HHS Public Access

Author manuscript

Antiviral Res. Author manuscript; available in PMC 2024 February 01.

Published in final edited form as:

Antiviral Res. 2023 February ; 210: 105516. doi:10.1016/j.antiviral.2022.105516.

"Flavivirus Proteases: The viral Achilles heel to prevent future pandemics"

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Abstract

Flaviviruses are important human pathogens and include dengue (DENV), West Nile (WNV), Yellow fever virus (YFV), Japanese encephalitis (JEV) and Zika virus (ZIKV). DENV, transmitted by mosquitoes, causes diseases ranging in severity from mild dengue fever with non-specific flu-like symptoms to fatal dengue hemorrhagic fever and dengue shock syndrome. DENV infections are caused by four serotypes, DENV1–4, which interact differently with antibodies in blood serum. The incidence of DENV infection has increased dramatically in recent decades and the CDC estimates 400 million dengue infections occur each year, resulting in ~25,000 deaths mostly among children and elderly people. Similarly, ZIKV infections are caused by infected mosquito bites to humans, can be transmitted sexually and through blood transfusions. If a pregnant woman is infected, the virus can cross the placental barrier and can spread to her fetus, causing severe brain malformations in the child including microcephaly and other birth defects. It is noteworthy that the neurological manifestations of ZIKV were also observed in DENV endemic regions, suggesting that pre-existing antibody response to DENV could augment ZIKV infection. WNV, previously unknown in the US (and known to cause only mild disease in Middle East), first arrived in New York city in 1999 (NY99) and spread throughout the US and Canada by Culex mosquitoes and birds. WNV is now endemic in North America. Thus, emerging and re-emerging flaviviruses are significant threat to human health. However, vaccines are available for only a limited number of flaviviruses, and antiviral therapies are not available for any flavivirus. Hence, there is an urgent need to develop therapeutics that interfere with essential enzymatic steps, such as protease in the flavivirus lifecycle as these viruses possess significant threat to future pandemics. In this review, we focus on our E. coli expression of NS2B hydrophilic domain (NS2BH) covalently linked to NS3 protease domain (NS3Pro) in their natural context which is processed by the combined action of both subunits of the NS2B-NS3Pro precursor. Biochemical activities of the viral protease such

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

as solubility and autoproteolysis of NS2BH-NS3Pro linkage depended on the C-terminal portion of NS2BH linked to the NS3Pro domain. Since 2008, we also focus on the use of the recombinant protease in high throughput screens and characterization of small molecular compounds identified in these screens.

1. Introduction

Flaviviruses are important human pathogens and include dengue (DENV), West Nile (WNV), Yellow fever virus (YFV), Japanese encephalitis (JEV), tick-borne encephalitis virus (TBEV), and Zika virus (ZIKV). In the review, we will focus on flaviviruses that are transmitted by mosquitoes. DENV, transmitted by Aedes aegypti and Aedes albopictus mosquitoes, causes diseases ranging in severity from mild dengue fever with non-specific flu-like symptoms to fatal dengue hemorrhagic fever and dengue shock syndrome (Clyde et al., 2006; Gubler, 1998; Halstead, 1988). DENV infections are caused by four serotypes, DENV1–4, which interact differently with antibodies in blood serum. Prior infections of a DENV generate the antibodies that recognize to a DENV of a different serotype but are unable to prevent infection. Instead, these antibodies allow DENV to get into the cells and cause more severe diseases by a process known as antibody-dependent enhancement mechanism (Halstead, 1988). Furthermore, due to a lack of cross-protection by the immune response to one DENV serotype to another serotype, a vaccine requires to be equally protective against all serotypes, which has been difficult to develop. The incidence of DENV infection has increased dramatically in recent decades and the CDC estimates 400 million dengue infections occur each year, resulting in ~25,000 deaths mostly among children and elderly people (Bhatt et al., 2013; Gubler, 2004). Thus, an anti-virus drug treatment effective against all four serotypes as well as pan flavivirus inhibitors are ideal for preventing fatal complications. These broad-spectrum inhibitors would be particularly desirable to prepare for the next flavivirus epidemic, which could emerge from yet unknown or neglected viruses.

Similarly, ZIKV can be transmitted not only by infected mosquitoes, but also from infected people via sexual transmission and through blood transfusion; ZIKV can spread from a pregnant woman to her fetus, causing severe brain malformations in the new-born child including microcephaly and other birth defects (Lazear and Diamond, 2016). It is noteworthy that the neurological manifestations of ZIKV were also observed in DENV endemic regions, suggesting that pre-existing antibody response to DENV could augment ZIKV infection. WNV was previously unknown in the US, and was known to cause only mild disease in the Middle East, first arrived in New York city in 1999 (NY99 strain) and spread throughout the US and Canada by Culex mosquitoes and infected birds. WNV is now endemic in North America (Morrison and Diamond, 2017). Although emerging and re-emerging flaviviruses are significant threat to human health, they have been neglected and did not attract any commercial interest for drug development. This scenario is changing now due to the climatic change and frequent hurricanes and increased prevalence of DENV, WNV, and ZIKV diseases in the US and US territories. Currently, vaccines are available for only a limited number of flaviviruses, and antiviral therapies are not available for any

flaviviruses. Hence, there is a grave need to develop therapeutics that interfere with essential steps in the flavivirus lifecycle.

2. Genomic Organization of Flavivirus RNA

Flavivirus RNA genome consists of the 5’ untranslated region (5’ UTR), single open-reading frame (ORF), and 3’ untranslated region (3’ UTR). The 5’ and 3’ UTRs contain RNA stem-loop structures that are essential for viral replicaton (Funk et al., 2010; Goertz and Pijlman, 2015). Flavivirus ORF encodes a single polyprotein which is processed by the host-encoded signalase and the heterodimeric viral NS2B-NS3 protease cotranslationally and post-translationally into mature proteins (reviewed in (Brinton, 2014; Padmanabhan and Strongin, 2010)) (Fig. 1A). Three structural proteins; C, prM and E as well as seven mature non-structural (NS) proteins; NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 are cleaved by either host signal peptidase or NS3 viral protease. A novel interaction between NS1 and NS4A-2K-NS4B which is required for viral replication but not for formation of the membranous replication organelle, has also been reported by Plaszczyca et al. (Plaszczyca et al., 2019) (see Fig. 1). NS3 and NS5 form a multi-enzyme complex of proteins involved in positive and negative viral RNA syntheses and 5’ capping. Flavivirus NS3 protease has multiple functional domains. The N-terminal domain (aa 1–170) of NS3 codes for the trypsin-like serine protease (Bazan and Fletterick, 1989; Gorbalenya et al., 1989a), followed by a flexible linker of ~20 aa and conserved domains found in RNA helicases (Bartelma and Padmanabhan, 2002; Benarroch et al., 2004; Frick, 2003; Frick et al., 2004; Li et al., 1999; Wu et al., 2005). Similarly, NS5 protein also has multiple functional domains. The N-terminal domain of NS5, has guanine N-7 and ribose 2’-O methylation activities that can methylate GpppA-capped and m(7) GpppA-capped RNAs sequentially, yielding m(7) GpppA and m(7)GpppAm RNA products in the presence of S-adenosyl methionine methyl donor. The conserved motif, K(61)-D(146)-K(182)-E(218) is important for guanine N-7 (Ray et al., 2006) and ribose 2’-O methylation reactions (Egloff et al., 2002; Selisko et al., 2010). N-7 methylation requires only D(146) although other amino acids of the motif facilitate the reaction. The C-terminal domain forms an RNA-dependent RNA polymerase (Gorbalenya et al., 1989b; Issur et al., 2009; Koonin, 1993; Ray et al., 2006; Tay and Vasudevan, 2018; Teramoto et al., 2017; Teramoto et al., 2014; Yon et al., 2005). The viral proteins perform a variety of functions such as viral replication, assembly into mature virions (Apte-Sengupta et al., 2014; Chatel-Chaix and Bartenschlager, 2014; Hodge et al., 2019; Klema et al., 2015; Lescar et al., 2018; Mukhopadhyay et al., 2005; Pierson and Diamond, 2020), evasion of immune response, and disruption cellular homeostatesis in the infected cells (reviewed in (Fischl and Bartenschlager, 2011; Heaton and Randall, 2011; Kirkegaard et al., 2004; Wileman, 2006).

3. Flavivirus protease.

In this regard, the viral protease is an excellent target for drug development. The discovery that NS2B and the N-terminal region of NS3 (NS3Pro) functions as a serine protease involved in the clevage of the polyprotein precursor was made in early studies by sequence homology to the N-terminal region of NS3 (Bazan and Fletterick, 1989; Gorbalenya et al., 1989a), transfection and translation of YFV and DENV2 RNA, and site-directed

mutagenesis studies (Chambers et al., 1991; Chambers et al., 1990; Zhang et al., 1992; Zhang and Padmanabhan, 1993). The active viral protease is formed from a hetero-dimeric complex of a 130 amino acid (aa) integral memebrane protein, NS2B, containing four transmembrane (TM) helices linked to the NS3Pro domain (Fig. 1B), which is then self-cleaved at the NS2B-NS3 juction site and dilution-insensitive cleavage of NS2A-NS2B site (Chambers et al., 1990).

In vitro, the active protease complex can be formed from NS2B-NS3Pro precursor with the addition of microsomal membrane in an *in vtro* coupled transcription/translation system resulting in the cotranslational insertion of NS2B-NS3 precursor into membranes, necessary for the self-cleavage of NS2B-NS3 site. When the central hydrophilic amino acids of NS2B (NS2BH) alone was used as a cofactor for the NS3Pro (Fig. 1B, bottom), addition of membranes was not required for the *cis* cleavage of NS2BH-NS3Pro site (Clum et al., 1997). Using this information, the NS2BH-NS3Pro precursor containing 49aa of hydrophilic region with the C-terminal 10aa) of NS2B (Fig. 2; shown in red) and the NS3Pro domain, was expressed in *E. coli* and the recombinant protein was purified by denaturation and refolding. Within the C-terminal 10aa region of NS2B, the hydrophobicity of underlined aa, WYLW, were likely to have contributed to aggregation and localization as insoluble inclusion bodies in *E. coli* which required denaturation and refolding to enzymatically active protease complex. The *in vitro* protease assay was established using fluorogenic peptide substrates as well as [^{35}S-Met]-labeled authentic target precursor protein, NS4B$_{FL}$-NS5(95aa) (Yusof et al., 2000). In subsequent studies, the C-terminal region of NS2BH was shortened to 2aa in DENV2(QR) and ZIKV (KR), and 5aa in WNV (QYTKR) proteases (Abrams et al., 2020; Balasubramanian et al., 2016; Ezgimen et al., 2012; Lai et al., 2013; Mueller et al., 2008; Mueller et al., 2007; Tiew et al., 2012), respectively, for expression, purification, biochemical characterization, and high-throughput screening (HTS) of compound libraries. The recombinant proteins had optimal solubility, yield, and enzymic properties. In another approach, Leung et al described the expression and purification of DENV protease in which NS2BH and NS3Pro were covalently linked by a non-cleavable Gly-rich linker as a single precursor protein, NS2BH-(Gly$_4$-S-Gly$_4$)-NS3Pro (referred here as a “linked protease”) (Leung et al., 2001). The linked protease was highly soluble and enzymatically active in cleaving chromogenic *para*-nitroanilide (pNA) hexapeptide substrates containing a Lys or Arg at P1 and P2 positions as well as a decapeptide substrate containing four P’ residues which was cleaved between Arg-Ala at the P1 and P1’ positions (Leung et al., 2001). The NS2BH-(Gly$_4$-S-Gly$_4$)-NS3Pro was used in several laboratories for biochemical characterization such as substrate specificity, kinetic parameters of DENV, WNV, and ZIKV as well as for HTS for identification of inhibitors (Abrams et al., 2020; Bera et al., 2007; Chappell et al., 2005; Li et al., 2005; Nall et al., 2004; Niyomrattanakit et al., 2004).

4. Structure Determinations of “linked” and “unlinked” proteases

The crystal structures of NS2BH-G$_4$SG$_4$-NS3pro were determined in several laboratories (Aleshin et al., 2007; Assenberg et al., 2009; Chandramouli et al., 2010; Chen et al., 2014; Erbel et al., 2006; Noble et al., 2012). The DENV2 NS2BH-(G$_4$-S-G$_4$)-NS3Pro structure was determined in the absence of an inhibitor, whereas the WNV structure contained the substrate peptide-based, Bz-Nle-Lys-Arg-Arg-aldehyde which was covalently linked to

catalytic triad Ser135 inhibitor. Comparison of these structures revealed that the NS3Pro domains adopt a chymotrypsin-like fold in both structures with two β-barrels, each formed by six β-strands, with the catalytic triad (His51-Asp75-Ser135) located at the cleft between the two β-barrels (Fig. 1C). The striking difference was observed in the DENV2 structure in the absence of the inhibitor in which the C-terminal aa of NS2BH beyond the residue 76 was disordered and assumed an “open” conformation. On the other hand, in the presence of the inhibitor, the C-terminal region of NS2BH wrapped around the inhibitor to form a closed conformation. Aleshin et al. determined the crystal structure of WNV NS2BH-Gly4-S-Gly4-NS3Pro in the presence and absence of the bovine pancreatic trypsin inhibitor, aprotinin (BPTI) confirming that NS2BH could adopt two distinct conformations (Aleshin et al., 2007). Chandramouli et. al reported the crystal structure of DENV1 NS2BH (aa 49–95 hydrophilic domain) fused to Gly_4-S-Gly_4-NS3Pro domain which also had the N-terminal deletion of NS3ProΔ11–20 aa (Chandramouli et al., 2010). Assenberg et al. reported the crystal structure of full-length NS3 protein linked to the NS2BH-Gly4-S-Gly4 hydrophilic domain of MVEV (Assenberg et al., 2009). From the biochemical characterization of the protease and the helicase, the authors concluded that the protease domain had little influence on the helicase activity and vice versa. This conclusion from MVEV was not applicable to DENV2, DENV4 and WNV (Chernov et al., 2008; Xu et al., 2005; Yon et al., 2005). The protease domain did seem to influence the helicase activity depending on the native inter-domain region between the protease and the helicase domains. The relative orientations of the protease and the helicase of DENV4 NS3 were also determined by crystallography (Luo et al. 2010; Phoo et al. 2020). For the structure determined by Luo et al. (Luo et al., 2010), only the C-terminal 18 aa of NS2B was linked to the full-length DENV4 NS3 and hence it is inactive as protease. However, the structure of the full-length NS3 protein showed that there were two conformations (I and II) differing with respect to the flexible linker region (169–179aa) between the protease and helicase domains. In conformation II, the helicase domain is more exposed and is active in binding RNA and ATP hydrolysis steps involved in RNA helicase activity (Luo et al., 2010). In the study reported by Phoo et al. (Phoo et al., 2020), the authors compared for the first time the structural features of DENV4 NS2BH and NS3 full-length complexes formed in three different ways: (1) bNS2B47-NS3 (2) cleavable eNS2B47-NS3, and (3) gNS2B47-NS3 containing Gly linker. The authors reported the crystal structures of linked and unlinked NS3 proteases in their free states and in complex with BPTI. Their results showed that NS2BH essentially adopted a closed conformation in the absence of Gly linker which promoted the shift toward an open conformation interfering with the protease activity with little effect on ATPase and helicase activities. In parallel studies, our group used the cleavable NS2BH-NS3Pro domains of DENV and WNV for biochemical and HTS studies in the active ‘closed’ conformation (Balasubramanian et al., 2016; Clum et al., 1997; Mueller et al., 2008; Mueller et al., 2007; Yon et al., 2005; Yusof et al., 2000).

Structure studies performed by NMR in solution have revealed a more complete picture of the dynamics of NS2B-NS3Pro in post-proteolysis state of the enzyme during polyprotein processing. For example, the structure of the last four amino acids of NS2BH cofactor bound at the NS3 active site agrees with the NMR studies and protease activity assays (Phoo et al., 2016). The structure determinations by NMR in solution also revealed that the

NS2BH-NS3Pro predominantly exist in closed conformation which offer valuable insight into the conformational changes of the proteases in the absence and presence of substrates and inhibitors which could be useful for the development of potent inhibitors of flavivirus infections (Li and Kang, 2020). NMR spectroscopy has been used to study complexes formed between low molecular weight inhibitors and the WNV NS2B-NS3Pro. These studies revealed that inhibitor binding near the substrate binding site rather than an allosteric site(s) (Su et al., 2009).

5. Identification of direct-acting antivirals against flavivirus proteases.

Direct-acting antivirals would offer specificity, safety and enhanced efficacy for treatment of flavivirus infections compared to non-specific therapeutics such as interferon and ribavirin-based treatments to inhibit flavivirus replication and infection. Viral proteases are an excellent target for anti-viral therapeutics due to their essential function in production of viral proteins (i.e., polyprotein cleavage) and assembly of viral replication complex. Several viral protease inhibitors have been developed into successful therapeutics to treat viral infections. FDA-approved viral inhibitors include for HIV (Lv et. al., 2015) and hepatitis C virus protease inhibitors (telaprevir) (Bukhtiyarova et al., 2001). The flavivirus NS2B-NS3 protease has been a major target for developing inhibitors against flavivirus infections. Peptide-based compounds often exhibit nanomolar affinities for flavivirus proteases but suffer from limited antiviral activities in cellular assays and animal models, which prevents further development into antiviral therapeutics (Abrams et al., 2020; Balasubramanian et al., 2016; Jiang et al., 2022; Lima et al., 2021; Mirza et al., 2022; Nitsche, 2018). Alternatively, HTS of small molecules using in vitro protease activity assays and in silico binding screens have been used to identify inhibitors. We and others have used HTS of large compound libraries and virtual screening, targeting DENV2, WNV, and ZIKV proteases, and identified several compounds with novel chemical scaffolds that not only inhibit the viral protease in vitro but also viral replication in cultured mammalian cells (Mueller et al., 2007). Several of these compounds show a broad-spectrum inhibition for DENV1–4 and WNV NS3 protease (Balasubramanian et al., 2016; Li et al., 2017). Since the activity of NS2B-NS3 protease requires formation of an active protein complex between NS2B and NS3 in the viral replication complex, small molecule inhibitors that target flavivirus NS2B and NS3 interaction is a strategy to treat viral infections.

WNV NS3 protease HTS.

For identification WNV NS3 protease inhibitors, we screened ~32K compounds using WNV NS2BH-NS3Pro in our first HTS (Ezgimen et al., 2012; Mueller et al., 2008). We identified compounds containing the 8-hydroxyquinoline (8-OHQ) scaffold which inhibited both WNV and DENV2 proteases *in vitro* and Renilla luciferase (Rluc) reporter replicon assays (Mueller et al., 2008) (Fig. 3). The 8-OHQ containing compounds were validated by structure-activity relationships (SAR), and their IC_{50} values for the 8-OHQ derivatives for the purified DENV2 and WNV protease are shown in Fig. 3. Compound 26 which lacks N1 nitrogen was inactive, confirming that the 8-OHQ ring is required for the inhibitory activity.

DENV2 NS3 protease HTS.

In the second HTS campaign, we screened ~120K compounds in the 384-well HTS format using DENV2 NS2BH-NS3Pro (Balasubramanian et al., 2016). After secondary validation in the 96-well format, we selected 8 'hit' compounds (A-H), 7 of which have the general structure of catechols (1,2-dihydroxybenzne), and analyzed their activity to inhibit the NS3 protease from four DENV serotypes and WNV (Fig 4A–B). The IC_{50} values range from 120 nM to 10 μM; Five compounds (C, E, F, G, H) had IC_{50} < 5 μM for all DENV1–4 and WNV protease. We then determined their kinetic constants (Ki) using DENV2 protease, and EC_{50} by plaque assay and *Renilla* luciferase (Rluc) reporter replicon assay (Fig. 4C). Compounds C, D, F, G, and H efficiently suppressed virus and replicon replication in cell-based assays (Fig. 4C) (Balasubramanian et al., 2016). All compounds had the cytotoxic concentration (CC_{50}) > 29 μM, and therapeutic index (TI = CC_{50}/EC_{50}) > 7 in DENV2 infection. Although G (tannic acid) and H (Suramin) were identified as protease inhibitors in the HTS and inhibit DENV1–4 and WNV proteases in vitro, they are considered as promiscuous inhibitors and reported to inhibit a variety of viruses (Balasubramanian et al., 2016) and illnesses affecting human health. For example, Suramin is also described as an anti-cancer agent (Marutsuka et al., 1995; Vogelzang et al., 2004), as an anti-HIV reverse transcriptase agent (Mahoney et al., 1990), and that blocked the entry of DENV to target cells; (Chen et al., 1997).

DENV NS3 protease virtual screen.

Identification of small molecules that inhibit all four DENV proteases would be desirable for development of pan flavivirus protease inhibitors. Although DENV1–4 NS3 proteases share high sequence identity (67–72 %), the residues near the active site are not identical (Fig 5). Previous DENV2 protease structures were determined using proteins with linked (uncleavable glycine-rich G_4-S-G_4 linker) NS2B-NS3 protease and did not show electron density for the entire NS2B peptide (open conformation), likely represent inactive conformation. In contrast, the cleaved form of protease (using the construct similar to our group) is in a closed conformation, where entire NS2BH was visible.

If the crystal structures of the targets are known, then virtual screens can be employed to identify new compounds that bind to the protein targets by using computational methods. For example, in one study, the search was carried out by using Swiss Similarity tool and molecular docking calculations, molecular dynamics simulations (MD) and free energy calculations with the compounds in the ZINC databases that led to the identification of the compounds with favorable druglike properties (Costa et al., 2022). Rarey et al. described a fast, flexible, and automatic method for docking organic ligands into protein binding sites (Rarey et al., 1996). A new software module, FlexX-Scan, is described which can perform a high throughput, structure-based virtual screening (Schellhammer and Rarey, 2004), which is based on the incremental construction docking tool, FlexX, described by Rarey et al. (Rarey et al., 1996).

Inhibitors targeting ZIKV protease

ZIKV infects and disrupts development in human neural stem cell (NSC) and fetal infection can result in congenital defects and microcephaly. Small molecule inhibitors show different

activities in Vero vs. NSC, and thus, compounds need to be screened for their ability to inhibit ZIKV in human NSCs. We have used a combination of HTS and virtual screen to identify ZIKV protease inhibitors (Fig. 6). Selected compounds were tested using *in vitro* protease assay, Vero cell assay, and NSC assay, and a total of 15 compounds were identified with diverse chemical scaffolds. Thus far, we have characterized three groups of compounds, tetracycline, MK-591, and JNJ404 for their ability to inhibit viral protease and viral infection. All three groups of compounds show activity in Vero cells and NSC.

To identify small molecule inhibitors against ZIKV protease, we used three strategies (Fig 6). *First,* a pilot HTS was performed using the ZIKV (Brazilian strain) NS2BH-NS3Pro as the target at the National Center for Advancing Translational Sciences (NCATS).(Abrams et al., 2020) In the study, ~2,000 compounds were screened using an AMC-labeled hexapeptide, Ac-VKTGKR-AMC (Ac: acetyl). Three classes of compounds were identified, flavonoids, anthraquinones and tetracyclines. Due to their commercial availability and ability to cross the blood-brain barrier, 11 tetracycline family of drugs were chosen for further study using human NSC assay. Members of the tetracycline family were not effective in inhibiting protease activity *in vitro*, but four compounds (Fig 6A) suppressed ZIKV replication in NSC with EC_{50} of 7.3 – 13.1 μM, suggesting that they may have multiple mechanisms of action. Methacycline was most potent (EC_{50} = 7.3 μM) and reduced the ZIKV in the brain and the ZIKV-induced motor deficits in an immunocompetent mouse model (Abrams et al., 2020) Since the tetracyclines are FDA-approved drugs, it could be quickly brought to clinical practice. *Second*, in a concurrent quantitative HTS (qHTS) assay in 1054-well plates, a total of 10,807 compounds were screened using *in vitro* protease assays, from which 272 compounds were selected. Additionally, 83 of known protease inhibitors were tested in the protease assay, and 35 were active. Finally, we used an AI-based QSAR model for virtual screening of 137,083 compounds, and selected 277 predicted inhibitors for protease assay, of which 153 compounds showed inhibition. Thus, a total of 460 hit compounds were identified from the ZIKV protease assay. The hit compounds were next used in consecutive Rluc replicon assay, mCherry infectivity assays, and qRT-PCR in Vero cells. This step eliminated ~95% of the selected compounds, leaving 18 compounds for further analysis in a more physiologically relevant human NSC. *Third*, the 460 compounds were directly taken to NSC assays. We found that 11 compounds were active in NSC assays. The most active compounds were MK-591 and JNJ-40418677, which have similar IC_{50} and EC_{50} values measured in protease activity and NSC-based assays, respectively (3.0 and 3.1 μM for MK-591 and 3.9 and 3.2 μM for JNJ-40418677), suggesting that the compounds inhibit viral replication by blocking viral protease activity (Fig 6B–C) (Abrams et al., 2020). MK-591 was previously identified as a five-lipoxygenase–activating protein (FLAP) inhibitor that would potentially treat a wide range of inflammatory diseases such as asthma (Gurusamy and Abdul, 2019). With its good safety profile, MK-591 is a promising lead compound to treat a ZIKV infection (Abrams et al., 2020)

Within the identified 15 hit compounds from HTS (Fig 6), except four tetracyclines, the 11 compounds show good correlation between the ZIKV protease inhibition (IC_{50} of 1.0–9.3 μM) and viral replication in NSC (EC_{50} of 1.2 –10.8 μM), suggesting that they are direct-acting inhibitors of viral replication via the inhibition of viral protease. A new software

module, FlexX-Scan has been described in literature to facilitate the structure-based high throughput virtual screening process.

6. Survey of literature between 2018 and 2022 on flavivirus protease functions

Martinez et al. performed a WNV NS2B-NS3Pro enzymatic screen of NIH clinical compound library and identified Zafirlukast, an FDA-approved drug for asthma, and its derivatives, as an inhibitor of WNV NS2B-NS3Pro with an IC_{50} value of 32 μM. A limited SAR study revealed that replacing the cyclopentenyl with a phenyl moiety improved the inhibition. The mode of inhibition was studied by experimental and computational methods to be by allosteric mechanism by blocking the binding of the NS2B cofactor to NS3pro (Martinez et al., 2018). Yao et al. reported by compound screening and medicinal chemistry, several drug-like broad spectrum inhibitors of flavivirus proteases with IC_{50} values of ~120 nM, and exhibited antiviral activities in cultured cells with EC_{68} of ~300–600 nM and in a mouse model for Zika virus. The X-ray structural studies revealed that the inhibitors bind to mostly to an allosteric site, a hydrophobic pocket of DENV NS3 protease and keep it in an open, catalytically inactive conformation (Yao et al., 2019). The placement of the inhibitor in the allosteric pocket is perhaps debatable as discussed by Behnam and Klein (Behnam and Klein, 2020). Bharadwaj et al using virtual screening followed by in vitro assay reported that triterpenoids from the medicinal fungus, Ganodermalucidum, are inhibitors of DENV NS2B-NS3pro. Their analysis was followed by binding affinities and stability calculations using the molecular mechanics / Born surface area method and MD simulations, respectively. Inhibition of viral infection in vitro suggested that Ganodermanontriol is a potent bioactive triterpenoid (Bharadwaj et al., 2019). Kuhl et al. (Kuhl et al., 2021) reported a new class of DENV and WNV inhibitors using chemical synthesis, in vitro SAR studies, DENV2 protease, *Renilla remiformis luciferase* reporter assays in Hela cells containing $NS2B_{FL}$-NS3pro.

The DENV protease inhibitor development efforts between 2015–2020 have been reviewed (Murtuja et al., 2021). Kaptein et al. (Kaptein et al., 2021) reported the identification and mode of action of a compound, JNJ-A07, de novo formation of NS3 and NS4B complex which is required for viral replication. The viral protease is indirectly involved in the formation of mature NS3 and NS4B components of the replication complex. The authors show that once the mature NS3/NS4B complex is formed, JNJ-A07 had no effect. JNJ-A07 is a highly potent inhibitor with nanomolar to picomolar potency and high selectivity in various cell lines including the immature dendritic cells (Kaptein et al., 2021). Analogs of JNJ-A07 are being investigated for their antiviral effects. Kuhl et al. reported the discovery of non-basic benzamide derivatives as inhibitors of DENV2pro having sub micromolar efficacy (EC50 = 0.24 μM in plaque reduction assay with a cytotoxicity (CC50) of > 100μM. The compound was stable against liver microsomes and pancreatic enzymes (Kuhl et al., 2021). In a subsequent study, the same group presented synthesis and analysis of benzoxaborole inhibitors of DENV NS2B-NS3pro. The most active compound had an EC50 of 0.54 μM in inhibition of viral replication with no relevant cytotoxicity. The active compound also inhibited SARS-CoV-2 Mpro with a single digit micromolar EC50 (Kuhl et

al., 2022). In one study, targeting the NS2B-NS3pro of TBEV, which causes neurological complications like WNV, Akaberi et al., reported peptide inhibitors of the viral protease. The IC50 values of 0.92 μM and 0.25 μM for two tripeptides in the in vitro enzymatic assays. No cell culture experiments like viral infection and cytotoxicity assays were performed (Akaberi et al., 2021). Li et. al. identified niclosamide as an inhibitor of ZIKV NS3pro interaction with NS2B by targeting the interface between the protease subunits. The drug discovery and development efforts for ZIKV and WNV NS2B-NS3pro between 2015–2021 has been recently reviewed (Samrat et al., 2022). Cheng et al. described the discovery of potent DENV NS2B-NS3pro covalent inhibitors containing phenoxymethylphenyl residue (Cheng et al., 2022). They previously reported a covalent inhibitor WSL-01 (IC_{50}=129 nM) and with SAR studies, they improved the potency in two analogs WSL-75 with IC_{50} of 24.8 nM and WSL-84, IC_{50} of 32.89 nM. This class of compounds exhibited passive membrane permeability using the precoated tri-layer parallel artificial membrane. The authors presented detailed SAR analysis and MD simulations that reveal binding modes of covalent inhibitors (Cheng et al., 2022). The cytotoxicity of the compounds was measured at 5 μM concentrations although no CC50 values were reported. The same group also identified Montelukast as a competitive inhibitor of DENV and ZIKV replicon replication with EC_{50} values of 1.03 nM for DENV and 1.14 nM for ZIKV by using an in silico approach (Jiang et al., 2022) involving MD simulations and binding free energy calculations followed by in vitro protease assays and replicon replication assays. Since the potency of this compound is weak in the in vitro protease assays compared to replicon replication assays in DENV and ZIKV-infected cells, Montelukast may not act as a direct-acting antiviral compound as an inhibitors of DENV and ZIKV proteases (Jiang et al., 2022). In another study, using the crystal structure of ZIKV NS2B-NS3pro, a computer-aided structure-based approach was used for screening diverse library of compounds. The top hits were selected based on free energy calculations followed by per-residue decomposition analysis. The selected hits were evaluated for their biological potency using ZIKV protease inhibition assays as well as antiviral activity assays (Mirza et al., 2022). Of the 26 selected compounds, 8 showed inhibition of ZIKV protease >25% at 10μM. Of these selected compounds, only one showed anti-ZIKV activity in cells. The CC50 values were reportedly >100 μM and the EC50 value of 9.79 ± 1.20 μM. Colarusso et al., (2022) described using a SAR study on a series of substrate-like linear tripeptides as non-covalent inhibitors of ZIKV NS2B-NS3Pro. They optimized residues at P1, P2, P3, and N- and C-terminal portions of tripeptides. The reported identification of inhibitors with sub-micromolar potency. Their results indicated phenyl glycine as Arg-mimicking group and benzamide as the C-terminal fragment. Extending the SAR studies, the authors found a series of peptides having a 4-substituted phenylglycine residue at the P1 position gave rise to potent tripeptides showing low nanomolar inhibition of ZIKV protease (IC50=30 nM) with high selectivity against trypsin-like serine proteases with no inhibition of thrombin and low inhibition of trypsin (IC50=203 μM) as well as other flaviviruses such as DENV2 and WNV proteases. In spite of the highly potent in vitro protease activity, the two tri-peptides showed weak activities in inhibiting ZIKV replication in Vero cells with EC50 values of 8.5 μM and 11.2 μM which the authors attributed to low permeability contributed by basic side chains of two Lys residues. In vitro assays against DENV2 and WNV proteases showed good selectivity of the

most potent tripeptides as the IC50 values were 0.2 μM and 0.8 μM against DENV2 and WNV proteases.

7. Concluding remarks and future directions.

The race is on to discover potent pan flavivirus protease inhibitors to be ready before a future pandemic strikes the world population. We are seeing a dramatic increase in the number of natural calamities brought by global warming in the US as well as globally. The number of cases and the severity of the disease are likely to increase in future pandemics. It is also heartening to know the research community realizes the dire need in bringing antiviral therapies with the collaboration with big pharmas as seen from the increase in the number of research articles and reviews. We apologize for any omission of other important citations in this review. Research to date focused on identification and development of flavivirus NS3 protease inhibitors using HTS and in silico assays mostly using the isolated NS2BH and NS3 protease domains; thus, other functions of full-length NS2B and NS3 have not been explored as a drug target. In virus-infected cells, the NS3 protein is not cleaved into protease and helicase domains, and thus the full-length NS2B-NS3 is the more authentic target than the NS2BH and NS3 protease domains.

Acknowledgments:

The research in the authors laboratories had been supported by NIH grants, R01 AI32078 (R.P.), U01 AI54776 (Craig E. Cameron and R.P.), R01 AI087856 (K.H.C. and R.P.), R01 AI05985 (K.H.C.), R21 AI154088 (T.T.) and U19 AI171413 (K.H.C).

Abbreviations:

aa	amino acid
DENV	Dengue Virus
DENV1, −2, −3, −4	DENV serotypes 1–4
HTS	high-throughput screening
MVEV	Murray Valley Encephalitis Virus
NS protein	Non-structural protein
NS2BH	NS2B hydrophilic region
NS3Pro	NS3 protease domain
SAR	structure-activity relationships
TBEV	Tick-borne Encephalitis Virus
WNV	West Nile Virus
YFV	Yellow Fever Virus

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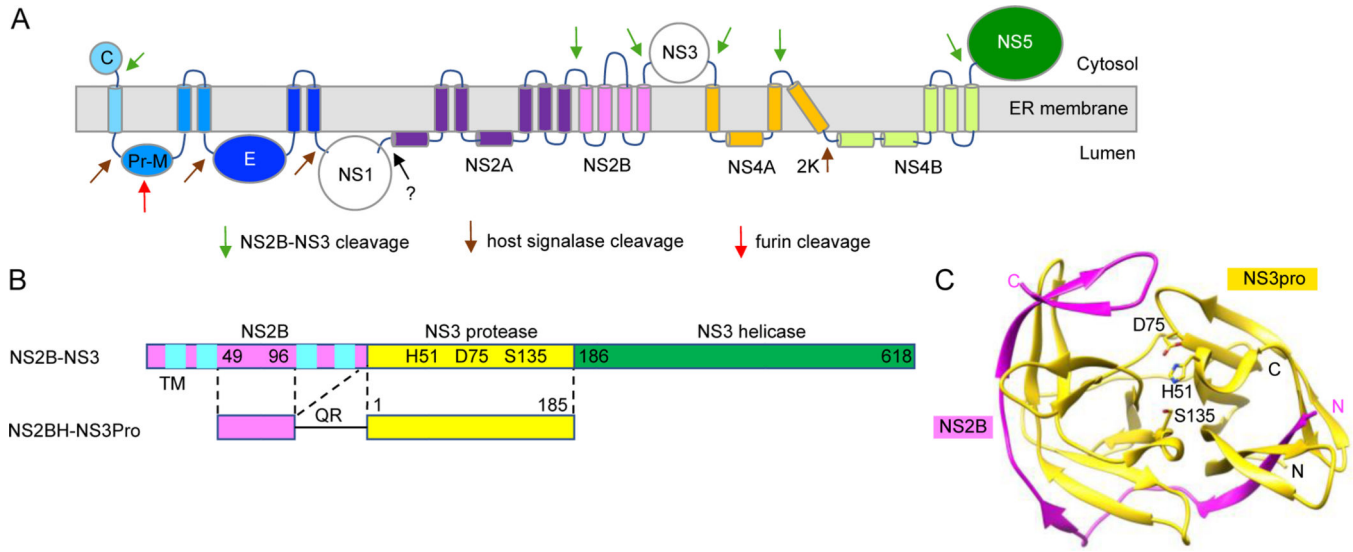

Fig. 1. Flavivirus NS2B-NS3 protease.

A. Flavivirus polyprotein topology and predicted transmembrane domains. Viral protease NS3 cleaves polyprotein from the cytoplasmic side. **B.** Arrangement of flavivirus DENV2 NS2B-NS3 protease. NS3 protease uses NS2B, a transmembrane (TM) protein with four helices (cyan), as a cofactor. The conserved hydrophilic region of NS2B (NS2BH; 49–96 aa), linked to NS3Pro (yellow), is required for protease activity. The NS2BH-NS3Pro construct used in the HTS is described below. **C.** Crystal structure of ZIKV protease (PDB code: 5GPI; (Zhang et al., 2016). The protease consists of NS2B peptide (49 aa, pink) and NS3 protease domain (yellow). The catalytic triad, H51, D75 and S135 are shown.

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DENV1 SWPLNEGIMA VGIVSILLSS LLKNDVPLAG PLIAGGMLIA CYVISGSSAD LSLEKAAEVS WEEEAEHSGA
DENV2 SWPLNEAIMA VGMVSILASS LLKNDIPMTG PLVAGGLLTV CYVLTGRSAD LELERAADVK WEDQAEISGS
DENV3 SWPLNEGVMA VGLVSILASS LLRNDVPMAG PLVAGGLLIA CYVITGTSAD LTVEKAADVT WEEEAEQTGV
DENV4 SWPLNEGIMA VGLVSLLGSA LLKNDVPLAG PMVAGGLLLA AYVMSGSSAD LSLEKAANVQ WDEMADITGS

DENV1 SHNILVEVQD DGTMKIKDEE RDDTLTILLK ATLLAVSGVY PLSIPATLFV WYFWQKKKQR SGVLWDTPSP
DENV2 SPILSITISE DGSMSIKNEE EEQTLTILIR TGLLVISGLF PVSIPITAAA WYLWEVKKQR AGVLWDVPSP
DENV3 SHNLMITVDD DGTMRIKDDE TENILTVLLK TALLIVSGIF PYSIPATMLV WHTWQKQTQR SGVLWDVPSP
DENV4 SPIIEVKQDE DGSFSIRDVE ETNMITLLVK LALITVSGLY PLAIPVTMTL WYMWQVKTQR SGALWDVPSP


DENV1 PEVERAVLDD GIYRIMQRGL LGRSQVGVGV FQENVFHTMW HVTRGAVLMY QGKRLEPSWA SVKKDLISYG
DENV2 PPVGKAELED GAYRIKQKGI LGYSQIGAGV YKEGTFHTMW HVTRGAVLMH KGKRIEPSWA DVKKDLISYG
DENV3 PETQKAELEE GVYRIKQQGI FGKTQVGVGV QKEGVFHTMW HVTRGAVLTH NGKRLEPNWA SVKKDLISYG
DENV4 AATKKAALSE GVYRIMQRGL FGKTQVGVGI HMEGVFHTMW HVTRGSVICH ETGRLEPSWA DVRNDMISYG

DENV1 GGWRLQGSWN TGEEVQVIAV EPGKNPKNVQ TAPGTFKTPE GEVGAIALDF KPGTSGSPIV NREGKIVGLY
DENV2 GGWKLEGEWK EGEEVQVLAL EPGKNPRAVQ TKPGLFKTNA GTIGAVSLDF SPGTSGSPII DKKGKVVGLY
DENV3 GGWRLSAQWQ KGEEVQVIAV EPGKNPKNFQ TMPGIFQTTT GEIGAIALDF KPGTSGSPII NREGKVVGLY
DENV4 GGWRLGDKWD KEEDVQVLAI EPGKNPKHVQ TKPGLFKTLT GEIGAVTLDF KPGTSGSPII NRKGKVIGLY

DENV1 GNGVVTTSGT YVSAIAQAKA SQEGPLPEIE
DENV2 GNGVVTRSGA YVSAIAQTEK SIEDNPEIED
DENV3 GNGVVTKNGG YVSGIAQTNA EPDGPTPELE
DENV4 GNGVVTKSGD YVSAITQAER
```

Fig. 2. Alignment of NS2B-NS3pro of DENV1, −2, −3, and −4.

The NS2B is 130aa and NS3Pro domain shown is 180aa after QR (the N-terminal 10 residues of NS3Pro are underlined). The sequences in red represent conserved hydrophilic domain essential for protease activity. The amino acid residues in bold of NS2B upstream of the C-terminus of NS2B are hydrophobic and contribute to insolubility in E, coli expression of DENV2 NS2BH (Yusof et al., 2000).

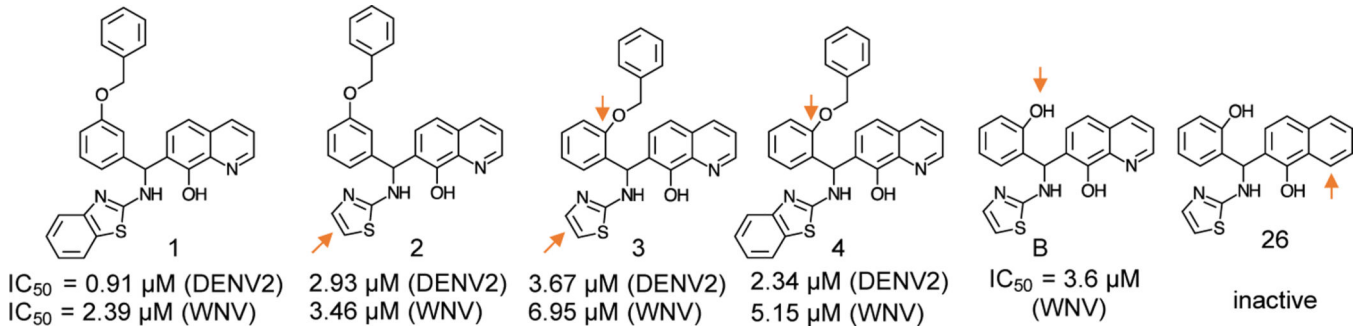
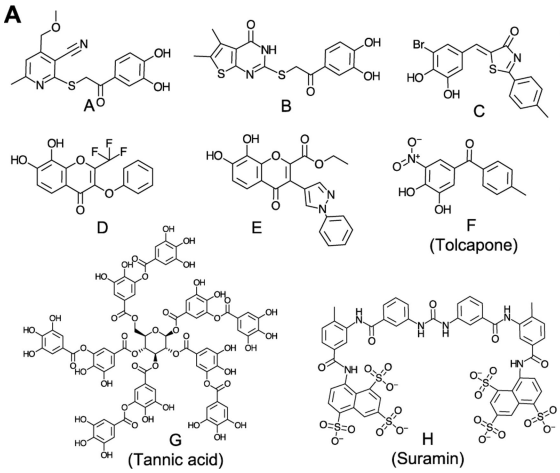


Fig 3. WNV protease inhibitors with 8-OHQ scaffold.

IC_{50} was determined by protease assay using DENV2 and WNV NS2BH-NS3Pro. The positions of substitutions in comparison to compound 1 are indicated by an arrow.



B

IC_{50} values against DENV1-4 and WNV protease*

protease	A	B	C	D	E	F	G	H
DENV1	9.65 ±0.32	11.3 ±0.33	4.06 ±0.21	10.8 ±0.37	6.20 ±1.20	1.15 ±0.1	0.77 ±0.05	2.76 ±0.25
DENV2	8.22 ±0.24	10.0 ±0.28	4.05 ±0.18	10.5 ±0.40	4.84 ±0.60	0.98 ±0.06	0.56 ±0.04	2.66 ±0.19
DENV3	9.95 ±0.45	9.66 ±0.63	2.94 ±0.18	11.1 ±0.38	5.0 ±1.40	0.91 ±0.06	0.40 ±0.03	3.25 ±0.18
DENV4	2.22 ±0.21	12.1 ±0.47	3.40 ±0.11	11.0 ±0.37	5.32 ±0.60	0.64 ±0.03	0.13 ±0.01	3.73 ±0.14
WNV	6.43 ±0.38	10.1 ±0.33	2.53 ±0.10	6.56 ±0.24	4.31 ±1.20	0.70 ±0.04	0.12 ±0.01	3.24 ±0.11

* IC_{50} values are averages of duplicate experiments and reported with the standard errors of the mean

C

compound	Pro inhibition Ki (μM)	DENV2 EC_{50} (μM) Plaque	DENV2 EC_{50} (μM) Replicon	CC_{50} (μM)	Therapeutic index plaque	Therapeutic index replicon
A	6.9 ±0.80	> 20	> 20	>100.0	-	-
B	2.7 ±0.33	> 20	> 20	62.4	-	-
C	1.0 ±0.15	8.97 ±0.05	6.97 ±0.6	76.1	8.5	11
D	3.6 ±0.39	14.2 ±0.06	> 20	> 100.0	> 7.0	-
E	1.6 ±0.16	> 20	10.4 ±4.2	80.0	-	7.7
F	0.22 ±0.03	2.03 ±0.1	2.29 ±0.3	29.2	14.4	12.7
G	0.34 ±0.05	0.084 ±0.01	n.d	68.1	810	n.d
H	0.87 ±0.14	8.62 ±1.32	n.d	> 100.0	> 11.6	n.d

* n.d., not determined

Fig 4. Hit compounds identified by DENV2 NS3 protease HTS.

A. Chemical structures of hit compounds A-H. **B.** IC_{50} values of the compounds A-H against DENV1,2,3,4 and WNV NS3 protease. **C.** Ki, EC_{50}, CC_{50}, and selective index for compound A-H. Ki was measured by *in vitro* protease assay and EC_{50} was measured by plaque and replicon assays.

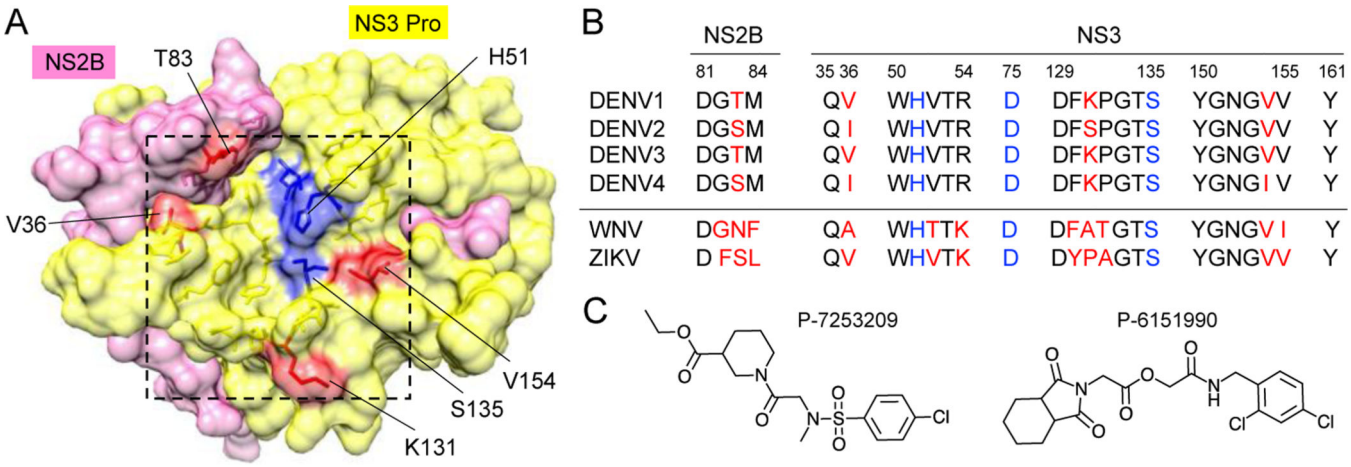


Fig 5. Substrate binding site of flavivirus protease.

A. Substrate binding site of DENV NS2B-NS3 protease (boxed) is shown in the same orientation as Fig 1C. The substrate-binding site residues that differ in DENV1–4 protease (**B**) are colored in red. The active site residues are shown in blue. **C.** DENV2 protease inhibitors identified by virtual screen.

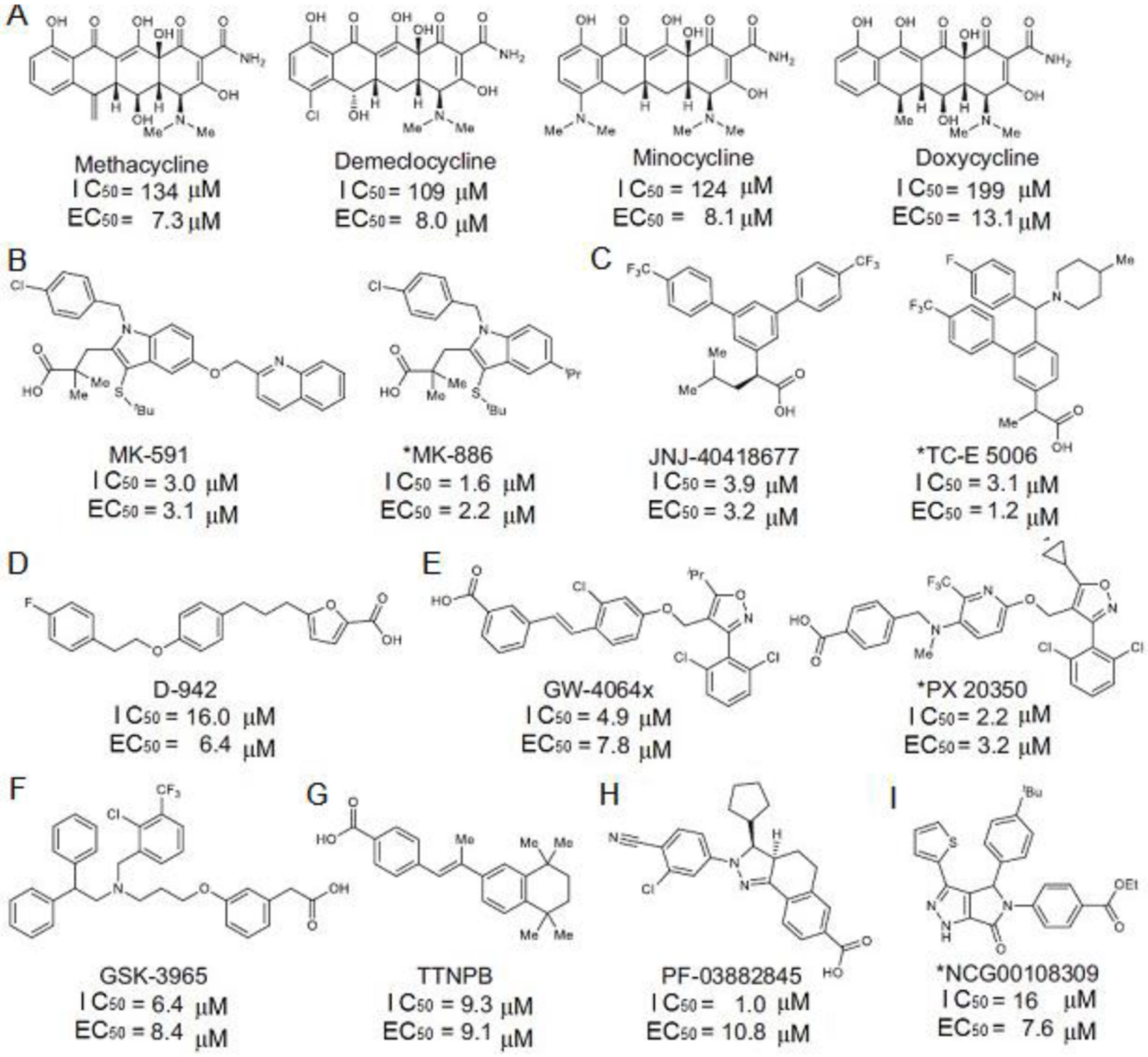


Fig 6. Hits identified from ZIKV protease HTS and viral infection assays in neural stem cells (NSC).

The 15 compounds can be divided into 9 classes of chemicals, A-I. The IC_{50} values were determined using ZIKV protease and EC_{50} determined in ZIKV infection assay in NSC.