

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

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16  
17 **Abbreviations:** NS nonstructural protein; NS3pro, protease domain of NS3; NS3hel, helicase  
18 domain of NS3; RdRP, RNA-dependent RNA polymerase

19  
20 **The flavivirus NS3 protein is associated with the endoplasmic reticulum membrane via**  
21 **its close interaction with the central hydrophilic region of the NS2B integral membrane**  
22 **protein. The multiple roles played by the NS2B-NS3 protein in the virus life cycle makes**  
23 **it an attractive target for antiviral drug discovery: The N-terminal region of NS3 and its**  
24 **cofactor NS2B constitute the protease that cleaves the viral polyprotein. The NS3 C-**  
25 **terminal domain possesses RNA helicase, nucleoside and RNA triphosphatase activities**  
26 **and is involved both in viral RNA replication and virus particle formation. In addition,**  
27 **NS2B-NS3 serves as a hub for the assembly of the flavivirus replication complex and**  
28 **also modulates viral pathogenesis and the host immune response. Here, we review**

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

4

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

7

## 8 **INTRODUCTION**

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

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

1 (Lindenbach et al., 2007). Long untranslated regions at both 5' and 3' ends play important  
2 roles in virus replication, viral protein translation and virion assembly (Alvarez et al., 2006;  
3 Filomatori et al., 2006; Khromykh et al., 2003; Markoff, 2003; Polacek et al., 2009; Villordo  
4 and Gamarnik, 2008; Yu et al., 2008a). Viral protein translation results in a single  
5 polypeptide precursor spanning across the endoplasmic reticulum (ER)-derived membrane.  
6 This polypeptide is processed into three structural proteins, capsid (C), envelope (E), and  
7 membrane protein (M) and seven non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A  
8 NS4B, and NS5 (**Fig. 1A**). Both host ER-derived proteases and the viral NS2B-NS3 protease  
9 specifically cleave the precursor to release the individual viral proteins (**Fig. 1A**)  
10 (Lindenbach et al., 2007). The C-terminal region of NS3 is a nucleoside triphosphatase  
11 (NTPase), a 5' terminal RNA triphosphatase (RTPase) and a RNA helicase (Xu et al., 2005).  
12 NS5 possesses RNA methyl-transferase (Dong et al., 2012; Dong et al., 2014; Egloff et al.,  
13 2002; Ray et al., 2006) and RNA-dependent RNA polymerase (RdRp) activities (Yap et al.,  
14 2007, Malet et al., 2008). Together with the viral RNA, viral cofactors, and host cell  
15 cofactors, NS3 and NS5 form the virus replication complex (RC) that assembles on the  
16 intracellular membrane to amplify the viral genome (Murray et al., 2008; Paul and  
17 Bartenschlager, 2013). Therefore, in principle, functional inhibition of the viral NS proteins  
18 and/or disruption of the RC underlie target-based anti-flavivirus drug development (Bollati et  
19 al., 2010; Lim et al., 2013; Noble and Shi, 2012; Sampath and Padmanabhan, 2009).

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

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

15

## 16 **NS2B-NS3 PROTEASE (NS2B-NS3PRO)**

17 The N-terminal domain of NS3 (aa 1–169) is a chymotrypsin-like serine protease that  
18 cleaves the viral polyprotein both in *cis* and in *trans* (Chambers et al., 1990b; Li et al., 2005).  
19 To function as an active enzyme, the NS3 protease requires the NS2B cofactor (Falgout et al.,  
20 1991; Jan et al., 1995; Yusof et al., 2000; Zhang et al., 1992). NS2B is an integral membrane  
21 protein of 14 kDa that contains three domains: two trans-membrane segments located at the N  
22 and C termini and a central region of 47 amino acids (spanning amino-acids 49–96) that acts  
23 as an essential protein cofactor of the NS3 protease (**Fig. 1B**) (Clum et al., 1997). The  
24 flavivirus NS3 protein is neither soluble nor catalytically active as a protease *in vitro*,  
25 suggesting that it does not fold properly without the NS2B protein, that must be either

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

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

1 Robin et al., 2009). Thus, the NS2B cofactor is able to regulate NS3pro activity both by  
2 stabilizing the correct protein fold of the core structure and also by directly participating in  
3 substrate cleavage (Erbel et al., 2006; Noble et al., 2012; Robin et al., 2009). This is in  
4 contrast to HCV NS3pro, where only a short segment of ~11 aa from the NS4A cofactor is  
5 required to form the active protease. Thus, a key difference between flaviviruses and  
6 hepaciviruses is that the NS4A protein cofactor of HCV plays a structural role but does not  
7 participate in substrate recognition or catalysis directly (Kim et al., 1996; Tomei et al., 1996;  
8 Urbani et al., 1998). Besides Gly<sub>4</sub>SerGly<sub>4</sub>-linked NS2B-NS3pro constructs, a protease  
9 complex containing a segment of 50-residues of the NS2B cofactor region and NS3pro  
10 without the glycine linker was recently reported using a coexpression system. Remarkably,  
11 this unlinked protease complex was catalytically active and exhibited an enzymatically active  
12 structure as determined using NMR (Kim et al., 2013). The classical Serine-protease catalytic  
13 triad (His51, Asp75, Ser135) is found in the central cleft of the NS3 protease domain (**Fig.**  
14 **2A and B**). The protease recognizes the positively charged residues Arg/Lys at the P1 and P2  
15 positions, followed by a small or polar amino acid at P1' – first residue after the cleavage,  
16 although exceptions are found (e.g., Glu is found at the P2 position at the NS2B-NS3 junction)  
17 (Gouvea et al., 2007; Li et al., 2005; Shiryayev et al., 2007). Fluorogenic peptide substrates are  
18 commonly used to study protease activities and for inhibitor screening purposes (Lai et al.,  
19 2014; Li et al., 2005; Niyomrattanakit et al., 2006; Yusof et al., 2000). Structures of NS2B-  
20 NS3pro bound to substrate-derived peptide inhibitors or aprotinin (bovine pancreatic trypsin  
21 inhibitor, BPTI) have revealed the substrate specificity and the catalysis mechanism (**Fig. 2D**).  
22 NMR structures of NS2B-NS3pro indicates that the disordered linker regions between NS2B  
23 cofactor and NS3 N terminus may be involved in substrate/inhibitor binding (Chen et al.,  
24 2014; de la Cruz et al., 2014, 2011) (**Fig. 2C**). Nonetheless, caution has to be exercised when

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

3

#### 4 **INHIBITORS OF NS2B-NS3Pro**

5 NS3 protease inhibitors are currently designed by either competing with substrate binding  
6 or by disrupting the interaction between NS2B and the NS3 protease domain, for a recent  
7 review see (Lim et al., 2013). Despite a wealth of structural and biochemical information  
8 available on the NS2B-NS3pro substrate-binding pocket, no compound has progressed to the  
9 preclinical stage yet. Researchers are still facing multiple challenges in identifying effective  
10 drugs that target NS2BNS3pro: The substrate-binding pocket is shallow and largely exposed  
11 to the solvent, rendering interactions between peptide mimics and enzyme labile. Moreover,  
12 the dibasic nature of residues at the P1 and P2 positions hinder peptidomimetic permeability  
13 and stability. Researchers have begun to study the structure and activity of the native-state-  
14 like NS2B-NS3pro in the context of the membrane, which could lead to better screening  
15 assays to identify protease inhibitors (Choksupmanee et al., 2012; Huang et al., 2013).

16 Several studies have identified novel non-peptidic inhibitors: Very recently, using a  
17 computational approach that included elaboration of a pharmacophore model, the NS2B-  
18 NS3pro X-ray crystallographic structure and a docking protocol, Li et al have identified a  
19 competitive non peptidic inhibitor (compound 14, **Fig. 3A**) with an EC<sub>50</sub> (Compound  
20 concentration that gives half-maximal response) of 5  $\mu$ M and a CC<sub>50</sub> (concentration of drug  
21 that results in *toxicity* to 50% of the cells compared with untreated control cells) greater than  
22 300  $\mu$ M (Li et al., 2014b). In another study, flavonoids (**Fig. 3B**) were found to be non-  
23 competitive inhibitors of DENV NS2B-NS3pro serotypes 2 and 3 with IC<sub>50</sub> (Compound  
24 concentration giving a 50% inhibition in enzymatic assay) values 15-44  $\mu$ M (de Sousa et al.,  
25 2015). Direct binding of compound 5 (**Fig. 3B**) to DENV serotype 3 NS2B-NS3pro was also

1 demonstrated ( $K_d=20\ \mu\text{M}$ ) using microscale thermophoresis (de Sousa et al., 2015). Diaryl  
2 (thio)ethers were discovered showing selective and noncompetitive inhibition towards the  
3 serotype 2 and 3 DENV NS2B-NS3pro in vitro and in cells. Some benzothiazole derivatives  
4 exhibited  $\text{IC}_{50}$ s in the low-micromolar range (Wu et al., 2015). Starting from a commercial  
5 compound library, Liu et al have identified a novel class of thiadiazoloacrylamide derivatives  
6 with potent inhibitory activity against the NS2B-NS3pro (Liu et al., 2014). The most potent  
7 compound in a series of analogues had an  $\text{IC}_{50}$  at  $2.24\ \mu\text{M}$  based on in vitro DENV2 NS2B-  
8 NS3pro assays. Finally, an interesting study provided evidence for the possibility of targeting  
9 the interaction between NS2B and NS3 as an effective antiviral strategy (Pambudi et al.,  
10 2013). In this study, Pambudi et al., report compound SK-12 (**Fig. 3C**) that acts by blocking  
11 the NS2B/NS3 protease interaction; SK-12 significantly inhibited DENV4 replication with an  
12  $\text{EC}_{50}$  of  $3.8\ \mu\text{M}$  as well as JEV with  $\text{EC}_{50}$  of  $14.4\ \mu\text{M}$  (Pambudi et al., 2013). For each of  
13 these novel non-peptidic inhibitors, putative modes of binding were proposed. However,  
14 further development will be greatly facilitated once the respective cocrystal structures  
15 become available to confirm the predicted interactions.

16

### 17 **NS3 NTPASE/RNA HELICASE (NS3HEL)**

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

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

12 Like most eukaryotic DExx helicase proteins, NS3 recognizes RNA largely in a sequence-  
13 independent manner. During the duplex RNA unwinding process, one strand is inserted into  
14 the RNA binding groove of NS3hel and the sugar-phosphate backbone makes either direct or  
15 water-mediated contacts to the helicase residues, whereas the other strand is separated by a  
16 hydrophobic  $\beta$ -hairpin that protrudes from subdomain 2. This  $\beta$ -hairpin acts as a “helix  
17 opener” to disrupt base stacking and stabilize the unwound duplex, while the basic concave  
18 region between subdomain 2 and 3 works as “the translocator” by binding the 3' overhang  
19 (Buttner et al., 2007; Luo et al., 2008c; Myong et al., 2007; Pyle, 2008). NS3hel binds to the  
20 3' overhang sequence of the duplex RNA and unwinds in the 3' to 5' direction (**Fig. 2C**)  
21 (Benarroch et al., 2004; Li et al., 1999; Xu et al., 2005). NTP hydrolysis provides the  
22 chemical energy to power the translocation and unwinding mechanical process, although the  
23 precise mechanism coupling these two activities remains unclear. Single-molecule and  
24 structural biology studies on the homologous HCV NS3hel suggested that the basic step of  
25 translocation and unwinding is one base per molecule of ATP hydrolysed (Appleby et al.,

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

5

## 6 **NS3HEL INHIBITORS**

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

23

## 24 **THE FULL-LENGTH NS3 PROTEIN**

1       Why apparently disconnected enzymatic activities hosted by two separate protein domains  
2   – protease and NTPase/helicase – are linked within a single NS3 polypeptide remains elusive  
3   and probably requires a better understanding of how the whole flavivirus replication complex  
4   (RC) functions. However, there are several obvious potential advantages for flaviruses to  
5   encode such fusion genes. Polyprotein processing occurs co- and post-translationally in ER-  
6   derived intracellular membranes (Chambers et al., 1991; Chambers et al., 1990a). NS3 may  
7   thus help bring NS4A-NS4B-NS5 in close proximity to NS2BNS3pro for the efficient  
8   cleavage and release of individual viral proteins (Amberg et al., 1994; Arias et al., 1993;  
9   Cahour et al., 1992; Lin et al., 1993a; Lin et al., 1993b). In addition, NS2B anchors NS3 in  
10   the membrane, which is a prerequisite for viral replication complex maturation. Flavivirus  
11   RNA replication is performed inside a vesicular-like compartment made of remodeled  
12   intracellular membranes (Miorin et al., 2013; Paul and Bartenschlager, 2013; Welsch et al.,  
13   2009). Therefore, NS2B together with other small viral membrane proteins might provide the  
14   membrane platform for the replicative enzymes NS3 and NS5 and viral RNA to assemble  
15   into a higher-order structure, flaviviral RC, that also includes NS1 (Miorin et al., 2013; Paul  
16   and Bartenschlager, 2013; Welsch et al., 2009). A more integrated view of the flavivirus RC,  
17   including the definition of its molecular interactions requires multiple approaches and the  
18   present understanding is summarized in an excellent review by Paul & Bartenschlager, 2013.  
19   Recently the molecular contacts between NS3hel and the integral membrane protein NS4B  
20   was mapped (Zou et al., 2015b), leading to a model for interactions between NS4B-NS3 and  
21   NS5 (**Fig. 5**) (Tay et al., 2015).

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

1 demonstrated that full-length NS3 has much higher unwinding activity but a lower ATP  
2 hydrolysis rate (Xu et al., 2005; Yon et al., 2005). The affinity of dengue virus serotype 4  
3 (DENV4) NS2B<sub>18</sub>NS3 to ATP analogs is 10-fold higher than that of the truncated helicase  
4 (Luo et al., 2008a). Intriguingly, no influence of the protease on helicase activity has been  
5 observed (or vice versa) in an MVEV NS2B<sub>47</sub>NS3 construct (Assenberg et al., 2009). More  
6 recently, the Gamarnik group also reported comparable ATPase and helicase activities for  
7 his<sub>6</sub>-tagged DENV2 NS2B<sub>47</sub>NS3 and NS3hel, respectively (Gebhard et al., 2012). These  
8 discrepancies may derive from variations in protein constructs, enzyme preparation, and  
9 experimental conditions for the assay. Furthermore, the correct folded protease domain might  
10 assist NS3 to select the RNA substrate: WNV NS3hel but not NS2B<sub>48</sub>NS3, unwinds both  
11 DNA and RNA (Chernov et al., 2008). Studying the biochemistry of a native-state-like  
12 NS2B-NS3 protein in a membrane context is likely to clarify these issues and shed more light  
13 on the various roles played by NS3 during virus replication (Choksupmanee et al., 2012;  
14 Huang et al., 2013).

15 Intriguingly, structural studies of the full-length NS3 protein have revealed three distinct  
16 configurations that differ from each other in the relative positioning of the NS3pro with  
17 respect to NS3hel and are made possible by a flexible inter-domain linker (**Fig. 4**) (Assenberg  
18 et al., 2009; Li et al., 2014a; Luo et al., 2012; Luo et al., 2010; Luo et al., 2008a). We  
19 reported two different conformations of the DENV4 NS2B<sub>18</sub>NS3 protein where the protease  
20 domain has rotated by approximately 161° (Fig. A and B) with respect to the helicase  
21 domain (Luo et al., 2010; Luo et al., 2008a). The structure of the MVEV NS2B<sub>47</sub>NS3 (**Fig.**  
22 **4C**) presented a third and a more radically different conformation of the NS3 protease-  
23 helicase (Assenberg et al., 2009). Interestingly, small-angle X-ray scattering (SAXS)  
24 experiments showed that both DENV4 NS2B<sub>18</sub>NS3 protein and Kunjin NS3 adopted similar  
25 elongated shapes in solution (Luo et al., 2008a; Mastrangelo et al., 2007). Given the close fit

1 of the crystal structures to the hydrated envelope determined *ab initio* from SAXS data, it is  
2 evident that isolated flaviviral NS3 protease-helicase enzymes maintain an elongated  
3 conformation, with the two domains loosely connected through a flexible linker (**Fig. 4**). In  
4 contrast, the HCV NS3NS4A protease-helicase has been shown to adopt a globular  
5 conformation in solution (**Fig. 4D**) (Yao et al., 1999). In the first crystal structure of the  
6 similarly engineered NS4A<sub>11</sub>NS3 fusion protein of HCV genotype 1b, the protease domain  
7 binds the C terminus of the NS3hel, mimicking the post-*cis*-cleavage state at the NS3-NS4A  
8 junction (**Fig. 4D**) (Yao et al., 1999). NS4A<sub>11</sub>NS3 fusion proteins from the same or different  
9 genotypes have also been crystallized as apo-enzyme in a similar globular  
10 conformation(Appleby et al., 2011), in complex with ssRNA and ATP analogs (Appleby et  
11 al., 2011), in complex with a macrocyclic protease inhibitor (Schiering et al., 2011), and in  
12 complex with allosteric inhibitors (Saalau-Bethell et al., 2012). Sequence analysis revealed  
13 that the linker (aa 169–179) is less conserved compared to the two functional domains of the  
14 flavivirus NS3 (**Fig. 1C**). The flavivirus NS3 linker is also rich in acidic residues(Luo et al.,  
15 2008a) (**Fig. 1C**). Conversely, the linker region of HCV NS3 (aa 181–194) is very conserved  
16 and rich in proline residues (Kohlway et al., 2014; Li et al., 2014a). The sequence divergence  
17 between flavivirus and hepatitis virus NS3 correlates with the distinct protease-helicase  
18 conformations: the flexible and less conserved linker allows the flavivirus protein to adopt  
19 extended conformations, whilst the conserved and proline-rich linker imposes a globular  
20 conformation for HCV NS3 (**Fig. 1C and 4**). In flaviviruses, the NS3 linker may have  
21 evolved to span an optimum length to endow the protein with the flexibility needed for  
22 polyprotein processing and RNA replication. It will be interesting to assess the impact of the  
23 linker on Flaviviridae virus replication and targeting these regions could lead to the design of  
24 attenuated viruses (Luo et al., 2010). Thus, studies on the full-length NS3 protein have led to  
25 a better understanding of the flavivirus life cycle and suggested new avenues for the

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

11

## 12 **NS3 ACTS AS A HUB IN THE FLAVIVIRUS REPLICATION COMPLEX**

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

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

9

## 10 **NS3 REGULATES THE HOST IMMUNE RESPONSE**

11 Studies using yeast two-hybrid systems and viral revertant analysis have begun to reveal  
12 the flavivirus NS proteins interactome (Khadka et al., 2011; Le Breton et al., 2011; Zou et al.,  
13 2011) leading to the identification of more than 100 human proteins that interact with NS3,  
14 NS5, or both. Many interacting proteins are involved in modulating transcription or the host  
15 immune response (Krishnan and Garcia-Blanco, 2014). Type I interferon (IFN) production in  
16 human monocyte-derived dendritic cells was inhibited as the cytoplasmic adaptor protein –  
17 stimulator of interferon genes (STING) (Ishikawa and Barber, 2008) or mediator of IRF3  
18 activation (MITA) (Zhong et al., 2008) – was found to be cleaved and thus inactivated by  
19 NS2BNS3pro from DENV (Aguirre et al., 2012). Moreover NS2BNS3 from WNV induces  
20 apoptosis through the activation of caspases 3 and 8 (Ramanathan et al., 2006). Another study  
21 reported that NS2BNS3pro from JEV inhibits the signaling pathway of activator protein 1  
22 (AP-1), probably also through proteolysis. The authors suggested that NS2BNS3pro may  
23 contribute to JEV-induced neurotropic pathogenesis (Lin et al., 2006). Thus, a better  
24 understanding of how the various enzymatic and non-enzymatic activities of NS3 are

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

3

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12

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46

1 **Figure Legends**

2 **Figure 1. Schematic representation of the flaviviral genome, polyprotein, and functional**

3 **domain partition along the NS2B-NS3 gene.** (A) Flavivirus genome and polyprotein. The

4 viral genome contains a 5' cap and untranslated regions at both the 5' and 3' termini. The

5 polyproteins are processed by both NS2BNS3 protease indicated as filled arrows and the host

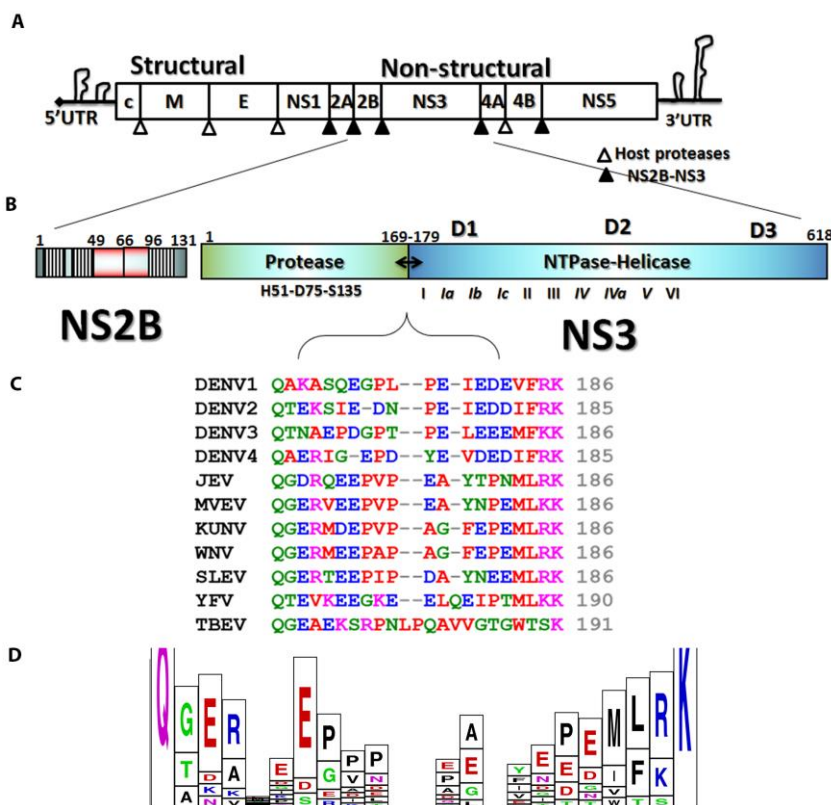
6 proteases by unfilled arrows. (B) The schematic representation of the NS2B and NS3 genes.

7 The central cofactor region of NS2B is in red, and the putative membrane associate regions of

8 NS2B are displayed as blocks. (C) The sequence alignment of the linker regions between the

9 protease and helicase domains of NS3 from DENV1-4 and various flaviviruses. (D) Sequence

10 conservation of the linker region generated with WebLogo (<http://weblogo.berkeley.edu/>).

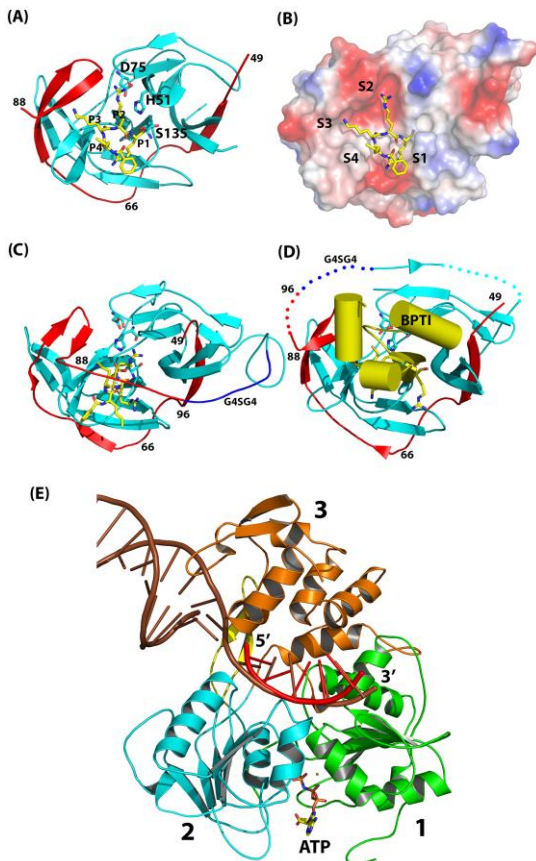


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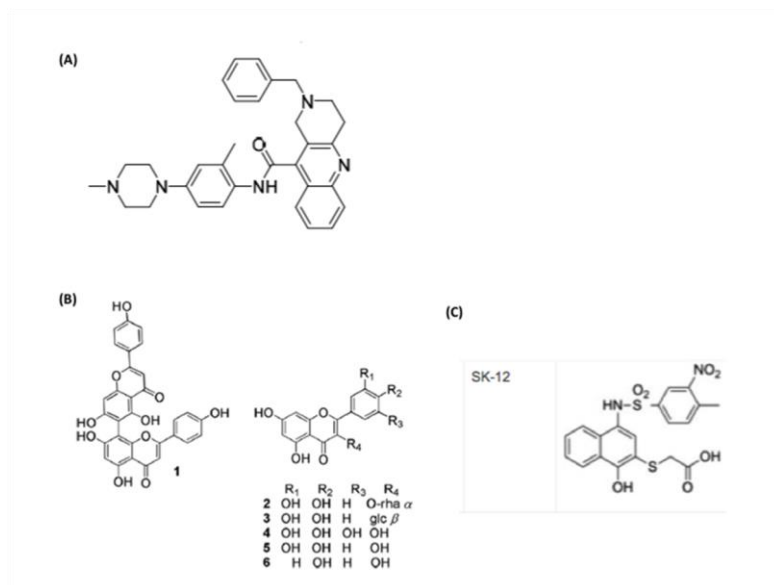
13

1 **Figure 2. 3D structures of NS2B-NS3pro and NS3hel.** (A) Overall fold of NS2B<sub>47</sub>NS3pro  
 2 from WNV. NS3Pro is shown in cyan, the NS2B<sub>47</sub> region in red, and the tetra-peptide  
 3 inhibitor is in yellow, labelled as P1-P2-P3-P4. PDB code: 2FP7. (B) Surface view of the  
 4 substrate binding site of WNV NS2BNS3pro. S1-S4 correspond to the pocket for P1-P4 of  
 5 the peptide inhibitor. (C) NMR structure of NS2B<sub>49-96</sub>G<sub>4</sub>SG<sub>4</sub>NS3pro bound to a protease  
 6 inhibitor BEZ-NLe-Lys-Arg-M9P (in yellow). PDB code: 2M9P. (D) Crystal structure of  
 7 WNV NS2B<sub>49-96</sub>G<sub>4</sub>SG<sub>4</sub>NS3pro complexed with BPTI (in yellow). PDB code: 2IJO. (E)  
 8 Ternary complex of DENV4 NS3hel-ssRNA-AMPPNP. NS3hel is colored according to the  
 9 domain boundaries: hel1 is in green, hel2 in cyan, the putative duplex separating beta-hairpin  
 10 motif in yellow, and hel3 in orange. Duplex RNA with a 3' overhang (in brown) is overlaid  
 11 to the ssRNA (in red) to model the unwinding activity of NS3hel. AMPPMP, a  
 12 nonhydrolyzable ATP analog, was used to mimic ATP binding at the ATPase active site (Luo  
 13 et al., 2008c). PDB code: 2JLV.



14

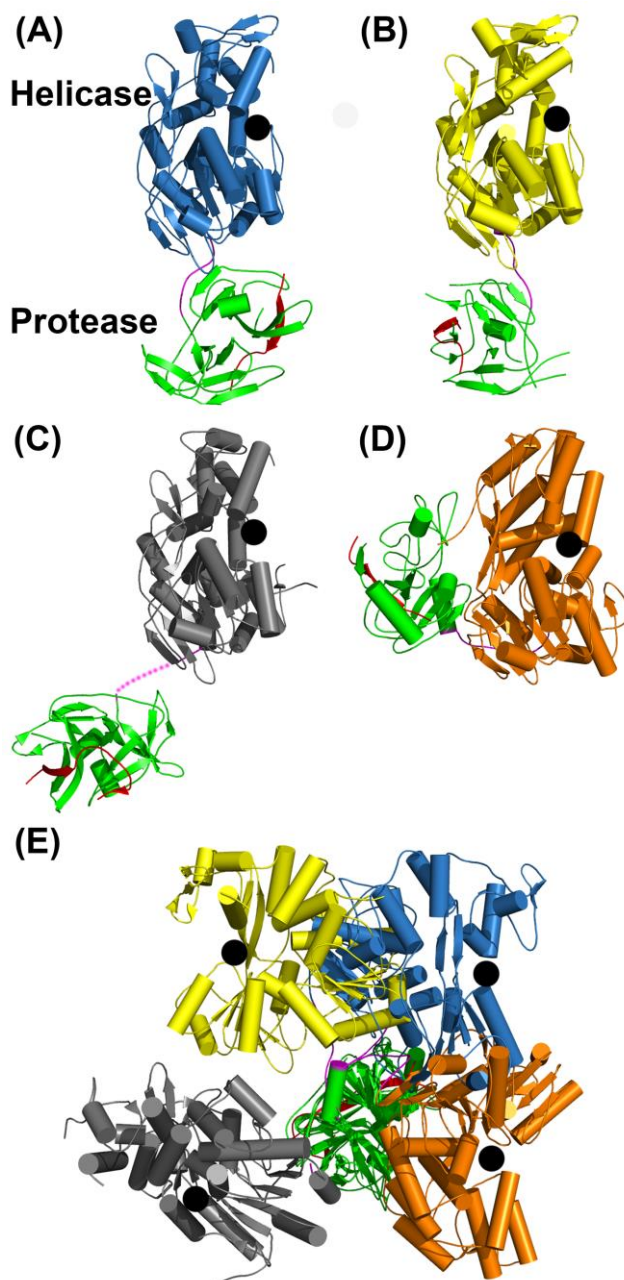
1 **Figure 3. Structures of NS2B-NS3pro non-peptidic inhibitors.** (A) Compound 14  
 2 identified by Li et al (2014) through the use of a new pharmacophore. This competitive  
 3 inhibitor has an IC<sub>50</sub> of 5 μM (B) A series of flavonoids that show non-competitive  
 4 inhibition activities against NS2B-NS3pro from serotypes 2 and 3 (de Sousa et al., 2015) (C)  
 5 Structure of a small compound targeting the interaction between nonstructural proteins 2B  
 6 and 3 that inhibits dengue virus replication (Pambudi et al., 2013).



7

8

1 **Figure 4. Structural views of the full-length NS3 protein.** Structures of (A) and (B)  
2 NS2B<sub>18</sub>NS3 from DENV4, (C) NS2B<sub>47</sub>NS3 from MVEV, and (D) NS3NS4A<sub>11</sub> from HCV.  
3 NS2B (or NS4A) cofactor peptide is in red; NS3pro in green; linker in purple; and NS3hel is  
4 in blue, yellow, gray, and orange. In (A-D), the helicase domains are aligned, and the black  
5 dots represent the ssRNA binding tunnel. (E) When the protease domains are superimposed,  
6 the helicase domains display various orientations. Adapted from Li et al., 2014 (Li et al.,  
7 2014a).





1

2 **Table Legend**

3 **Table 1. List of flavivirus NS2BNS3 structures.** Reported here are PDB codes for crystal  
4 structures of the flavivirus NS3 protein comprising the protease domain of NS3 with 47  
5 residues of the NS2B cofactor (NS2B<sub>47</sub>G<sub>4</sub>SG<sub>4</sub>NS3pro), the helicase domain (NS3hel) or both  
6 the protease and helicase domains of NS3 (NS2B<sub>18</sub> G<sub>4</sub>SG<sub>4</sub>NS3) and (NS2B<sub>47</sub>G<sub>4</sub>SG<sub>4</sub>NS3).

7

Table 1. List of flavivirus NS2BNS3 structures.				
Domains	Virus	PDB code	Ligands	Reference
NS2B <sub>47</sub> G <sub>4</sub> S G <sub>4</sub> NS3pro	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 <sub>4</sub>	(Xu et al., 2005)
		2BMF	-	
	DENV4	2JLQ	-	(Luo et al., 2008c)
		2JLR	AMPPNP_Mn <sup>2+</sup>	
		2JLU	ssRNA <sub>12</sub>	
		2JLV	ssRNA <sub>12</sub> , AMPPNP_Mn <sup>2+</sup>	
		2JLW	ssRNA <sub>13</sub>	
		2JLX	ssRNA <sub>12</sub> , ADP-Vandate_Mn <sup>2+</sup>	
		2JLY	ssRNA <sub>12</sub> , ADP-PO <sub>4</sub> _Mn <sup>2+</sup>	
	2JLZ	ssRNA <sub>12</sub> , ADP_Mn <sup>2+</sup>		
	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)
1YKS		-		
YFV	1YMF	ADP	(Wu et al., 2005)	
	2WHX	ADP_Mn <sup>2+</sup>	(Luo et al., 2010)	
DENV4	2WZQ	-		
	2VBC	-	(Luo D, et al., 2008)	
NS2B <sub>47</sub> G <sub>4</sub> SG <sub>4</sub> NS3	MVEV	2WV9	-	(Assenberg et al., 2009)