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The flavivirus NS2B-NS3 protease-helicase as a target for antiviral drug development

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Abbreviations: NS nonstructural protein; NS3pro, protease domain of NS3; NS3hel, helicase domain of NS3; RdRP, RNA-dependent RNA polymerase

The flavivirus NS3 protein is associated with the endoplasmic reticulum membrane via its close interaction with the central hydrophilic region of the NS2B integral membrane protein. The multiple roles played by the NS2B-NS3 protein in the virus life cycle makes it an attractive target for antiviral drug discovery: The N-terminal region of NS3 and its cofactor NS2B constitute the protease that cleaves the viral polyprotein. The NS3 C-terminal domain possesses RNA helicase, nucleoside and RNA triphosphatase activities and is involved both in viral RNA replication and virus particle formation. In addition, NS2B-NS3 serves as a hub for the assembly of the flavivirus replication complex and also modulates viral pathogenesis and the host immune response. Here, we review

biochemical and structural advances on the NS2B-NS3 protein, including the network of interactions it forms with NS5 and NS4B and highlight recent drug development efforts targeting this protein.

KEYWORDS: dengue virus NS2B-NS3 protease; crystal structures; antiviral drug target; serine protease; RNA helicase, replication complex

INTRODUCTION

The rationale and approaches taken to discover dengue antivirals, particularly the targeting of the viral protease and polymerase activities were laid out in some early work carried out in several academic laboratories and have been captured in a monograph arising from the Novartis Foundation Symposium held in September 2005 (Xu et al., 2006). The intensive efforts over the last decade to discover novel antivirals against dengue virus (DENV), was reviewed recently (Lim et al., 2013; Noble and Shi, 2012). At the same time the efforts to produce vaccines against DENV which started more than 40 years ago is only now starting to bear fruits with several vaccines now finally reaching various stages of human clinical trials (Capeding et al., 2014; Sabchareon et al., 2012). This long road for developing a dengue vaccine, long after vaccines have become available against YFV (Verma et al., 2013), JEV (Yun and Lee, 2013) and TBEV (Rendi-Wagner, 2008), illustrates some of the challenges specific to DENV. Here we extend previous reviews by adding some of the new developments on the biology of the flavivirus NS2B-NS3 enzyme, including its interactions with viral proteins NS5 and NS4B and recent antiviral drug development not covered in (Lescar et al., 2008b; Li et al., 2014a; Lim et al., 2013).

The flavivirus genome is approximately 11 kilobases long with a 5' end cap structure similar to that of cellular mRNA but devoid of a 3' end polyadenylation tail (**Fig. 1A**)

(Lindenbach et al., 2007). Long untranslated regions at both 5' and 3' ends play important roles in virus replication, viral protein translation and virion assembly (Alvarez et al., 2006; Filomatori et al., 2006; Khromykh et al., 2003; Markoff, 2003; Polacek et al., 2009; Villordo and Gamarnik, 2008; Yu et al., 2008a). Viral protein translation results in a single polypeptide precursor spanning across the endoplasmic reticulum (ER)-derived membrane. This polypeptide is processed into three structural proteins, capsid (C), envelope (E), and membrane protein (M) and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A NS4B, and NS5 (**Fig. 1A**). Both host ER-derived proteases and the viral NS2B-NS3 protease specifically cleave the precursor to release the individual viral proteins (**Fig. 1A**) (Lindenbach et al., 2007). The C-terminal region of NS3 is a nucleoside triphosphatase (NTPase), a 5' terminal RNA triphosphatase (RTPase) and a RNA helicase (Xu et al., 2005). NS5 possesses RNA methyl-transferase (Dong et al., 2012; Dong et al., 2014; Egloff et al., 2002; Ray et al., 2006) and RNA-dependent RNA polymerase (RdRp) activities (Yap et al., 2007, Malet et al., 2008). Together with the viral RNA, viral cofactors, and host cell cofactors, NS3 and NS5 form the virus replication complex (RC) that assembles on the intracellular membrane to amplify the viral genome (Murray et al., 2008; Paul and Bartenschlager, 2013). Therefore, in principle, functional inhibition of the viral NS proteins and/or disruption of the RC underlie target-based anti-flavivirus drug development (Bollati et al., 2010; Lim et al., 2013; Noble and Shi, 2012; Sampath and Padmanabhan, 2009).

Flavivirus NS3 (69 kDa) is the second-largest viral protein after NS5 in the flavivirus genome and plays several essential roles in the viral life cycle (**Fig. 1B**). NS3 has a N-terminal protease chymotrypsin-like domain, that cleaves the viral polyprotein precursor to release individual NS proteins and a C-terminal NTPase-dependent RNA helicase (with a SF2 like fold) involved in genome replication and viral RNA synthesis (Lescar et al., 2008a; Luo et al., 2012). A recent report has also described an ATP-independent RNA annealing

activity for the DENV NS3 helicase (Gebhard et al., 2012). These two functional domains are connected by a flexible linker of about 10 amino acids (**Fig. 1C**) (Luo et al., 2010). Why a single polypeptide encompasses two domains with seemingly disconnected activities remains elusive. In addition to the advantages afforded by the colocalization of two different enzymatic activities (see below), several studies suggested that both enzymatic domains are functionally coupled and that the helicase activity is enhanced by the presence of the protease domain (Luo et al., 2010; Luo et al., 2008b; Xu et al., 2005; Yon et al., 2005). If confirmed, this "cross-talk" between domains would be reminiscent of the HCV NS3 protein, also a fusion between a protease and a ATPase/helicase (Lindenbach et al., 2007) whose activity is regulated by the protease domain (Frick and Lam, 2006). Recently, several drugs targeting the NS3 protease of HCV have received U.S. Food & Drug Administration (FDA) approval, and a few promising candidates are in clinical trials (Salam and Akimitsu, 2013). This should further stimulate the development of antiviral drugs targeting the flavivirus NS2B-NS3 protein.

NS2B-NS3 PROTEASE (NS2B-NS3PRO)

The N-terminal domain of NS3 (aa 1–169) is a chymotrypsin-like serine protease that cleaves the viral polyprotein both in *cis* and in *trans* (Chambers et al., 1990b; Li et al., 2005). To function as an active enzyme, the NS3 protease requires the NS2B cofactor (Falgout et al., 1991; Jan et al., 1995; Yusof et al., 2000; Zhang et al., 1992). NS2B is an integral membrane protein of 14 kDa that contains three domains: two trans-membrane segments located at the N and C termini and a central region of 47 amino acids (spanning amino-acids 49–96) that acts as an essential protein cofactor of the NS3 protease (**Fig. 1B**) (Clum et al., 1997). The flavivirus NS3 protein is neither soluble nor catalytically active as a protease *in vitro*, suggesting that it does not fold properly without the NS2B protein, that must be either

provided in *cis* (Xu et al., 2005) or in *trans* (Phong et al., 2011; Wu et al., 2003) . In 1999, a crystal structure of the Dengue virus NS3 protease (PDB code: 1BEF, now obsolete) allegedly using refolded NS3 protease domain was published. However, the inability of several academic and industrial laboratories engaged in drug discovery research to repeat this work (cited more than 97 times) eventually resulted in the paper being retracted by the publisher (THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 49, p. 34468, December 4, 2009). The complex between the NS3pro and the Bowman-Birk inhibitor published in the JOURNAL OF MOLECULAR BIOLOGY (2000) 301,759-767) has now also been retracted. The important point is that NS3pro is expressed as an insoluble protein in the absence of NS2B and it is likely that the PDB entry 1BEF may have been modeled based on HCV NS3 protease domain as a template.

The flaviviral protease field benefitted immensely when Paul Young and colleagues provided a remedy for the lack of solubility of the sole protease domain of NS3, by engineering a synthetic fusion protein, where the NS2B hydrophilic region (residues 49 – 95) was connected to the NS3 protease domain via a nine amino-acids (Gly4-Ser-Gly4) linker. When expressed in bacteria, the fusion protein becomes soluble and catalytically active *in vitro* (Arakaki et al., 2002; Leung et al., 2001). Not only did this advance greatly facilitate subsequent structure determination of NS3 (Table 1 and **Fig. 2 ABCD**), but it also revealed a dual function for the central region of NS2B: residues 49–67 of the NS2B cofactor N-terminal hydrophilic region are sufficient to render NS3 protease soluble but the resulting protein construct remains enzymatically inactive (Erbel et al., 2006; Luo et al., 2008a). The C-terminal part of the central region of NS2B (aa 68–96) forms a β- hairpin that contributes to shape the hydrophobic S2 and S3 pockets in the substrate-binding site of NS3pro. Thus, this region of NS2B directly interacts with either the substrate or substrate-based-inhibitors, supporting a direct catalytic role for NS2B (**Fig. 2**) (Erbel et al., 2006; Noble et al., 2012;

Robin et al., 2009). Thus, the NS2B cofactor is able to regulate NS3pro activity both by stabilizing the correct protein fold of the core structure and also by directly participating in substrate cleavage (Erbel et al., 2006; Noble et al., 2012; Robin et al., 2009). This is in contrast to HCV NS3pro, where only a short segment of ~11 aa from the NS4A cofactor is required to form the active protease. Thus, a key difference between flaviviruses and hepaciviruses is that the NS4A protein cofactor of HCV plays a structural role but does not participate in substrate recognition or catalysis directly (Kim et al., 1996; Tomei et al., 1996; Urbani et al., 1998). Besides $Gly_4SerGly_4$-linked NS2B-NS3pro constructs, a protease complex containing a segment of 50-residues of the NS2B cofactor region and NS3pro without the glycine linker was recently reported using a coexpression system. Remarkably, this unlinked protease complex was catalytically active and exhibited an enzymatically active structure as determined using NMR (Kim et al., 2013). The classical Serine-protease catalytic triad (His51, Asp75, Ser135) is found in the central cleft of the NS3 protease domain (**Fig. 2A and B**). The protease recognizes the positively charged residues Arg/Lys at the P1 and P2 positions, followed by a small or polar amino acid at P1' – first residue after the cleavage, although exceptions are found (e.g., Glu is found at the P2 position at the NS2B-NS3 junction) (Gouvea et al., 2007; Li et al., 2005; Shiryaev et al., 2007). Fluorogenic peptide substrates are commonly used to study protease activities and for inhibitor screening purposes (Lai et al., 2014; Li et al., 2005; Niyomrattanakit et al., 2006; Yusof et al., 2000). Structures of NS2B-NS3pro bound to substrate-derived peptide inhibitors or aprotinin (bovine pancreatic trypsin inhibitor, BPTI) have revealed the substrate specificity and the catalysis mechanism (**Fig. 2D**). NMR structures of NS2B-NS3pro indicates that the disordered linker regions between NS2B cofactor and NS3 N terminus may be involved in substrate/inhibitor binding (Chen et al., 2014; de la Cruz et al., 2014, 2011) (**Fig. 2C**). Nonetheless, caution has to be exercised when

using such artificial single-chain NS2B-G4SG4-NS3 construct for the development of protease inhibitors, in terms of their relevance to the in vivo situation.

INHIBITORS OF NS2B-NS3Pro

NS3 protease inhibitors are currently designed by either competing with substrate binding or by disrupting the interaction between NS2B and the NS3 protease domain, for a recent review see (Lim et al., 2013). Despite a wealth of structural and biochemical information available on the NS2B-NS3pro substrate-binding pocket, no compound has progressed to the preclinical stage yet. Researchers are still facing multiple challenges in identifying effective drugs that target NS2BNS3pro: The substrate-binding pocket is shallow and largely exposed to the solvent, rendering interactions between peptide mimics and enzyme labile. Moreover, the dibasic nature of residues at the P1 and P2 positions hinder peptidomimetic permeability and stability. Researchers have begun to study the structure and activity of the native-state-like NS2B-NS3pro in the context of the membrane, which could lead to better screening assays to identify protease inhibitors (Choksupmanee et al., 2012; Huang et al., 2013). Several studies have identified novel non-peptidic inhibitors: Very recently, using a computational approach that included elaboration of a pharmacophore model, the NS2B-NS3pro X-ray crystallographic structure and a docking protocol, Li et al have identified a competitive non peptidic inhibitor (compound 14, **Fig. 3A**) with an EC50 (Compound concentration that gives half-maximal response) of 5 μM and a CC50 (concentration of drug that results in *toxicity* to 50% of the cells compared with untreated control cells) greater than 300 μM (Li et al., 2014b). In another study, flavonoids (**Fig. 3B**) were found to be non-competitive inhibitors of DENV NS2B-NS3pro serotypes 2 and 3 with IC50 (Compound concentration giving a 50% inhibition in enzymatic assay) values 15-44 μM (de Sousa et al., 2015). Direct binding of compound 5 (**Fig. 3B**) to DENV serotype 3 NS2B-NS3pro was also

demonstrated (Kd=20 μM) using microscale thermophoresis (de Sousa et al., 2015). Diaryl (thio)ethers were discovered showing selective and noncompetitive inhibition towards the serotype 2 and 3 DENV NS2B-NS3pro in vitro and in cells. Some benzothiazole derivatives exhibited IC50s in the low-micromolar range (Wu et al., 2015). Starting from a commercial compound library, Liu et al have identified a novel class of thiadiazoloacrylamide derivatives with potent inhibitory activity against the NS2B-NS3pro (Liu et al., 2014). The most potent compound in a series of analogues had an IC50 at 2.24 μM based on in vitro DENV2 NS2B-NS3pro assays. Finally, an interesting study provided evidence for the possibility of targeting the interaction between NS2B and NS3 as an effective antiviral strategy (Pambudi et al., 2013). In this study, Pambudi et al., report compound SK-12 (**Fig. 3C**) that acts by blocking the NS2B/NS3 protease interaction; SK-12 significantly inhibited DENV4 replication with an EC50 of 3.8 μM as well as JEV with EC50 of 14.4 μM (Pambudi et al., 2013). For each of these novel non-peptidic inhibitors, putative modes of binding were proposed. However, further development will be greatly facilitated once the respective cocrystal structures become available to confirm the predicted interactions.

NS3 NTPASE/RNA HELICASE (NS3HEL)

The C-terminal domain of the NS3 protein (aa 180–618) belongs to the helicase superfamily 2 (SF2) (Fairman-Williams et al., 2010; Gorbalenya and Koonin, 1993). The overall structure can be broken up into three subdomains (**Fig. 2C**). Subdomain 1 and 2 adopt the RecA-like fold (Rao and Rossmann, 1973; Story and Steitz, 1992) and contain eight conserved motifs essential for RNA binding, ATP hydrolysis and communication between both binding sites (Fairman-Williams et al., 2010; Gorbalenya and Koonin, 1993; Pyle, 2008). The third subdomain forms the single-stranded RNA binding tunnel. There is also solid evidence suggesting that subdomain 3 mediates the interaction between NS3 and NS5 and

disrupting this interaction could constitute a strategy for the design of antiviral compounds (Brooks et al., 2002; Fang et al., 2013; Tay et al., 2015). NS3 also has RNA 5' triphosphatase activity (RTPase), which shares the same active site for ATP binding and hydrolysis (**Fig. 2C**) (Wang et al., 2009). RNA 5' triphosphate hydrolysis is the first step for viral RNA capping (Decroly et al., 2012). Viruses carrying a defective or impaired NS3 helicase gene cannot replicate properly, indicating an essential role for NS3 helicase/RTPase activity in virus replication (Matusan et al., 2001). It is speculated that NS3hel could resolve secondary structures of the genomic RNA, displace transacting protein cofactors, and/or separate the dsRNA intermediates that are transiently formed during the polymerization reaction catalyzed by NS5 RdRP into single-strand form amenable for further rounds of amplification (Malet et al., 2007; Yu et al., 2008b).

Like most eukaryotic DExx helicase proteins, NS3 recognizes RNA largely in a sequence-independent manner. During the duplex RNA unwinding process, one strand is inserted into the RNA binding groove of NS3hel and the sugar-phosphate backbone makes either direct or water-mediated contacts to the helicase residues, whereas the other strand is separated by a hydrophobic β-hairpin that protrudes from subdomain 2. This β-hairpin acts as a "helix opener" to disrupt base stacking and stabilize the unwound duplex, while the basic concave region between subdomain 2 and 3 works as "the translocator" by binding the 3' overhang (Buttner et al., 2007; Luo et al., 2008c; Myong et al., 2007; Pyle, 2008). NS3hel binds to the 3' overhang sequence of the duplex RNA and unwinds in the 3' to 5' direction (**Fig. 2C**) (Benarroch et al., 2004; Li et al., 1999; Xu et al., 2005). NTP hydrolysis provides the chemical energy to power the translocation and unwinding mechanical process, although the precise mechanism coupling these two activities remains unclear. Single-molecule and structural biology studies on the homologous HCV NS3hel suggested that the basic step of translocation and unwinding is one base per molecule of ATP hydrolysed (Appleby et al.,

2010; Dumont et al., 2006; Gu and Rice, 2010; Myong et al., 2007). ComparaFtive studies on flavivirus NS3 are not yet available but will be of great interest. To date, the 3D structures of several flaviviral NS3hel domain have been reported (**Table 1**) providing a firm molecular basis for its various enzymatic activities.

NS3HEL INHIBITORS

Helicase inhibitors could serve as a pan anti-viral therapy against flaviviruses and were recently reviewed (Lim et al., 2013). While HCV NS3hel has been extensively studied, and several inhibitors have been reported (Lim et al., 2013), the lack of specific pockets at the RNA and at the NTP binding sites is likely to lead to significant toxicity, as compounds targeting these sites might also bind to many similar cellular proteins with helicase/NTPase activities. The intrinsic flexibility of motor proteins also makes it difficult to identify high-affinity and high potency compounds, although allosteric inhibition trapping the protein in one conformation, remains an attractive concept for inhibitor design (Li et al., 2010; Noble and Shi, 2012; Saalau-Bethell et al., 2012). Interestingly, several NS3hel inhibitors have been reported including the antiparasitic drug ivermectin (Mastrangelo et al., 2012) and suramin that was shown to inhibit DENV NS3hel activity with a Ki of 0.75 μM as a non-competitive inhibitor (Basavannacharya and Vasudevan, 2014). Rather disappointingly, an extensive fragment based screen targeting NS3hel and NS5 did not identify any fragment binding to NS3hel, while several fragments binding to the Methyltransferase domain of NS5 were found and further confirmed by careful crystallographic studies (Coutard et al., 2014). To date, no helicase inhibitors have been approved for clinical trials or usage.

THE FULL-LENGTH NS3 PROTEIN

Why apparently disconnected enzymatic activities hosted by two separate protein domains – protease and NTPase/helicase – are linked within a single NS3 polypeptide remains elusive and probably requires a better understanding of how the whole flavivirus replication complex (RC) functions. However, there are several obvious potential advantages for flaviruses to encode such fusion genes. Polyprotein processing occurs co- and post-translationally in ER-derived intracellular membranes (Chambers et al., 1991; Chambers et al., 1990a). NS3 may thus help bring NS4A-NS4B-NS5 in close proximity to NS2BNS3pro for the efficient cleavage and release of individual viral proteins (Amberg et al., 1994; Arias et al., 1993; Cahour et al., 1992; Lin et al., 1993a; Lin et al., 1993b). In addition, NS2B anchors NS3 in the membrane, which is a prerequisite for viral replication complex maturation. Flavivirus RNA replication is performed inside a vesicular-like compartment made of remodeled intracellular membranes (Miorin et al., 2013; Paul and Bartenschlager, 2013; Welsch et al., 2009). Therefore, NS2B together with other small viral membrane proteins might provide the membrane platform for the replicative enzymes NS3 and NS5 and viral RNA to assemble into a higher-order structure, flaviviral RC, that also includes NS1 (Miorin et al., 2013; Paul and Bartenschlager, 2013; Welsch et al., 2009). A more integrated view of the flavivirus RC, including the definition of its molecular interactions requires multiple approaches and the present understanding is summarized in an excellent review by Paul & Bartenschlager, 2013. Recently the molecular contacts between NS3hel and the integral membrane protein NS4B was mapped (Zou et al., 2015b), leading to a model for interactions between NS4B-NS3 and NS5 (**Fig. 5)** (Tay et al., 2015).

The study of enzymatic activities of the two domains (i.e. protease and helicase) on their own or in the context of the full-length NS3 protein, have led to some conflicting results. This may be partly due to the fact that the earlier work compared the enzymatic activities of full-length DENV2 NS3 (without NS2B cofactor) with that of NS3 helicase (aa171-618) and

demonstrated that full-length NS3 has much higher unwinding activity but a lower ATP hydrolysis rate (Xu et al., 2005; Yon et al., 2005). The affinity of dengue virus serotype 4 (DENV4) $NS2B_{18}NS3$ to ATP analogs is 10-fold higher than that of the truncated helicase (Luo et al., 2008a). Intriguingly, no influence of the protease on helicase activity has been observed (or vice versa) in an MVEV $NS2B_{47}NS3$ construct (Assenberg et al., 2009). More recently, the Gamarnik group also reported comparable ATPase and helicase activities for his_6-tagged DENV2 $NS2B_{47}NS3$ and NS3hel, respectively (Gebhard et al., 2012). These discrepancies may derive from variations in protein constructs, enzyme preparation, and experimental conditions for the assay. Furthermore, the correct folded protease domain might assist NS3 to select the RNA substrate: WNV NS3hel but not $NS2B_{48}NS3$, unwinds both DNA and RNA (Chernov et al., 2008). Studying the biochemistry of a native-state-like NS2B-NS3 protein in a membrane context is likely to clarify these issues and shed more light on the various roles played by NS3 during virus replication (Choksupmanee et al., 2012; Huang et al., 2013).

Intriguingly, structural studies of the full-length NS3 protein have revealed three distinct configurations that differ from each other in the relative positioning of the NS3pro with respect to NS3hel and are made possible by a flexible inter-domain linker (**Fig. 4**) (Assenberg et al., 2009; Li et al., 2014a; Luo et al., 2012; Luo et al., 2010; Luo et al., 2008a). We reported two different conformations of the DENV4 $NS2B_{18}NS3$ protein where the protease domain has rotated by approximately 161° (Fig. A and B) with respect to the helicase domain(Luo et al., 2010; Luo et al., 2008a). The structure of the MVEV $NS2B_{47}NS3$ (**Fig. 4C**) presented a third and a more radically different conformation of the NS3 protease-helicase (Assenberg et al., 2009). Interestingly, small-angle X-ray scattering (SAXS) experiments showed that both DENV4 $NS2B_{18}NS3$ protein and Kunjin NS3 adopted similar elongated shapes in solution (Luo et al., 2008a; Mastrangelo et al., 2007). Given the close fit

of the crystal structures to the hydrated envelope determined *ab initio* from SAXS data, it is evident that isolated flaviviral NS3 protease-helicase enzymes maintain an elongated conformation, with the two domains loosely connected through a flexible linker (**Fig. 4**). In contrast, the HCV NS3NS4A protease-helicase has been shown to adopt a globular conformation in solution (**Fig. 4D**) (Yao et al., 1999). In the first crystal structure of the similarly engineered $NS4A_{11}NS3$ fusion protein of HCV genotype 1b, the protease domain binds the C terminus of the NS3hel, mimicking the post-*cis*-cleavage state at the NS3-NS4A junction (**Fig. 4D**) (Yao et al., 1999). $NS4A_{11}NS3$ fusion proteins from the same or different genotypes have also been crystallized as apo-enzyme in a similar globular conformation(Appleby et al., 2011), in complex with ssRNA and ATP analogs (Appleby et al., 2011), in complex with a macrocyclic protease inhibitor (Schiering et al., 2011), and in complex with allosteric inhibitors (Saalau-Bethell et al., 2012). Sequence analysis revealed that the linker (aa 169–179) is less conserved compared to the two functional domains of the flavivirus NS3 (**Fig. 1C**). The flavivirus NS3 linker is also rich in acidic residues(Luo et al., 2008a) (**Fig. 1C**). Conversely, the linker region of HCV NS3 (aa 181–194) is very conserved and rich in proline residues (Kohlway et al., 2014; Li et al., 2014a). The sequence divergence between flavivirus and hepatitis virus NS3 correlates with the distinct protease-helicase conformations: the flexible and less conserved linker allows the flavivirus protein to adopt extended conformations, whilst the conserved and proline-rich linker imposes a globular conformation for HCV NS3 (**Fig. 1C and 4**). In flaviviruses, the NS3 linker may have evolved to span an optimum length to endow the protein with the flexibility needed for polyprotein processing and RNA replication. It will be interesting to assess the impact of the linker on Flaviviridae virus replication and targeting these regions could lead to the design of attenuated viruses (Luo et al., 2010). Thus, studies on the full-length NS3 protein have led to a better understanding of the flavivirus life cycle and suggested new avenues for the

development of antivirals and vaccines (**Fig. 4**) (Luo et al., 2010; Saalau-Bethell et al., 2012). Very recently, we determined the crystal structure of the full length NS5 protein from Dengue virus serotype 3 comprising both its Methyltransferase and RNA dependent RNA polymerase domains (Zhao et al., 2015). Compared to NS5 from JEV, DENV3 NS5 displayed a different domain orientation, where the MTase domain has rotated by ~ 115 degrees relative to the RdRp domain. An examination of the linker region of the NS5 MTase-RdRp revealed that it is also the least evolutionary conserved region (Lu and Gong, 2013; Zhao et al., 2015). Thus, one role played by linker regions in flaviviruses appears to impart intrinsic conformational flexibility to the multifunctional NS3 and NS5 proteins; this might facilitate the inherently dynamic replication process (Zhao et al., 2015).

NS3 ACTS AS A HUB IN THE FLAVIVIRUS REPLICATION COMPLEX

Apart from its various enzymatic roles, NS3 plays non-enzymatic roles mediated by its capacity to interact with other proteins from the RC, but also by its ability to recruit host cell proteins of cellular pathways involved in autophagy, actin polymerization and fatty acid biosynthesis (Heaton et al., 2010). Interestingly, YFV NS3 has been suggested to be involved in virus assembly in a role independent of its enzymatic functions. A W349A mutation within the subdomain 2 of NS3hel did not result in viral replication defects. However, no infectious viruses but only capsidless subviral particles could be detected from cells infected by the mutant virus (Patkar and Kuhn, 2008). The Fatty Acid Synthase colocalizes with NS3 at sites of viral RNA replication, with which it was shown to interact using a Yeast two-hybrid assay (Heaton et al., 2010). The mapping at atomic resolution of the interactions established by NS3 in the RC must await structural determination of relevant complexes; however, several residues involved in the interactions were identified by genetic interaction studies, mutagenesis in the context of infectious clones and revertant analysis and biochemical assays

(Tay et al., 2015; Umareddy et al., 2006; Zou et al., 2011; Zou et al., 2015a). A graphical summary of these interactions is given in **Fig. 5**. Lys330 of NS5 is required for the interaction between NS5 and NS3 and this involves the C-terminal 50 amino-acids residues of NS3 around N570, within subdomain 3 of its helicase domain (**Fig. 5**) (Tay et al., 2015). Interestingly, subdomains 2 and 3 of the NS3 helicase were shown to bind to the cytoplasmic loop of the integral membrane protein NS4B using a direct SPR-based biochemical assay (Zou et al., 2015a). Thus, these advances provide assays and reagents to screen for molecules able to disrupt the flavivirus RC, as possible antiviral compounds.

NS3 REGULATES THE HOST IMMUNE RESPONSE

Studies using yeast two-hybrid systems and viral revertant analysis have begun to reveal the flavivirus NS proteins interactome (Khadka et al., 2011; Le Breton et al., 2011; Zou et al., 2011) leading to the identification of more than 100 human proteins that interact with NS3, NS5, or both. Many interacting proteins are involved in modulating transcription or the host immune response (Krishnan and Garcia-Blanco, 2014). Type I interferon (IFN) production in human monocyte-derived dendritic cells was inhibited as the cytoplasmic adaptor protein – stimulator of interferon genes (STING) (Ishikawa and Barber, 2008) or mediator of IRF3 activation (MITA) (Zhong et al., 2008) – was found to be cleaved and thus inactivated by NS2BNS3pro from DENV (Aguirre et al., 2012). Moreover NS2BNS3 from WNV induces apoptosis through the activation of caspases 3 and 8 (Ramanathan et al., 2006). Another study reported that NS2BNS3pro from JEV inhibits the signaling pathway of activator protein 1 (AP-1), probably also through proteolysis. The authors suggested that NS2BNS3pro may contribute to JEV-induced neurotropic pathogenesis (Lin et al., 2006). Thus, a better understanding of how the various enzymatic and non-enzymatic activities of NS3 are

regulated is needed. This might inform antiviral drug discovery and also gives clues on how to mitigate their immunopathogenicity.

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Figure Legends

Figure 1. Schematic representation of the flaviviral genome, polyprotein, and functional domain partition along the *NS2B-NS3* gene. (A) Flavivirus genome and polyprotein. The viral genome contains a 5' cap and untranslated regions at both the 5' and 3' termini. The polyproteins are processed by both NS2BNS3 protease indicated as filled arrows and the host proteases by unfilled arrows. (B) The schematic representation of the *NS2B* and *NS3* genes. The central cofactor region of *NS2B* is in red, and the putative membrane associate regions of *NS2B* are displayed as blocks. (C) The sequence alignment of the linker regions between the protease and helicase domains of NS3 from DENV1-4 and various flaviviruses. (D) Sequence conservation of the linker region generated with WebLogo (http://weblogo.berkeley.edu/).

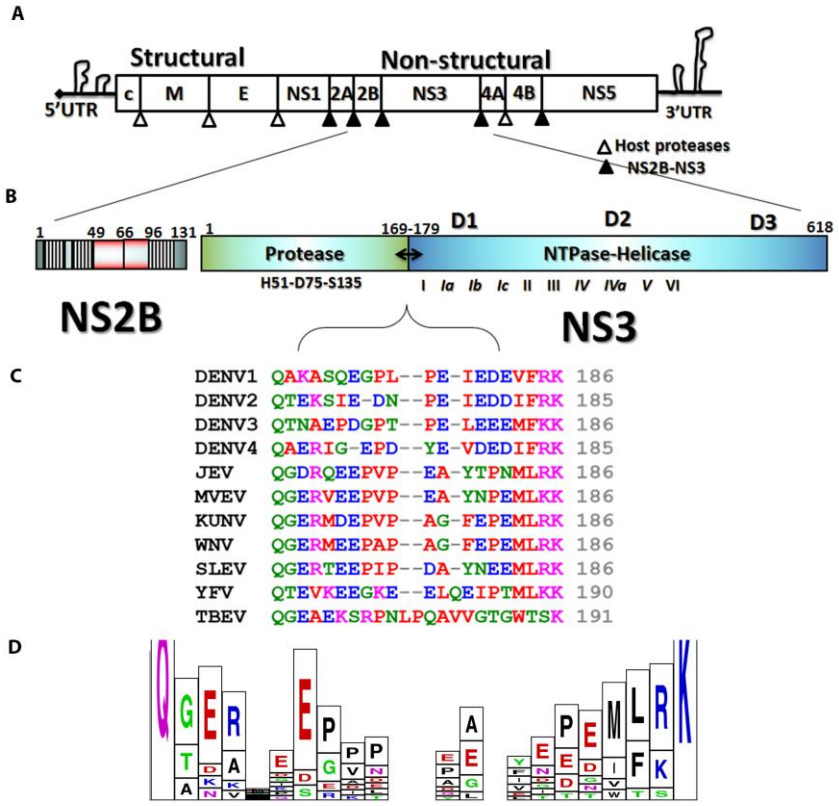

Figure 2. 3D structures of NS2B-NS3pro and NS3hel. (A) Overall fold of $NS2B_{47}$NS3pro from WNV. NS3Pro is shown in cyan, the $NS2B_{47}$ region in red, and the tetra-peptide inhibitor is in yellow, labelled as P1-P2-P3-P4. PDB code: 2FP7. **(B)** Surface view of the substrate binding site of WNV NS2BNS3pro. S1-S4 correspond to the pocket for P1-P4 of the peptide inhibitor. **(C)** NMR structure of $NS2B_{49\text{-}96}G_4SG_4$NS3pro bound to a protease inhibitor BEZ-NLe-Lys-Arg-M9P (in yellow). PDB code: 2M9P. **(D)** Crystal structure of WNV $NS2B_{49\text{-}96}G_4SG_4$NS3pro complexed with BPTI (in yellow). PDB code: 2IJO. **(E)** Ternary complex of DENV4 NS3hel-ssRNA-AMPPNP. NS3hel is colored according to the domain boundaries: hel1 is in green, hel2 in cyan, the putative duplex separating beta-hairpin motif in yellow, and hel3 in orange. Duplex RNA with a 3' overhang (in brown) is overlaid to the ssRNA (in red) to model the unwinding activity of NS3hel. AMPPMP, a nonhydrolyzable ATP analog, was used to mimic ATP binding at the ATPase active site (Luo et al., 2008c). PDB code: 2JLV.

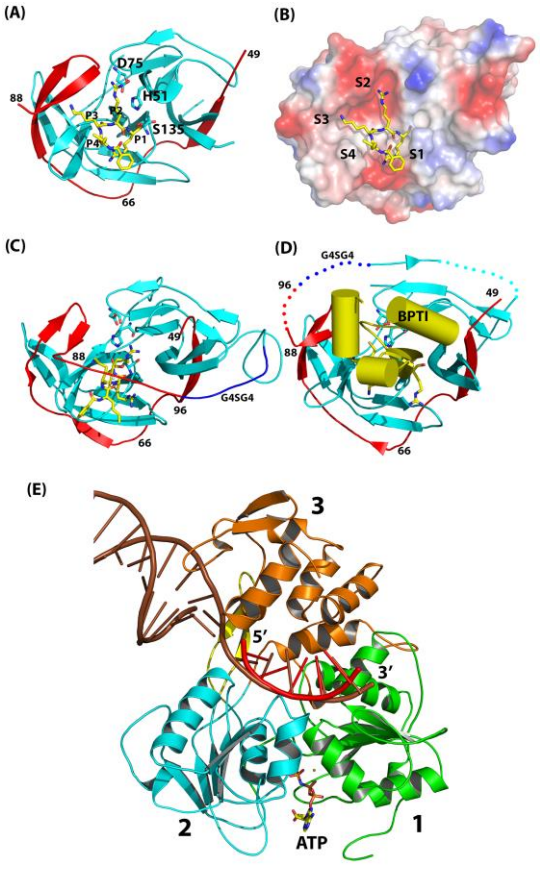

Figure 3. Structures of NS2B-NS3pro non-peptidic inhibitors. (A) Compound 14 identified by Li et al (2014) through the use of a new pharmacophore. This competitive inhibitor has an IC50 of 5 μM **(B)** A series of flavonoids that show non-competitive inhibition activities against NS2B-NS3pro from serotypes 2 and 3 (de Sousa et al., 2015) (**C**) Structure of a small compound targeting the interaction between nonstructural proteins 2B and 3 that inhibits dengue virus replication (Pambudi et al., 2013).

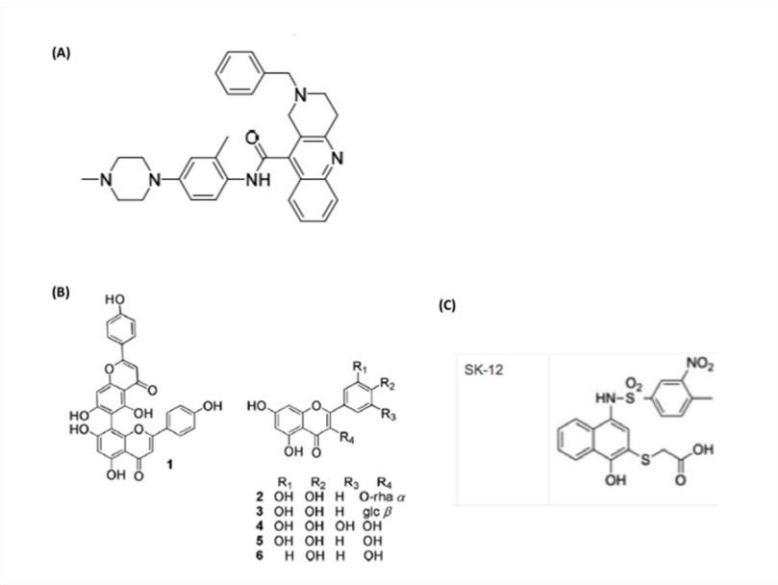

Figure 4. Structural views of the full-length NS3 protein. Structures of (A) and (B) $NS2B_{18}NS3$ from DENV4, (C) $NS2B_{47}NS3$ from MVEV, and (D) $NS3NS4A_{11}$ from HCV. NS2B (or NS4A) cofactor peptide is in red; NS3pro in green; linker in purple; and NS3hel is in blue, yellow, gray, and orange. In (A-D), the helicase domains are aligned, and the black dots represent the ssRNA binding tunnel. (E) When the protease domains are superimposed, the helicase domains display various orientations. Adapted from Li et al., 2014 (Li et al., 2014a).

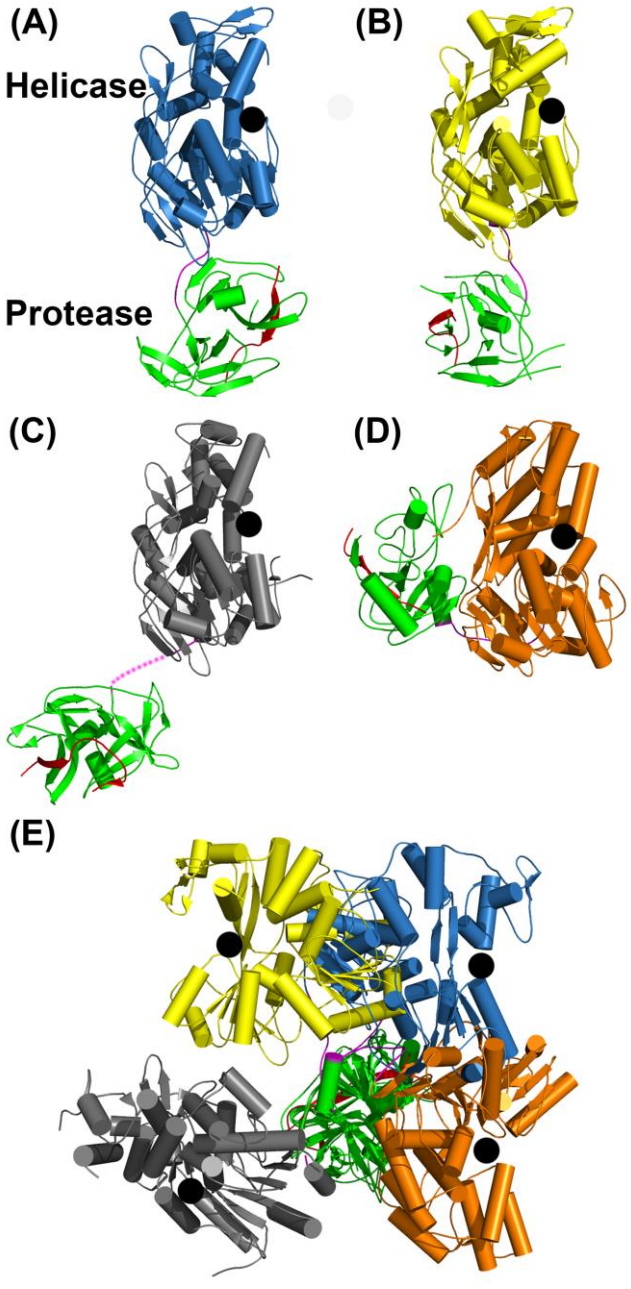

Figure 5. Putative model of the membrane-associated state of the NS2B-NS3 complex and its interactions with NS4B and NS5. NS2B structure is modeled based on its predicted TM regions (Luo et al., 2010). Residues 49–96 are the central hydrophilic cofactor region of NS2B; residues 83–85 directly participate in substrate binding; membrane-associated regions include N-49 and 96-C. NS3pro is colored in brown, NS3hel in green, cyan (RecA like subdomains 1 and 2, and ssRNA is depicted as a black line. The components of the viral replication complex, including NS5 and NS4B, are drawn across the ER membrane. Information about the intermolecular interfaces is taken from Tay et al., 2015 and Zou et al., 2015. Disrupting either NS3-NS5 (Takahashi et al., 2012), NS3-NS4B (Zou et al., 2015a) or NS3-NS2B interactions (Pambudi et al., 2013) constitute original approaches to identify compounds with antiviral activity. Interestingly, the RTPase activity of NS3 implies that NS3 must interact with the 5' end of the RNA genome before AND after RNA synthesis. This might be facilitated by the cyclization sequences identified in the genome. Adapted from Zou et al., 2015a.

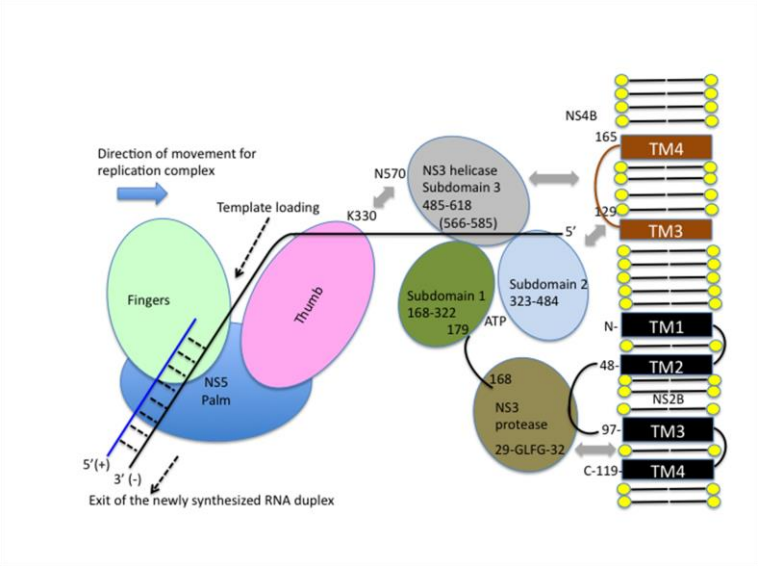

Table Legend

Table 1. List of flavivirus NS2BNS3 structures. Reported here are PDB codes for crystal structures of the flavivirus NS3 protein comprising the protease domain of NS3 with 47 residues of the NS2B cofactor ($NS2B_{47}G_4SG_4NS3pro$), the helicase domain (NS3hel) or both the protease and helicase domains of NS3 ($NS2B_{18}$ G_4SG_4NS3) and ($NS2B_{47}G_4SG_4NS3$).

Table 1. List of flavivirus NS2BNS3 structures.				
Domains	Virus	PDB code	Ligands	Reference
$NS2B_{47}G_4SG_4NS3pro$	DENV1	3L6P	-	(Chandramouli et al., 2010)
		3LKW	-	
	DENV2	2FOM	-	(Erbel et al., 2006)
		2M9P	BEZ-NLE-LYS-ARG-M9P	(Gibbs et al., 2014)
		2M9Q	BEZ-NLE-LYS-ARG-M9P	
	DENV3	3U1I	Bz-Nle-Lys-Arg-Arg-H	(Noble et al., 2012)
		3U1J	Aprotinin	
	WNV	2YOL	3,4-dichlorophenylacetyl-Lys-Lys-GCMA	(Hammamy et al., 2013)
		3E90	2-naphthoyl-Lys-Lys-Arg-H	(Robin et al., 2009)
		2FP7	Bz-Nle-Lys-Arg-Arg-H	(Erbel et al., 2006)
		2IJO	Aprotinin	(Aleshin et al., 2007)
		2GGV	-	
NS3hel	DENV2	2BHR	SO_4	(Xu et al., 2005)
		2BMF	-	
	DENV4	2JLQ	-	(Luo et al., 2008c)
		2JLR	AMPPNP_Mn^{2+}	
		2JLU	$ssRNA_{12}$	
		2JLV	$ssRNA_{12}$, AMPPNP_Mn^{2+}	
		2JLW	$ssRNA_{13}$	
		2JLX	$ssRNA_{12}$, ADP-Vandate_Mn^{2+}	
		2JLY	$ssRNA_{12}$, ADP-PO_4_Mn^{2+}	
		2JLZ	$ssRNA_{12}$, ADP_Mn^{2+}	
	Kokobera Virus	2V6I	-	(Speroni et al., 2008)
		2V6J	-	
	JEV	2Z83	-	(Yamashita et al., 2008)
	MVEV	2V8O	-	(Mancini et al., 2007)
	Kunjin Virus	2QEQ	-	(Mastrangelo et al., 2007)
	YFV	1YKS	-	(Wu et al., 2005)
		1YMF	ADP	
$NS2B_{18}G_4SG_4NS3$	DENV4	2WHX	ADP_Mn^{2+}	(Luo et al., 2010)
		2WZQ	-	
		2VBC	-	(Luo D, et al., 2008)
$NS2B_{47}G_4SG_4NS3$	MVEV	2WV9	-	(Assenberg et al., 2009)

1