Proteases from dengue, West Nile and Zika viruses as drug targets

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Abstract

Proteases from flaviviruses have gained substantial interest as potential drug targets to combat infectious diseases caused by dengue, West Nile, Zika and related viruses. Despite nearly two decades of drug discovery campaigns, promising lead compounds for clinical trials have not yet been identified. The main challenges for successful lead-compound development are associated with limited drug-likeness of inhibitors and structural ambiguity of the protease target. This brief review focuses on the available information on the structure of flavivirus proteases and their interactions with inhibitors, and attempts to point the way forward for successful identification of future lead compounds.

Keywords

Flavivirus, serine protease, NS2B-NS3, protease inhibitor

Introduction

The genus flavivirus includes over 50 viruses and belongs to the family of *Flaviviridae* (Barrows et al. 2018). Each year, millions of people worldwide are infected by members of the flavivirus genus (Boldescu et al. 2017). Some, like yellow fever, Japanese encephalitis or tick-borne encephalitis can be prevented through use of established vaccines. Others, like dengue, West Nile or Zika represent major health burdens without available specific antiviral treatments or effective and safe vaccines. Flaviviruses are usually vector-transmitted (e.g. by the mosquitos *Aedes aegypti* and *Aedes albopictus*), restricting their distribution to the continuously expanding vector habitats. Additional transmission routes for Zika virus involving sexual contact have recently been proposed (Baud et al. 2017; Poland et al. 2018).

Estimates of the annual dengue virus infections are between 284 and 528 million, of which between 67 and 136 million cases manifest clinically (Bhatt et al. 2013). The efficacy of the recently approved vaccine CYD-TDV (Dengvaxia) differs amongst the four known dengue serotypes and age groups of those receiving the vaccine. In addition, the vaccine performs differently in individuals with evidence of prior dengue infection (seropositive) and those without (seronegative), with an increased risk for hospitalisation in the latter group (WHO 2018). Consequently, the WHO recommends the current vaccine only for seropositive patients.

West Nile virus affects animals (e.g. birds and horses) and humans (Suthar et al. 2013). Approximately 80% of human West Nile virus infections are asymptomatic, 20% cause self-limiting symptoms (West Nile fever) and less than 1% are characterised by neuroinvasive disease, with 10% of this subgroup resulting in fatality (Burki 2018). Although candidates are in clinical trials, no West Nile virus vaccine for humans has yet been approved. During 2018, Europe registered an alarming increase of West Nile cases with more than 2000 reports of autochthonous infections and 180 deaths, exceeding the total number of cases reported during the previous seven years (ECDC 2018).

The Zika virus has emerged very recently as a health-threatening pathogen after epidemic outbreaks in Latin America (Baud et al. 2017). Most infections are asymptomatic, however, neurological complications in patients and severe fetal disorders (microcephaly) prompted the WHO to declare Zika virus a Public Health Emergency of International Concern in 2016 (Baud et al. 2017). Since that time, several vaccine candidates have been developed, of which four are

currently in clinical trials (Poland et al. 2018). Potential cross-reactions between Zika and dengue virus antibodies (antibody-dependent enhancements) may lead to increased viremia and severity of the disease and thus challenge vaccination campaigns where dengue and Zika co-circulate (Poland et al. 2018).

These examples describing the challenges of vaccine development, illustrate that anti-flaviviral drugs must be pursued in addition to vaccination campaigns to present therapeutic options for the treatment of symptomatic patients and individuals where vaccination is not recommended (e.g. dengue seronegative individuals). In what follows, the current state of play in targeting the protease of flaviviruses is described.

Function of the NS2B-NS3 protease

All members of the flavivirus genus contain a single-stranded RNA genome that is translated by the host cell into a single polyprotein (Barrows et al. 2018). Embedded into the membrane of the endoplasmatic reticulum, this precursor protein is post-translationally processed by host cell proteases and the viral protease NS2B-NS3 into three structural and various non-structural (NS) proteins (Barrows et al. 2018; Boldescu et al. 2017). Consequently, the NS2B-NS3 protease is essential for viral replication and thus presents itself as a promising drug target (Nitsche 2018; Nitsche et al. 2014). The catalytically active protease complex consists of the actual protease unit located at the N-terminal part of the non-structural protein 3 (NS3) and requires approximately 40 residues of a hydrophilic domain of the membrane-associated non-structural protein 2B (NS2B) as a cofactor. NS2B-NS3 is a serine protease with the highly conserved catalytic triad (serine 135, histidine 51 and aspartate 75) (Nitsche et al. 2014). According to the nomenclature for protease subsites suggested by Schechter and Berger (1967), the NS2B-NS3 proteases show a common preference to cleave peptidic backbones after two basic residues (arginine or lysine) in P_1 and P_2. In contrast to other viral protease drug targets that recognise more hydrophobic substrate residues in these key positions (e.g. HIV, HCV), this dibasic recognition motif has manifested as a major challenge to the drug discovery process against flaviviruses.

Constructs for drug discovery

Protease constructs with full-length NS2B that rely on lipid support (e.g. micelles) have been reported (Choksupmanee et al. 2012; Huang et al. 2013; Ng et al. 2019), but the most commonly

used constructs for biochemical and structural studies comprise only the hydrophilic core segment of NS2B, excluding the hydrophobic membrane-associated domains. These truncated constructs have been designed with and without covalent linkage between NS2B and NS3 (Nitsche et al. 2014). The most frequently used truncated construct includes a covalent Gly_4-Ser-Gly_4 linker between NS2B and NS3, as initially introduced for dengue serotype 2 (Leung et al. 2001), followed by West Nile (Nall et al. 2004), the other dengue serotypes (Li et al. 2005) and most recently Zika virus protease (Lei et al. 2016). Unlinked constructs rely either on an NS2B/NS3 autocleavage site (de la Cruz et al. 2014; Phoo et al. 2016) or co-expression systems (Kim et al. 2013; Zhang et al. 2016). There is an ongoing debate in the literature whether the linked or unlinked protease constructs are more suitable for drug discovery campaigns (Kang et al. 2017; Nitsche 2018; Nitsche et al. 2014). The linked constructs tend to be more stable, whereas the unlinked constructs are thought to resemble a state that is closer to the native one (de la Cruz et al. 2014; Hill et al. 2018; Li et al. 2017a). Recent studies with Zika virus protease suggest that the covalent linker may prevent the formation of the catalytically active complex to a certain degree (Li et al. 2017a), whereas no significant differences for inhibitor binding could be observed between linked and unlinked Zika virus protease constructs (Nitsche et al. 2019).

Structure of the NS2B-NS3 protease

Fig. 1 shows the NS2B-NS3 proteases from dengue, West Nile and Zika viruses in their catalytically active form (Lei et al. 2016; Nitsche et al. 2017; Noble et al. 2012). These structures were produced by co-crystallising substrate-derived ligands in the active site. In case of dengue, an active structure could so far only be solved for serotype 3 (Noble et al. 2012). These protease structures share a high degree of similarity with several conserved structural features, as illustrated by superimposition (Fig. 1D). This has raised hopes that once a promising lead compound is discovered, it could be considered as a potential pan-flaviviral protease inhibitor to treat dengue, West Nile, Zika and related flaviviral infectious diseases. In its active form, the protease adopts a chymotrypsin-like fold where the N-terminal β-strand of NS2B integrates into the N-terminal domain of NS3 and the C-terminal β-hairpin of NS2B wraps around the active site of NS3. The latter interaction forms the S_2 pocket and is thus essential for substrate recognition and catalytic activity. Only the protease from Zika virus can engage in a salt-bridge between the P_2 side chain and the NS2B aspartate residue D83* (Fig. 1C), which is not conserved in West Nile and all four

dengue serotypes. It has been suggested that this additional ionic interaction between substrate (P_2) and NS2B (S_2) is partially responsible for the observed hyperactivity of Zika virus protease (Lei et al. 2016). A second substantial interaction involves the highly conserved NS3 aspartate residue D129 and the basic P_1 substrate side chain, which form a salt-bridge in all structures (Fig. 1A-C).

Open or closed?

Two major groups of X-ray crystal structures have been solved for each viral protease (dengue, West Nile, Zika) that differ substantially in the C-terminal NS2B domain (Fig. 2). One group (crystallised in absence of inhibitor) observes the C-terminal part of NS2B, either dissociated from NS3, or not defined by electron density (Fig. 2A, C, E). The second group (usually crystallised in presence of an inhibitor) defines the C-terminal NS2B domain as a β-hairpin wrapped around the active site (Fig. 2B, D, F). The former inactive state is often referred to as open, whereas the latter active one is known as closed. These crystallographic observations indicated substantial conformational changes of NS2B upon activation by substrate or inhibitor. Particularly, the early unliganded open crystal structures from dengue (Erbel et al. 2006) and West Nile (Aleshin et al. 2007) proteases imply this conclusion. However, recent observations of a crystal structure of Zika virus protease in the closed conformation were made for the unlinked protease construct in absence of a ligand (Zhang et al. 2016). In contrast, unliganded structures from linked constructs of dengue (Erbel et al. 2006), West Nile (Aleshin et al. 2007) and Zika (Chen et al. 2016) proteases all revealed inactive open states, suggesting that the covalent $Gly_4SerGly_4$ linker between NS2B and NS3 prevents the formation of the closed conformation in the absence of substrate or inhibitor.

Various biomolecular NMR studies were conducted to analyse the conformational ambiguity of flavivirus proteases in solution, particularly with respect to the localisation of the C-terminal part of NS2B. After initial challenges with heterogeneity in sample preparations, most reports concluded that both, the dengue and West Nile virus proteases, predominately adopt the closed conformation in solution, regardless of the construct (linked or unlinked) or the presence of an inhibitor (de la Cruz et al. 2014; de la Cruz et al. 2011; Kim et al. 2013; Su et al. 2009). Observed conformational flexibilities may relate to exchange phenomena between open and closed states, the extent of which can differ between constructs (Hill et al. 2018). In the case of Zika virus protease, NMR experiments indicated sample heterogeneity promoted by the $Gly_4SerGly_4$ linker and ligand-binding events (Li et al. 2017a; Mahawaththa et al. 2017). Distances measured by

double electron-electron resonance in the linked construct of the Zika virus protease matched the closed conformation in presence and absence of a ligand (Mahawaththa et al. 2018). In case of the unlinked Zika virus protease constructs, the closed conformation was found to be dominant in solution with a tendency of additional stabilisation in presence of a ligand (Li et al. 2018; Li et al. 2017b; Phoo et al. 2016; Zhang et al. 2016).

Most importantly, all studies showed that regardless of the protease construct, the enzymes are folded correctly in solution. Ambiguities are mainly attributed to partial dissociations between NS2B and NS3 as well as inhibitor-binding events. A certain degree of conformational flexibility of NS2B-NS3, particularly in the C-terminal region of NS2B is not surprising, as the substrate must enter the active site prior cleavage reaction. Substrate binding stabilises the active conformation but is not an essential requirement to assemble the active closed NS2B-NS3 complex. Therefore, the closed state should be the preferred template for rational drug discovery attempts. There is a general tendency that the unlinked constructs resemble the closed and active state more reliably than the linked variants.

Inhibitors

Several inhibitors of flaviviral proteases have been described and comprehensively reviewed (Behnam et al. 2016; Boldescu et al. 2017; Lim and Shi 2013; Lim et al. 2013; Nitsche 2018; Nitsche et al. 2014; Poulsen et al. 2014; Timiri et al. 2016). They can generally be categorised as either substrate-derived peptides/peptidomimetics or small molecules without substrate character (Nitsche 2018). The former group is usually characterised by high affinity and limited drug-likeness, whereas the latter group implies the opposite properties: improved drug-likeness, but lower affinity. Only few inhibitors have been reported that display dissociation constants with flaviviral proteases in the desirable lower nanomolar range, i.e. clearly below 100 nM, (Behnam et al. 2015; Nitsche et al. 2017; Schüller et al. 2011; Shiryaev et al. 2006; Stoermer et al. 2008; Yin et al. 2006) and all of them are peptidic-based substrate mimetics with the majority bearing warheads that allow covalent modification of the catalytically active serine residue 135.

Most campaigns focused on the discovery of competitive inhibitors that bind to the active site. As a consequence, the undesirable dibasic substrate recognition motif is reflected in several compounds. Out of those inhibitors that have been proven to bind to the active site by X-ray crystallography (Fig. 3), only two are non-peptidic and do not comprise at least two basic side

chains. Only a few attempts were made to discover allosteric inhibitors that do not interact with the active site (Brecher et al. 2017; Nitsche et al. 2019; Roy et al. 2017; Shiryaev et al. 2017; Wu et al. 2015; Yildiz et al. 2013). To date, a distinct binding mode has not been reported for any. Once structural data will become available, more rational attempts towards allosteric inhibitors can be pursued.

Co-crystal structures of NS2B-NS3 and inhibitors

Over the past three years, massive efforts in X-ray crystallography have generated substantial structural information about the activation of flavivirus proteases and their interactions with inhibitors.

Many crystal structures of dengue virus NS2B-NS3 protease have been solved for serotypes 1 (3L6P, 3LKW) (Chandramouli et al. 2010), 2 (2FOM, 4M9K, 4M9M, 4M9I, 4M9F, 4M9T) (Erbel et al. 2006; Yildiz et al. 2013), 3 (3U1I, 3U1J) (Noble et al. 2012) and for the full-length protease-helicase complex of serotype 4 (2VBC, 2WHX, 2WZQ) (Luo et al. 2010; Luo et al. 2008). Recently, additional NS2B-NS3 structures with full-length NS3 (protease and helicase) of serotype 4 have been deposited in the protein data base (5YVU, 5YVJ, 5YVW, 5YVY, 5YW1). Two structures were solved in complex with the 6.5 kDa broad-spectrum protease inhibitor aprotinin (also referred to as BPTI) for serotypes 3 (3U1J) (Noble et al. 2012) and 4 (5YW1). Despite the large total number of available crystal structures, only one describes a small molecule inhibitor (**1**) in complex with the dengue serotype 3 protease (Fig. 3A) (Noble et al. 2012). Tetrapeptide **1** resembles the non-prime site of the substrate recognition sequence from P_1 to P_4 (Nle-Lys-Arg-Arg), which was previously identified from a large screening campaign (Li et al. 2005). An additional aldehyde function at the peptide C-terminus allows covalent linkage to serine 135.

In stark contrast to dengue, the majority of crystal structures of the West Nile virus protease have been solved in complex with inhibitors. One unliganded (2GGV) and one structure in complex with aprotinin (2IJO) have been reported (Aleshin et al. 2007), all others feature small molecules (Fig. 3B-E). Compound **2** represents a short peptide mimetic that binds covalently to the catalytic serine 135 via a boronic acid warhead (Fig. 3B), while its two basic side chains occupy the S_1 and S_2 pockets (Nitsche et al. 2017). Compound **3** is the only co-crystallised small molecule that does not covalently modify the West Nile virus protease (Fig. 3C) (Hammamy et al. 2013).

Similar to the complex of dengue protease with compound **1**, the three basic side chains of inhibitor **3** occupy S_1-S_3, which is a common feature in several structures of flavivirus proteases in complex with peptide inhibitors (e.g. Fig 3A, C, D, E, J, K, L). Compounds **4** and **1** are tri- and tetrapeptide aldehydes, respectively, that bind covalently to serine 135 (Fig. 3D, E) (Erbel et al. 2006; Robin et al. 2009). The binding mode of compound **1** to West Nile protease (Fig. 3E) is similar to the interaction of **1** with dengue protease serotype 3 (Fig. 3A).

Recently, several crystal structures of Zika virus protease were reported, of which some lack ligands, like 5GXJ (Chen et al. 2016), 5GPI (Zhang et al. 2016), 5T1V, 5TFN and 5TFO. Others revealed active-site complexes with terminal peptide chains from NS2B (5GJ4) (Phoo et al. 2016) or NS3 5GPI (Zhang et al. 2016). Additional structures have been solved in complex with small molecule inhibitors bound to the active site (Fig. 3F-L). The first crystal structure became available in complex with compound **2** covalently linked to S135 (Lei et al. 2016). The binding mode of **2** is similar to that one observed in complex with West Nile virus protease (Fig. 1B, Fig. 3B), except the additional salt bridge between P_2 and D83* of NS2B (Fig. 1C, Fig. 3F). Compound **5** is a short dipeptide aldehyde that binds covalently to S135 and occupies S_1 and S_2 with its two basic side chains, (Fig. 3G) (Li et al. 2018; Li et al. 2017b) similarly to boronate **2**. The low-affinity fragment **6** represents the first very small molecule co-crystallised with a flavivirus protease (Fig. 3H) (Zhang et al. 2016). It is involved in stacking interactions with Y161 and hydrogen-bonding with Y150 in the S_1 pocket without any contacts to NS2B. Activated pyrazole ester **7** and analogues are known to inhibit the proteases from dengue, West Nile and Zika viruses by transesterification (Johnston et al. 2007; Koh-Stenta et al. 2015; Li et al. 2018; Sidique et al. 2009). In the final inhibition state, the benzoate is covalently linked to S135, engaging in π-π stacking with Y161 and hydrogen-bonding with H51 in the S_1 pocket (Fig. 3I) (Li et al. 2018). Finally, the peptidic inhibitors **8**-**10** have been co-crystallised with Zika virus protease (Fig. 3J-L) (Phoo et al. 2018). Only compound **10** was found be resistant to proteolysis. With two glycines in P_1' and P_2', compound **8** acts as a substrate of Zika virus protease and only the non-prime site residues remain bound to the protease, indicating the possibility of product inhibition.

Conclusion

Despite two decades of active research, the suitability of flaviviral proteases to serve as drug targets is yet to be proven. For a long time, the debate was dominated by subtle differences and

structural uncertainties in artificial model systems that were mainly designed for screening campaigns and only reflected the *in vivo* situation to a limited degree. This has constrained efforts to identify promising lead compounds and distracted the attention from the actual main challenge: How drug-like and high-affinity compounds can be generated, given the fact of such an unfortunate dibasic substrate-recognition motif. Options are available to address this dilemma: First, active-site inhibitors that are structurally unrelated to the substrate should be pursued more intensively. Fragment-based techniques could be one way of doing this and the recently solved first co-crystal structure of a flavivirus protease with an active-site fragment (Zhang et al. 2016) may be a promising step in the right direction. Second, highly modified, metabolically stable and target-selective substrate mimetics might reach the protease *in vivo*, if modern drug delivery concepts are explored. Third, yet underexplored allosteric inhibitors may offer a convenient option to circumvent the difficulties associated with active-site ligands. The upcoming decade of research will show whether one of these avenues will provide the first clinical candidate to combat diseases associated with dengue, West Nile and Zika virus infections.

Compliance with ethical standards

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Conflict of interest: Christoph Nitsche declares that he has no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

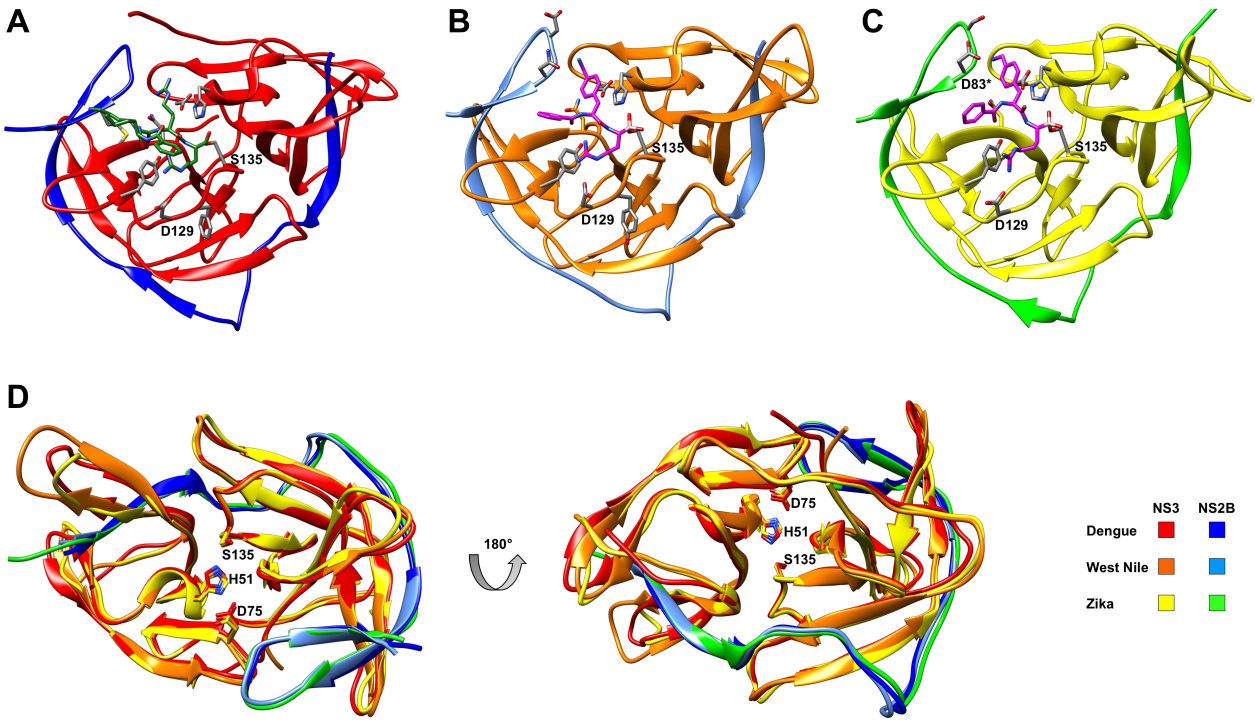

Fig. 1 Ligand-bound X-ray co-crystal structures of active flavivirus NS2B-NS3 proteases. **A**: Dengue protease serotype 3 with the covalently bound aldehyde ligand **1** (3U1I) (Noble et al. 2012). **B**: West Nile protease with the covalently bound boronate ligand **2** (5IDK) (Nitsche et al. 2017). **C**: Zika protease with the covalently bound boronate ligand **2** (5LC0) (Lei et al. 2016). **D**: Superimposition of active conformations of NS2B-NS3 proteases from dengue (3U1I), West Nile (5IDK) and Zika (5LC0) viruses. Co-crystallised ligands have been removed from the structures. Residues of the catalytic triad are indicated. Residues marked with an asterisk indicated NS2B. This Figure has been generated with Chimera (Pettersen et al. 2004).

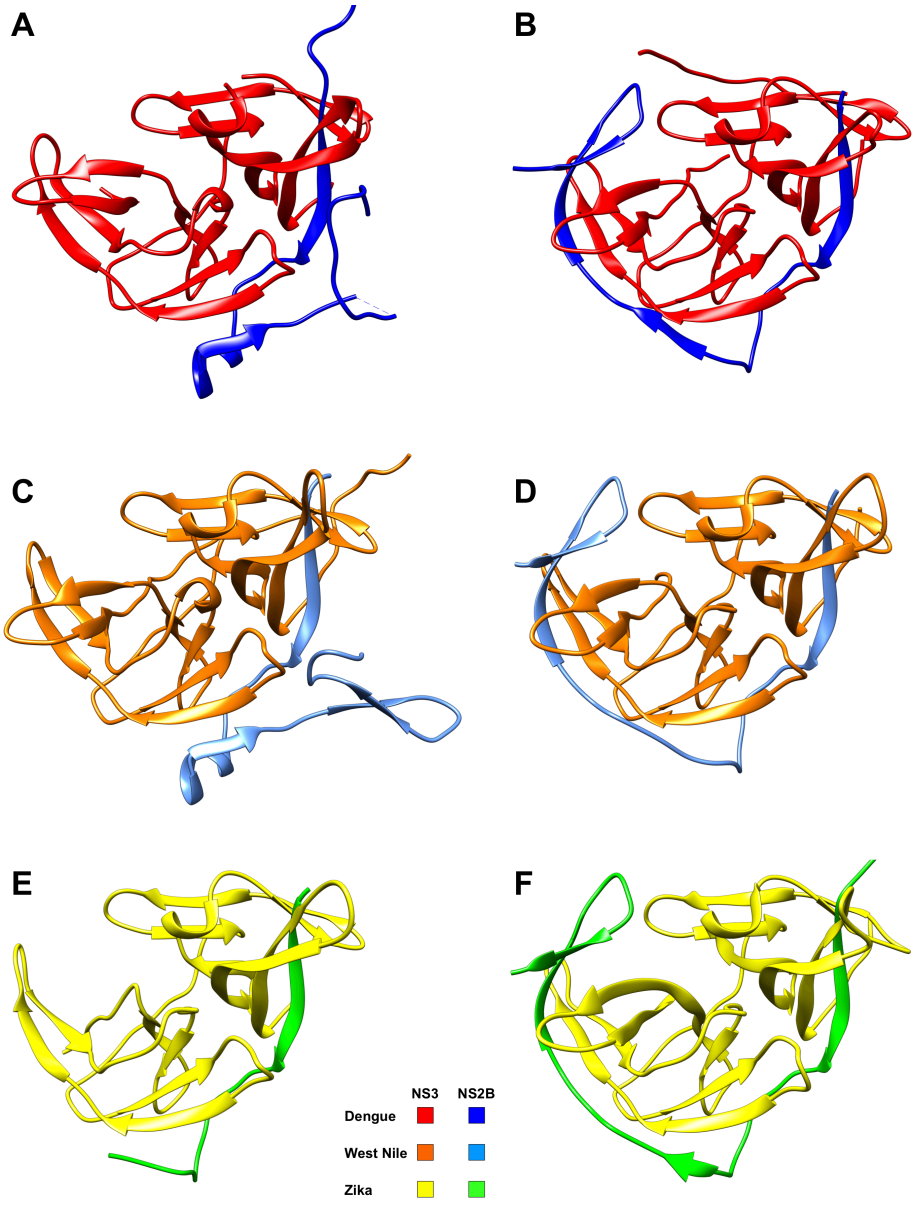

Fig. 2 X-ray crystal structures of inactive (open) and active (closed) conformations of flavivirus NS2B-NS3 proteases. Co-crystallised ligands have been removed from the active (closed) structures. **A**: Inactive (open) conformation of the dengue protease serotype 2 (2FOM) (Erbel et al. 2006). **B**: Active (closed) conformation of the dengue protease serotype 3 (3U1I) (Noble et al. 2012). **C**: Inactive (open) conformation of the West Nile protease mutant H51A (2GGV) (Aleshin et al. 2007). **D**: Active (closed) conformation of the West Nile protease (5IDK) (Nitsche et al. 2017). **E**: Inactive (open) conformation of the Zika protease (5GXJ) (Chen et al. 2016). **F**: Active (closed) conformation of the Zika protease (5LC0) (Lei et al. 2016). This Figure has been generated with Chimera (Pettersen et al. 2004).

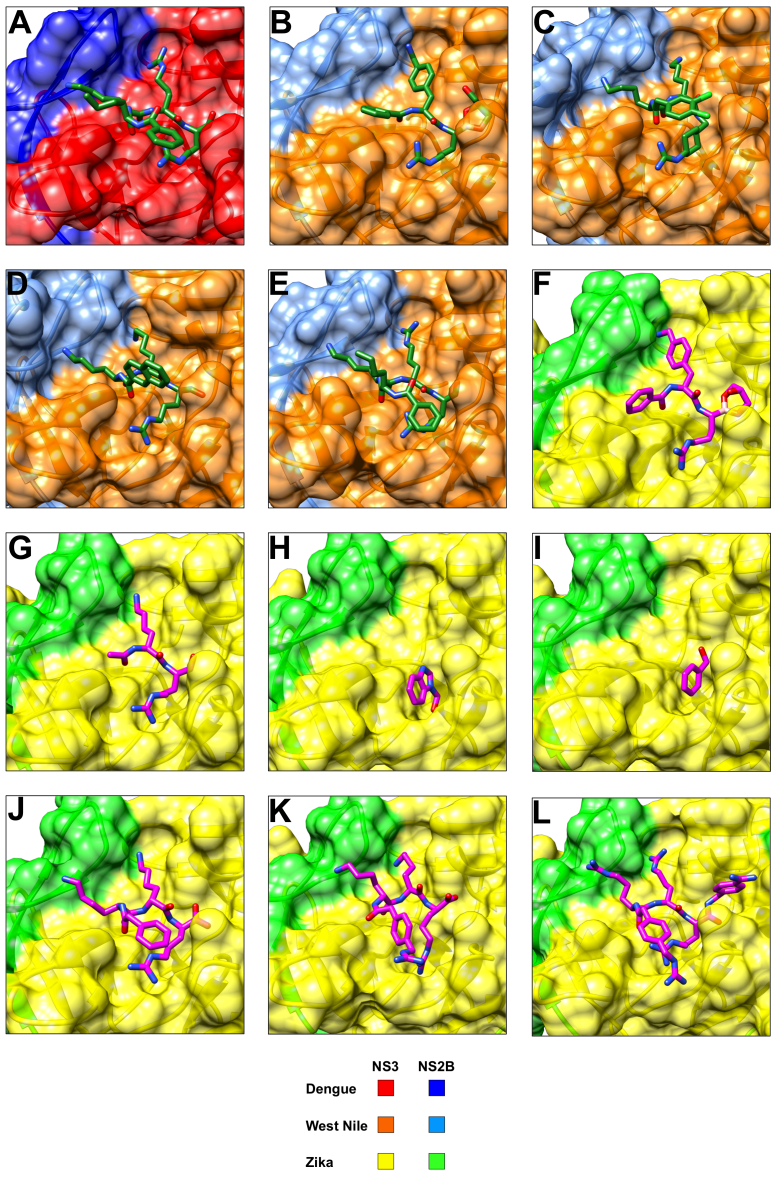

Fig. 3 X-ray co-crystal structures of flavivirus NS2B-NS3 proteases in complex with active-site inhibitors. **A**: Dengue protease serotype 3 in complex with compound **1** (3U1I) (Noble et al. 2012). The aldehyde in **1** forms a covalent hemiacetal with S135 (not shown). **B**: West Nile protease in complex with compound **2** (5IDK) (Nitsche et al. 2017). The boronic acid in **2** forms a cyclic ester adduct with glycerol (as shown) and a covalent boronate with S135 (not shown). **C**: West Nile protease in complex with compound **3** (2YOL) (Hammamy et al. 2013). **D**: West Nile protease in complex with compound **4** (3E90) (Robin et al. 2009). The aldehyde in **4** forms a covalent hemiacetal with S135 (not shown). **E**: West Nile protease in complex with compound **1** (2FP7) (Erbel et al. 2006). The aldehyde in **1** forms a covalent hemiacetal with S135 (not shown). **F**: Zika

protease in complex with compound **2** (5LC0) (Lei et al. 2016). The boronic acid in **2** forms a cyclic ester adduct with glycerol (as shown) and a covalent boronate with S135 (not shown). **G**: Zika protease C143S mutant in complex with compound **5** (5YOF) (Li et al. 2018). The aldehyde in **5** forms a covalent hemiacetal with S135 (not shown). A similar structure of lower resolution has also been reported for compound **5** in complex with the Zika protease wildtype (5H6V) (Li et al. 2017b). **H**: Zika protease in complex with fragment **6** (5H4I) (Zhang et al. 2016). **I**: Zika protease in complex with a benzoyl fragment (5YOD) (Li et al. 2018). Transesterification between Zika protease and compound **7** results in the formation of a S135 benzoate (covalent bond not shown). **J**: Zika protease C143S mutant in complex with proteolytically cleaved compound **8** (5ZMQ) (Phoo et al. 2018). **K**: Zika protease C143S mutant in complex with proteolytically hydrolysed compound **9** (5ZMS) (Phoo et al. 2018). **L**: Zika protease C143S mutant in complex with compound **10** (5ZOB) (Phoo et al. 2018). This Figure has been generated with Chimera (Pettersen et al. 2004).

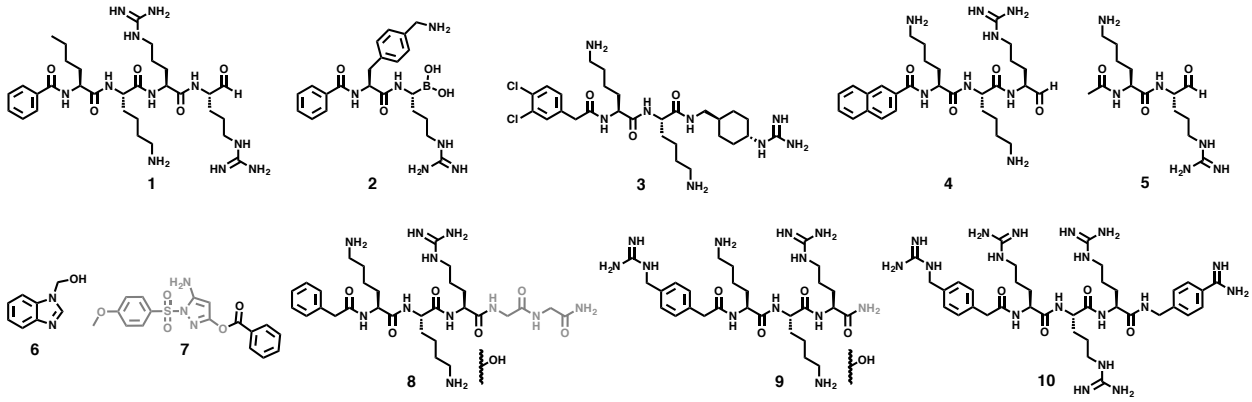

Fig. 4 Active-site inhibitors that were co-crystallised with NS2B-NS3 proteases from dengue, West Nile and Zika viruses. The actual observed ligand is highlighted in black. Inhibitor moieties

that have been cleaved or hydrolysed by proteolytic digest are shown in grey. Compounds **1**, **2**, **4**, **5** and **7** bind covalently to the catalytically active residue S135.

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