VIROLOGICA SINICA, December 2013, 28 (6): 326-336
DOI 10.1007/s12250-013-3390-x
www.virosin.org
Email: info@virosin.org

Review

The Flavivirus Protease As a Target for Drug Discovery

Matthew Brecher[1], Jing Zhang[1] and Hongmin Li[1, 2]✉

1. Wadsworth Center, New York State Department of Health, 120 New Scotland Ave, Albany NY 12208, USA
2. Department of Biomedical Sciences, School of Public Health, State University of New York, Empire State Plaza, PO Box 509, Albany, New York 12201-0509, USA

Many flaviviruses are significant human pathogens causing considerable disease burdens, including encephalitis and hemorrhagic fever, in the regions in which they are endemic. A paucity of treatments for flaviviral infections has driven interest in drug development targeting proteins essential to flavivirus replication, such as the viral protease. During viral replication, the flavivirus genome is translated as a single polyprotein precursor, which must be cleaved into individual proteins by a complex of the viral protease, NS3, and its cofactor, NS2B. Because this cleavage is an obligate step of the viral life-cycle, the flavivirus protease is an attractive target for antiviral drug development. In this review, we will survey recent drug development studies targeting the NS3 active site, as well as studies targeting an NS2B/NS3 interaction site determined from flavivirus protease crystal structures.

Flavivirus, Inhibitor, Protease

Introduction

Flaviviruses belong to the viral family *Flaviviridae* that include about 70 viruses (Brinton M A, 1981; Brinton M A, 2002; Westaway E G, et al., 1985). Many flaviviruses are significant human pathogens. Dengue virus (DENV) serotypes 1-4, Yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and tick-borne encephalitis complex virus (TBEV) are categorized as global emerging pathogens and are NIAID Priority Pathogens as well (Burke D S, et al., 2001). Flaviviruses cause significant human disease, some of which are fatal such as dengue hemorrhagic syndromes and various encephalitides (Asnis D S, et al., 2001; Asnis D S, et al., 2000; Kramer L D, et al., 2001; Shi P Y, et al., 2002; Shi P Y, et al., 2002; Shi P Y, et al., 2001).

The World Health Organization has estimated annual human cases of 50,000 for JE (WHO, 2009), 200,000 for YF (WHO, 2009), and more than 50 million for Dengue fever (WHO, 2009). WNV is now the leading cause of arboviral encephalitis in the US, leading to more than a thousand human deaths (CDC, 2010; USGS, 2010). Morbidity and mortality rates are waning for WNV in the US, but are expected to increase for DENV. Currently, approximately 2.5 billion people are at risk of DENV infection, with an estimated 500,000 cases in the form of life-threatening disease such as dengue hemorrhagic fever and dengue shock syndrome (WHO, 2009). However, vaccines for humans currently are available only for YFV, JEV, and TBEV (Burke D S, et al., 2001); and more importantly no clinically approved antiviral therapy is available for treatment of flavivirus infection. Therefore, it is a public health priority to develop antiviral agents for post-infection treatment (Kramer L D, et al., 2007).

This article will review recent advances in flavivirus drug development targeting the essential viral protease.

The flaviviral genome structure

The flavivirus genome RNA, approximately 11 kb in length, is single-stranded and of positive (i.e., mRNA-sense) polarity. The viral genome consists of a 5' untranslated region (UTR), a single long open reading frame (ORF), and a 3' UTR (Fig. 1) (Rice C M, et al., 1985; Shi P Y, et al., 2001). A cap is present at the 5' end, followed by

Received: 2013-10-08, Accepted: 2013-11-01
Published Online: 2013-11-14
✉ Corresponding author.
Phone: +1-518-486-9154, Fax: +1-518-408-2190
Email: lih@wadsworth.org

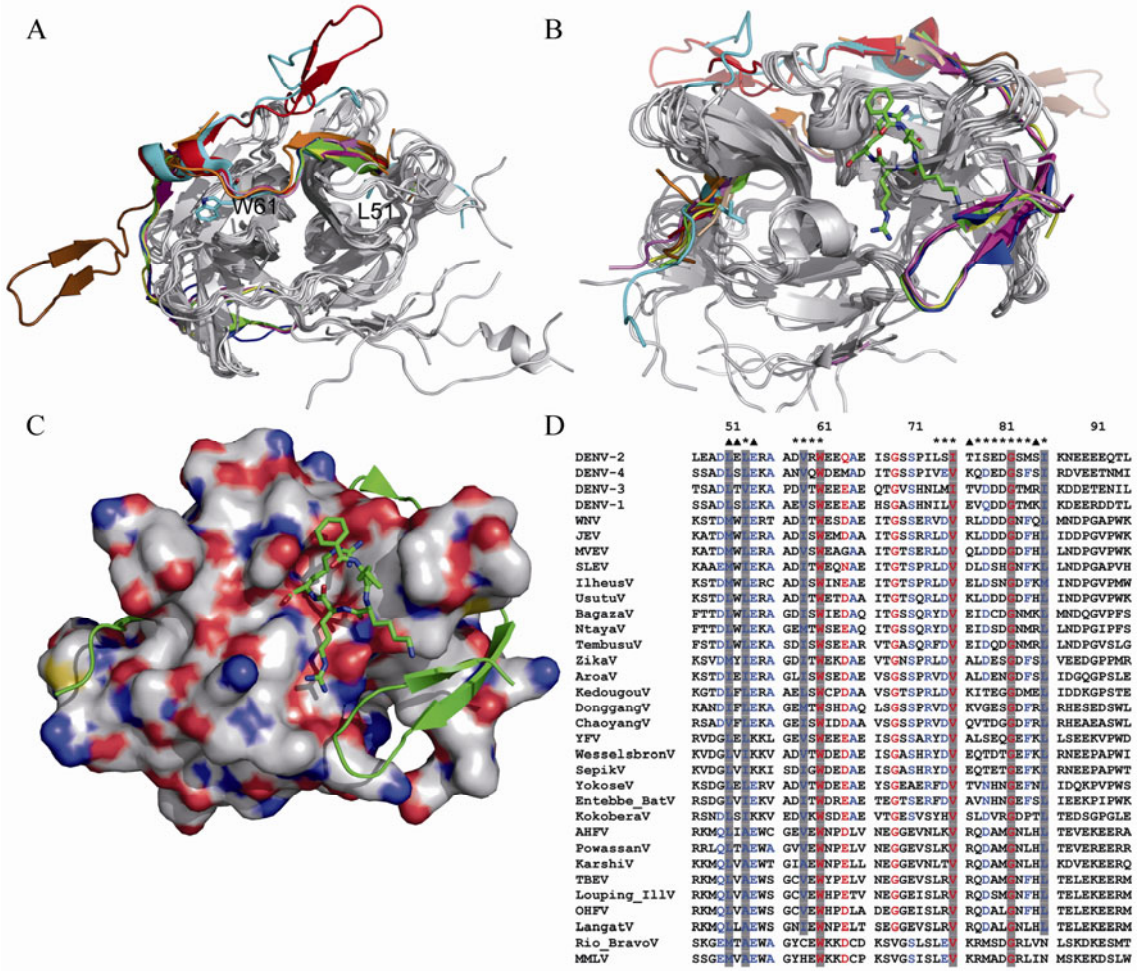

Fig. 1 Crystal structures and sequence alignment of flavivirus NS2B-NS3 protease complexes. **(A)** Superposition of all available crystal structures of the NS2B-NS3 protease complex, in the absence or presence of inhibitors. All NS3 chains were colored gray, with NS2B in different colors. PDB codes: 2FP7 (WNV, with peptide inhibitor, green) (Erbel P, et al., 2006), 2FOM (DENV-2, apo form, cyan) (Erbel P, et al., 2006), 2GGV (WNV, apo form, red) (Aleshin A, et al., 2007), 2IJO (WNV, aprotinin bound, yellow)(Aleshin A, et al., 2007), 3E90 (WNV, with peptide inhibitor, blue) (Robin G, et al., 2009), 2WV9 (MVEV, NS3 full-length, apo form, orange) (Assenberg R, et al., 2009), 3LKW (DENV-1, apo form, brown) (Chandramouli S, et al., 2010), 2WHX (DENV-4, NS3 full-length, apo form, gray) (Luo D, et al., 2010), 3U1I (DENV-3, with peptide inhibitor, magenta)(Noble C G, et al., 2012). L51 and W61 were labeled and shown in stick representation. (B) As in **(**A**)** with approximate 180º rotation, showing the active site of the superimposed NS2B-NS3 protease complexes and a bound inhibitor (PDB: 2FP7), with atom colors as: carbon (green), oxygen (red), and nitrogen (blue). (C) Surface representation of the NS3 protease active site (PDB: 2FP7), with atom colors as: carbon (gray), oxygen (red), and nitrogen (blue). The bound inhibitor was shown in stick representation, with atomic colors as in (B), and sulfur in yellow. NS2B was shown in ribbon representation (green). (D) Alignment of sequences of the NS2B cofactor region of representative flaviviruses with known sequences. Conserved hydrophobic residues and other strictly conserved residues that are essential or important for the protease function were shaded. Residues were colored according to the extent of their sequence conservation: >90% conserved (red); 50-90% conservation (blue); <50% less or not conserved (black). Residues essential for the protease function (Chappell K J, et al., 2008) were marked with a star above the sequences; residues less essential but still important for the protease function (Chappell K J, et al., 2008) were marked with a solid triangle symbol above the sequences. Abbreviations used here include: SLEV, Saint Louis encephalitis Virus; AHFV: Alkhumra hemorrhagic fever virus; OHFV: Omsk hemorrhagic fever virus; MMLV: Montana myotis leukoencephalitis virus. All other viruses were either defined with abbreviations in the main text or abbreviated here with their full-name prior to a "V" representing for virus.

the conserved dinucleotide sequence 5'-AG-3' (Cleaves G R, et al., 1979). The 3' end of the genome terminates with 5'-CU_{OH}-3' (Wengler G, 1981) rather than with a poly(A) tract. The single ORF of flavivirus encodes a polyprotein precursor of about 3,430 amino acids (Fig. 1A). The polyprotein is co- and post-translationally processed by viral and cellular proteases into three structural proteins (capsid [C], premembrane [prM] or membrane [M], and envelope [E]) and seven nonstructural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Chambers

T J, et al., 1990). The structural proteins form the viral particle and are involved in viral fusion with host cells including monocytes, macrophages and dendritic cells (Li L, et al., 2008; Lindenbach B D, et al., 2007; Marianneau P, et al., 1999; Tassaneetrithep B, et al., 2003). Low pH in the endosomal compartment triggers fusion of the viral and host cell membrane, which leads to the release of the nucleocapsid and viral RNA into the cytoplasm. This process is mediated by the viral E protein which is able to switch among different oligomeric states: as a trimer of prM-E heterodimers in immature particles, as a dimer in mature virus, and as a trimer when fusing with a host cell (Bressanelli S, et al., 2004; Modis Y, et al., 2004). The virus prM glycoprotein can be cleaved by furin protease to release the N-terminal "pr" residues during maturation, leaving only the ectodomain and C-terminal transmembrane region of "M" in the virion. The pr peptide protects immature virions against premature fusion with the host membrane (Guirakhoo F, et al., 1992; Li L, et al., 2008; Zhang Y, et al., 2003).

The NS proteins participate in RNA replication, virion assembly, and evasion of innate immune responses (Lindenbach B D, et al., 2007). The majority of the flavivirus NS proteins are multifunctional. NS1 is a large glycoprotein which is required for negative strand RNA synthesis (Lindenbach B D, et al., 1997; Lindenbach B D, et al., 1999; Muylaert I R, et al., 1997). NS2A has been reported to function in the generation of virus-induced membranes during virus assembly and/or release of infectious flavivirus particles (Kummerer B M, et al., 2002; Leung J Y, et al., 2008). NS2B is a required cofactor for the protease activity of NS3 (Arias C F, et al., 1993; Chambers T J, et al., 1991; Chambers T J, et al., 1993; Falgout B, et al., 1993). NS3 is a large multi-functional protein with the activities of a serine protease (with NS2B as a cofactor), a 5′-RNA triphosphatase (RTPase), a nucleoside triphosphatase (NTPase), and a helicase (Li H, et al., 1999; Warrener P, et al., 1993; Wengler G, 1991). NS4A is an integral membrane protein involved in membrane rearrangements required to form the viral replication complex (Miller S, et al., 2007; Roosendaal J, et al., 2006). NS4B has been reported to inhibit the type I interferon response of host cells, and might modulate viral RNA synthesis (Grant D, et al., 2011; Munoz-Jordan J L, et al., 2005; Umareddy I, et al., 2006). NS5 is the largest flaviviral protein with multiple enzymatic activities, namely the RNA-dependent RNA polymerase (RdRp) (Ackermann M, et al., 2001; Guyatt K J, et al., 2001; Tan B H, et al., 1996), the N-7 guanine and 2'-O ribose methyltransferase (Dong H, et al., 2012; Egloff M P, et al., 2002; Koonin E V, 1993; Ray D, et al., 2006; Zhou Y, et al., 2007), and the RNA guanylyltransferase (GTase) (Issur M, et al., 2009). Several NS proteins such as NS2A, NS4A, NS4B, and NS5 are thought to interfere with host immune responses (Ashour J, et al., 2009; Best S M, et al., 2005; Daffis S, et al., 2010; Guo J, et al., 2005; Munoz-Jordan J L, et al., 2003; Munoz-Jordan J L, et al., 2005).

The NS3/NS2B protease

The NS3 protein (~618 amino acids (aa)) is the second largest protein encoded by flavivirus. The N-terminal 170 aa of NS3 displays protease activity, and a hydrophobic core of about 40 aa in length within NS2B provides an essential cofactor function (Chambers T J, et al., 1991; Chambers T J, et al., 1990; Falgout B, et al., 1991). The NS3 protease belongs to the trypsin serine protease superfamily with a catalytic triad (e.g. His51-Asp75-Ser135 for the DENV NS3) (Bazan J F, et al., 1989). The NS2B/NS3 protease complex prefers a substrate with basic residues (Arg or Lys) at the P1 and P2 sites and a short side-chain amino acid (Gly, Ser, or Ala) at the P1′ site (Chambers T J, et al., 1990; Gouvea I E, et al., 2007). The central function of the NS2B/NS3 protease complex is to process the flavivirus polyprotein precursor. As shown in Fig. 1, the peptide bonds between capsid, NS2A-NS2B, NS2B-NS3, NS3-NS4A and NS4B-NS5 are cleaved by the NS2B/NS3 protease complex, leading to the release of mature individual NS proteins.

The NS2B/NS3 protease complex is essential for the flavivirus replication and virion assembly, as evidenced by the lack of production of infectious virions in mutants carrying inactivating viral proteases (Chambers T J, et al., 1993).

Crystal structure of the NS3/NS2B protease complex

The development of protease inhibitor began with the determination of the three-dimensional (3D) structures of the flavivirus NS3 protease, the NS2B/NS3 protease complex, and the protease-inhibitor complexes (Aleshin A, et al., 2007; Assenberg R, et al., 2009; Chandramouli S, et al., 2010; Erbel P, et al., 2006; Hammamy M Z, et al., 2013; Luo D, et al., 2008; Luo D, et al., 2010; Luo D, et al., 2008; Noble C G, et al., 2012; Robin G, et al., 2009). Currently, fourteen crystal structures of the NS2B/NS3 protease complex are available for the flavivirus NS2B/NS3 protease complexes, including the apo structures of proteases of WNV, DENV-1, DENV-2, DENV-4, and Murray Valley encephalitis virus (MVEV), the structures of proteases of WNV and DENV3 in complex peptide substrate-based inhibitors, and the broad-spectrum serine

protease inhibitor aprotinin-bound structures of proteases of WNV and DENV-3.

In general, the flavivirus NS3 proteases display a chymotrypsin-like fold (Erbel P, et al., 2006). In all these structures, a NS2B fragment composed of about 44-47 amino acids, which provides an essential cofactor function (Chambers T J, et al., 1991; Chambers T J, et al., 1990; Falgout B, et al., 1990), is associated with NS3. When no substrate or inhibitor is present, the N-terminal (residues 51-61 in DENV-2) but not the C-terminal portion of NS2B is bound to NS3 (Erbel P, et al., 2006) (Fig. 1A). The central portion of this N-terminal part forms a β-strand and is part of the β-barrel of NS3 (Erbel P, et al., 2006). Consistent with the important structural role of this part of NS2B, structural comparison indicates that the NS2B residues within the N-terminal portion display similar conformations in all structures, regardless of presence or absence of inhibitors (Fig. 1A). It has also been reported that the N-terminal portion of NS2B (aa 49-66 only) is sufficient to bind and stabilize the NS3 conformation (Luo D, et al, 2008; Luo D, et al., 2010), although such a complex lacks protease activity (Luo D, et al., 2008; Luo D, et al., 2010; Phong W Y, et al., 2011). Mutagenesis studies demonstrated that two NS2B regions are critical for the protease function (Chappell K J, et al., 2008; Niyomrattanakit P, et al., 2004; Phong W Y, et al., 2011; Radichev I, et al., 2008) (Fig. 1D). Region one corresponds to the N-terminal region mentioned above, whereas region two is referred to a C-terminal region composed of residues 74-86 of NS2B. Residues within region one show great sequence conservation, especially for several hydrophobic residues at positions 51, 53, 59, and 61 (in DENV-2 order), with Trp61 strictly conserved (Fig. 1D). Functional studies indicated that three of these residues are essential, and the remaining one is also important, for the protease function (Chappell K J, et al., 2008). Structure comparison indicated that these conserved hydrophobic residues bind deeply into several pockets of NS3 (Fig. 1A). In contrast, residues within region two display greater sequence variation than those within region one, which may contribute to their fine substrate specificities as region two is part of the protease active site (see below) (Fig. 1B, 1C). In addition, in contrast to the N-terminal region which shows similar conformations, the C-terminal portion (beyond aa 61) of NS2B displays significantly large conformational differences between inhibitor-bound and inhibitor-free structures, and even between inhibitor-free structures (Fig. 1A). These results suggest that the N-terminal portion, but not the C-terminal portion, of NS2B is essential for NS2B to bind and stabilize NS3.

The C-terminal portion of NS2B has an integral role in active site formation in WNV and DENV. Although the C-terminal portions of NS2B display significantly different conformation in various apo crystal structures, the C-terminal portions of bound structures show remarkable conformational similarity when the complex is bound either to substrate analogs or the protease inhibitor aprotinin (Fig. 1B). In the structure of inhibitor-bound form, the C-terminal portion of NS2B forms a β-hairpin and "wraps around" the NS3 core, closing the NS3 active site. Several residues within this region make direct interactions, including hydrogen bonds, with substrate analogs or aprotinin inhibitors. Unsurprisingly, results from mutagenesis studies have demonstrated the importance of this region in protease function (Chappell K J, et al., 2008; Niyomrattanakit P, et al., 2004), likely due to its structural role in formation of the protease active site. The active site of the flavivirus NS2B/NS3 protease complex is quite flat and hydrophilic (Fig. 1C) and requires several basic residues as substrates, potentially hampering the development of potent competitive inhibitors.

Inhibitors for the NS3/NS2B protease

Viral proteases are proven antiviral targets. Numerous inhibitors against the HIV protease have been successfully developed and used in treatment of AIDS (Menendez-Arias L, 2010). Two HCV protease inhibitors have been recently approved to treat chronic HCV infections by FDA (Lin C, et al., 2006; Lin K, et al., 2006; Sarrazin C, et al., 2007). The success of protease inhibitors in other viruses has put the flavivirus protease in the focus of development for anti-flavivirus therapy. Both high throughput screening (HTS) and structure-based drug design have been explored to identify inhibitors against flavivirus protease.

Leung et al. reported the first inhibition studies using a recombinant covalently-linked NS2B/NS3 protease complex of DENV2 (Leung D, et al., 2001). Of sixteen standard serine protease inhibitors tested, however, only aprotinin, a basic pancreatic trypsin inhibitor, was shown to inhibit the enzyme with nanomolar IC_{50} (Drug concentration required to reduce enzyme activity by 50%) (Leung D, et al., 2001; Mueller N H, et al., 2007). Aprotinin was found to bind the NS2B/NS3 proteases of all four serial types of DENV with high affinity (picomolar) (Li J, et al., 2005); the *in vivo* efficacy of aprotinin in reduction of flavivirus has not been reported. Nevertheless, although aprotinin is a potent inhibitor for the flavivirus NS3 protease, severe safety issues prevent it from being used as a drug. Aprotinin is a small protein which inhibits

trypsin and related proteolytic enzymes and has been administered by injection, under the trade name Trasylol (Bayer) as a medication to reduce bleeding during complex surgery, such as heart and liver surgery, before 2007. The drug was permanently withdrawn worldwide in 2008 after studies suggested that its use increased the risk of complications or death (Mangano D T, et al., 2006; Mangano D T, et al., 2007).

Besides standard serine protease inhibitors, several peptidic α-keto amide inhibitors were also explored (Leung D, et al., 2001). Two peptidic inhibitor candidates showed inhibition activity for the protease with low micromolar IC_{50}. Several similar peptidic inhibitor candidates, including cyclopeptides (Gao Y, et al., 2010; Xu S, et al., 2012), were found to be active for the NS2B/NS3 protease complex of DENV2, WNV, and YFV with K_i (the absolute inhibition constant) as low as 43 nM (Chanprapaph S, et al., 2005; Knox J E, et al., 2006; Nall T A, et al., 2004; Nitsche C, et al., 2012; Schuller A, *et al*., 2011; Yin Z, et al., 2006; Yin Z, et al., 2006). Although the *in vivo* efficacy of these inhibitor candidates has not been verified, the highly charged nature of these peptidic inhibitors may indicate poor bioavailability. The following studies seemed to verify this notion. Shiryeav et al. reported that the D-arginine-based peptides are potent inhibitors for the WNV NS3 protease, with K_i as low as 1 nM in an *in vitro* biochemical protease assay (Shiryaev S, et al., 2006). However, in a cell-based virus reduction assay, the inhibitor only showed micromolar inhibitory activity against the WNV (Shiryaev S, et al., 2006). In another study, Stoermer et al. reported that a peptidic inhibitor candidate showed high potency (K_i = 9 nM) for the WNV protease (Stoermer M J, et al., 2008). The inhibitor, composed of cationic tripeptide (KKR) with a phenacetyl-cap at the N-terminus and an aldehyde at the C-terminus, is cell permeable and stable in serum, but displays a much reduced antiviral activity (EC_{50} (concentration required for 50% viral reduction)=1.6 μM) (Stoermer M J, et al., 2008). The poor activities of these peptide-based inhibitors in cell-based assays may be explained by the poor penetration of charged peptides across the cell membrane. Nevertheless, the low bioavailability of these substrate inhibitors could limit their potential as effective chemotherapeutics (Chappell K J, et al., 2008; Noble C G, et al., 2010).

In addition to the standard inhibitors based on substrates, attempts to use protein as inhibitor has been explored (Rothan H A, et al., 2012). Rothan et al. reported that retrocyclin-1 (RC-1) can inhibit the NS2B/NS3 protease activity *in vitro* with IC_{50} in micromolar range. However, it only moderately reduced the virus growth even at 150 μM concentration.

Nonsubstrate based inhibitors were also investigated, though only moderate inhibition activity (IC_{50} in low micromolar range) was observed (Cregar-Hernandez L, et al., 2011; Ganesh V K, et al., 2005; Jia F, et al., 2010; Kiat T S, et al., 2006). To explore more small molecular inhibitors for the protease, both *in silico*-based and protein-based HTS has been developed (Aravapalli S, et al., 2012; Deng J, et al., 2012; Ekonomiuk D, et al., 2009; Ekonomiuk D, et al., 2009; Ezgimen M, et al., 2012; Gao Y, et al., 2013; Johnston P A, et al., 2007; Knehans T, et al., 2011; Lai H, et al., 2013; Lai H, et al., 2013; Mueller N H, et al., 2008; Nitsche C, et al., 2011; Samanta S, et al., 2012; Steuer C, et al., 2011; Tiew K C, et al., 2012; Tomlinson S M, et al., 2012; Tomlinson S M, et al., 2009). Several small molecule inhibitors were identified possessing low micromolar or high nanomolar inhibition activities for the WNV and DENV proteases (Bodenreider C, et al., 2009; Cregar-Hernandez L, et al., 2011; Ekonomiuk D, et al., 2009; Johnston P A, et al., 2007; Knehans T, et al., 2011; Lai H, et al., 2013; Mueller N H, et al., 2008; Sidique S, et al., 2009; Tomlinson S M, et al., 2011; Tomlinson S M, et al., 2009; Yang C C, et al., 2011). Although some of these compounds are potent inhibitors (IC_{50} up to 0.105 μM) for the flavivirus NS3 protease, some of them show poor stability with half life of only 1-2 h in solution (Johnston P A, et al., 2007). In addition, the majority of these studies, except the three discussed below (Mueller N H, et al., 2008; Tomlinson S M, et al., 2009; Yang C C, et al., 2011), did not use cell-based assays to evaluate the antiviral efficacy of identified compounds. In two studies (Mueller N H, et al., 2008; Tomlinson S M, et al., 2009), several compounds were found to inhibit the growth of WNV and DENV with EC_{50} in the low micromolar range. Furthermore, Yang et al. showed that a compound could inhibit the DENV NS3 protease with IC_{50} of 15 μM (Yang C C, et al., 2011). Encouragingly, this compound appeared much more potent in a replicon-based antiviral assay (EC_{50} of 0.17 M) than in the enzyme-based protease assay, possibly due to additional cellular targets.

All current approaches to identify inhibitors for the NS3 protease focus on the protease active site. However, only limited success has been achieved. This could be because the active site of the flavivirus NS3 protease is quite flat and highly charged (Aleshin A, et al., 2007; Assenberg R, et al., 2009; Chandramouli S, et al., 2010; Erbel P, et al., 2006; Luo D, et al., 2008; Luo D, et al., 2010; Luo D, et al., 2008; Robin G, et al., 2009), which

makes it difficult to find small-molecule inhibitors of the NS2B/NS3 protease. Therefore, alternative approaches should be considered. Notably, the flavivirus NS3 protease requires NS2B as a co-factor for function. Therefore, the NS2B-NS3 association site may be targeted for identification and development of compounds that inhibit flavivirus NS3 protease function by blocking NS2B-NS3 association. The crystal structures of the NS2B/NS3 complex (Aleshin A, et al., 2007; Assenberg R, et al., 2009; Chandramouli S, et al., 2010; Erbel P, et al., 2006; Luo D, et al., 2008; Luo D, et al., 2010) and ample data from functional studies (Chambers T J, et al., 2005; Chappell K J, et al., 2006; Chappell K J, et al., 2008; Niyomrattanakit P, et al., 2004; Radichev I, et al., 2008) provide solid bases for HT screening of compound libraries to identify allosteric inhibitors. Currently, this approach has not been extensively explored. Only two reports indicated that a non-competitive inhibitor was identified, through a protein-based HTS assay, to have high potency against the NS3 protease, although one of the compounds was very unstable in solution (Johnston P A, et al., 2007; Pambudi S, et al., 2013). Docking experiments suggested that the compound binds to a site on the NS3 surface that may interfere with the binding between NS3 and the cofactor NS2B (Johnston P A, et al., 2007; Pambudi S, et al., 2013). Although a crystal structure of the inhibitor-NS3 complex is required to confirm the mode of action of this type of inhibitor, *in vitro* virus inhibition studies indicated that the compound identified by Pambudi et al. that targets the NS2B-NS3 interactions can efficiently inhibit all four serotypes of DENV with EC_{50} of 0.74-4.92 μM (Pambudi S, et al., 2013). This compound also showed moderate inhibition activity toward YFV, indicating a potentially broad antiviral spectrum. Mutagenesis studies further revealed that mutations of DENV4 and YFV residues that were predicted to interact with the inhibitor candidate affected the sensitivity of viruses to this compound (Pambudi S, et al., 2013). These results strongly support the hypothesis that the interaction between NS2B and NS3 is a valid therapeutic target for anti-DENV drugs and argue that greater effort should be put towards developing allosteric inhibitors targeting the NS2B-NS3 interactions.

Future directions

Historically, the most straightforward approach to developing inhibitors of an enzyme target has been to screen for compounds that competitively bind the enzyme's active site and displace native substrate. The advantage of such an approach is that characterization of the properties of a particular enzyme's substrate is often a sufficient starting point for selecting compounds that mimic or exceed the substrate in its affinity for the enzyme. Unfortunately, this approach might be unlikely to yield effective compounds in the case of flavivirus NS2B/NS3 protease for three reasons: First, NS2B/NS3 has a flat and hydrophilic active site which decreases the likelihood that compounds can bind specifically with high affinity. Second, the NS2B/NS3 active site is similar enough to those of host serine proteases that toxic effects in the host are likely for many compounds, as has been observed in the case of aprotinin. Third, the active site preferentially binds positively charged moieties; this charge can have deleterious effects on compound bioavailability.

In addition, lessons should be learned from the development of active site inhibitors for the HCV protease. Although two HCV protease substrate-based inhibitors were developed, resistant mutations occurred quickly (Wyles D L, 2013). This is because the active site of the HCV protease is shallow and solvent exposed. The featureless property of the active site of the HCV protease implies that inhibitors would rely on relatively few interactions with the enzyme for tight binding, resulting in a low barrier to resistance and extensive cross-resistance (Romano K P, et al., 2010; Wegzyn C M, et al., 2012). It has been reported that as few as a single key mutation resulted in a significant loss of inhibition and cross-resistance (Romano K P, et al., 2010; Wyles D L, 2012; Wyles D L, 2013). Similar to that of the HCV protease, the active site of flavivirus NS2B/NS3 protease complex is also flat and featureless, in addition to the hydrophilic nature. Therefore, potential drug resistance should be taken into account, when development of active-site inhibitors for flavivirus protease complex is considered.

Fortunately, the solved crystal structures of flavivirus protease in both substrate bound and unbound states has yielded mechanistic insight into protease function. Details of the interaction of the NS2B cofactor, critical for enzyme function, with NS3 have suggested an allosteric approach to inhibition through disruption of NS2B/NS3 binding. Lead compounds developed by this approach are less likely to have the drawbacks observed with active site inhibitors, and are amenable to both computational and HTS screening methods. In the future, this "structure-guided" approach may suggest additional allosteric sites in flavivirus protease and has the potential to open broad avenues to drug discovery in other disease target proteins.

Acknowledgements

This research was partially supported by grants

(AI094335) from the National Institute of Health and from the Wadsworth Center Scientific Interaction Group.

Author Contributions

All authors carried out the work presented here. MB, JZ, and HML wrote the paper the paper. MB and HML defined, reviewed and edited the theme of this review.

References

Ackermann M, and Padmanabhan R. 2001. **De novo synthesis of RNA by the dengue virus RNA-dependent RNA polymerase exhibits temperature dependence at the initiation but not elongation phase.** J Biol Chem, 276: 39926-39937.

Aleshin A, Shiryaev S, Strongin A, and Liddington R. 2007. **Structural evidence for regulation and specificity of flaviviral proteases and evolution of the Flaviviridae fold.** Protein Sci., 16: 795-806.

Aravapalli S, Lai H, Teramoto T, Alliston K R, Lushington G H, Ferguson E L, Padmanabhan R, and Groutas W C. 2012. **Inhibitors of Dengue virus and West Nile virus proteases based on the aminobenzamide scaffold**. Bioorg Med Chem, 20: 4140-4148.

Arias C F, Preugschat F, and Strauss J H. 1993. **Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain**. Virology, 193: 888-899.

Ashour J, Laurent-Rolle M, Shi P Y, and Garcia-Sastre A. 2009. **NS5 of dengue virus mediates STAT2 binding and degradation**. J Virol, 83: 5408-5418.

Asnis D S, Conetta R, Waldman G, and Teixeira A A. 2001. **The West Nile virus encephalitis outbreak in the United States (1999-2000): from Flushing, New York, to beyond its borders**. Ann N Y Acad Sci, 951: 161-171.

Asnis D S, Conetta R, Teixeira A A, Waldman G, and Sampson B A. 2000. **The West Nile Virus outbreak of 1999 in New York: the Flushing Hospital experience**. Clin Infect Dis, 30: 413-418.

Assenberg R, Mastrangelo E, Walter T S, Verma A, Milani M, Owens R J, Stuart D I, Grimes J M, and Mancini E J. 2009. **Crystal structure of a novel conformational state of the flavivirus NS3 protein: implications for polyprotein processing and viral replication**. J Virol, 83: 12895-12906.

Bazan J F, and Fletterick R J. 1989. **Detection of a trypsin-like serine protease domain in flaviviruses and pestiviruses**. Virology, 171: 637-639.

Best S M, Morris K L, Shannon J G, Robertson S J, Mitzel D N, Park G S, Boer E, Wolfinbarger J B, and Bloom M E. 2005. **Inhibition of interferon-stimulated JAK-STAT signaling by a tick-borne flavivirus and identification of NS5 as an interferon antagonist**. J. Virol., 79: 12828-12839.

Bodenreider C, Beer D, Keller T H, Sonntag S, Wen D, Yap L, Yau Y H, Shochat S G, Huang D, Zhou T, Caflisch A, Su X C, Ozawa K, Otting G, Vasudevan S G, Lescar J, and Lim S P. 2009. **A fluorescence quenching assay to discriminate between specific and nonspecific inhibitors of dengue virus protease**. Anal Biochem, 395: 195-204.

Bressanelli S, Stiasny K, Allison S L, Stura E A, Duquerroy S, Lescar J, Heinz F X, and Rey F A. 2004. **Structure of a flavivirus envelope glycoprotein in its low-pH-induced membrane fusion conformation**. EMBO J., 23: 728-738.

Brinton M A. 1981. **Isolation of a replication-efficient mutant of West Nile virus from a persistently infected genetically resistant mouse cell culture**. J Virol, 39: 413-421.

Brinton M A. 2002. **THE MOLECULAR BIOLOGY OF WEST NILE VIRUS: A New Invader of the Western Hemisphere**. Annu Rev Microbiol, 56: 371-402.

Burke D S, and Monath T P. 2001. **Flaviviruses**. Lippincott William & Wilkins.

CDC. 2010. **CDC West Nile virus homepage**. http://www.cdc.gov/ncidod/ dvbid/ westnile/surv&controlCaseCount03.htm**.**

Chambers T J, Grakoui A, and Rice C M. 1991. **Processing of the yellow fever virus nonstructural polyprotein: a catalytically active NS3 proteinase domain and NS2B are required for cleavages at dibasic sites.** J. Virol., 65: 6042-6050.

Chambers T J, Hahn C S, Galler R, and Rice C M. 1990. **Flavivirus genome organization, expression, and replication**. Annu Rev Microbiol, 44: 649-688.

Chambers T J, Nestorowicz A, Amberg S M, and Rice C M. 1993. **Mutagenesis of the yellow fever virus NS2B protein: effects on proteolytic processing, NS2B-NS3 complex formation, and viral replication**. J Virol, 67: 6797-6807.

Chambers T J, Droll D A, Tang Y, Liang Y, Ganesh V K, Murthy K H, and Nickells M. 2005. **Yellow fever virus NS2B-NS3 protease: characterization of charged-to-alanine mutant and revertant viruses and analysis of polyprotein-cleavage activities**. J Gen Virol, 86: 1403-1413.

Chandramouli S, Joseph J S, Daudenarde S, Gatchalian J, Cornillez-Ty C, and Kuhn P. 2010. **Serotype-specific structural differences in the protease-cofactor complexes of the dengue virus family**. J Virol, 84: 3059-3067.

Chanprapaph S, Saparpakorn P, Sangma C, Niyomrattanakit P, Hannongbua S, Angsuthanasombat C, and Katzenmeier G. 2005. **Competitive inhibition of the dengue virus NS3 serine protease by synthetic peptides representing polyprotein cleavage sites**. Biochem Biophys Res Commun, 330: 1237-1246.

Chappell K J, Stoermer M J, Fairlie D P, and Young P R. 2006. **Insights to substrate binding and processing by West Nile Virus NS3 protease through combined modeling, protease mutagenesis, and kinetic studies**. J Biol Chem, 281: 38448-38458.

Chappell K J, Stoermer M J, Fairlie D P, and Young P R. 2008. **West Nile Virus NS2B/NS3 protease as an antiviral target**. Curr Med Chem, 15: 2771-2784.

Chappell K J, Stoermer M J, Fairlie D P, and Young P R. 2008. **Mutagenesis of the West Nile virus NS2B cofactor domain reveals two regions essential for protease activity**. J Gen Virol, 89: 1010-1014.

Cleaves G R, and Dubin D T. 1979. **Methylation status of intracellular dengue type 2 40 S RNA**. Virology, 96: 159-165.

Cregar-Hernandez L, Jiao G S, Johnson A T, Lehrer A T, Wong T A, and Margosiak S A. 2011. **Small molecule pan-dengue and West Nile virus NS3 protease inhibitors**. Antivir Chem Chemother, 21: 209-217.

Daffis S, Szretter K J, Schriewer J, Li J, Youn S, Errett J, Lin T Y, Schneller S, Zust R, Dong H, Thiel V, Sen G C, Fensterl V, Klimstra W B, Pierson T C, Buller R M, Gale M, Jr., Shi P Y, and Diamond M S. 2010. **2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members**. Nature, 468: 452-456.

Deng J, Li N, Liu H, Zuo Z, Liew O W, Xu W, Chen G, Tong X, Tang W, Zhu J, Zuo J, Jiang H, Yang C G, Li J, and Zhu W. 2012. **Discovery of novel small molecule inhibitors of dengue viral NS2B-NS3 protease using virtual screening and scaffold hopping**. J Med Chem, 55: 6278-6293.

Dong H, Chang D C, Hua M H, Lim S P, Chionh Y H, Hia F, Lee Y H, Kukkaro P, Lok S M, Dedon P C, and Shi P Y. 2012. **2'-O**

methylation of internal adenosine by flavivirus NS5 methyltransferase. PLoS pathogens, 8: e1002642.

Egloff M P, Benarroch D, Selisko B, Romette J L, and Canard B. 2002. **An RNA cap (nucleoside-2'-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization**. Embo J, 21: 2757-2768.

Ekonomiuk D, Su X C, Ozawa K, Bodenreider C, Lim S P, Otting G, Huang D, and Caflisch A. 2009. **Flaviviral protease inhibitors identified by fragment-based library docking into a structure generated by molecular dynamics**. J Med Chem, 52: 4860-4868.

Ekonomiuk D, Su X C, Ozawa K, Bodenreider C, Lim S P, Yin Z, Keller T H, Beer D, Patel V, Otting G, Caflisch A, and Huang D. 2009. **Discovery of a non-peptidic inhibitor of west nile virus NS3 protease by high-throughput docking**. PLoS Negl Trop Dis, 3: e356.

Erbel P, Schiering N, D'Arcy A, Renatus M, Kroemer M, Lim S, Yin Z, Keller T, Vasudevan S, and Hommel U. 2006. **Structural basis for the activation of flaviviral NS3 proteases from dengue and West Nile virus**. Nat. Struct. Mol. Biol., 13: 372-373.

Ezgimen M, Lai H, Mueller N H, Lee K, Cuny G, Ostrov D A, and Padmanabhan R. 2012. **Characterization of the 8-hydroxyquinoline scaffold for inhibitors of West Nile virus serine protease**. Antiviral Res, 94: 18-24.

Falgout B, Miller R H, and Lai C J. 1993. **Deletion analysis of dengue virus type 4 nonstructural protein NS2B: identification of a domain required for NS2B-NS3 protease activity**. J Virol, 67: 2034-2042.

Falgout B, Bray M, Schlesinger J J, and Lai C J. 1990. **Immunization of mice with recombinant vaccinia virus expressing authentic dengue virus nonstructural protein NS1 protects against lethal dengue virus encephalitis**. J Virol, 64: 4356-4363.

Falgout B, Pethel M, Zhang Y M, and Lai C J. 1991. **Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins**. J Virol, 65: 2467-2475.

Ganesh V K, Muller N, Judge K, Luan C H, Padmanabhan R, and Murthy K H. 2005. **Identification and characterization of nonsubstrate based inhibitors of the essential dengue and West Nile virus proteases**. Bioorg Med Chem, 13: 257-264.

Gao Y, Cui T, and Lam Y. 2010. **Synthesis and disulfide bond connectivity-activity studies of a kalata B1-inspired cyclopeptide against dengue NS2B-NS3 protease**. Bioorg Med Chem, 18: 1331-1336.

Gao Y, Samanta S, Cui T, and Lam Y. 2013. **Synthesis and in vitro Evaluation of West Nile Virus Protease Inhibitors Based on the 1,3,4,5-Tetrasubstituted 1H-Pyrrol-2(5H)-one Scaffold**. ChemMedChem, 8: 1554-1560.

Gouvea I E, Izidoro M A, Judice W A, Cezari M H, Caliendo G, Santagada V, dos Santos C N, Queiroz M H, Juliano M A, Young P R, Fairlie D P, and Juliano L. 2007. **Substrate specificity of recombinant dengue 2 virus NS2B-NS3 protease: influence of natural and unnatural basic amino acids on hydrolysis of synthetic fluorescent substrates**. Arch Biochem Biophys, 457: 187-196.

Grant D, Tan G K, Qing M, Ng J K, Yip A, Zou G, Xie X, Yuan Z, Schreiber M J, Schul W, Shi P Y, and Alonso S. 2011. **A Single Amino Acid in Nonstructural Protein NS4B Confers Virulence to Dengue Virus in AG129 Mice through Enhancement of Viral RNA Synthesis**. J Virol, 85: 7775-7787.

Guirakhoo F, Bolin R A, and Roehrig J T. 1992. **The Murray Valley encephalitis virus prM protein confers acid resistance to virus particles and alters the expression of epitopes within the R2 domain of E glycoprotein**. Virology, 191: 921-931.

Guo J, Hayashi J, and Seeger C. 2005. **West nile virus inhibits the signal transduction pathway of alpha interferon**. J. Virol., 79: 1343-1350.

Guyatt K J, Westaway E G, and Khromykh A A. 2001. **Expression and purification of enzymatically active recombinant RNA-dependent RNA polymerase (NS5) of the flavivirus Kunjin**. J Virol Methods, 92: 37-44.

Hammamy M Z, Haase C, Hammami M, Hilgenfeld R, and Steinmetzer T. 2013. **Development and characterization of new peptidomimetic inhibitors of the West Nile virus NS2B-NS3 protease**. ChemMedChem, 8: 231-241.

Issur M, Geiss B J, Bougie I, Picard-Jean F, Despins S, Mayette J, Hobdey S E, and Bisaillon M. 2009. **The flavivirus NS5 protein is a true RNA guanylyltransferase that catalyzes a two-step reaction to form the RNA cap structure**. Rna, 15: 2340-2350.

Jia F, Zou G, Fan J, and Yuan Z. 2010. **Identification of palmatine as an inhibitor of West Nile virus**. Arch Virol, 155: 1325-1329.

Johnston P A, Phillips J, Shun T Y, Shinde S, Lazo J S, Huryn D M, Myers M C, Ratnikov B I, Smith J W, Su Y, Dahl R, Cosford N D, Shiryaev S A, and Strongin A Y. 2007. **HTS identifies novel and specific uncompetitive inhibitors of the two-component NS2B-NS3 proteinase of West Nile virus**. Assay Drug Dev Technol, 5: 737-750.

Kiat T S, Pippen R, Yusof R, Ibrahim H, Khalid N, and Rahman N A. 2006. **Inhibitory activity of cyclohexenyl chalcone derivatives and flavonoids of fingerroot, Boesenbergia rotunda (L.), towards dengue-2 virus NS3 protease**. Bioorg Med Chem Lett, 16: 3337-3340.

Knehans T, Schuller A, Doan D N, Nacro K, Hill J, Guntert P, Madhusudhan M S, Weil T, and Vasudevan S G. 2011. **Structure-guided fragment-based in silico drug design of dengue protease inhibitors**. J Comput Aided Mol Des, 25: 263-274.

Knox J E, Ma N L, Yin Z, Patel S J, Wang W L, Chan W L, Ranga Rao K R, Wang G, Ngew X, Patel V, Beer D, Lim S P, Vasudevan S G, and Keller T H. 2006. **Peptide inhibitors of West Nile NS3 protease: SAR study of tetrapeptide aldehyde inhibitors**. J Med Chem, 49: 6585-6590.

Koonin E V. 1993. **Computer-assisted identification of a putative methyltransferase domain in NS5 protein of flaviviruses and lambda 2 protein of reovirus**. J Gen Virol, 74: 733-740.

Kramer L D, and Bernard K A. 2001. **West Nile virus infection in birds and mammals**. Ann N Y Acad Sci, 951: 84-93.

Kramer L D, Li J, and Shi P Y. 2007. **West Nile virus**. Lancet Neurol, 6: 171-181.

Kummerer B M, and Rice C M. 2002. **Mutations in the yellow fever virus nonstructural protein NS2A selectively block production of infectious particles**. J. Virol., 76: 4773-4784.

Lai H, Sridhar Prasad G, and Padmanabhan R. 2013. **Characterization of 8-hydroxyquinoline derivatives containing aminobenzothiazole as inhibitors of dengue virus type 2 protease in vitro**. Antiviral Res, 97: 74-80.

Lai H, Dou D, Aravapalli S, Teramoto T, Lushington G H, Mwania T M, Alliston K R, Eichhorn D M, Padmanabhan R, and Groutas W C. 2013. **Design, synthesis and characterization of novel 1,2-benzisothiazol-3(2H)-one and 1,3,4-oxadiazole hybrid derivatives:**

potent inhibitors of Dengue and West Nile virus NS2B/NS3 proteases. Bioorg Med Chem, 21: 102-113.

Leung D, Schroder K, White H, Fang N-X, Stoermer M, Abbenante G, Martin J, PR Y, and Fairlie D. 2001. **Activity of recombinant dengue 2 virus NS3 protease in the presence of a truncated NS2B co-factor, small peptide substrates, and inhibitors**. J. Biol. Chem., 276: 45762-45771.

Leung J Y, Pijlman G P, Kondratieva N, Hyde J, Mackenzie J M, and Khromykh A A. 2008. **Role of nonstructural protein NS2A in flavivirus assembly**. J Virol, 82: 4731-4741.

Li H, Clum S, You S, Ebner K E, and Padmanabhan R. 1999. **The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids**. J Virol, 73: 3108-3116.

Li J, Lim S P, Beer D, Patel V, Wen D, Tumanut C, Tully D C, Williams J A, Jiricek J, Priestle J P, Harris J L, and Vasudevan S G. 2005. **Functional profiling of recombinant NS3 proteases from all four serotypes of dengue virus using tetrapeptide and octapeptide substrate libraries**. J Biol Chem, 280: 28766-28774.

Li L, Lok S M, Yu I M, Zhang Y, Kuhn R J, Chen J, and Rossmann M G. 2008. **The flavivirus precursor membrane-envelope protein complex: structure and maturation**. Science, 319: 1830-1834.

Lin C, Kwong A D, and Perni R B. 2006. **Discovery and development of VX-950, a novel, covalent, and reversible inhibitor of hepatitis C virus NS3.4A serine protease**. Infect Disord Drug Targets, 6: 3-16.

Lin K, Perni R B, Kwong A D, and Lin C. 2006. **VX-950, a novel hepatitis C virus (HCV) NS3-4A protease inhibitor, exhibits potent antiviral activities in HCv replicon cells**. Antimicrob Agents Chemother, 50: 1813-1822.

Lindenbach B D, and Rice C M. 1997. **trans-Complementation of yellow fever virus NS1 reveals a role in early RNA replication**. J. Virol., 71: 9608-9617.

Lindenbach B D, and Rice C M. 1999. **Genetic interaction of flavivirus nonstructural proteins NS1 and NS4A as a determinant of replicase function**. J Virol, 73: 4611-4621.

Lindenbach B D, Thiel H J, and Rice C M. 2007. **Flaviviridae: The Virus and Their Replication**, Fourth ed. Lippincott William & Wilkins.

Luo D, Xu T, Hunke C, Gruber G, Vasudevan S G, and Lescar J. 2008. **Crystal structure of the NS3 protease-helicase from dengue virus**. J Virol, 82: 173-183.

Luo D, Wei N, Doan D N, Paradkar P N, Chong Y, Davidson A D, Kotaka M, Lescar J, and Vasudevan S G. 2010. **Flexibility between the protease and helicase domains of the dengue virus NS3 protein conferred by the linker region and its functional implications**. J Biol Chem, 285: 18817-18827.

Luo D, Xu T, Watson R P, Scherer-Becker D, Sampath A, Jahnke W, Yeong S S, Wang C H, Lim S P, Strongin A, Vasudevan S G, and Lescar J. 2008. **Insights into RNA unwinding and ATP hydrolysis by the flavivirus NS3 protein**. Embo J, 27: 3209-3219.

Mangano D T, Tudor I C, and Dietzel C. 2006. **The risk associated with aprotinin in cardiac surgery**. N Engl J Med, 354: 353-365.

Mangano D T, Miao Y, Vuylsteke A, Tudor I C, Juneja R, Filipescu D, Hoeft A, Fontes M L, Hillel Z, Ott E, Titov T, Dietzel C, and Levin J. 2007. **Mortality associated with aprotinin during 5 years following coronary artery bypass graft surgery**. JAMA, 297: 471-479.

Marianneau P, Steffan A M, Royer C, Drouet M T, Jaeck D, Kirn A, and Deubel V. 1999. **Infection of primary cultures of human Kupffer cells by Dengue virus: no viral progeny synthesis, but cytokine production is evident**. J Virol, 73: 5201-5206.

Menendez-Arias L. 2010. **Molecular basis of human immunodeficiency virus drug resistance: an update**. Antiviral Res, 85: 210-231.

Miller S, Kastner S, Krijnse-Locker J, Buhler S, and Bartenschlager R. 2007. **The non-structural protein 4A of dengue virus is an integral membrane protein inducing membrane alterations in a 2K-regulated manner**. J Biol Chem, 282: 8873-8882.

Modis Y, Ogata S, Clements D, and Harrison S C. 2004. **Structure of the dengue virus envelope protein after membrane fusion**. Nature, 427: 313-319.

Mueller N H, Yon C, Ganesh V K, and Padmanabhan R. 2007. **Characterization of the West Nile virus protease substrate specificity and inhibitors**. Int J Biochem Cell Biol, 39: 606-614.

Mueller N H, Pattabiraman N, Ansarah-Sobrinho C, Viswanathan P, Pierson T C, and Padmanabhan R. 2008. **Identification and biochemical characterization of small-molecule inhibitors of west nile virus serine protease by a high-throughput screen**. Antimicrob Agents Chemother, 52: 3385-3393.

Munoz-Jordan J L, Sanchez-Burgos G G, Laurent-Rolle M, and Garcia-Sastre A. 2003. **Inhibition of interferon signaling by dengue virus**. Proc. Natl. Acad. Sci. USA, 100: 14333-14338.

Munoz-Jordan J L, Laurent-Rolle M, Ashour J, Martinez-Sobrido L, Ashok M, Lipkin W I, and Garcia-Sastre A. 2005. **Inhibition of Alpha/Beta Interferon Signaling by the NS4B Protein of Flaviviruses**. J. Virol., 79: 8004-8013.

Muylaert I R, Galler R, and Rice C M. 1997. **Genetic analysis of the yellow fever virus NS1 protein: identification of a temperature-sensitive mutation which blocks RNA accumulation.** J. Virol., 71: 291-298.

Nall T A, Chappell K J, Stoermer M J, Fang N X, Tyndall J D, Young P R, and Fairlie D P. 2004. **Enzymatic characterization and homology model of a catalytically active recombinant West Nile virus NS3 protease**. J Biol Chem, 279: 48535-48542.

Nitsche C, Steuer C, and Klein C D. 2011. **Arylcyanoacrylamides as inhibitors of the Dengue and West Nile virus proteases**. Bioorg Med Chem, 19: 7318-7337.

Nitsche C, Behnam M A, Steuer C, and Klein C D. 2012. **Retro peptide-hybrids as selective inhibitors of the Dengue virus NS2B-NS3 protease**. Antiviral Res, 94: 72-79.

Niyomrattanakit P, Winoyanuwattikun P, Chanprapaph S, Angsuthanasombat C, Panyim S, and Katzenmeier G. 2004. **Identification of residues in the dengue virus type 2 NS2B cofactor that are critical for NS3 protease activation**. J Virol, 78: 13708-13716.

Noble C G, Seh C C, Chao A T, and Shi P Y. 2012. **Ligand-bound structures of the dengue virus protease reveal the active conformation**. Journal of Virology, 86: 438-446.

Noble C G, Chen Y L, Dong H, Gu F, Lim S P, Schul W, Wang Q Y, and Shi P Y. 2010. **Strategies for development of Dengue virus inhibitors**. Antiviral Res, 85: 450-462.

Pambudi S, Kawashita N, Phanthanawiboon S, Omokoko M D, Masrinoul P, Yamashita A, Limkittikul K, Yasunaga T, Takagi T, Ikuta K, and Kurosu T. 2013. **A Small Compound Targeting the Interaction between Nonstructural Proteins 2B and 3 Inhibits Dengue Virus Replication**. Biochem Biophys Res Commun: in press (doi: 10.1016/j.bbrc.2013.1009.1078).

Phong W Y, Moreland N J, Lim S P, Wen D, Paradkar P N, and Vasudevan S G. 2011. **Dengue protease activity: the structural integrity and interaction of NS2B with NS3 protease and its**

potential as a drug target. Bioscience reports.

Radichev I, Shiryaev S A, Aleshin A E, Ratnikov B I, Smith J W, Liddington R C, and Strongin A Y. 2008. **Structure-based mutagenesis identifies important novel determinants of the NS2B cofactor of the West Nile virus two-component NS2B-NS3 proteinase**. J Gen Virol, 89: 636-641.

Ray D, Shah A, Tilgner M, Guo Y, Zhao Y, Dong H, Deas T, Zhou Y, Li H, and Shi P. 2006. **West nile virus 5'-cap structure is formed by sequential guanine N-7 and ribose 2'-O methylations by nonstructural protein 5**. J. Virol., 80: 8362-8370.

Rice C M, Lenches E M, Eddy S R, Shin S J, Sheets R L, and Strauss J H. 1985. **Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution**. Science, 229: 726-733.

Robin G, Chappell K, Stoermer M J, Hu S H, Young P R, Fairlie D P, and Martin J L. 2009. **Structure of West Nile virus NS3 protease: ligand stabilization of the catalytic conformation**. J Mol Biol, 385: 1568-1577.

Romano K P, Ali A, Royer W E, and Schiffer C A. 2010. **Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding**. Proc Natl Acad Sci U S A, 107: 20986-20991.

Roosendaal J, Westaway E G, Khromykh A, and Mackenzie J M. 2006. **Regulated cleavages at the West Nile virus NS4A-2K-NS4B junctions play a major role in rearranging cytoplasmic membranes and Golgi trafficking of the NS4A protein**. J Virol, 80: 4623-4632.

Rothan H A, Han H C, Ramasamy T S, Othman S, Rahman N A, and Yusof R. 2012. **Inhibition of dengue NS2B-NS3 protease and viral replication in Vero cells by recombinant retrocyclin-1**. BMC Infect Dis, 12: 314.

Samanta S, Cui T, and Lam Y. 2012. **Discovery, synthesis, and in vitro evaluation of West Nile virus protease inhibitors based on the 9,10-dihydro-3H,4aH-1,3,9,10a-tetraazaphenanthren-4-one scaffold**. ChemMedChem, 7: 1210-1216.

Sarrazin C, Rouzier R, Wagner F, Forestier N, Larrey D, Gupta S K, Hussain M, Shah A, Cutler D, Zhang J, and Zeuzem S. 2007. **SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders**. Gastroenterology, 132: 1270-1278.

Schuller A, Yin Z, Brian Chia C S, Doan D N, Kim H K, Shang L, Loh T P, Hill J, and Vasudevan S G. 2011. **Tripeptide inhibitors of dengue and West Nile virus NS2B-NS3 protease**. Antiviral Res, 92: 96-101.

Shi P Y, Tilgner M, and Lo M K. 2002. **Construction and characterization of subgenomic replicons of New York strain of West Nile virus**. Virology, 296: 219-233.

Shi P Y, Tilgner M, Lo M K, Kent K A, and Bernard K A. 2002. **Infectious cDNA clone of the epidemic west nile virus from New York City**. J Virol, 76: 5847-5856.

Shi P Y, Kauffman E B, Ren P, Felton A, Tai J H, Dupuis A P, 2nd, Jones S A, Ngo K A, Nicholas D C, Maffei J, Ebel G D, Bernard K A, and Kramer L D. 2001. **High-throughput detection of West Nile virus RNA**. J Clin Microbiol, 39: 1264-1271.

Shiryaev S, Ratnikov B, Chekanov A, Sikora S, Rozanov D, Godzik A, Wang J, Smith J, Huang Z, Lindberg I, Samuel M, Diamond M, and Strongin A. 2006. **Cleavage targets and the D-arginine-based inhibitors of the West Nile virus NS3 processing proteinase**. Biochem J., 393: 503-511.

Sidique S, Shiryaev S A, Ratnikov B I, Herath A, Su Y, Strongin A Y, and Cosford N D. 2009. **Structure-activity relationship and improved hydrolytic stability of pyrazole derivatives that are allosteric inhibitors of West Nile Virus NS2B-NS3 proteinase**. Bioorg Med Chem Lett, 19: 5773-5777.

Steuer C, Gege C, Fischl W, Heinonen K H, Bartenschlager R, and Klein C D. 2011. **Synthesis and biological evaluation of alpha-ketoamides as inhibitors of the Dengue virus protease with antiviral activity in cell-culture**. Bioorg Med Chem, 19: 4067-4074.

Stoermer M J, Chappell K J, Liebscher S, Jensen C M, Gan C H, Gupta P K, Xu W J, Young P R, and Fairlie D P. 2008. **Potent cationic inhibitors of West Nile virus NS2B/NS3 protease with serum stability, cell permeability and antiviral activity**. J Med Chem, 51: 5714-5721.

Tan B H, Fu J, Sugrue R J, Yap E H, Chan Y C, and Tan Y H. 1996. **Recombinant dengue type 1 virus NS5 protein expressed in Escherichia coli exhibits RNA-dependent RNA polymerase activity**. Virology, 216: 317-325.

Tassaneetrithep B, Burgess T H, Granelli-Piperno A, Trumpfheller C, Finke J, Sun W, Eller M A, Pattanapanyasat K, Sarasombath S, Birx D L, Steinman R M, Schlesinger S, and Marovich M A. 2003. **DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells**. J Exp Med, 197: 823-829.

Tiew K C, Dou D, Teramoto T, Lai H, Alliston K R, Lushington G H, Padmanabhan R, and Groutas W C. 2012. **Inhibition of Dengue virus and West Nile virus proteases by click chemistry-derived benz[d]isothiazol-3(2H)-one derivatives**. Bioorg Med Chem, 20: 1213-1221.

Tomlinson S M, and Watowich S J. 2011. **Anthracene-based inhibitors of dengue virus NS2B-NS3 protease**. Antiviral Res, 89: 127-135.

Tomlinson S M, and Watowich S J. 2012. **Use of parallel validation high-throughput screens to reduce false positives and identify novel dengue NS2B-NS3 protease inhibitors**. Antiviral Res, 93: 245-252.

Tomlinson S M, Malmstrom R D, Russo A, Mueller N, Pang Y P, and Watowich S J. 2009. **Structure-based discovery of dengue virus protease inhibitors**. Antiviral Res, 82: 110-114.

Umareddy I, Chao A, Sampath A, Gu F, and Vasudevan S G. 2006. **Dengue virus NS4B interacts with NS3 and dissociates it from single-stranded RNA**. J Gen Virol, 87: 2605-2614.

USGS. 2010. **Disease Maps 2010**. http://diseasemaps.usgs.gov/.

Warrener P, Tamura J K, and Collett M S. 1993. **RNA-stimulated NTPase activity associated with yellow fever virus NS3 protein expressed in bacteria**. J Virol, 67: 989-996.

Wegzyn C M, and Wyles D L. 2012. **Antiviral drug advances in the treatment of human immunodeficiency virus (HIV) and chronic hepatitis C virus (HCV)**. Curr Opin Pharmacol, 12: 556-561.

Wengler G. 1981. **Terminal sequences of the genome and replicative-from RNA of the flavivirus West Nile virus: absence of poly(A) and possible role in RNA replication**. Virology, 113: 544-555.

Wengler G. 1991. **The carboxy-terminal part of the NS 3 protein of the West Nile flavivirus can be isolated as a soluble protein after proteolytic cleavage and represents an RNA-stimulated NTPase**. Virology, 184: 707-715.

Westaway E G, Brinton M A, Gaidamovich S Y, Horzinek M C, Igarashi A, Kaariainen L, Lvov D K, Porterfield J S, Russell P K, and Trent D W. 1985. **Flaviviridae**. Intervirol., 24: 183-192.

WHO. 2009. **Immunization, vaccines and biologicals: Japanese encephalitis**. <http://www.who.int/nuvi/je/en/>>.

WHO. 2009. **Dengue factsheet.** <http://www.who.int/mediacentre/factsheets/fs117/en/>.

WHO. 2009. **Yellow fever factsheet**. <http://www.who.int/mediacentre/factsheets/fs100/en/>.

Wyles D L. 2012. **Beyond telaprevir and boceprevir: resistance and new agents for hepatitis C virus infection**. Top Antivir Med, 20: 139-145.

Wyles D L. 2013. **Antiviral resistance and the future landscape of hepatitis C virus infection therapy**. J Infect Dis, 207 Suppl 1: S33-39.

Xu S, Li H, Shao X, Fan C, Ericksen B, Liu J, Chi C, and Wang C. 2012. **Critical effect of peptide cyclization on the potency of peptide inhibitors against Dengue virus NS2B-NS3 protease**. J Med Chem, 55: 6881-6887.

Yang C C, Hsieh Y C, Lee S J, Wu S H, Liao C L, Tsao C H, Chao Y S, Chern J H, Wu C P, and Yueh A. 2011. **Novel dengue virus-specific NS2B/NS3 protease inhibitor, BP2109, discovered by a high-throughput screening assay**. Antimicrob Agents Chemother, 55: 229-238.

Yin Z, Patel S J, Wang W L, Wang G, Chan W L, Rao K R, Alam J, Jeyaraj D A, Ngew X, Patel V, Beer D, Lim S P, Vasudevan S G, and Keller T H. 2006. **Peptide inhibitors of Dengue virus NS3 protease. Part 1: Warhead**. Bioorg Med Chem Lett, 16: 36-39.

Yin Z, Patel S J, Wang W L, Chan W L, Ranga Rao K R, Wang G, Ngew X, Patel V, Beer D, Knox J E, Ma N L, Ehrhardt C, Lim S P, Vasudevan S G, and Keller T H. 2006. **Peptide inhibitors of dengue virus NS3 protease. Part 2: SAR study of tetrapeptide aldehyde inhibitors**. Bioorg Med Chem Lett, 16: 40-43.

Zhang Y, Corver J, Chipman P R, Zhang W, Pletnev S V, Sedlak D, Baker T S, Strauss J H, Kuhn R J, and Rossmann M G. 2003. **Structures of immature flavivirus particles**. EMBO J., 22: 2604-2613.

Zhou Y, Ray D, Zhao Y, Dong H, Ren S, Li Z, Guo Y, Bernard K A, Shi P Y, and Li H. 2007. **Structure and function of flavivirus NS5 methyltransferase.** J Virol, 81: 3891-3903.