been reported: (i) molecules that block virus−receptor interaction; (ii) compounds that prevent conformational change of viral envelope protein during virus−host membrane fusion. We also discuss the advantages and disadvantages of targeting viral entry for treatment of flavivirus infection as compared to targeting viral replication proteins.



KEYWORDS: *flavivirus, virus entry, antivirals*

INTRODUCTION

The Unmet Medical Need for Flavivirus Antiviral Therapy. Flavivirus infections have increased significantly in tropical and subtropical regions of the world in the past 40 years, mainly due to reduced mosquito control, widespread international travel, and dense urbanization. There are more than 50 viruses in the *Flavivirus* genus within the *Flaviviridae* family, of which more than 40 are human pathogens with a global distribution.[1] Pathogenic mosquito- and/or tick-borne flaviviruses cause a variety of clinical diseases in a wide range of vertebrate species. These disease syndromes include mild/severe febrile illness, "flu-like" syndromes with a rash, or, in other cases, severe encephalitis or hemorrhagic disease. Yellow fever virus (YFV), the prototype flavivirus, was the first human virus shown to be experimentally transferred via the filtered serum of an infected individual, whereas naturally the infectious agent is transmitted to humans by mosquitoes.[2] Although an efficient vaccine exists against YFV, this virus is still a leading cause of hemorrhagic fever and related mortality worldwide. There are an estimated 200,000 human cases of yellow fever, leading to 30,000 deaths globally each year.[3] Another flavivirus, dengue virus (DENV), threatens up to 2.5 billion people and is now spreading in many regions of the world where it was not previously endemic. A recent study estimated 390 million human infections annually, with 96 million infections exhibiting disease symptoms.[4] A third important member of the *Flavivirus* genus is Japanese encephalitis virus (JEV). This mosquito-borne virus is the leading cause of viral encephalitis worldwide with an estimated 67,900 annual cases.[5] Approximately 20−30% of JEV cases are fatal and 30−50% of survivors have significant neurologic sequelae.[6] Since its introduction in New York City in 1999, West Nile virus (WNV) has spread throughout the country and is now endemic in the continental United States, causing thousands of human infections.[7] There is no established drug therapy for any flavivirus infection. Treatment is symptomatic, aimed at preventing complications and reducing the symptoms for the comfort of the patients.

Flavivirus Genome Organization and Proteins. Flavivirus genomes consist of a single, positive-strand RNA of about 11,000 nucleotides in length, encoding a 5′ untranslated region (UTR), a single open-reading-frame (ORF), and a 3′ UTR. Translation of the ORF produces a large polyprotein that is co- and post-translationally processed into 10 proteins. The N-terminal one-fourth of this polyprotein contains three structural proteins [capsid (C), premembrane (PrM), envelope (E) protein], followed by seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The nonstructural proteins are essential for virus replication, virion assembly, and invasion of host immune response.[2,8−10] Among the 10 viral proteins, only NS3 and NS5 have enzymatic activities. The N-terminal domain of NS3 contains a serine protease activity; the C-terminal domain functions as an RNA helicase, an RNA triphosphatase (RTPase), and an NTP phosphatase (NTPase). The N-terminal domain of NS5 has a methyltransferase activity and a possible guanylyltransferase; the C-terminal domain

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serves as an RNA-dependent RNA polymerase (RdRp). Besides the enzymatic NS3 and NS5, other viral proteins are also required for RNA replication, among which NS2A, NS2B, NS4A, and NS4B are transmembrane proteins that form the scaffold and components of the viral replication complex.

The structural proteins form the viral particle, with the C protein interacting with viral genomic RNA to form nucleocapsid. The nucleocapsid is enveloped by a lipid bilayer in which viral prM and E proteins are embedded.[11] The prM protein functions as a chaperone for the folding of viral E protein and prevents E from being prematurely activated by low pH during the transport of progeny virions through the acidic compartments and vesicles of the trans-Golgi network.[12−14] The E protein mediates virus entry into the cell through receptor binding and low-pH-induced fusion in endosomes after receptor-mediated endocytosis. The E protein also carries the major antigenic epitopes, leading to a protective immune response.[15,16] In this review, we describe the current understanding of flavivirus cell entry and review the progress in identification of entry inhibitors against flaviviruses. This review focuses on small molecule inhibitors and will not discuss therapeutic antibodies in detail. Readers who are interested in therapeutic antibodies are encouraged to refer to an excellent review on this topic.[17]

FLAVIVIRUS ENTRY

Putative Cellular Receptors. The flavivirus infection cycle begins with binding of the E protein to a yet undefined specific cellular receptor. This interaction allows the internalization of the virus particle via receptor-mediated endocytosis and subsequent fusion of the viral envelope and endosomal membrane. In recent years several attachment factors important for flavivirus entry have been identified. Negatively charged glycosaminoglycans (GAGs), such as heparan sulfate, act as attachment factors that concentrate virions at the cell surface and allow the interaction with primary receptors.[18] C-type lectins, such as dendritic cell-specific intercellular adhesion molecule-3 (ICAM3)-grabbing nonintegrin (DC-SIGN) and liver/lymph node-specific ICAM-3 grabbing nonintegrin (L-SIGN), are able to bind and promote infection of both DENV and WNV by interacting with the carbohydrate residues on E protein.[19−21] Crystallographic studies on DENV−DC-SIGN complexes revealed that the interaction is mediated via the glycosylated Asn67 on the E protein.[22] The mannose receptor of macrophages is another C-type lectin that has been described as a functional receptor of DENV.[23] In addition, several other cell surface proteins have been proposed to be the receptors, including the lipopolysaccharide receptor CD14, heat-shock proteins 70 and 90, the ER chaperonin GRP78, and phosphatidylserine receptors TIM and TAM.[24−27] For more details, interested readers could refer to a recent review by Perara-Lecoin et al.[28] Collectively, a large number of molecules have been described as flavivirus candidate receptors in different cell types, but their precise role in virus endocytosis remains obscure.

Flavivirus E Protein. Flavivirus E protein is a class II fusion protein sharing about 40% amino acid identity among different members of the genus.[29] X-ray crystallography and cryo-electron microscopy (cryo-EM) studies revealed that flavivirus consists of 180 copies of E associated as homodimers and arranged in a herringbone or "raft" pattern on a host-derived lipid membrane (reviewed in ref 30). Each monomer of E ectodomain consists of three distinct domains: the central β-barrel domain I (DI), the elongated fingerlike domain II (DII), and the immunoglobin-like domain III (DIII)[31−38] (Figure 1).

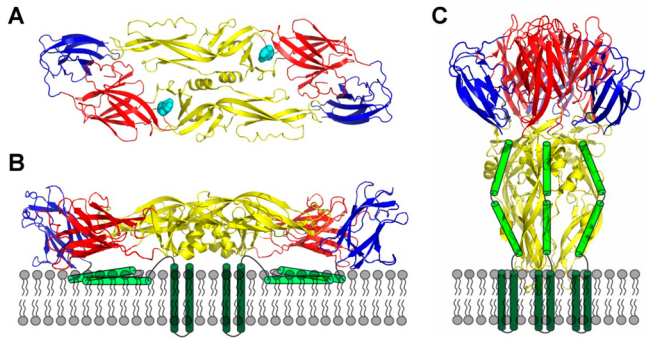

Figure 1. Oligomeric structure of the flavivirus E glycoprotein in its pre- and postfusion conformations. (A) Top view of the soluble ectodomian (sE) of DENV-2 in the dimeric prefusion conformation found on mature virions.[33] Domain I is in red, domain II in yellow, domain III in blue, and β-OG in cyan. (B) Side view of the sE dimer presented on the virion surface prior to low-pH exposure. Domain colors are as in (A). The "stem" region in green is known as a helix−loop−helix, modeled from a cryo-EM reconstruction.[43] (C) Postfusion E trimer.[31] Domain colors are as in (A) and (B).

DI contains the amino terminus and carries one N-linked carbohydrate side chains. DII mediates dimerization of E and bears a hydrophobic and well-conserved fusion peptide loop at its tip. DIII constitutes the carboxyl terminus and is predicted to be involved in receptor binding.[39,40] All three domains have epitopes involved in neutralization (reviewed in ref 41). Those in DI and DII tend to be conformational and elicit weak neutralization, whereas epitopes in DIII elicit very potent neutralization and tend to be relatively linear in sequence. The junctions between the three domains are flexible and capable of hinge-like motions that are important in the structural rearrangements of the E protein for its conversions between immature, mature, and fusogenic forms of the virus.[32] Between DIII and the transmembrane anchor are about 50 residues called the "stem" region.[42] Cryo-EM image reconstructions revealed that the stem is a pair of α-helices with an intervening loop, sandwiched between the main part of the E protein and the outer leaflet of the lipid bilayer (Figure 1).[43]

TARGETING FLAVIVIRUS ENTRY

Three features of flavivirus E protein and membrane fusion could be used for design of entry inhibitors. First, between DI and DII is a hydrophobic pocket that was found to interact with detergent β-*N*-octyl-glucoside (β-OG).[33] Inhibitors that occupy this pocket could sterically block the conformational changes between domains I and II; such conformational change is essential for virus−host membrane fusion. Second, during the course of membrane fusion, the highly conserved stem region needs to fold back onto the trimer of E ectodomain to form a six-helix bundle, reminiscent of the hairpin-like postfusion structures of class I fusion proteins found in HIV and influenza virus.[44] Therefore, following the successful example of the development of HIV T20/enfuvirtide,[45] designing a peptide containing sequences derived from the stem is expected to competitively block membrane fusion. One caveat of this approach is that HIV fuses with the plasma membrane on the cell surface, whereas flaviviruses fuse with the endosome membrane inside the cell. Therefore, a peptide inhibitor needs

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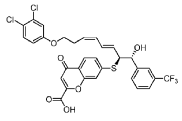
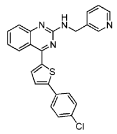
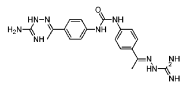
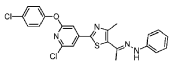
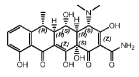
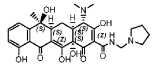
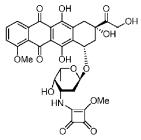
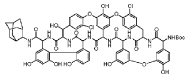
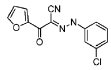
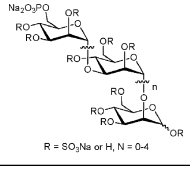
Table 1. Entry Inhibitors of Flavivirus

Compound	Structure	Mode-of-action	Cellular activity against DENV (EC_{50}/CC_{50}) μM	Reference
NITD-448		E protein β-OG pocket	9.8 / 48.7	46
Compound 6		E protein β-OG pocket	0.119 / >20	47
P02		E protein β-OG pocket	13 / 371	48
A5		E protein β-OG pocket	1.2 / >100	49
Doxycycline		E protein β-OG pocket	55.6/ >500	50
Rolitetracycline		E protein β-OG pocket	67.1/ >500	50
SA-17		Possibly through E β-OG pocket	0.52 / 43	51
LCTA-949		Entry	6.9 / >25	52
1662G07		E stem and E trimer interaction	16.9 / >100	53
DN59 (stem peptide)	MAILGDTAWDF GSLGGVFTSIGK ALHQVFGAIY	Bind to E trimer to block stem binding	10 / > 30	54
DN57opt (DII hinge region peptide)	RWMVWRHWF HRLRLPYNPGK NKQNQQWP	Bind to pre-fusion E to block virus:cell binding	8 / 40	57
1OAN1 (DI/DII conneting β sheet peptide)	FWFTLIKTQAK QPARYRRFC	Bind to pre-fusion E to block virus:cell binding	7 / >50	57
PI-88		Heparin mimetic to block virus attachment	200 μg/mL	64

to enter the cell to exert its antiviral activity against flaviviruses. Third, the GAG-binding motifs within DIII of E protein are responsible for virus attachment to target cells.[18] GAGs, such as heparin or its mimetics that bind with the E protein, can

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ACS Infect. Dis. 2015, 1, 428−434

thereby sterically prevent virus adsorption and infection. Furthermore, the use of carbohydrate moieties on E protein for receptor DC-SIGN binding also suggests a strategy of using carbohydrate-binding agents to block virus entry. In the following sections, we will describe briefly the progress in searching for entry inhibitors of flaviviruses on the basis of these rationales.

Targeting a Hydrophobic Pocket of E Protein. Virtual screens of small molecule libraries have been performed to identify compounds that bind to the hydrophobic pocket for β-OG in DENV E protein. It should be noted that the in silico hits identified so far have yet to be shown to bind in the β-OG pocket. Two classes of compounds were identified from the Novartis compound library. The first class of compounds, represented by **NITD-448** (Table 1), inhibited E protein-mediated membrane fusion (IC_{50} = 6.8 μM) as well as DENV-2 infection (EC_{50} = 9.8 μM) in cell culture.[46] Due to the large molecular weight, poor selectivity, and pharmacokinetic properties of this compound, this inhibitor series was not further pursued. The second class of compounds (e.g., compound **6**; Table 1), contained a quinazoline core and inhibited various laboratory and clinical isolates of DENV with micromolar to nanomolar EC_{50} values.[47] After significant chemistry efforts to improve the pharmacokinetic properties of this scaffold, its low potency and high plasma protein-binding activity (due to lipophilicity) excluded the compound from further development.

In silico docking with the NCI library identified a highly basic compound, **P02** (Table 1), which inhibited YFV production at micromolar concentrations.[48] This compound was confirmed to bind DENV virions by NMR; in addition, the compound competed with β-OG for binding to the E protein. Kampmann and co-workers[49] also performed a virtual screen using the Maybridge database. One compound, **A5** (Table 1), was found to have low micromolar activity against several flaviviruses, including DENV, WNV, and YFV. The compound prevented syncytium formation in mosquito cells C6/36 expressing DENV envelope. Several tetracycline derivatives were discovered from a virtual docking of the Comprehensive Medicinal Chemistry database. The compounds doxycycline and rolitetracycline showed moderate antiviral potency (EC_{50} = 55 and 67 μM, respectively) against DENV-2 in cell culture.[50] It has not yet been shown, however, whether the compounds found in this way indeed bind to the pocket of E protein as predicted, and the binding of these molecules to the pocket will disrupt the dimer-to-trimer transition of E and thereby block membrane fusion.

In addition to virtual screening, Neyts and colleagues identified two classes of antibiotics that inhibit DENV with micromolar activities in cell culture by using DENV infection assay. The two classes of antibiotic inhibitors are **SA-17** (a doxorubicin derivate;[51] Table 1) and **LCTA-949** (aglycon analogue of teicoplanin;[52] Table 1). A time-of-addition experiment suggests both compounds inhibit an early step of viral infection cycle. In addition, LCTA-949 was shown to interfere with antibody-mediated cell entry of DENV particles. In silico docking suggests that SA-17 fits into the β-OG pocket of E protein and interacts with specific amino acids (Ala50, Tyr137, and Gln200) that are crucial for the fusion of the membranes. However, no follow-up studies have been reported for these compounds.

Targeting the Interaction between E Stem and E Trimer. Because the stem region of E needs to interact with E trimer during endosomal membrane fusion, Schmidt and co-workers[53] developed a fluorescence polarization (FP) assay that detects the binding of an E stem-derived peptide to a stem-less E trimer. Using this assay, they discovered a series of compounds (e.g., **1662G07**, Table 1) that inhibit DENV-2 in cell culture. Furthermore, they showed that the compounds block low-pH-triggered fusion of virus with liposomes. Structure−activity relationship (SAR) studies led to analogues with submicromolar IC_{90} values against DENV-2, moderate activity against DENV-4, but weak activity against DENV-1 and -3. The antiviral spectrum of the compounds needs to be broadened to achieve pan-dengue-serotypic inhibition.

As mentioned earlier, peptides could be designed to inhibit E-mediated viral entry. Peptide **DN59** (Table 1), representing the stem region (amino acids 412−444) of DENV-2 E, inhibits DENV-2 and cross-inhibited WNV fusion/infectivity.[54] Subsequent studies demonstrated that **DN59** induced the formation of pores in the viral membrane to release the genome, resulting in empty noninfectious virions. However, the mechanism of peptide-induced nucleocapsid ejection remains to be determined.[55] Similar to **DN59**, peptides derived from the sequences of the stem region of DENV E protein bind specifically to the postfusion conformation of soluble E and exhibit antiviral activity.[56]

In addition to designing peptides based on E stem sequence, Costin et al. used the prefusion DENV-2 E protein for rational design of small peptides via biologically validated computer modeling techniques.[57] The two most active peptides, **DN57opt** and **1OAN1**, were designed to mimic the DII hinge region and the first β sheet strand connecting DI and DII of E protein, respectively. DENV-2 viral particles treated with these peptides displayed rough outer surface morphology, contrary to the smooth outer surface of mature virus, indicating that the E proteins were likely rearranged. Further optimization is needed to improve the potency of these peptide inhibitors. Interestingly, these peptides could also inhibit the antibody-enhanced DENV infection.[58] The drawbacks of peptidic inhibitors are the need for intravenous administration and their short shelf life. A significant amount of medicinal chemistry optimization effort is required to turn peptides into drug candidates that can be administered by oral route.

Targeting the Receptor Binding Site of E Protein. A number of studies have reported the antiviral activity of heparan sulfate (HS) mimetics.[59−67] These heparin-like molecules bind to DENV E protein with dissociation constants in the range of 10^{-7}−10^{-9} M and can competitively inhibit binding of E protein to heparin, suggesting the antiviral effect of HS mimetics involving steric hindrance of virus attachment.[66] Two sulfated polysaccharides have previously been reported to have both in vitro and in vivo antiviral effect. One is sulfated galactomannans isolated from plants that are active against YFV in a mosquito cell culture; the inhibitors protected virus-infected mice from death only when administered simultaneously with virus inoculation.[65] The other sulfated polysaccharide, **PI-88** (Table 1), which is currently in clinical trials for antitumor activity, inhibits DENV-2 infection both in cell culture and in vivo.[64] In JEV and DENV challenge mouse models, intraperitoneal administration of **PI-88** increased animal survival time. Despite the fact that heparin analogues demonstrated in vivo efficacy in mouse models, these compounds often have anticoagulant activities. Such anticoagulant activity represents a major liability for their use as an

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antiviral candidate because plasma leakage is the disease hallmark in dengue patients.

Plant lectins, generally designated carbohydrate-binding agents (CBAs), are shown to exhibit antiviral activities against all four serotypes of DENV in monocyte-derived dendritic cells.[68,69] However, plant lectins are not orally bioavailable, sensitive for proteolytic cleavage, and expensive to produce. These shortcomings pose a significant challenge with respect to their development.

CONCLUSIONS AND FUTURE DIRECTIONS

Inhibition of viral entry is a validated antiviral approach, as exemplified by clinically approved T20/enfuvirtide[45] and Maraviroc[70] (a small molecule inhibitor of CCR5 coreceptor) for HIV treatment. Compared with inhibitors targeting viral enzymes (e.g., polymerase and protease), inhibitors targeting viral entry have two potential advantages. First, inhibitors that bind to viral E protein may exert a dual antiviral activity through blocking viral entry as well as virion assembly/maturation. Second, entry inhibitors can directly bind to virions in blood without the requirement of penetration into cells. In contrast, inhibitors of viral enzymes need to get into infected cells before exerting their antiviral activities. This latter advantage allows treatment of patients by transfusion of therapeutic antibodies and peptide mimetic inhibitors.

Due to the plastic nature of viral surface proteins, two disadvantages could be perceived for targeting flavivirus E protein. First, the amino acid sequence variation of the four serotypes of DENV E protein reaches 40%, higher than those of enzymatic proteins (variations up to 30%).[71] Conceptually, this higher variation of flaviviral E protein makes it challenging to achieve small molecule inhibitors with pan-serotype activity. However, this challenge could be overcome by designing inhibitors that specifically target conserved regions of the E protein. A range of human and mouse monoclonal antibodies recognize epitopes that are shared by all mosquito-borne flaviviruses. The epitopes of these antibodies are conformational and located around the fusion peptide region, as prototyped by the 4G2 antibody. Screaton's group has recently reported a set of human monoclonal antibodies that can neutralize all four serotypes of DENV in cell culture.[72] Structural analysis of DENV-2 E protein in complex with these antibodies mapped the binding sites to a serotype-conserved region at the E−dimer interface, including the exposed main chain of the E fusion loop and the two conserved glycan chains.[73] These results clearly indicate the feasibility of antibodies to achieve pan-serotype activity by targeting conserved regions of flavivirus E protein. It remains to be demonstrated that these antibodies could achieve pan-serotype in vivo efficacy (e.g., AG129 mouse). Second, the barrier of resistance emergence might be low for entry inhibitors. Although no resistance studies have been reported for small molecular inhibitors of flavivirus E protein, resistant DENV could rapidly emerge in cell culture and in non-human primates when treated with monoclonal antibodies.[74] The weakness of low-resistance barrier could be overcome through combination therapy with compounds of other modes of action. Therefore, the ongoing antiviral effort should be encouraged to pursue inhibitors of viral replication components (e.g., polymerase or NS4B) in parallel with inhibitors of viral entry.[75]

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Moureau, G., Cook, S., Lemey, P., Nougairede, A., Forrester, N. L., Khasnatinov, M., Charrel, R. N., Firth, A. E., Gould, E. A., and de Lamballerie, X. (2015) New Insights into Flavivirus Evolution, Taxonomy and Biogeographic History, Extended by Analysis of Canonical and Alternative Coding Sequences. *PLoS One 10*, e0117849.

(2) Lindenbach, B. D., Thiel, H.-J., and Rice, C. M. (2007) *Flaviviridae: the viruses and their replication*, 5th ed. (Knipe, D. M., Howley, P. M., Eds.), pp 1101−1151, Lippincott Williams & Wilkins, Philadelphia, PA, USA.

(3) Gardner, C. L., and Ryman, K. D. (2010) Yellow Fever: A Reemerging Threat. *Clin. Lab. Med. 30*, 237−260.

(4) Bhatt, S., Gething, P. W., Brady, O. J., Messina, J. P., Farlow, A. W., Moyes, C. L., Drake, J. M., Brownstein, J. S., Hoen, A. G., Sankoh, O., Myers, M. F., George, D. B., Jaenisch, T., Wint, G. R. W., Simmons, C. P., Scott, T. W., Farrar, J. J., and Hay, S. I. (2013) The global distribution and burden of dengue. *Nature 496*, 504−507.

(5) Campbell, G. L., Hills, S. L., Fischer, M., Jacobson, J. A., Hoke, C. H., Hombach, J. M., Marfin, A. A., Solomon, T., Tsai, T. F., Tsu, V. D., and Ginsburg, A. S. (2011) Estimated global incidence of Japanese encephalitis: a systematic review. *Bull. WHO 89*, 766−774.

(6) Fischer, M., Hills, S., Staples, E., Johnson, B., Yaich, M., and Solomon, T. (2008) Japanese Encephalitis Prevention and Control: Advances, Challenges, and New Initiatives. In *Emerging Infections*, Vol. 8, American Society of Microbiology, Washington, DC, USA.

(7) CDC. (2013) West Nile virus and other arboviral diseases − United States, 2012. *MMWR Morbidity Mortality Weekly Rep. 62*, 513−517.

(8) Bollati, M., Alvarez, K., Assenberg, R., Baronti, C., Canard, B., Cook, S., Coutard, B., Decroly, E., de Lamballerie, X., Gould, E. A., Grard, G., Grimes, J. M., Hilgenfeld, R., Jansson, A. M., Malet, H., Mancini, E. J., Mastrangelo, E., Mattevi, A., Milani, M., Moureau, G., Neyts, J., Owens, R. J., Ren, J., Selisko, B., Speroni, S., Steuber, H., Stuart, D. I., Unge, T., and Bolognesi, M. (2010) Structure and functionality in flavivirus NS-proteins: Perspectives for drug design. *Antiviral Res. 87*, 125−148.

(9) Kümmerer, B. M., and Rice, C. M. (2002) Mutations in the Yellow Fever Virus Nonstructural Protein NS2A Selectively Block Production of Infectious Particles. *J. Virol. 76*, 4773−4784.

(10) Muñoz-Jordán, J. L., Sánchez-Burgos, G. G., Laurent-Rolle, M., and García-Sastre, A. (2003) Inhibition of interferon signaling by dengue virus. *Proc. Natl. Acad. Sci. U. S. A. 100*, 14333−14338.

(11) Kuhn, R. J., Zhang, W., Rossmann, M. G., Pletnev, S. V., Corver, J., Lenches, E., Jones, C. T., Mukhopadhyay, S., Chipman, P. R., Strauss, E. G., Baker, T. S., and Strauss, J. H. (2002) Structure of Dengue Virus: Implications for Flavivirus Organization, Maturation, and Fusion. *Cell 108*, 717−725.

(12) Yu, I. M., Zhang, W., Holdaway, H. A., Li, L., Kostyuchenko, V. A., Chipman, P. R., Kuhn, R. J., Rossmann, M. G., and Chen, J. (2008) Structure of the Immature Dengue Virus at Low pH Primes Proteolytic Maturation. *Science 319*, 1834−1837.

(13) Li, L., Lok, S. M., Yu, I. M., Zhang, Y., Kuhn, R. J., Chen, J., and Rossmann, M. G. (2008) The Flavivirus Precursor Membrane-Envelope Protein Complex: Structure and Maturation. *Science 319*, 1830−1834.

(14) Lorenz, I. C., Allison, S. L., Heinz, F. X., and Helenius, A. (2002) Folding and Dimerization of Tick-Borne Encephalitis Virus Envelope Proteins prM and E in the Endoplasmic Reticulum. *J. Virol. 76*, 5480−5491.

DOI: 10.1021/acsinfecdis.5b00066
ACS Infect. Dis. 2015, 1, 428−434

(15) Nybakken, G. E., Oliphant, T., Johnson, S., Burke, S., Diamond, M. S., and Fremont, D. H. (2005) Structural basis of West Nile virus neutralization by a therapeutic antibody. *Nature 437*, 764−769.
(16) Wu, K.-P., Wu, C.-W., Tsao, Y.-P., Kuo, T.-W., Lou, Y.-C., Lin, C.-W., Wu, S.-C., and Cheng, J.-W. (2003) Structural Basis of a Flavivirus Recognized by Its Neutralizing Antibody: Solution Structure of the Domain III of the Japanese Encephalitis Virus Envelope Protein. *J. Biol. Chem. 278*, 46007−46013.
(17) Diamond, M. S., Pierson, T. C., Roehrig, J. T. (2012) Chapter 11, Antibody therapeutics against flaviviruses. In *Molecular Virology and Control of Flaviviruses*, pp 231−256, Caister Academic Press, Norfolk, UK.
(18) Chen, Y., Maguire, T., Hileman, R. E., Fromm, J. R., Esko, J. D., Linhardt, R. J., and Marks, R. M. (1997) Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat. Med. 3*, 866−871.
(19) Davis, C. W., Nguyen, H. Y., Hanna, S. L., Sanchez, M. D., Doms, R. W., and Pierson, T. C. (2006) West Nile virus discriminates between DC-SIGN and DC-SIGNR for cellular attachment and infection. *J. Virol. 80*, 1290−1301.
(20) Navarro-Sanchez, E., Altmeyer, R., Amara, A., Schwartz, O., Fieschi, F., Virelizier, J. L., Arenzana-Seisdedos, F., and Despres, P. (2003) Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. *EMBO Rep. 4*, 723−728.
(21) Tassaneetrithep, B., Burgess, T. H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M. A., Pattanapanyasat, K., Sarasombath, S., Birx, D. L., Steinman, R. M., Schlesinger, S., and Marovich, M. A. (2003) DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. *J. Exp. Med. 197*, 823−829.
(22) Pokidysheva, E., Zhang, Y., Battisti, A. J., Bator-Kelly, C. M., Chipman, P. R., Xiao, C., Gregorio, G. G., Hendrickson, W. A., Kuhn, R. J., and Rossmann, M. G. (2006) Cryo-EM reconstruction of dengue virus in complex with the carbohydrate recognition domain of DC-SIGN. *Cell 124*, 485−493.
(23) Miller, J. L., de Wet, B. J., Martinez-Pomares, L., Radcliffe, C. M., Dwek, R. A., Rudd, P. M., and Gordon, S. (2008) The mannose receptor mediates dengue virus infection of macrophages. *PLoS Pathog. 4*, e17.
(24) Chen, Y. C., Wang, S. Y., and King, C. C. (1999) Bacterial lipopolysaccharide inhibits dengue virus infection of primary human monocytes/macrophages by blockade of virus entry via a CD14-dependent mechanism. *J. Virol. 73*, 2650−2657.
(25) Meertens, L., Carnec, X., Lecoin, M. P., Ramdasi, R., Guivel-Benhassine, F., Lew, E., Lemke, G., Schwartz, O., and Amara, A. (2012) The TIM and TAM Families of Phosphatidylserine Receptors Mediate Dengue Virus Entry. *Cell Host Microbe 12*, 544−557.
(26) Reyes-del Valle, J., Chavez-Salinas, S., Medina, F., and del Angel, R. M. (2005) Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. *J. Virol. 79*, 4557−4567.
(27) Jindadamrongwech, S., Thepparit, C., and Smith, D. R. (2004) Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. *Arch. Virol. 149*, 915−927.
(28) Perera-Lecoin, M., Meertens, L., Carnec, X., and Amara, A. (2014) Flavivirus Entry Receptors: An Update. *Viruses 6*, 69−88.
(29) Modis, Y. (2013) Class II fusion proteins. *Adv. Exp. Med. Biol. 790*, 150−166.
(30) Mukhopadhyay, S., Kuhn, R. J., and Rossmann, M. G. (2005) A structural perspective of the flavivirus life cycle. *Nat. Rev. Microbiol. 3*, 13−22.
(31) Modis, Y., Ogata, S., Clements, D., and Harrison, S. C. (2004) Structure of the dengue virus envelope protein after membrane fusion. *Nature 427*, 313−319.
(32) Zhang, Y., Zhang, W., Ogata, S., Clements, D., Strauss, J. H., Baker, T. S., Kuhn, R. J., and Rossmann, M. G. (2004) Conformational Changes of the Flavivirus E Glycoprotein. *Structure 12*, 1607−1618.
(33) Modis, Y., Ogata, S., Clements, D., and Harrison, S. C. (2003) A ligand-binding pocket in the dengue virus envelope glycoprotein. *Proc. Natl. Acad. Sci. U. S. A. 100*, 6986−6991.
(34) Modis, Y., Ogata, S., Clements, D., and Harrison, S. C. (2005) Variable Surface Epitopes in the Crystal Structure of Dengue Virus Type 3 Envelope Glycoprotein. *J. Virol. 79*, 1223−1231.
(35) Nybakken, G. E., Nelson, C. A., Chen, B. R., Diamond, M. S., and Fremont, D. H. (2006) Crystal Structure of the West Nile Virus Envelope Glycoprotein. *J. Virol. 80*, 11467−11474.
(36) Rey, F. A., Heinz, F. X., Mandl, C., Kunz, C., and Harrison, S. C. (1995) The envelope glycoprotein from tick-borne encephalitis virus at 2 A resolution. *Nature 375*, 291−298.
(37) Kanai, R., Kar, K., Anthony, K., Gould, L. H., Ledizet, M., Fikrig, E., Marasco, W. A., Koski, R. A., and Modis, Y. (2006) Crystal Structure of West Nile Virus Envelope Glycoprotein Reveals Viral Surface Epitopes. *J. Virol. 80*, 11000−11008.
(38) Luca, V. C., AbiMansour, J., Nelson, C. A., and Fremont, D. H. (2012) Crystal Structure of the Japanese Encephalitis Virus Envelope Protein. *J. Virol. 86*, 2337−2346.
(39) Chu, J. J., Rajamanonmani, R., Li, J., Bhuvanakantham, R., Lescar, J., and Ng, M. L. (2005) Inhibition of West Nile virus entry by using a recombinant domain III from the envelope glycoprotein. *J. Gen. Virol. 86*, 405−412.
(40) Huerta, V., Chinea, G., Fleitas, N., Sarria, M., Sanchez, J., Toledo, P., and Padron, G. (2008) Characterization of the interaction of domain III of the envelope protein of dengue virus with putative receptors from CHO cells. *Virus Res. 137*, 225−234.
(41) Pierson, T. C., Fremont, D. H., Kuhn, R. J., and Diamond, M. S. (2008) Structural Insights into the Mechanisms of Antibody-Mediated Neutralization of Flavivirus Infection: Implications for Vaccine Development. *Cell Host Microbe 4*, 229−238.
(42) Allison, S. L., Stiasny, K., Stadler, K., Mandl, C. W., and Heinz, F. X. (1999) Mapping of functional elements in the stem-anchor region of tick-borne encephalitis virus envelope protein E. *J. Virol. 73*, 5605−5612.
(43) Zhang, W., Chipman, P. R., Corver, J., Johnson, P. R., Zhang, Y., Mukhopadhyay, S., Baker, T. S., Strauss, J. H., Rossmann, M. G., and Kuhn, R. J. (2003) Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. *Nat. Struct. Biol. 10*, 907−912.
(44) Jardetzky, T. S., and Lamb, R. A. (2004) Virology: a class act. *Nature 427*, 307−308.
(45) Kilby, J. M., Hopkins, S., Venetta, T. M., DiMassimo, B., Cloud, G. A., Lee, J. Y., Alldredge, L., Hunter, E., Lambert, D., Bolognesi, D., Matthews, T., Johnson, M. R., Nowak, M. A., Shaw, G. M., and Saag, M. S. (1998) Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. *Nat. Med. 4*, 1302−1307.
(46) Poh, M. K., Yip, A., Zhang, S., Smit, J. M., Wilschut, J., Priestle, J. P., P.-Y, S., Wenka, M. R., and Schul, W. (2009) A small molecule fusion inhibitor of dengue virus. *Antiviral Res. 84*, 260−266.
(47) Wang, Q. Y., Patel, S. J., Vangrevelinghe, E., Xu, H. Y., Rao, R., Jaber, D., Schul, W., Gu, F., Heudi, O., Ma, N. L., Poh, M. K., Phong, W. Y., Keller, T. H., Jacoby, E., and Vasudevan, S. G. (2009) A small-molecule dengue virus entry inhibitor. *Antimicrob. Agents Chemother. 53*, 1823−1831.
(48) Zhou, Z., Khaliq, M., Suk, J. E., Patkar, C., Li, L., Kuhn, R. J., and Post, C. B. (2008) Antiviral compounds discovered by virtual screening of small-molecule libraries against dengue virus E protein. *ACS Chem. Biol. 3*, 765−775.
(49) Kampmann, T., Yennamalli, R., Campbell, P., Stoermer, M. J., Fairlie, D. P., Kobe, B., and Young, P. R. (2009) In silico screening of small molecule libraries using the dengue virus envelope E protein has identified compounds with antiviral activity against multiple flaviviruses. *Antiviral Res. 84*, 234−241.
(50) Yang, J.-M., Chen, Y.-F., Tu, Y.-Y., Yen, K.-R., and Yang, Y.-L. (2007) Combinatorial Computational Approaches to Identify Tetracycline Derivatives as Flavivirus Inhibitors. *PLoS One 2*, e428.

DOI: 10.1021/acsinfecdis.5b00066
ACS Infect. Dis. 2015, 1, 428−434

(51) Kaptein, S. J. F., De Burghgraeve, T., Froeyen, M., Pastorino, B., Alen, M. M. F., Mondotte, J. A., Herdewijn, P., Jacobs, M., de Lamballerie, X., Schols, D., Gamarnik, A. V., Sztaricskai, F., and Neyts, J. (2010) A Derivate of the Antibiotic Doxorubicin Is a Selective Inhibitor of Dengue and Yellow Fever Virus Replication In Vitro. *Antimicrob. Agents Chemother. 54*, 5269−5280.
(52) De Burghgraeve, T., Kaptein, S. J. F., Ayala-Nunez, N. V., Mondotte, J. A., Pastorino, B., Printsevskaya, S. S., de Lamballerie, X., Jacobs, M., Preobrazhenskaya, M., Gamarnik, A. V., Smit, J. M., and Neyts, J. (2012) An Analogue of the Antibiotic Teicoplanin Prevents Flavivirus Entry In Vitro. *PLoS One 7*, e37244.
(53) Schmidt, A. G., Lee, K., Yang, P. L., and Harrison, S. C. (2012) Small-molecule inhibitors of dengue-virus entry. *PLoS Pathog. 8*, e1002627.
(54) Hrobowski, Y., Garry, R., and Michael, S. (2005) Peptide inhibitors of dengue virus and West Nile virus infectivity. *Virol. J. 2*, 49.
(55) Lok, S. M., Costin, J. M., Hrobowski, Y. M., Hoffmann, A. R., Rowe, D. K., Kukkaro, P., Holdaway, H., Chipman, P., Fontaine, K. A., Holbrook, M. R., Garry, R. F., Kostyuchenko, V., Wimley, W. C., Isern, S., Rossmann, M. G., and Michael, S. F. (2012) Release of dengue virus genome induced by a peptide inhibitor. *PLoS One 7*, e50995.
(56) Schmidt, A. G., Yang, P. L., and Harrison, S. C. (2010) Peptide Inhibitors of Flavivirus Entry Derived from the E Protein Stem. *J. Virol. 84*, 12549−12554.
(57) Costin, J. M., Jenwitheesuk, E., Lok, S.-M., Hunsperger, E., Conrads, K. A., Fontaine, K. A., Rees, C. R., Rossmann, M. G., Isern, S., Samudrala, R., and Michael, S. F. (2010) Structural Optimization and De Novo Design of Dengue Virus Entry Inhibitory Peptides. *PLoS Neglected Trop. Dis. 4*, e721.
(58) Nicholson, C. O., Costin, J. M., Rowe, D. K., Lin, L., Jenwitheesuk, E., Samudrala, R., Isern, S., and Michael, S. F. (2011) Viral entry inhibitors block dengue antibody-dependent enhancement in vitro. *Antiviral Res. 89*, 71−74.
(59) Hidari, K. I., Takahashi, N., Arihara, M., Nagaoka, M., Morita, K., and Suzuki, T. (2008) Structure and anti-dengue virus activity of sulfated polysaccharide from a marine alga. *Biochem. Biophys. Res. Commun. 376*, 91−95.
(60) Ichiyama, K., Gopala Reddy, S. B., Zhang, L. F., Chin, W. X., Muschin, T., Heinig, L., Suzuki, Y., Nanjundappa, H., Yoshinaka, Y., Ryo, A., Nomura, N., Ooi, E. E., Vasudevan, S. G., Yoshida, T., and Yamamoto, N. (2013) Sulfated polysaccharide, Curdlan sulfate, efficiently prevents entry/fusion and restricts antibody-dependent enhancement of dengue virus infection in vitro: a possible candidate for clinical application. *PLoS Neglected Trop. Dis. 7*, e2188.
(61) Pujol, C. A., Ray, S., Ray, B., and Damonte, E. B. (2012) Antiviral activity against dengue virus of diverse classes of algal sulfated polysaccharides. *Int. J. Biol. Macromol. 51*, 412−416.
(62) Vervaeke, P., Alen, M., Noppen, S., Schols, D., Oreste, P., and Liekens, S. (2013) Sulfated Escherichia coli K5 polysaccharide derivatives inhibit dengue virus infection of human microvascular endothelial cells by interacting with the viral envelope protein E domain III. *PLoS One 8*, e74035.
(63) Talarico, L. B., Pujol, C. A., Zibetti, R. G., Faria, P. C., Noseda, M. D., Duarte, M. E., and Damonte, E. B. (2005) The antiviral activity of sulfated polysaccharides against dengue virus is dependent on virus serotype and host cell. *Antiviral Res. 66*, 103−110.
(64) Lee, E., Pavy, M., Young, N., Freeman, C., and Lobigs, M. (2006) Antiviral effect of the heparan sulfate mimetic, PI-88, against dengue and encephalitic flaviviruses. *Antiviral Res. 69*, 31−38.
(65) Ono, L., Wollinger, W., Rocco, I. M., Coimbra, T. L. M., Gorin, P. A. J., and Sierakowski, M.-R. (2003) In vitro and in vivo antiviral properties of sulfated galactomannans against yellow fever virus (BeH111 strain) and dengue 1 virus (Hawaii strain). *Antiviral Res. 60*, 201−208.
(66) Marks, R. M., Lu, H., Sundaresan, R., Toida, T., Suzuki, A., Imanari, T., Hernáiz, M. J., and Linhardt, R. J. (2001) Probing the Interaction of Dengue Virus Envelope Protein with Heparin: Assessment of Glycosaminoglycan-Derived Inhibitors. *J. Med. Chem. 44*, 2178−2187.
(67) Rees, C. R., Costin, J. M., Fink, R. C., McMichael, M., Fontaine, K. A., Isern, S., and Michael, S. F. (2008) In vitro inhibition of dengue virus entry by p-sulfoxy-cinnamic acid and structurally related combinatorial chemistries. *Antiviral Res. 80*, 135−142.
(68) Alen, M. M. F., De Burghgraeve, T., Kaptein, S. J. F., Balzarini, J., Neyts, J., and Schols, D. (2011) Broad Antiviral Activity of Carbohydrate-Binding Agents against the Four Serotypes of Dengue Virus in Monocyte-Derived Dendritic Cells. *PLoS One 6*, e21658.
(69) Alen, M. M. F., Kaptein, S. J. F., De Burghgraeve, T., Balzarini, J., Neyts, J., and Schols, D. (2009) Antiviral activity of carbohydrate-binding agents and the role of DC-SIGN in dengue virus infection. *Virology 387*, 67−75.
(70) Emmelkamp, J. M., and Rockstroh, J. K. (2007) CCR5 antagonists: comparison of efficacy, side effects, pharmacokinetics and interactions − review of the literature. *Eur. J. Med. Res. 12*, 409−417.
(71) Shrestha, B., Brien, J. D., Sukupolvi-Petty, S., Austin, S. K., Edeling, M. A., Kim, T., O'Brien, K. M., Nelson, C. A., Johnson, S., Fremont, D. H., and Diamond, M. S. (2010) The development of therapeutic antibodies that neutralize homologous and heterologous genotypes of dengue virus type 1. *PLoS Pathog. 6*, e1000823.
(72) Dejnirattisai, W., Wongwiwat, W., Supasa, S., Zhang, X., Dai, X., Rouvinsky, A., Jumnainsong, A., Edwards, C., Quyen, N. T., Duangchinda, T., Grimes, J. M., Tsai, W. Y., Lai, C. Y., Wang, W. K., Malasit, P., Farrar, J., Simmons, C. P., Zhou, Z. H., Rey, F. A., Mongkolsapaya, J., and Screaton, G. R. (2015) Corrigendum: A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus. *Nat. Immunol. 16*, 544.
(73) Rouvinski, A., Guardado-Calvo, P., Barba-Spaeth, G., Duquerroy, S., Vaney, M. C., Kikuti, C. M., Navarro Sanchez, M. E., Dejnirattisai, W., Wongwiwat, W., Haouz, A., Girard-Blanc, C., Petres, S., Shepard, W. E., Despres, P., Arenzana-Seisdedos, F., Dussart, P., Mongkolsapaya, J., Screaton, G. R., and Rey, F. A. (2015) Recognition determinants of broadly neutralizing human antibodies against dengue viruses. *Nature 520*, 109−113.
(74) Zou, G., Kukkaro, P., Lok, S. M., Ng, J. K., Tan, G. K., Hanson, B. J., Alonso, S., MacAry, P. A., and Shi, P. Y. (2012) Resistance analysis of an antibody that selectively inhibits dengue virus serotype-1. *Antiviral Res. 95*, 216−223.
(75) Lim, S. P., Wang, Q. Y., Noble, C. G., Chen, Y. L., Dong, H., Zou, B., Yokokawa, F., Nilar, S., Smith, P., Beer, D., Lescar, J., and Shi, P. Y. (2013) Ten years of dengue drug discovery: progress and prospects. *Antiviral Res. 100*, 500−519.

DOI: 10.1021/acsinfecdis.5b00066
ACS Infect. Dis. 2015, 1, 428−434