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Review

Biological characteristics of dengue virus and potential targets for drug design

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Dengue infection is a major cause of morbidity in tropical and subtropical regions, bringing nearly 40% of the world population at risk and causing more than 20,000 deaths per year. But there is neither a vaccine for dengue disease nor antiviral drugs to treat the infection. In recent years, dengue infection has been particularly prevalent in India, Southeast Asia, Brazil, and Guangdong Province, China. In this article, we present a brief summary of the biological characteristics of dengue virus and associated flaviviruses, and outline the progress on studies of vaccines and drugs based on potential targets of the dengue virus.

Keywords dengue virus; NS3 protease; polyprotein processing; drug target

Introduction

The family *Flaviviridae* is a large group of viral pathogens responsible for causing severe disease and mortality in humans and animals. The family consists of three genera, *Flavivirus*, *Pestivirus*, and *Hepacivirus*. The flaviviruses (Latin "flavus" meaning yellow, because of the jaundice induced by yellow fever virus) comprise a large genus of medically important, arthropod-transmitted, enveloped viruses with more than 70 members that include dengue virus, Japanese encephalitis virus (JEV), tick-borne encephalitis virus (TBEV), West Nile virus (WNV), and yellow fever virus (YFV). Symptoms of flavivirus infection can range from mild fever and malaise to fatal encephalitis and haemorrhagic fever [1,2].

Dengue virus is responsible for the highest rate of disease and mortality among members of the *Flavivirus* genus. Global epidemics of dengue virus have occurred over the past few years. Dengue virus infects 50 to 100 million people each year, with 500,000 patients developing the more severe disease, namely, dengue hemorrhagic fever (DHF), leading to hospitalizations and resulting in approximately 20,000 deaths, mainly in children [3–5]. Dengue viruses are transmitted to humans by the bite of infective female mosquitoes of the genus *Aedes*. Throughout tropical and subtropical regions around the world, over 2.5 billion people live in areas where dengue virus and its mosquito vectors, the *Aedes aegypti* and *Aedes albopictus*, are endemic. The most efficient epidemic vector is *A. aegypti*, although *A. albopictus* and *A. polynesiensis* are also involved in dengue outbreaks [6]. Several factors have been implicated in the global resurgence of dengue: failure to control the *Aedes* population; increased airplane travel to dengue endemic areas; uncontrolled urbanization; unprecedented population growth; and global climate warming [7,8].

Infection of dengue virus is usually characterized by fever and severe joint pain, but more serious syndromes, DHF or dengue shock syndrome, sometimes occur following dengue infection. DHF was mostly confined to Southeast Asia until the 1960s, then it also became endemic in Central America, and more recently in South America. There are four antigenically related but distinct serotypes of dengue virus, designated DEN-1, DEN-2, DEN-3, and DEN-4, and infection by any one serotype does not protect the individual from infection by the remaining three serotypes [9,10]. It has been postulated that hemorrhagic fever or shock syndrome is usually the result of sequential infection with multiple serotypes. Although vaccines have been developed for several flaviviruses, control of dengue virus through the use of vaccination has proven to be elusive [3].

The dengue viruses share many characteristics with other flaviviruses, such as a single-stranded RNA genome that is packaged by the virus capsid protein in a host-derived lipid bilayer, and surrounded by 180 copies of two glycoproteins. The complete virion is approximately 50

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nm in diameter and contains an approximately 10.7 kb positive-sensed RNA genome that has one open reading frame encoding a single polyprotein [11]. The 5′-end of the genomic RNA has a type 1 cap, and the 3′-end is devoid of a poly(A) tail. The 5′-end encodes three structural proteins: capsid (C); membrane precursor protein (prM) proteolytically cleaved by the host protease furin to form the membrane protein in mature virions; and envelope (E) constituting the enveloped virus particle [11,12]. Seven non-structural (NS) proteins essential for viral replication are encoded by the remainder of the genome. The order of proteins encoded is 5′-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3′ [12] [**Fig. 1(A)**].

Structure

The dengue virus surface is composed of 180 copies of the envelope glycoprotein and the membrane protein. The E protein of dengue virus contains a class II fusion peptide sequence that is important for viral invasion of a host cell. There are remarkable structural deviations between the immature and mature dengue envelopes as revealed by elegant cryo-electron microscopy studies [11,13]. The immature dengue virus particle is covered with 60 asymmetric trimers of prM-E heterodimers that stick out like spikes from its surface [**Fig. 2(A)**]. The prM protein

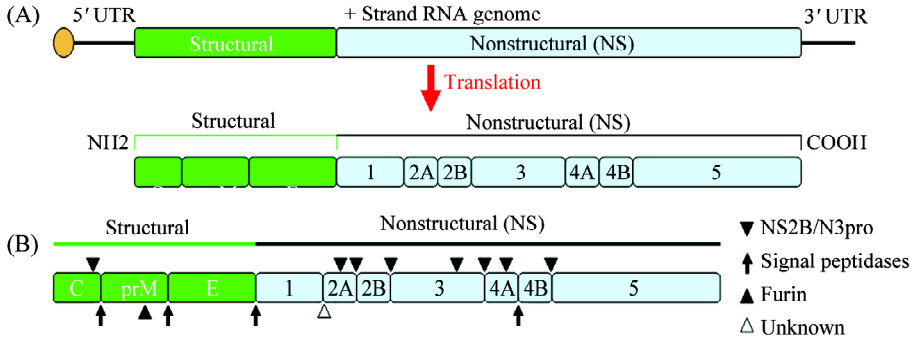

Fig. 1 Genome of dengue virus and its polyprotein processing (A) The dengue virus genome encodes a single large open reading frame that is translated to form a viral polyprotein. The structural domain and nonstructural domain are colored green and light blue, respectively. The 5′-end of the genomic RNA has a type 1 cap shown in orange, and the 3′-end is devoid of a poly(A) tail. (B) Proteolytic processing sites in the dengue virus polyprotein. The dengue virus polyprotein is cleaved by viral and host proteases to produce three structural proteins (C, capsid or core protein; E, envelope protein; prM, precursor to membrane protein) shown in green, and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) shown in light blue. The identified cleavages in the regions of the structural and nonstructural proteins that are mediated by the host cell proteases and the virus-encoded NS2B/NS3 protease are indicated. UTR, untranslated region.

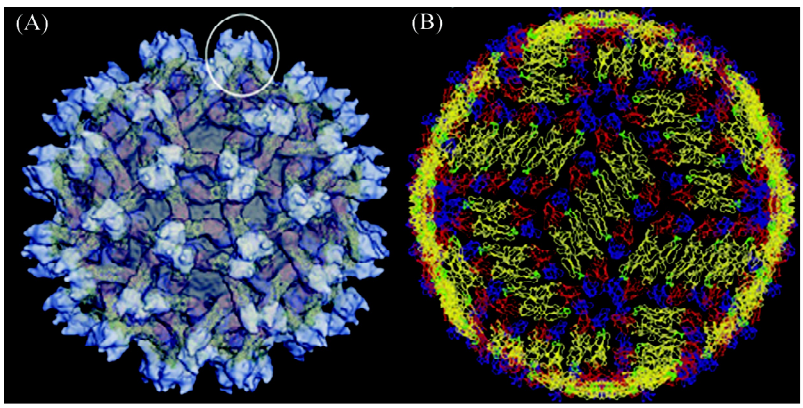

Fig. 2 Immature and mature dengue viruses [11] (A) The immature dengue particle. It has 60 protein "spikes" (circled) that jut from its surface, making it far less smooth than the mature form. (B) The structure of the mature dengue virus. The virus surface is unusually smooth and its membrane is completely enclosed by a protein shell. One raft consists of three parallel dimers of the envelope protein, the different domains of which are represented by different colors (domains I, II, and III are colored red, yellow, and blue, respectively) and the fusion peptide is shown in green.

protects E from premature fusion while passing through the acidic environment of the trans-Golgi network (TGN) during morphogenesis [13]. During maturation, the N-terminal part of the prM protein is released by the host cell furin that induces a rearrangement of the E proteins essential for fusion. In the mature virus, the E proteins exist as homodimers that lie on the viral membrane in the form of 30 so-called "rafts". Each raft contains three parallel dimers arranged in icosahedral symmetry and organized into a herringbone pattern [11] [**Fig. 2(B)**].

Life Cycle

Virions attach to the surface of a host cell and subsequently enter the cell by receptor-mediated endocytosis. Acidification of the endosomal vesicle triggers an irreversible trimerization of the E protein in the virion that results in fusion of the viral and cell membranes [14]. After fusion has occurred, the nucleocapsid (NC) is released into the cytoplasm, leading to the dissociation of the C protein and RNA. Once the genome is released into the cytoplasm, the positive-sense RNA is translated into a single polyprotein that is processed cotranslationally and post-translationally by viral and host proteases. Genome replication occurs on intracellular membranes. Assembly and formation of immature virus particles occur on the surface of the endoplasmic reticulum (ER) when the structural proteins and newly synthesized RNA bud into the lumen of ER [14–16]. Although these particles contain E and prM, lipid membrane and NC, they cannot induce host-cell fusion, remaining non-infectious, because the prM protein is needed to be further processed [17,18]. Subviral particles are also produced in ER, but only contain the glycoproteins and membrane, and lack the C protein and genomic RNA, making them also non-infectious [19]. The resultant non-infectious, immature viral and subviral particles are transported through the TGN. The immature virion particles are then cleaved by the host protease furin, resulting in mature, infectious particles. Subviral particles are also cleaved by furin. The mature virions and subviral particles are subsequently released from the host cell by exocytosis (**Fig. 3**) [20].

Entry, Fusion, and Infection

The structure of soluble E protein elucidated by X-ray crystallography consists of three domains: domain I, the N-terminal part structurally located in the central part; domain II, the fusion domain containing a hydrophobic fusion peptide; domain III, the putative receptor binding

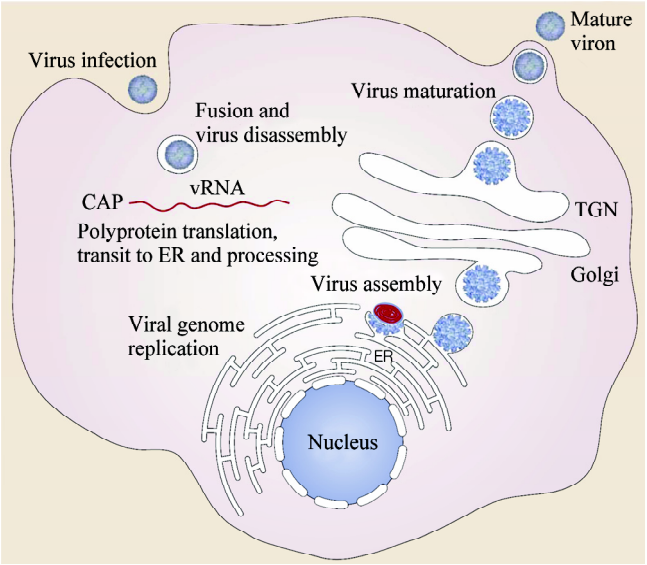

Fig. 3 Life cycle of dengue and associated flaviviruses [20] Virions attach to the surface of a host cell and subsequently enter the cell by receptor-mediated endocytosis. Acidification of the endosomal vesicle triggers conformational changes in the virion, fusion of the viral and cell membranes, and particle disassembly. Once the genome is released into the cytoplasm, the positive-sense RNA is translated into a single polyprotein that is processed cotranslationally and post-translationally by viral and host proteases. Genome replication occurs on intracellular membranes. Virus assembly occurs on the surface of the endoplasmic reticulum (ER) when the structural proteins and newly-synthesized RNA bud into the lumen of ER. The resultant non-infectious, immature viral and subviral particles are transported through the trans-Golgi network. The immature virion particles are cleaved by the host protease furin, resulting in mature, infectious particles. Subviral particles are also cleaved by furin. Mature virions and subviral particles are subsequently released by exocytosis.

domain [21,22]. Cryo-electron microscopy revealed the presence of a C-terminal "stem" and two transmembrane sequences through which the E protein is anchored to the viral surface [23]. During endocytosis, under the acid condition in endosome, the E proteins undergo a dramatic structural change from dimer into trimer. These trimers cluster on the viral surface and induce curvature that might promote fusion. In the E trimer, the fusion peptide is exposed at the tip of the trimer, leading the virus and endosomal membranes to merge [24–26].

Dengue virus is known to enter cells through receptor-mediated endocytosis [14,27–37]. Several primary cellular receptors and low-affinity coreceptors for flaviviruses have been identified. Dendritic cell-specific ICAM-grabbing non-integrin and CD-14-associated molecules have been suggested as the primary receptors for dengue virus [28, 31,36]. Heparin and other glycosaminoglycans act as low-affinity coreceptors for several flaviviruses [27,29,30,32–

35,37].

Enzymatic Activities and Processing

Once dengue virus enters cells, the viral genome consisting of a single positive-strand RNA is liberated into the cytoplasm, and is used as a template for translation into a large polyprotein precursor. The cotranslational and post-translational processing of the polyprotein precursor by the host cell proteases (e.g. signalase, furin) within the ER and by the viral protease (NS3pro) in cytoplasm gives rise to three structural proteins of the enveloped virus particle (C-prM-E) and seven nonstructural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5), most of which are thought to be required for assembling together with yet poorly-defined host proteins to form a replication machine in the cytoplasm of the infected cells that catalyze copying of the viral RNA [38] [**Fig. 1(B)**]. The newly-generated RNAs are then used for translation to produce more viral proteins and for copying more viral RNAs of virus particles.

During translation of the polyprotein, the structural proteins are translocated and anchored in ER by various signal sequences and membrane anchor domains (**Fig. 4**). And the C-terminal region of the C protein, serving as a hydrophobic signal sequence, anchors the C protein into the ER membrane, and thus translocates prM into the lumen of ER. Subsequently, this signal sequence is cleaved off by the host cell signalase, liberating the N-terminus of prM, whereas the C protein remains closely associated with the ER membrane [39]. This association is present in all flaviviruses, and promotes viral assembly [40]. The prM protein has two transmembrane-spanning domains, a stop transfer sequence and a signal sequence (**Fig. 4**), and the sequentially linked E protein is then also translocated into the lumen of ER. After the appropriate proteolytic cleavages, the C protein remains associated with the ER membrane, whereas the viral RNA is released into the cytoplasm after replication. On the lumenal side of ER, the prM and E proteins form a stable heterodimer within a few minutes of translation [41–43].

NS3 is a multifunctional protein. Based on sequence comparison with known proteases, a classic trypsin-like serine protease with a catalytic triad (His51, Asp75, and Ser135) was identified in the N-terminal 180 amino acid residues of NS3 [38,44–48]. The enzyme requires NS2B as a cofactor for activation of protease activity [46,49]. The minimum sequence for protease activity was mapped to the first 167 residues of NS3 [50]. The C-terminal part of NS3 carries three other enzymatic activities: an RNA-stimulated nucleoside triphosphatase (NTPase); an RNA

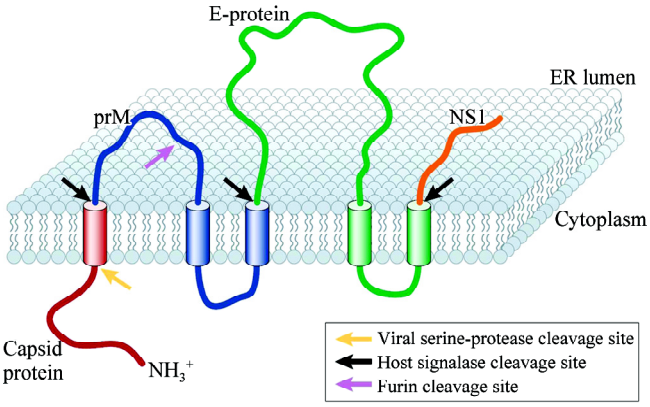

Fig. 4 Membrane topology of dengue and associated flavivirus structural proteins [20] The predicted orientation of the structural proteins across the endoplasmic reticulum (ER) membrane is shown. Transmembrane helices are indicated by cylinders, arrows indicating the sites of post-translational cleavage by specific enzymes are indicated by different colors. E, envelope; NS1, nonstructural protein 1; prM, precursor to membrane protein.

helicase; and an RNA 5′-triphosphatase (RTPase) [45,47, 50–55] [**Fig. 5(A)**]. The latter is most likely required for removal of the terminal phosphate group from the newly-synthesized RNA, and for formation of the viral cap structure at the 5′-end of the virus RNA genome [51,54, 55]. The helicase functions to unwind the double-stranded nucleic acids during viral RNA replication [45,50]. This activity is energy-dependent and is carried out by its NTPase activity that hydrolyses ATP to generate the necessary energy [50,52,53]. The minimal domain for helicase and NTPase activities was reported to comprise the full C-terminal part of NS3 [45,51,56]. It is most likely that the two different enzymatic activities (RTPase and NTPase) are exerted by one active site in the same protein, and are strictly Mg^{2+}-dependent [51,54].

Unlike trypsin, the NS3 protease has a marked preference for dibasic residues (e.g. Arg and Lys at positions P_1 and P_2 [57]) and requires a cofactor supplied by the non-structural protein NS2B for efficient cleavage of the dengue virus polyprotein [46]. The NS2B-NS3 protease catalyzes the cleavage of the viral polyprotein precursor in the non-structural region at the NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5 sites [58,59]. Additional proteolytic cleavages are within the viral C protein, NS2A, and NS4A, and at the C-terminal part of NS3 [49,60–62], whereas the host cell proteases (such as signalase and furin) act on the remaining cleavage sites [63–66] [**Fig. 1 (B)**]. Deletion studies have further shown that a central 40-amino acid conserved hydrophilic domain within NS2B

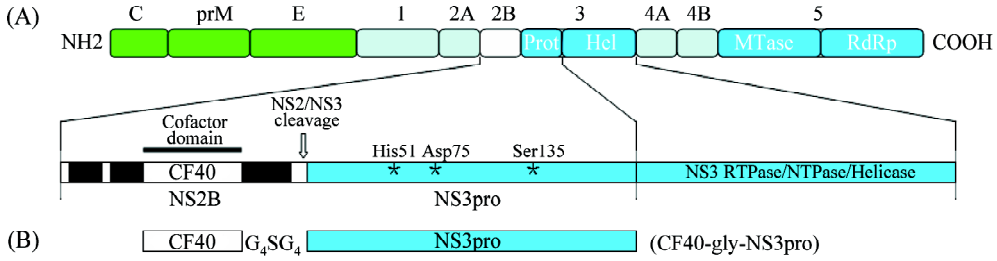

Fig. 5 Scheme for the enzymatic activities of dengue virus (A) Scheme for viral enzymes and non-structural (NS) protein NS2B. The cofactor NS2B is in white, and the protease (Prot) domain of NS3 (NS3pro), the RNA 5′-triphosphatase (RTPase)/RNA-stimulated nucleoside triphosphatase (NTPase)/helicase (Hel) domain of NS3, the methyltransferase (MTase) and RNA-dependent RNA polymerase (RdRp) domains of NS5 are in blue. The black regions represent the hydrophobic regions flanking the conserved hydrophilic domain of NS2B (cofactor domain). The protease catalytic triad (His51, Asp75, and Ser135) within the N-terminal 185 amino acid residues of NS3 is indicated. (B) The engineered recombinant NS2B-NS3 fusion protein is the complex of NS3pro and the central 40 amino acid conserved hydrophilic part of NS2B (CF40) linked through a flexible glycine-rich linker [$(Gly)_4Ser(Gly)_4$]. The construct is designated CF40-gly-NS3pro.

is sufficient for the cofactor activity [67]. The flanking hydrophobic residues of NS2B are likely to function to associate the protease complex and the infected cell membranes [68] [**Fig. 5(A)**]. The residues within the core hydrophilic segment of the cofactor NS2B responsible for binding the NS3 protease domain have been further identified [69].

The NS5 proteins of all flaviviruses consist of at least three very important enzymes that are essential for viral propagation [70,71]. Located at the NS5 N-terminal part, approximately 320 residues comprise the S-adenosyl-methionine-dependent methyltransferase (MTase), possessing the MTase and guanylyltransferase activities responsible for capping and methylation of the capped positive-strand genomic RNA at the 5′-end [**Fig. 5(A)**]. The structure of MTase of DEN-2 and its complex with relevant small molecules has been determined by X-ray crystallography [72]. As the RNA capping is an essential viral function, it provides a structural basis for the rational design of drugs against flaviviruses. The C-terminal part of NS5 is the RNA-dependent RNA polymerase (RdRp) at residue position 420–900, responsible for synthesis of the intermediate RNA template for further replication of the positive-strand genomic RNA [73,74] [**Fig. 5(A)**]. The RdRp activity of dengue virus has been shown for several other flaviviruses, including West Nile virus and Kunjin virus [71,75–77]. In all flavivirus RdRp, there is an essential and classical amino acid sequence signature, the Gly-Asp-Asp motif [71].

Assembly, Maturation, and Release

During the assembly and maturation of viral particles, the C protein of dengue virus is crucial. The C proteins of DEN-2 readily form dimers in solution, and can be regarded as building blocks for NC assembly [78–80]. The secondary structure of the C protein from residue 21 to 100 is composed of four α-helices: helix I, the N-terminal part; helix II, hydrophobic and essential for the ER membrane association; and helices III and IV, the C-terminal part containing the signal sequence for anchoring the ER membrane. The N-terminal 20 residue fragment is flexible [78,81]. The 3D picture of the dengue C protein shows a dimeric structure maintained by the homotypic binding domain, facilitating the interaction between RNA and the C protein by an asymmetric charge distribution, suggesting the membrane-associated C protein mediates viral assembly by a highly coordinated interaction with the prM-E heterodimer in ER [40,81]. Several copies of the C protein and one copy of the genomic RNA form the NC that finally buds into the lumen of ER and produces immature viral particles. It was shown that the C protein can also be found in the nucleus, and can possibly interact with heterogeneous nuclear ribonucleoprotein K, suggesting a role in regulation of the dengue life cycle probably by controlling apoptosis [82].

Virus maturation is a two-step process. First, during maturation in the TGN, under low pH conditions, the prM proteins are conformationally changed and cleaved by the host cell furin. As a result, the 60 “spikes” composed of the three prM-E heterodimers that project from the immature virus surface are dissociated, consequently forming a smooth surface of mature virus composed of 90 E homodimers (**Fig. 2**) [66,83,84]. Second, during exocytosis, a major rearrangement of the E protein occurs. The anti-parallel E homodimers dissociate into monomers,

that then re-associate into parallel homotrimers [14,85,86]. The mature viral particles are then eventually released from the host cell by exocytosis.

Potential Targets and Progress in Study of Vaccines and Drugs

The re-emerged dengue fever/DHF has becomes a global threat and endemic in more than 100 countries throughout the Americas, Southeast Asia, and western Pacific islands. It is an increasingly important public health concern, and challenges scientists to discover new vaccines and antiviral drugs. There are three strategies for the control of dengue virus disease: the control and elimination of the mosquito vector; the development of safe vaccines to prevent infection; and the search for specific antidengue drugs for treatment of disease. So far, the only way to prevent dengue transmission is to control the principal vector mosquito and reduce human-vector contact, because there is no approved vaccine or effective antiviral drug for dengue disease.

In the absence of effective antiviral drugs, vaccination offers a good option for decreasing the incidence of these diseases. An ideal dengue vaccine must be effective for all four virus serotypes, be safe in 9–12-month-old children, and provide a long-lasting protective immunity. Various strategies have been used to develop dengue vaccines [87–89]. Using primates for preclinical evaluation, chimeric tetravalent vaccines show a high level of neutralizing antibody against all serotypes, and clinical trials are in progress [88–90]. Another type of dengue vaccine is the DNA vaccine [91,92]. Recently, a new dengue tetravalent DNA vaccine against DEN-3 and DEN-4, based on prM/E and combined with two previously constructed DNA vaccines against DEN-1 and DEN-2, has been constructed [93]. Molecular biology techniques have facilitated the development of the recombinant subunit vaccines. The structural proteins (E and prM) and non-structural proteins (NS1 and NS3) are the dominant sources of cross-reactive $CD4^+$ and $CD8^+$ cytotoxic T-lymphocyte epitopes [94–97]. Some immunization studies show that these proteins are important for inducing protective immunity [98–105]. The combined DNA and protein vaccines have a synergistic effect on the antibody titers [106–109].

A tetravalent live attenuated vaccine was developed at the Walter Reed Army Institute of Research (Silver Spring, USA) and licensed to GlaxoSmithKline [87]. However, there are still several issues that make the live-attenuated vaccines problematic, including the phenomenon of antibody-dependent enhancement [87,110].

There are four stages of the viral life cycle, and each stage can be considered for the development of drugs. In stage 1, prevent viral entry or infection of the host cell, or inhibit fusion of the viral envelope with the host vesicles. The E protein can be taken as an ideal target. In stage 2, prevent maturation processing of the individual viral protein. The well-studied viral protease is considered a good target. In stage 3, prevent viral RNA synthesis by inhibiting the viral helicase and RdRp. Finally, in stage 4, target the host proteins such as furin and signalase that help the maturation and release of infectious viral particles.

It is well known that dengue virus NS3 is a multifunctional protein with an N-terminal protease domain (NS3pro), RTPase, an RNA helicase, and an RNA-stimulated NTPase domain in the C-terminal region [45,46] [**Fig. 5(A)**]. Thus, the dengue virus NS3 plays a crucial role in viral replication and represents an interesting target for the development of specific antiviral inhibitors/drugs.

NS3pro is required to process the polyprotein precursor into the individual functional proteins that are essential for viral replication, thus NS3pro is a promising drug target [111]. The 3D structure of the protease domain of NS3 was solved [112]. Several inhibitors targeting hepatitis C virus (HCV) NS3pro are now in different stages of clinical trial [113]. A recombinant NS2B-NS3 fusion protein has been engineered in which a 40 residue cofactor corresponding to the core part of NS2B is covalently connected through a flexible glycine-rich linker to DEN-2. NS3 protease has been successfully expressed in *Escherichia coli*, and the purified protein was found to be highly active on peptide substrates designed on the base of the polyprotein cleavage sites [114] [**Fig. 5(B)**]. The cofactor NS2B, which has three hydrophobic regions flanking a conserved hydrophilic domain of approximately 40 amino acid residues, revealed that this hydrophilic region is necessary and sufficient for activation of the NS3 protease domain *in vivo* and *in vitro* [67,68]. The substrate-based inhibitors with a natural dengue recognition sequence can inhibit the DEN-2 protease in a competitive manner [114–116]. Similar to the HCV NS3 protease, the small molecule inhibitors of NS2B-NS3pro, based on the peptide substrates, have been synthesized [114,115,117–119]. However, in contrast to HCV NS3 protease, some synthetic peptides representing the polyprotein cleavages sites do not show an appreciable inhibition on this protease [115] and some other small inhibitors (molecules) based peptide substrate have an apparent Ki value at μM and nM [114, 117–119]. According to the crystal structure of the NS3 protease complexed with the mung bean Bowman-Birk inhibitor [120], several non-substrate-based compounds

were developed [121]. The crystal structures of a dengue NS2B-NS3pro complex, and of a West Nile virus NS2B-NS3pro complex, with a substrate-based inhibitor Bz-Nle-Lys-Arg-Arg-H have also been solved [122]. The structures identify the key residues for NS3pro substrate recognition and clarify the mechanism of NS3pro activation [122].

The NS3 helicase essential for in viral replication also makes it an attractive target for the design of antiviral compounds [123,124]. The 3D structure of dengue virus helicase/NTPase shows that there are three domains: domains I and II situated at the N-terminal (the NTPase site resides between these two domains); and the C-terminal domain III bound to NS5 [125]. A tunnel that runs across the interface between domain III and the tip of domains I and II can accommodate a single-stranded nucleic acid tail along which the enzyme can translocate. This motion is triggered by NTP hydrolysis to provide the energy [125, 126]. There is no drug to target NS3 helicase. Several low molecular weight compounds that inhibit the NS3 helicase from the West Nile virus or the Japanese encephalitis virus have been described [127]. The regions crucial for the ATPase or nucleic acid duplex unwinding activity have been identified by mutagenesis that might be suitable for the design of allosteric inhibitors [124,128].

The viral polymerase NS5 (RdRp) is also a potential target for drug design [73,74]. The crystallographic structure of an active fragment of the dengue virus NS5 RdRp has been refined at 1.85 Å resolution [129]. This structural information of NS5 RdRp will facilitate the design of antiviral compounds because the host cells are devoid of this enzymatic activity. In fact, the selective inhibitors against HIV-1 reverse transcriptase, and the inhibitors against hepatitis B virus, cytomegalovirus, and herpes simplex virus polymerases have been approved as drugs for treatment of the associated viral infections [130]. In addition, the interaction between viral NS5 RdRp and the NS3 helicase also offers a possible target for drug design [131].

In conclusion, although there are no ideal vaccines or therapy for the prevention and treatment of DHF, the understanding of the life cycle of dengue virus has made great progress over the past few years, and all the life cycle stages can represent potential targets for antiviral drug discovery.

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