

Review

Antiviral agents against flavivirus protease: prospect and future direction

Subodh K Samrat¹, Jimin Xu², Zhong Li¹, Jia Zhou² and Hongmin Li^{1,3}

¹ Department of Pharmacology and Toxicology, College of Pharmacy, the University of Arizona, 1703 E Ma-bel St, Tucson, Arizona 85721-0207, United States of America

² Chemical Biology Program, Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas 77555, United States of America

³ BIO5 Institute, The University of Arizona, Tucson, Tucson, Arizona 85721, United States of America

* Correspondence: hli1@pharmacy.arizona.edu, Tel: 520-621-5728

Abstract: Flaviviruses cause a significant amount of mortality and morbidity, especially in the area where they are endemic. A recent example is the outbreak of Zika virus though out the world. Development of antiviral drugs against different viral targets is as important as development of vaccine. 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. Flavivirus protease is the most attractive target for development of therapeutic antivirals because it is essential for processing of viral polyprotein precursor and generation of functional viral proteins. In this review, we have summarized recent development in drug discovery targeting NS3-NS2B protease of flaviviruses, especially Zika, dengue and West Nile virus.

Keywords: Flavivirus; NS2B-NS3; ZIKA Virus; Dengue Virus; West Nile Virus; Inhibitors;

1. Introduction

Flavivirus genus of the Flaviviridae family includes more than 70 related arthropod-borne viruses [1-4]. The most common and representative members are the dengue virus (DENV) with four closely related but antigenically distinct serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), Zika (ZIKV), West Nile (WNV), Yellow Fever (YFV), Japanese-encephalitis (JEV), tick-borne-encephalitis (TBEV), and Usutu viruses [5-7]. These are the causative agents for viral hemorrhagic fever and encephalitis in human beings.

Flaviviruses are transmitted to susceptible hosts by infected arthropod vectors [8-11]. Many flaviviruses are restricted to a particular region. An occasional spill of viruses is frequently occurring, which is a significant challenge for international health care. For examples, WNV jumped from the Middle East into the Americas [12,13]; and ZIKV spread from Africa to Southeast Asia, the islands of Polynesia, and later to Brazil in 2015-2016, causing an epidemic [3,14,15].

There is no approved antiviral therapeutic or vaccine available to combat most flavivirus infections, except for YFV, DENV, JEV, Kyasanur forest disease virus (KFDV), and TBEV [11]. In the case of DENV, vaccine design and development have been hampered because the incomplete vaccine immunity against all four serotypes may lead to an antibody-dependent enhancement in the setting of subsequent natural infection [11,16]. As a result, the goal is to develop a vaccine that simultaneously elicits a balanced tetravalent neutralizing response against all four DENV serotypes. In 2019, the United States Food and Drug Administration (FDA) approved the live-attenuated, tetravalent vaccine, Dengvaxia (from Sanofi Pasteur), but only for individuals between 9–16 years of age who have laboratory-confirmed prior DENV infection and are living in endemic areas [16,17].

These challenges increase the demand to develop therapeutics and vaccines that targets different flaviviruses.

The development of antiviral therapeutics will enable new approaches for the management of flavivirus outbreaks due to their potential for use as treatment and prophylaxis [18,19]. In this review, we describe strategies and examples of current efforts to identify potential inhibitors against flavivirus NS3 protease activities that can be developed as therapeutic agents to combat flavivirus infections.

2. Flavivirus genome organization

Flavivirus genome consists of a positive-sense single-stranded RNA which is ~11 kb in length, consisting of a 5' untranslated region (UTR), a single long open reading frame (ORF), and a 3' UTR [3,4,20,21]. It is capped like cellular mRNAs, but not polyadenylated. The single ORF encodes a polyprotein precursor (PP) that is further processed with the help of host and viral proteases. The flavivirus protease, which works with host proteases to cleave the viral PP, is a highly conserved enzyme essential for virus replication. The viral PP is processed to three structural proteins (Capsid, pr-Membrane, and Envelope) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [4,21]. Three structural proteins form the virus coat, whereas seven NS proteins are part of the membrane-bound replication complex. Among these NS proteins, only NS3 and NS5 bear enzymatic functions [19,22,23] (Fig 1).

3. Flavivirus protease

NS3 is a protein with multiple functions, including protease, helicase, and triphosphatase activities [24,25]. The NS3 N-terminal domain (amino acids (aa) 1-180) is a trypsin-like serine protease with a classic catalytic triad (Ser135-His51-Asp75) [3,26]. It requires a small hydrophilic proportion of NS2B as a co-factor for activating the protease enzymatic function [27-29]. The NS3 C-terminus domain (aa 180-618) is a superfamily 2ATPase/helicase that binds to the 3' end of transient dsRNA and unwinds it in 3' → 5' direction [30]. In addition, the C-terminal domain of NS3 also has RNA 5'-triphosphatase activity for viral RNA 5'-capping [23,31].

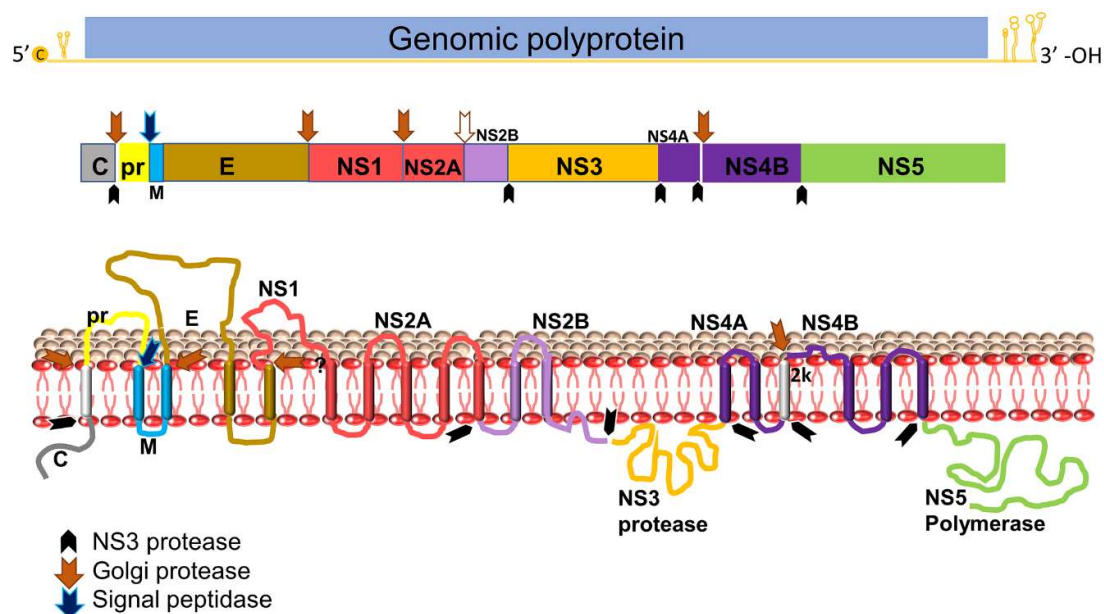


Figure 1. Schematic diagram of flavivirus polyprotein organization, processing, and predicted membrane topology of mature protein. Top, the representative flavivirus genome. c, RNA cap. Middle, linear organization of the structural and nonstructural proteins within the polyprotein. Black

arrows denote cleavage by the viral NS2B-NS3 protease complex, whereas the brown arrows indicate cleavage by the Golgi protease and blue arrow denotes cleavage by signal peptidase. Bottom, putative membrane topology of the polyprotein predicted from biochemical and cellular analyses, which is further processed by host and viral proteases (indicated by arrows).

NS2B is an integral membrane protein of 14 kDa with three domains: two transmembrane segments located at the N and C termini and a central region of 47 amino acids (spanning aa 49–96) that interacts with the NS3 protease as a co-factor (**Fig 1**) [29,32]. It has been reported that flavivirus NS3 protein is neither soluble nor catalytically active as a protease *in vitro*, without providing NS2B either in *cis* [33–35] or in *trans* [36–40]. Proteases among flaviviruses have significant similarity on both structural and sequence levels [41].

The flavivirus NS2B-NS3 protease is one of the most promising validated targets for developing a pan flavivirus treatment which is evidenced by the clinical availability of ten HIV-1 protease inhibitors (PIs) [42–44] and the two HCV Protease inhibitors [45–47]. Thus, it is plausible that a protease inhibitor for flaviviruses will be efficacious in the clinic. However, experience with both HIV-1 and HCV indicates that there are certain drawbacks associated with PIs, which are important considerations in developing a flavivirus protease inhibitor. Because both HIV and HCV cause chronic infections, PI drug-resistant viral strains were rapidly observed [48]. In contrast, most flaviviruses cause acute infections, drug resistance may be less of a concern.

Most attempts to develop flavivirus protease inhibitors have focused on the NS3 active site with limited success possibly due to flat and featureless active site and requirement of charged substrate to bind with P1 and P2 sites. Moreover, many of the active site inhibitors are only effective in biochemical assays but show poor bioavailability *in vivo* [48].

4. Structural insight of NS2B-NS3 protease

Structural study of NS2B-NS3 led to better screening assays to identify protease inhibitors [49,50]. The NS2B and NS3 protein complex adopts two different conformations [15]. In the absence of substrate or active-site inhibitor, the NS2B C-terminal portion adopts an “open” inactive conformation (**Fig 2A**) [15,51]. However, in the presence of inhibitor or substrate binding to the NS3 active site, the C-terminal portion of NS2B “wraps around” the NS3 core, closing the NS3 active site with the so-called active “closed” conformation [52] (**Fig 2B and 2C**). NS2B conformational changes are required for NS3 function; mutations that abrogate NS2B binding greatly reduce the proteolytic activity of the complex [15,53,54].

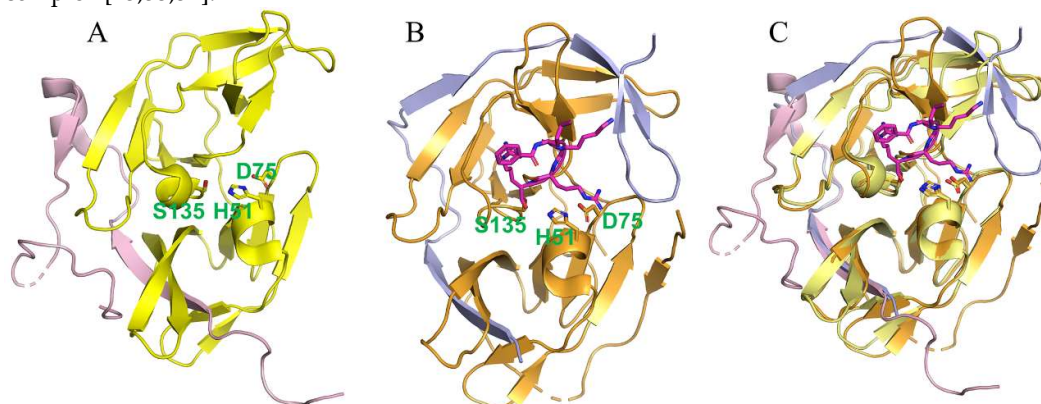


Figure 2. Crystal structures of the dengue NS2B-NS3 protease. **(A)** The open (inactive) conformation (PDB ID of 2FOM) in the unbound state. The NS2B cofactor is colored in purple and NS3 protease in yellow. **(B)** The closed (active) conformation (PDB ID of 3U1I) in complex with a substrate peptide analog (magenta sticks). The NS2B cofactor is colored in blue and NS3 protease in orange. **(C)**

Superimposed structure of inactive and active conformation. The catalytic triad His51-Asp75-Ser135 is displayed in stick representation.

5. NS2B-NS3 protease inhibitors:

The functional significance of NS2B-NS3 protease makes it an important target for drug development against flaviviruses. The structural and functional similarity among the proteases of different flaviviruses further indicates its significance. Several studies have reported the development of inhibitors of NS2B-NS3 protease that target either the active site or allosteric/orthosteric site of the enzyme. These inhibitors may be referred to competitive and non-competitive inhibitors, respectively. In this review, we have reviewed the inhibitors against the NS2B-NS3 protease of ZIKV, DENV, and WNV reported in literatures between 2015 and 2021.

5.1. Competitive inhibitors

The competitive inhibitor drugs are known to interact with NS2B-NS3 protease at the active site and inhibit the enzyme activity. Several drugs have been identified that work as competitive inhibitors against flavivirus NS2B-NS3 protease, as shown in **Fig 3** and listed in **Table 1**.

Table 1. Competitive inhibitors of flavivirus NS2B-NS3 protease.

Number (Name) of compound	Targeted virus	IC ₅₀ (or K _i) (μM)	EC ₅₀ (μM)	In vivo	Reference
1 (Novobiocin)	Zika	14.2 ± 1.1	42.63	Yes	[55]
2 (Asunaprevir)	Zika	6.0	4.7		[56]
3 (Simeprevir)	Zika	2.6	0.4		[56]
4 (carbazole-based amidines)	Zika	0.52	1.25		[57]
5 (MK-591)	Zika	3.0	3.1		[58]
6 (JNJ-40418677)	Zika	3.9	3.2		[58]
7 (4-CF ₃ -benzyl ether)	TBEV	0.92			[59]
	Zika	1.64			
8	Zika	0.25			[60]
	TBEV	0.97			
	DENV	0.05	3.4		
	WNV	0.018	15.5		
9	Zika	0.94			[60]
	TBEV	3.72			
10 (Policresulen)	DENV-2	0.48 μg/mL	4.99 μg/mL		[62]
11	DENV-2	0.95			[61,63]
12	DENV	1.1	2.0		[63]
13 PCRARIYGGCA	DENV-3	K _i = 2.9			[65]
14 (C30H25NO5)	DENV-2	17.46	14.9		[66]
15 (C34H23NO7S2)	DENV-2	9.09	11.8		[66]
16 (Peptide-hybrid inhibitors based on 2,4-thiazolidinedione scaffold)	WNV	0.75			[67]
	DENV	1.05			
17 (α-aminoalkylphosphonates)	DENV-2	K _i = 0.4			[68]
18 (Theaflavin-3,3'-digallate (ZP10))	Zika	7.65	3		[69]

5.1.1. Repurposed inhibitors

Yuan et. al., performed an *in silico* screening of more than 8,000 drugs, followed by biochemical and cellular assay. The FDA-approved drug **1** (Novobiocin) was found as the most promising ZIKV NS2B-NS3 protease inhibitor [55]. Animal studies suggest that Novobiocin treatment reduces viral load in blood and major organs of mice and increases the mouse survival rates [55].

Using structure-based pharmacophore anchor approach, Pathak et al. showed that the FDA-approved drugs, compounds **2** (Asunaprevir) and **3** (Simeprevir) inhibited the ZIKV protease with IC₅₀ values of 6.0 µM and 2.6 µM, respectively [56]. These molecules also showed anti-ZIKV activity with EC₅₀ values of 4.7 µM and 0.4 µM, respectively [56]. However, these molecules need further validation in animal model for its anti ZIKV activity.

5.1.2. Synthetic inhibitors

Initial efforts were made to develop peptides-based protease inhibitor with or without serine trap. However, peptides are in general unstable *in vivo* and mostly non-permeable to cross cell membranes, so focus now has changed to develop small molecule against flavivirus protease [57].

Rassias et al synthesized a series of novel N-substituted carbazole-based amidines as ZIKV protease inhibitors, and compound **4** (carbazole derivative) demonstrated its *in vitro* biochemical and cell-based inhibitory profiles against ZIKV with an IC₅₀ of 0.52 µM and EC₅₀ of 1.25 µM [57].

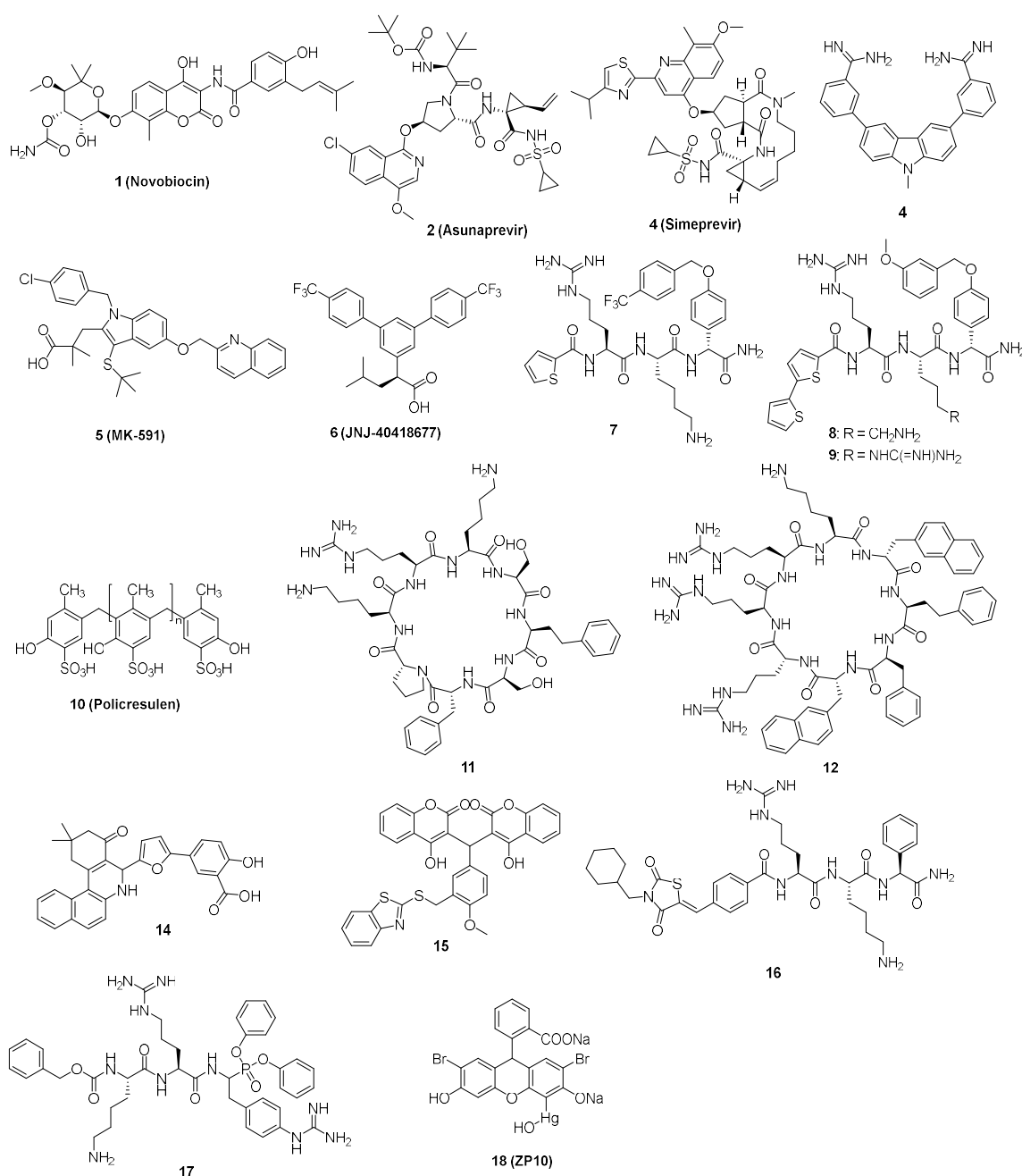


Figure 3. The chemical structures of compounds 1-18 as competitive inhibitors of flavivirus NS2B-NS3 protease.

Using quantitative high throughput screenings (qHTS) for small-molecule protease inhibitors, Abrams et al [58] found that compounds 5 (MK-591) and 6 (JNJ-40418677) showed antiviral effects primarily through inhibition of the Zika viral protease with IC_{50} values of 3.0 μ M and 3.9 μ M, respectively. Moreover, compounds 5 and 6 showed the ability to inhibit ZIKV in human neuronal stem cells using a ZIKV isolate from the 2013 French Polynesian outbreak with EC_{50} values of 3.1 μ M and 3.2 μ M respectively [58].

In another study using different *in silico* methods, compound 7 (4-CF₃-benzyl ether), a pan inhibitor of DENV, WNV and ZIKV proteases, was found as an active inhibitor against the TBEV protease [59]. *In vitro* protease assay confirmed that compound 7

inhibited the TBEV protease with an IC_{50} value of 0.92 μ M. Another two analogues of compound **7** named in our manuscript as compounds **8** and **9** show strong TBEV protease inhibition with IC_{50} values of 0.97 μ M and 3.72 μ M, respectively [60]. The antiviral efficacy of these compounds remains elusive.

Interestingly the compound **8** has also been described as an inhibitor of the NS2B-NS3 proteases of DENV and WNV by Behnam et al [61]. The compound **8** showed IC_{50} values of 50 nM and 18 nM against the DENV and WNV proteases, respectively. The inhibitor also displayed significant reduction of DENV and WNV titers in cell-based assays with EC_{50} of 3.4 μ M and 15.5 μ M respectively [61].

Several synthetic competitive inhibitors have been identified that target the active site of the DENV NS2B-NS3 protease and affect the cleavage of substrate polyprotein. Compound **10** (Policresulen) is one of the compounds predicted to interact with Gln106 and Arg133 of the DENV-2 NS2B-NS3 protease and affects its stability, leading to inhibition of its protease activity and virus replication at an IC_{50} of 0.48 μ g/mL and an EC_{50} of 4.99 μ g/mL, respectively [62].

Another approach for inhibiting the protease activity is to generate synthetic cyclic peptides as competitive inhibitors mimicking the substrate of NS2B-NS3 protease. Takagi et al. synthesized an array of cyclic peptides, showing that proper positioning of arginine and aromatic residues in these cyclic peptides enhanced the DENV-specific antiviral activity at an IC_{50} of 0.95 μ M for the most potent compound **11** [63]. Unfortunately, this compound does not have antiviral activity. Incorporation of L-2-naphthylalanine and adjacent positions of arginine led to discovery of compound **12**, which showed significant anti-DENV activity with EC_{50} of 2.0 μ M, although with slightly reduced protease inhibition of IC_{50} of 1.1 μ M [63].

In a recent study, Lin et al explored cyclic peptides based on aprotinin, a pan-serine protease inhibitor composed of a monomeric globular polypeptide of 58 amino acids [64,65]. Because targeting the P side of the enzyme can affect the human serine protease activity, the binding loop of aprotinin was engineered to identify the optimal hydrophobic amino acids for each of the P' positions. The cyclic peptides (CPs) to target both P and P' sites of the DENV protease active-site pocket were designed to optimize their sequence and length. The best binding cyclic peptide **13** (CP7) has a K_i value of 2.9 μ M against the DENV-3 protease [65]. However, the antiviral potency of these cyclic peptides was not evaluated.

Several non-peptide molecules with inhibitory effect on the DENV NS2B-NS3 protease have been identified and tested against DENV. Two of the tested compounds viz. **14** (C30H25NO5 (CID: 54692801)) and **15** (C34H23NO7S2 (CID: 54715399)) were shown to have significant inhibitory effect on the NS2B-NS3 protease as was observed in the fluorometric enzyme assay using synthetic peptide Boc-Gly-Arg-Arg-AMC as a fluorescent substrate with IC_{50} of 17.46 μ M and 9.09 μ M, respectively [66]. Assessment of anti-DENV activity revealed that these compounds have moderate antiviral activity with EC_{50} values of 14.9 μ M and 11.8 μ M, respectively [66].

WNV is another medically important flavivirus which need NS2B-NS3 protease for its replication. Several inhibitors have been designed till date to block the activity of viral protease. Bastos Lima et al. synthesized a group of novel reversible peptide-hybrid inhibitors based on compound **16** (2,4-thiazolidinedione scaffold) [67], with low micromolar inhibitory activities against the WNV and DENV proteases, although their antiviral efficacy was not investigated.

Recently, synthesis and application of α -aminoalkyl phosphonates and their peptidyl derivatives as the WNV NS2B-NS3 protease inhibitors have been reported [68]. These compounds belong to a class of irreversible inhibitors that specifically and exclusively react with the active site serine residue, leading to formation of a slow hydrolyzing protease-inhibitor complex. The most potent compound **17** was observed to have a K_i value of 0.4 μ M [68]. The antiviral efficacy of this compound remains elusive.

5.1.3. Natural compound inhibitors

Several studies have shown that compounds derived from natural sources viz. plants or microorganisms can exhibit broad range medicinal properties. In a study using fluorescence-based screening assay it was shown that natural active compound **18** (theaflavin-3,3'-digallate, ZP10), derived from black tea, acted as a potent ZIKV protease inhibitor with IC_{50} value of 3 μ M. ZP10 exhibited dose-dependent inhibitory effect on ZIKV replication (EC_{50} = 7.65 μ M) [69]. Compound **18** directly was predicted to bind to several critical residues at the proteolytic cavity of the NS2B-NS3 protease and inhibited the polyprotein processing [69].

5.2. Non-competitive inhibitors

The non-competitive inhibitors are known to interact with NS2B-NS3 protease at a site other than the active site (allosteric or orthosteric site) and modulate the enzyme activity. Unlike the flat and featureless active site, the NS3 pockets holding the NS2B N-terminal residues (such as key contact residues L51, L53, V59, and W61, DENV-2 number) are deep and hydrophobic. Since the NS2B binding is essential for protease function, an alternative strategy to target the NS2B-NS3 interaction interface to inhibit the NS3 protease function orthosterically has been investigated [15,70]. The novel approach avoided the active site's featureless nature and the necessity for charged inhibitors at the active site. Several recently identified non-competitive inhibitors are discussed in this as also shown in Fig 4 and listed in Table 2.

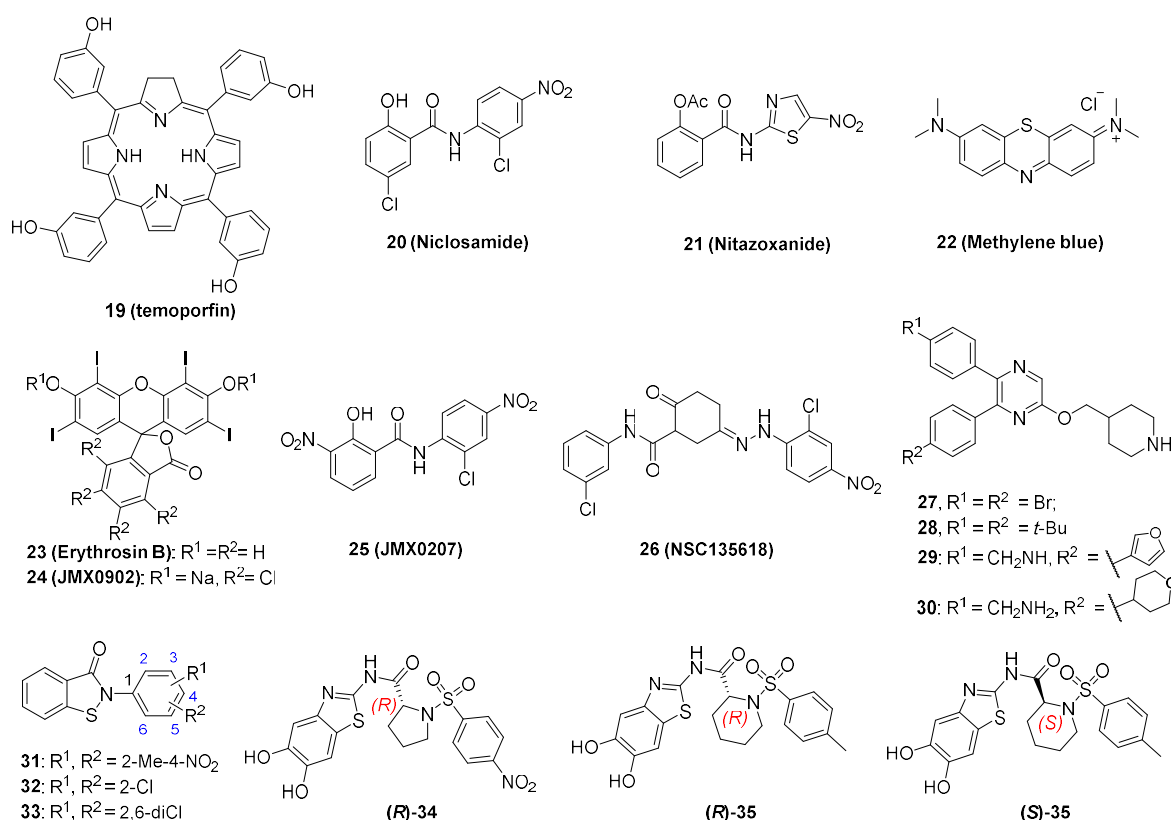


Figure 4. The chemical structures of compounds 19-35 as noncompetitive inhibitors of flavivirus NS2B-NS3 protease.

Table 2. Noncompetitive inhibitors of flavivirus NS2B-NS3 protease.

Number (Name) of compound	Targeted virus	IC ₅₀ (μM)	EC ₅₀ (μM)	<i>In vivo</i>	Reference
19 (Temoporfin)	Zika	1.1 ± 0.1	0.024	Yes	[70]
	DENV-2		0.020		
	WNV		0.010		
	JEV		0.011		
	YFV		0.006		
20 (Niclosamide)	Zika	12.3 ± 0.6	0.48		[70]
	DENV-2		0.55		
	WNV		0.54		
	JEV		1.02		
	YFV		0.84		
21 (Nitazoxanide)	Zika	15.9 ± 0.9	1.48		[70]
	JEV		0.39		
22 (methylene blue)	DENV-2	8.9	0.36	Yes	[71]
	ZIKV		0.087-0.2		[71]
23 (Erythrosin B)	ZIKV	1.7 1.9	0.62	Yes	[72]
	DENV-2		1.2		
	WNV		0.66		
	JEV		0.35		
	YFV		0.57		
24 (JMX0902)	Zika	2.6	0.3		[73]
25 (JMX0207)	ZIKV	8.2	0.3	Yes	[74]
	DENV		0.31		
26 (NSC135618)	DENV-2	1.8	0.81		[15]
	Zika		1.0		
	WNV		1.27		
	YFV		0.28		
27	Zika	21.7			44
28	Zika	3.1			44
29	ZIKV	0.20 ± 0.01	EC ₆₈ 0.3 or 0.6	Yes	[75,76]
	DENV-2	0.59 ± 0.02			
	DENV-3	0.52 ± 0.06			
	WNV	0.78 ± 0.02			
30	Zika	0.13	EC ₆₈ 0.6		[76]
	DENV-2	2.4			
	WNV	0.82			
31	DENV-2	2.56 ± 1.03	<30		[77]
32	DENV-2	2.01 ± 0.98	<30		[77]
33	DENV-2	5.28 ± 1.89	<30		[77]
(R)-34	DENV-2	0.32	< 3		[78]
(R)-35	DENV-2	0.51			[78]
(S)-35	DENV-2	0.58			[78]

Li et al developed a split luciferase complementation (SLC)-based protein-protein interaction assay to monitor NS2B-NS3 interactions [70]. A total of 2,816 approved and investigational drugs were screened. Several orthosteric inhibitors capable of abolishing the NS2B-NS3 interactions were identified. Among these, 23 compounds were found to have an IC₅₀ value below 15 μM. Three of them, including compounds **19** (temoporfin), **20** (niclosamide), and **21** (nitazoxanide), could inhibit the viral NS2B-NS3 protease activity with an IC₅₀ of 1.1 ± 0.1, 12.3 ± 0.6, 15.9 ± 0.9 μM and cytotoxicity CC₅₀ of 40.7 ± 0.7, 4.8 ± 1.0, 77 ± 7.2 μM in A549 cells, respectively. These molecules are broad-spectrum antivirals against multiple representative flaviviruses with EC₅₀ values in low micromolar to nanomolar range (Table 2). Temoporfin was further tested in animal model showing that it could inhibit viremia and protect 83% of mice from lethal challenge of ZIKV. Importantly, mice that survived did not show any signs of neurological disorder [70]. These three compounds inhibited the interaction between NS3 and the NS2B co-factor.

In addition to the compounds mentioned above, compound **22** (methylene blue (MB)) was found to significantly inhibit the NS2B and NS3 protease activity with

IC₅₀ values in micromolar range *via* a non-competitive mechanism [71]. In addition, it was shown that compound **22** significantly reduced titres of multiple strains of ZIKV and DENV-2 with low micromolar and nanomolar EC₅₀ in cell-based antiviral assays. Moreover, compound **22** was found to inhibit viral replication in primary cells relevant to ZIKV pathogenesis and protect 3D mini-brain organoids from ZIKV infection. Animal model study confirmed that compound **22** treatment significantly increased the survival rate of mice challenged by a lethal dose of ZIKV [71].

Furthermore, compound **23** (erythrosin B (EB)) was found as a potent orthosteric inhibitor of the NS2B-NS3 proteases of ZIKV and DENV-2 with IC₅₀ values of 1.7 μ M and 1.9 μ M, and EC₅₀ values of 0.62 μ M and 1.2 μ M, respectively [72]. SAR studies suggested that compound **23** derivative compound **24** (JMX0902, or Acid Red 94) was slightly more potent than the compound **23** in inhibition of ZIKV replication, with an IC₅₀ of 2.6 μ M and EC₅₀ of 0.3 μ M [73]. The SAR studies indicated that iodine substitutions at R₁ and R₃ positions of the xanthene ring were essential for compound **23**'s biological activities, whereas chlorine substitutions were allowed on the isobenzofuran ring of compound **23** [73].

Recently, a small library of niclosamide derivatives was screened, leading to identification of a new analogue with improved pharmacokinetic properties. The compound **25** (JMX0207) showed improved efficacy in inhibition of the molecular interaction between NS3 and NS2B, better inhibition of viral protease function at EC₅₀ of 0.3 μ M against DENV-2 and EC₅₀ of 0.31 μ M against ZIKV NS2B-NS3 interaction [74]. This compound also reduced ZIKV infection in 3D mini brain organoid and significantly reduced viremia in a ZIKV animal model [74].

In another study, Li et al developed an SLC-based conformational switch assay [15]. This assay monitors the conformational changes of NS2B after binding of inhibitor specific for NS2B-NS3 protease complex. Our aim was to identify and characterize allosteric inhibitors that prevent the formation of active conformation by NS2B [15]. From a virtual screening pipeline, twenty-nine compounds were selected for testing in a protease inhibition assay. One of these compounds, namely **26** (NSC135618), significantly inhibited the DENV-2 protease *in vitro*, with IC₅₀ values of 1.8 μ M. Compound **26** could also inhibit the protease conformational change in the SLC-based assay. Later it was found that compound **26** also inhibited viral replication of DENV, ZIKV, WNV, and YFV with EC₅₀ values in low micromolar range [15].

In a screening of 1,200 synthesized compounds targeting histone modifying enzymes including lysine specific demethylase1 (LSD1), Yao et al found compounds 2,3-bis(4-bromophenyl)-5-(piperidin-4-ylmethoxy) pyrazine **27** and 2,3-bis(4-(tert-butyl) phenyl)-5-(piperidin-4-ylmethoxy) pyrazine **28** as novel inhibitors with IC₅₀ of 21.7 and 3.1 μ M against the ZIKV protease [75]. Through further SAR studies, a series of 2,5,6-trisubstituted pyrazine compounds are discovered as broadly active inhibitors of flavivirus proteases. Among them, compounds **29** with a furan-3-yl group and **30** with a tetrahydropyran-3-yl ring exhibited IC₅₀ values of 200 nM and 130 nM, respectively, against the ZIKV protease and potently inhibited ZIKV replication with EC₆₈ values of 300–600 nM [75]. In addition, X-ray studies reveal that compound **29** binds to an allosteric pocket of NS3, providing a druggable pocket of the flavivirus protease, as contrasted to the shallow active site recognizing polar and positively charged Arg or Lys of the substrate [75]. Further studies confirmed that treatment with compound **29** at dose of 30 mg/kg for 3 days inhibited ZIKV viral loads in plasma and brain by up to 96 and 98% (at 24 h), respectively [75,76]. Compound **29** also prolonged the mice survival rate.

Hybrid pharmacophores generated by combination of two individual pharmacophores with 1,2-Benzisothiazol-3(2H)-one (BIT) as the skeleton have been proven to show synergistic effects in inhibition of the DENV-2 NS2B-NS3 [77]. It was observed that BITs bind to the protease in vicinity of the catalytic triad. The BITs containing aromatic

substitutions at the N-atom like compounds **31** with 2-methyl-4-nitrophenyl, **32** with 2-chlorophenyl, and **33** 2,6-dichlorophenyl showed the highest protease inhibition with IC₅₀ of $2.56 \pm 1.03 \mu\text{M}$, $2.01 \pm 0.98 \mu\text{M}$ and $5.28 \pm 1.89 \mu\text{M}$, respectively. The DENV-2 infection assay showed that two BITs, the compound **32** reduced viral infectivity by 1.4 orders of magnitude and the compound **33** reduce the viral infectivity by about six fold [77].

Impermeability of the competitive inhibitors led the researchers to look for non-competitive inhibitors having better properties in terms of affinity, hydrophilicity, and lipophilic ligand efficiency. Millies et al developed a series of proline-based enantiomers of previously identified non-competitive inhibitors by exchange of the aromatic ether moiety to the 1-tosylprolinyl fragment [78]. The substitution pattern of the (sulfone) amide moiety was also judged for their effect on activity of the zika virus protease. The most potent inhibitor was the 4-nitrophenyl substituted sulfonamide (*R*)-**34** with IC₅₀ value of 0.32 μM . Further replacing the proline moiety with a pipecolinic acid afforded compounds (*R*)-**35** and (*S*)-**35** with IC₅₀ value of 0.51 and 0.58 μM , respectively [78]. Unfortunately, only compound (*R*)-**34** showed moderate to weak antiviral efficacy against DENV and ZIKV.

6. Conclusion and future direction

This review reports the inhibitors of flavivirus NS2B-NS3 inhibitors evaluated in the last six years. Selected studies investigated the competitive and non-competitive inhibitors having the ability to block the activity of these proteases. Potential inhibitors were further investigated for antiviral activity against DENV, ZIKV and WNV. However, pharmacokinetic characteristics of majority of these compounds have not been reported, except for a few. One of the main barriers in the development of antiviral agents is the optimization of their absorption, distribution, metabolism, and excretion properties. Moreover, the flavivirus NS2B-NS3 protease is a serine protease similar to other host serine proteases. Therefore, inhibitors targeting the P side of protease could likely affect the activities important for host physiology. Inhibitors targeting the P' side of the protease or its allosteric and orthosteric sites may be better options to counter the flavivirus infection in human individuals. Furthermore, effective compounds found *in vitro* study using the NS2B-NS3 protease assay should not solely be used in determining the pharmaceutical potential of compounds. Thus, extensive studies are needed to determine the *in vivo* performance of selected inhibitors against flavivirus infections.

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Data Availability Statement: In this section, please provide details regarding where data supporting reported results can be found, including links to publicly archived datasets analyzed or generated during the study. Please refer to suggested Data Availability Statements in section “MDPI Research Data Policies” at <https://www.mdpi.com/ethics>. You might choose to exclude this statement if the study did not report any data.

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