Vector-Borne Diseases & Treatment

Chapter 1

Recent Advances in Inhibitors of Flavivirus NS2B-NS3 Protease from Dengue, Zika, and West Nile Viruses

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Abstract

Zoonotic viruses can be transmitted to humans by hematophagous insects, such as mosquitoes, biting midges, ticks, sand flies and thus they are called arthropod-borne viruses. Normally, flaviviruses are transmitted by mosquitoes from the *Aedes* genus, representing a significant health problem worldwide the development of small molecules targeting the NS2B-NS3 protease is extremely important in order to identify new anti-flaviviral agents, considering that it is an essential enzyme involved in the viral replication process. In this chapter, the flavivirus cycle of infection, morphological aspects, and the catalytic mechanism of proteolytic activity of NS2B-NS3 protease will be discussed. Additionally, all recent advances in the drug development against Dengue (DENV), Zika (ZIKV), and West Nile (WNV) viruses will be deeply addressed. Furthermore, these will be classified into categories and individually analyzed.

Keywords: NS2B-NS3, flavivirus, inhibitors, Dengue, Zika, West Nile virus.

1. Introduction

During the evolution of the human species, infectious diseases have also evolved in parallel. The emergence of new diseases and re-emergence of diseases considered as *old* are a challenge to humankind [1]. Emerging infectious diseases have quickly increased, in term of frequency and geographical territory. Among these, zoonotic diseases are responsible for billions of cases of human illness and millions of deaths every year, constituting a significant health problem worldwide [2].

Climate changes and intercontinental travels are considered as the most significant factors that contribute with the spread of flaviviruses from their natural habitats in tropical forests to the urban centers. In 2017, the World Health Organization (WHO) reported approximately 3.9 million people are living in risk zones for flaviviral infections in 128 countries worldwide [3]. From these, over 2 million people are living in regions with climates suitable to support flavivirus-transmitting vector [4].

In general, zoonotic viruses which are transmitted to humans by hematophagous insects, such as mosquitoes, biting midges, ticks, sandflies are called arthropod-borne viruses (or Arboviruses) [2,5]. Typically, flaviviruses are transmitted by female mosquitoes from *Aedes aegypti*, A. *albopictus*, A. *furcifer* [6–8], A. *africanus*, A. *apicoargenteus*, A. *hensilli*, A. *luteocephalus*, A. *vitattus* [7], although, insects from the Culex genus can act as vector as well [2,9–13] (**Figure 1**).

Figure 1: Most typical genres that act as vectors of flaviviral diseases.

In sense, mosquitoes are vectors of several pathogens of different diseases caused by flaviviruses, such as West Nile (WNV), Dengue (DENV), and Zika (ZIKV) [14]. This last is associated with cases of neurological defects, fetal growth restriction, microcephaly in newborns, and Guillain-Barre syndrome in adults [4,15]. In sense, the vector control of these diseases has global importance, especially in countries located in tropical and sub-tropical zones [14]. Although, the utilization of insecticides to eliminate these vector has led to the development of resistance, in several cases [14].

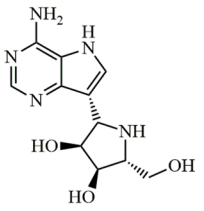
In 2016, the division of vaccines from Sanofi-Pasteur approved the first tetravalent vaccine against DENV1-4, namely Dengvaxia® (also known as CYD-TDV). However, its efficacy against the DENV1 and 2 serotypes is not completely clear [16,17]. Basically, Dengvaxia® is a prophylactic, tetravalent, live attenuated viral vaccine, which contains genes encoding the premembrane (prM) and E-protein of DENV1-4 serotypes inserted onto the chimeric backbone

of a live attenuated Yellow Fever virus (YFV) vaccine strain [18]. Its vaccination schedule consists of 3 injections of 0.5 mL at 6-month intervals. Nevertheless, it is only recommended in the age group ranging from 9 to 45 years old [17]. Additionally, each dose (0.5 mL) has 4.5-6.0 log10 IDCC50, where IDCC50 means the infectant dose for 50% of the cellular culture [19]. Finally, Dengvaxia® has been listed in more than 10 countries, including India, United States, Mexico, Australia, and Brazil [20].

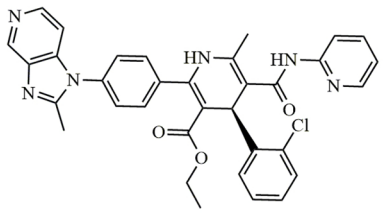
This recent advance can be considered as a product of the massive efforts into the development of vaccines against DENV, ZIKV, and WNV by several pharmaceutical companies, in collaboration with many research groups worldwide, as observed in [**Table 1**].

a: Compound initially designed to the target (indication), however, during the trials a possible different target (proposal) was observed; b: Status information and updates were collected from the databases: www.drugbank.ca; www.adisinsight.springer.com, and www.accessdata.fda.gov. Abbreviations - ZIKV: Zika virus; DENV: Dengue virus; WNV: West Nile virus; YFV: Yellow Fever virus; HCV: Hepatitis C virus.

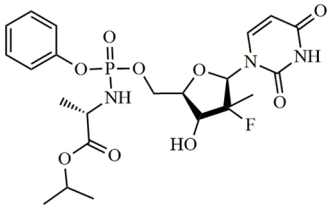
Table 1: Current status (and last updates) for vaccines and drugs/prototypes against DENV, ZIK, and WNV in the pipeline.



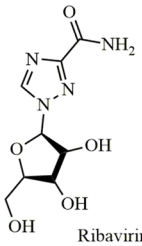
Galidesivir **(1)**



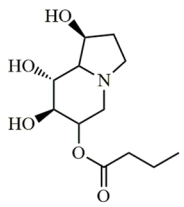
Modipafant **(2)**



Sofosbuvir **(3)**



Ribavirin **(4)**



Celgosivir **(5)**

Indication (Proposal)[a]	Inhibitor name (Synonymies)/Type	Company	Status (Year)b
ZIKV (ZIKV)	BBV 121/vaccine	Bharat Biotech®	Phase II trials for ZIKV in India (2018)
ZIKV (ZIKV)	Galidesivir/active compound (1)	BioCryst®	Preclinical trials for ZKIV in USA and United Kingdom (2018)
DENV (YFV and ZIKV)	EMX 001/vaccine	Emergex Vaccines®	Preclinical trials in DENV, YFV, and ZIKV in United Kingdom (2018)
Carcinoma (ZIKV and WNV)	Rintatolimod/vaccine adjuvant	Hemispherx Biopharma®	Preclinical trials for ZIKV and WNV in USA
YFV	vaccine	Oswaldo Cruz Foundation	Preclinical trials for YFV in Brazil (2018)
DENV (DENV)	KD-382/vaccine	Kaketsuken®	Phase I trials for Dengue in Australia and Japan (2018)

Asthma (DENV)	Modipafant/active compound (2)	Pfizer®	Discontinued in Clinical-Phase II trials for Asthma in the United Kingdom (2015). However, it is Phase II trials for DENV (2018)
HCV (YFV)	Sofosbuvir (GS-7977)/ active compound (3)	Pharmasset®/Gilead®	Approved for HCV (2013). However, it is in Phase II trials for YFV (2018).
HCV (DENV, YFV, ZIKV)	Ribavirin/active compound (4)	Roche®	Approved (2013)
DENV (HCV, HIV)	Celgosivir (5)	Sanofi-Aventis®	Phase I/II trials for Dengue in Singapore (2018)
DENV	Dengvaxia (CYD-TDV)/vaccine	Sanofi Pasteur®	Approved (2016). However, it was withdrawn in Philippines (2017).
DENV (DENV)	TAK 003/vaccine	Takeda®	Phase III trials for Dengue in United Kingdom and USA (2018)
ZIKV (ZIKV)	CSB 013/vaccine	Tianjin CanSino Biotechnology®	Preclinical trials for ZIKV in China (2018)
Ebola and Marburg (ZIKV and YFV)	Tyzivumab/vaccine	Tychan/WuXi Biologics®	Phase I trials for ZIKV and YFV in Singapore (2018)

In this chapter, the cycle of infection, morphological aspects, and the catalytic mechanism of proteolytic activity of flavivirus NS2B-NS3 proteases will be addressed. Moreover, all recent advances in the drug development against DENV, ZIKV, and WNV (considering the years of 2015 to 2018) will be presented and deeply discussed. Furthermore, all information will be classified into three different categories, such as compounds obtained from the: *(i) natural, (ii) synthetic, and (iii) virtual screening sources.*

2. Flavivirus

Flaviviruses belong to the *Flaviviridae* family (*Flavivirus* genus includes 53 species) that is related to several important pathogens, including YFV, Japanese encephalitis (JEV), WNV, Tick-borne encephalitis (TBE) [21,22], Hepatitis C virus (HCV) [20,23], and Pestivirus [20], as well as, DENV and ZIKV viruses, as previously cited. In general, viruses with the ability to cross different species and cause fatalities are extremely important to human health and humankind is not effectively prepared to deal with such emerging threats [24].

2.1. Cycle of Infection

The members of the flavivirus genus demonstrate a high similarity between their intracellular life cycles. In sense, the infection with arthropod-borne flaviviruses begins when the vector takes a blood meal and the virus is injected into the host's bloodstream. Initially, viral particles infect susceptible cells, such as monocytes, dendritic cells, and activate resident immune cells (such as mast cells). Basically, the cell tropism will determine the pathology of more severe cases, as well as the human-to-human transmission patterns [25]. The typical

flaviviral infection cycle is illustrated in [**Figure 2**].

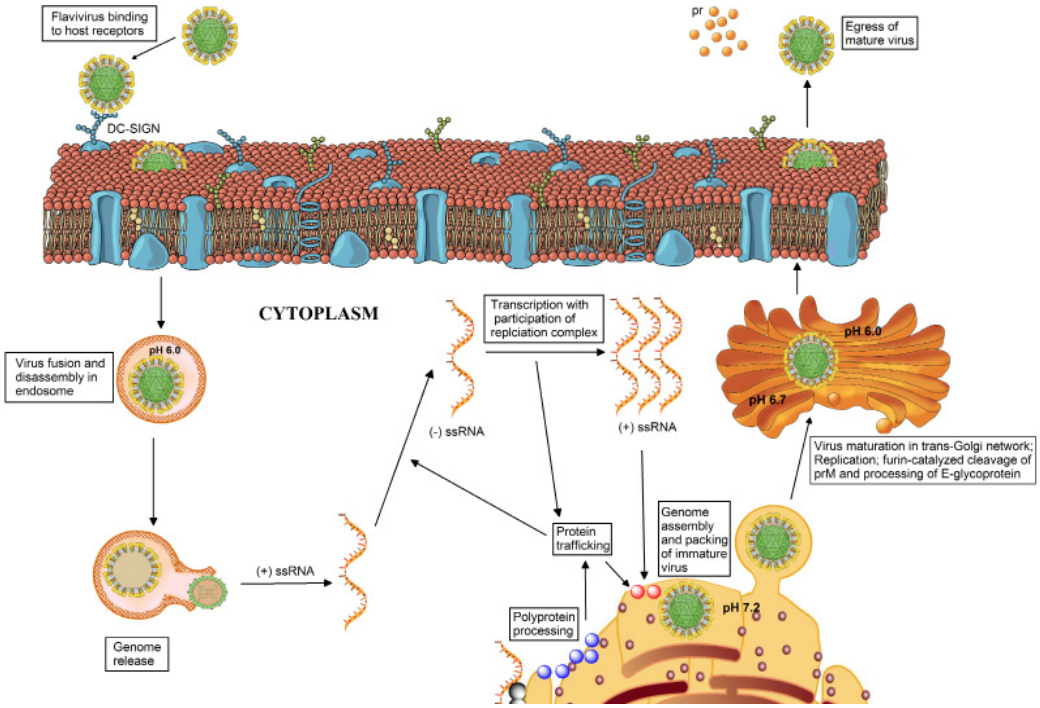


Figure 2: Overview of the infection cycle of flaviviruses on a cellular level.

The most typical receptors for flaviviruses involve C-type lectins, such as the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), as well as the liver-specific ICAM3-grabbing non-integrin L-SIGN (L-SIGN). Additionally, phosphatidylserine receptors (T-cell immunoglobulin and mucin), and tyrosine kinase receptors (Tyro3, MertK, and AXL) perform a similar function [26]. Typically, DC-SIGN is expressed on dendritic cells [27,28], while L-SIGN on endothelial cells [29,30]. Finally, these receptors interact with glycans of the flavivirus E-glycoprotein, during the viral entry [31].

Regarding Figure 2, different host cell receptors for the flaviviral E-glycoprotein are expressed at the cellular membrane (*i.e.* DC-SIGN). The cell surface receptor-mediated endocytosis is used by flaviviruses in a clathrin-dependent manner to entry on the host cell. Membrane fusion of the viral envelope with the endosome membrane, catalyzed by the acidic medium (pH: 6.0) of the endosomal environment, uncoating the nucleocapsid and releasing the viral genome into the host cell cytoplasm [32]. In sequence, genome replication occurs by action of a viral RNA polymerase [33]. In sequence, the RNA genome is translated as a single polyprotein by the host ribosomes, which is then translocated across the endoplasmic reticulum (ER) membrane. This polyprotein is subsequently cleaved into several components [34], such as three structural proteins (premembrane (prM), envelope (E), and capsid (C) proteins) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4, NS4B, and NS5) [22]. The immature virions (viral particles) formed in the ER are transported to the Golgi apparatus, where they are matured by NS2B-NS3 action, as well as by the host protease furin. Thus, these are released via the host secretory pathway [35]. Finally, the mature virus and immature viral particles (virions) are released from the host cell and start a new cycle of infection again.

2.2. Flaviviral NS2B-NS3 Protease

In general, the viral replication depends on the nonstructural protein 3 ($NS3^{pro}$), which is only fully active when it is associated to the NS2B, which acts as a cofactor (the central hydrophilic domain, more precisely), where it improves the activity of the enzyme towards peptide substrates from 300 to 7600-fold [34,36–38]. Furthermore, the NS3pro is the second-largest flaviviral protein (69 kDa), only losing for the NS5 (103 kDa) [38].

Based on this, the development of small molecules targeting the NS2B-NS3 protease complex is extremely important in order to identify new anti-flaviviral agents. Disruption of this protease activity has been shown to be lethal for the viral replication [39]. The assembled protease cleaves the viral polyprotein to release structural and nonstructural proteins that are essential for the viral replication and assembly of new virus particles. The 40 amino acid hydrophilic core domain of NS2B is binding the NS3*pro* at its protease domain and at the same time anchoring the NS2B-NS3 complex to the cytoplasmic leaflet of the ER-membrane via *N*- and *C*-terminal transmembrane domains. The NS3*pro* also incorporates other enzymatic activities at the C-terminal (helicase and RNA-endonuclease), all of these are tightly regulated by steric interactions with the ER membrane [40].

$NS3^{pro}$ consists of a chymotrypsin-like serine protease domain (two β-barrels, each containing 6 β-sheets) within the N-terminus of its 180 residues full-length protein [23,37,41], which cleaves the viral polyprotein both in *cis* and *trans* conformations [38]. Between both barrel-like lobes a catalytic cleft is formed, revealing four well-defined specificity pockets [21,42] (Figure 3). Finally, binding of a ligand to the active site is responsible to stabilize a determined conformational state of NS2B-NS3 protease [41].

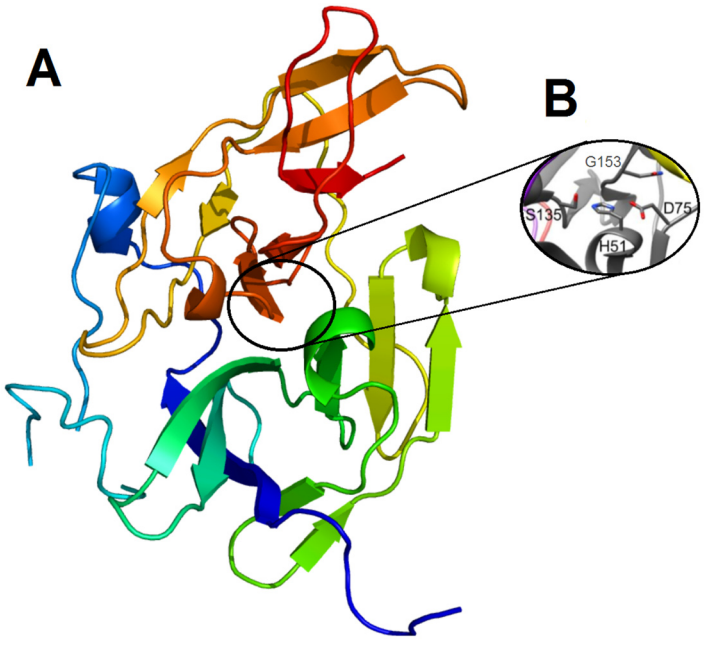


Figure 3: NS2B-NS3 protease from DENV-2. In (A), an overview of NS2B-NS3 (in open conformation, PDB ID: 2FOM) showing both barrel-like lobes (green and orange). In (B), maximization of the catalytic cleft evidencing the catalytic triad (H51, D75, and S135), as well as the auxiliary residue, G153. Amino acid code - H: Histidine; D: Aspartate; S: Serine; and G: Glycine.

2.2.1. NS2B-NS3 Protease Catalytic Mechanism

All flaviviruses possess the classical catalytic triad of serine proteases, composed by His51, Asp75, and Ser135 residues, located at the NS3pro N-terminus [22,34]. Additionally, the C-terminal (two-thirds of the protein) part is associated with the RNA-triphosphatase and NTPase/RNA helicase activities [21]. The hydrophobic domains of the NS2B cofactor, responsible for membrane tethering, are not essential for the proteolytic function of NS3pro [43]. Initially, Ser135 residue performs a nucleophilic attack on the P1 carbonyl atom of the substrate. The nucleophilicity of the hydroxyl group of the Ser135 residue is enhanced by the His51 residue which acts as a base (**Figure 4-I**).

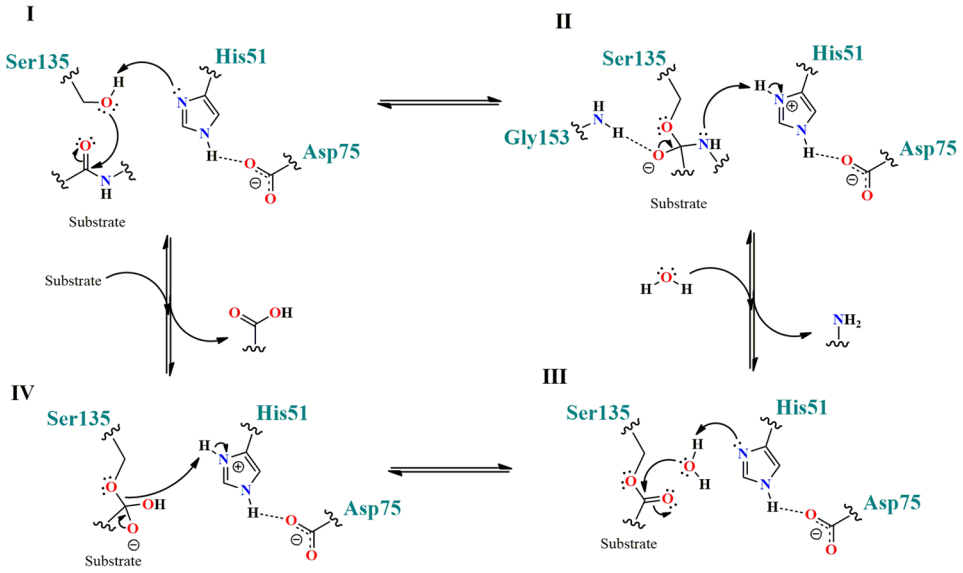


Figure 4: Enzymatic mechanism of the catalytic triad in NS2B-NS3 proteases. In (i), the nucleophilic attack performed by Ser135; In (ii), the oxyanion hole by Gly153, stabilizing the tetrahedral intermediate. Additionally, an amine derivative is released as a product; In (iii), the substrate (covalently bound) suffers a new nucleophilic attack by a single water molecule; In (iv), a rearrangement occurs and stabilizes the second tetrahedral intermediate, releasing a carboxylic acid as another product.

Consequently, the tetrahedral intermediate is stabilized by a hydrogen bond with the Gly153 residue in the oxyanion hole (**Figure 4-II**). Cleavage of the C-terminal part of this intermediate anion releases an amine derivative, as a product. In contrast, the N-terminal fragment remains covalently bound to the Ser135 residue via an ester group. In sequence, the His51 residue acts as a base, increasing the nucleophilicity of the water molecule by generating a hydroxyl anion that hydrolyzes the ester bond (**Figure 4-III**). Finally, a second cleavage occurs releasing a carboxylic acid as a product, by a re-protonation step [44] (**Figure 4-IV**).

3. Recent Advances in Drug Development Targeting NS2B-NS3 Protease from DENV, ZIKV, and WNV

Actually, the development of effective and safe anti-flaviviral drugs can be considered as the most important point in the flaviviral drug design [20]. However, it is known that an accurate processing of the viral polyprotein is a vital step for virus replication, and it is executed by the action of a combination of cellular proteases (co- and post-translation), and a

two-component serine protease (NS2B-NS3 complex) [21,37]. Additionally, this molecular target has different allosteric pockets surrounding the catalytic cleft [45]. Considering this, the NS2B-NS3 protease can be utilized as a primary target for the design and development of flaviviruses antiviral agents [45]. Furthermore, the NS2B-NS3 proteases from DENV, ZIKV, and WNV share 30-80% sequence homology. Thus, the development of 'pan-flaviviral' agents seems to be an excellent strategy against these arboviruses [46].

3.1. Natural Products as a Source of Novel NS2B-NS3 Inhibitors

Normally, plants can produce specific chemical compounds which are normally used against pathogens and predators. Among these, protease inhibitors are naturally occurring defensive proteins in plants [47]. These protease inhibitors from plants possess great endogenous importance, such as seed storage proteins, cell signaling, growth factor activation, and protease regulation [48]. In sense, a recent study demonstrated that the Kunitz or Bowman-Birk family of protease inhibitors was capable of inactivating most of the serine proteases, especially, trypsin. In addition, it was verified that the Lima bean trypsin inhibitor has a concentration-dependent amyloid fibril formation [49].

Considering these facts, Palayam *et al.* [50] reported the purification and structural characterization of a trypsin protease inhibitor from *Alocasia macrorrhizos* (popularly known as "giant elephant ear" and/or "giant Taro"). Additionally, the interactions of this inhibitor (AMTIN) with trypsin, chymotrypsin, Barley α-amylase, and NS2B-NS3 protease from DENV2 were individually evaluated. Basically, AMTIN is a glycoprotein composed of two monomers located in the asymmetric unit consisting of 12 antiparallel *β*-strands, that are connected by long loops forming a *β*-trefoil structure. Finally, the docking analyses showed that AMTIN can act as an inhibitor against amylases and proteases, taking into account that it is capable of interacting at the active binding site in these macromolecular targets. Furthermore, AMTIN can be considered as a promising multifunctional inhibitor.

Two compounds isolated from Boesenbergia rotunda, 4-hydroxypanduratin A (6) and panduratin A (7), α,β-unsaturated ketones, were found to be significantly actives against DENV2 serotype, demonstrating K_i values of 21 and 25 μM against the protease, respectively [51]. Based on this study, Osman *et al.* [52] synthesized nine novel 3,5-bis(arylidene)-4-piperidone derivatives containing the bioactive heterocyclic piperidone moiety inserted into the *α,β*-unsaturated ketone mimetic. Basically, this modification was performed in order to improve the character of hydrogen bond donor/acceptor properties of the final compound. In sequence, all derivatives were screened against the NS2B-NS3 of DENV2. Among these tested compounds, only two compounds (8 and 9) showed moderate effects (**Figure 5**). Finally, it was observed that these best compounds interact with the active site from NS2B-NS3 DENV2; where both of these compounds form hydrogen bonds with the residues Gly53, Arg54, Pro132,

and Gly153, including His51 and Ser135 from the catalytic triad.

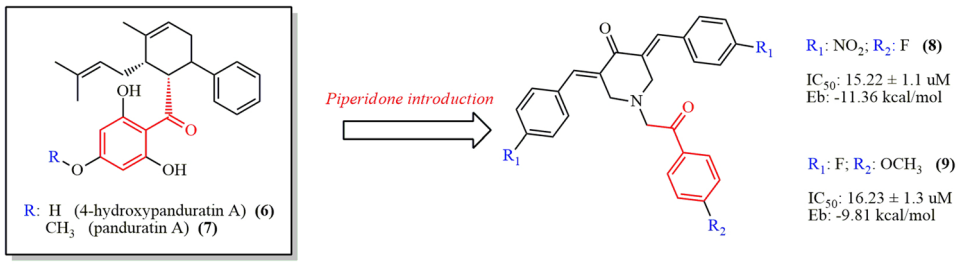


Figure 5: *α, β*-unsaturated ketones (and ketone mimetics) with activity against NS2B-NS3 of DENV2.

In a study performed by Srivarangkul *et al.* [17], it was observed that a novel flavanone derivative (10) inhibited pH-dependent DENV2 fusion in a cell-based assay. Additionally, it was capable of inhibiting DENV2 infectivity in LLC/MK2 and Vero cell lines with EC_{50} values of 15.99 ± 5.38 and 12.31 ± 1.64 µM, respectively. Against DENV4, this flavanone (10) inhibited the infectivity at 11.7 ± 6.04 µM concentration. Moreover, no cytotoxic effects were observed up to 100 µM concentration toward LCC/MK2, HEK-293, and HepG2 cell lines at 72 hours. Also, other phytochemical compounds [11-15] were found to be actives against DENV2 (**Figure 6**). Finally, it was verified that this natural compound has low activity upon NS2B-NS3 DENV2, up to 25 µM concentration.

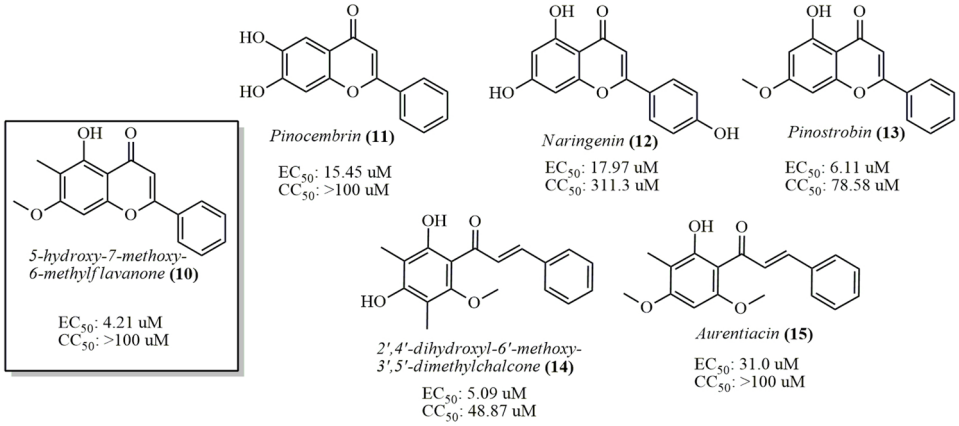


Figure 6: Phytochemical compounds with activity against DENV2 infection.

3.2. Synthetic NS2B-NS3 Inhibitors

Rational design of new NS2B-NS3 inhibitors (or substrate mimetics) is considered an immense challenge because this enzyme has a shallow and hydrophobic catalytic site [53,54]. However, small viral replication inhibitors should be able to reduce the viral load, as well as prevent disease progression [17]. In sense, cyclic peptides are considered as a privileged chemical class of molecules since they can address so-called undruggable targets, such as protein-protein interactions [55,56].

Based on this, Takagi *et al.* [57] have performed studies involving high-throughput synthesis of cyclic peptides. In total, 33 cyclic peptides were synthesized and evaluated toward the NS2B-NS3 protease from DENV2. Among these, 17 cyclic peptides demonstrated significant

inhibition, with IC_{50} values ranging from 0.95 to 15.1 μM.

The most active cyclic peptide (16) (**Figure 7**) has an IC50 value of 0.95 μM, and the molecular docking proposed that the side chains of P1 (L-Lys), P2 (L-Arg), and P3 (L-Lys) were perfectly inserted into the negatively-charged cleft of the binding pocket, forming hydrogen bond with Asp129 ($NS3^{pro}$), Met84 (NS2B), and Asp81 (NS2B) residues, respectively. Moreover, the phenyl ring at P2' L-Phe remains surrounding His51, a residue from the catalytic triad. Finally, the portion P4'-P4-P3-P2 forms a *β*-turn-like conformation.

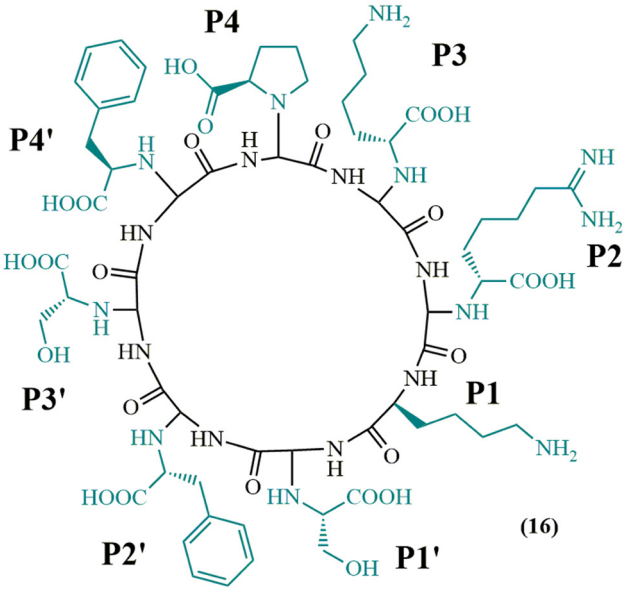


Figure 7: Most active cyclic peptide against NS2B-NS3 DENV2. P1: L-Lys; P2: L-Arg; P3: L-Lys; P4: D-Pro; P1': L-Ser; P2': L-hPe; P3': L-Ser; and P4': D-Phe.

Considering the good results from studies involving peptide inhibitors of NS2B-NS3 protease, a series of linear chain dipeptides was synthesized and screened against NS2B-NS3 from DENV2. From this, compound (17) was found to be the most active compound with IC50 and Ki values of 1.2 ± 0.4 and 4.9 μM, respectively [58]. Based on this previous information, Weng *et al*. [8] synthesized and biologically evaluated a new series of fused bicyclic pyrrolidine and imidazolidinone analogs against NS2B-NS3 DENV2. After all bioassays had been performed, the compound (18) showed exactly the same activity as its precursor compound (17) (**Figure 8**). Moreover, the cell-based assay was performed with this new analog (18), where it inhibited the DENV2 at 39.4 ± 6.2 μM concentration (EC_{50}). Finally, cytotoxic effects upon Huh-7 cells were not observed until 100 μM concentration. Furthermore, molecular docking suggested that this compound (18) interacts with Tyr150, Pro132, and Ser135.

Still within this research topic, several efforts have been addressed to the drug discovery of peptide-based inhibitors. However, these have failed in clinical trials because of their poor cellular uptake and pharmacokinetic parameters [40,54]. In sense, an interesting alternative to solve these problems is to reduce the molecular mass and converting these compounds into peptidomimetics [46]. Therefore, the introduction of electrophilic warheads, such as boronic acids have provided very potent inhibitors, with IC_{50} values in the nM range [59]. Additionally,

the P2 4-aminomethyl phenylalanine moiety forms a salt-bridge with Asp83 residue, which contributes to the enzyme's high catalytic efficiency.

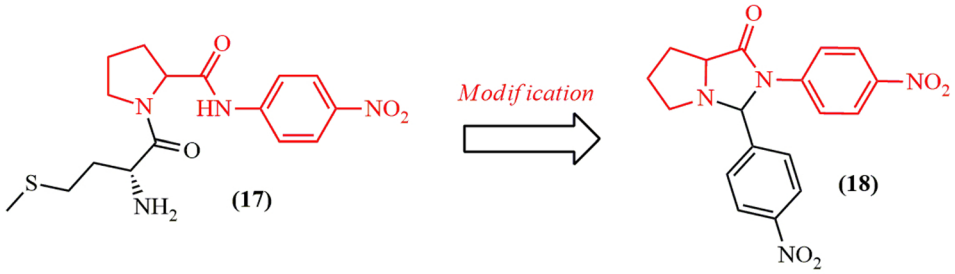


Figure 8: Dipeptide and pyrrolidine derivatives activities against NS2B-NS3 DENV2.

Based on these observations, Nitsche *et al.* [53] developed a series of dipeptide-boronic acid inhibitors of NS2B-NS3 WNV, DENV, and ZIKV. They observed that insertion of a boronic acid warhead into the peptidomimetic scaffolds provided a 1000-fold increase in the affinity. Among these, compound (19) was identified as the most potent inhibitor (**Figure 9**). Interestingly, it showed better inhibition of NS2B-NS3 ZIKV, suggesting that the lysine mimetics at P2 lead to better results of inhibition.

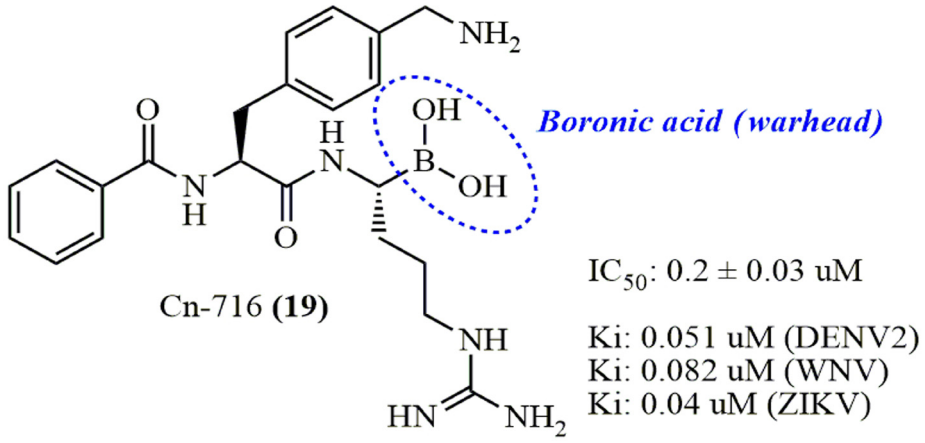


Figure 9: Peptidomimetic-boronic acid derivative highly active towards NS2B-NS3 ZIKV.

From a high-throughput phenotypic screening against DENV2, a novel hit compound (20) was identified as a potent DENV2 inhibitor, with an IC_{50} value of 0.64 µM towards DENV2 RNA replication *in vitro* [20,60]. Considering this result, Lu *et al.* [20] synthesized and biologically evaluated a series of pyridazinone derivatives in a DENV2 RNA-replication assay. Additionally, the CC_{50} values were determined on Huh-7 cells line. Different series of analogs were initially synthesized and screened, revealing IC_{50} values ranging from 22.89 to 0.6 µM and CC_{50} values between >80 and 8.51 µM concentrations. Furthermore, a potent series of pyridazinones containing a phenyl ring moiety were synthesized, considering previous structure-activity relationship (SAR) studies. After several modifications had been performed, compound (21) (**Figure 10**) was discovered as the most promising from this study, which demonstrated potent activity and a high selectivity index (SI equal to 89.2). Finally, docking studies proposed that it has a high affinity to the $NS3^{pro}$, interacting with the residues Leu128, Phe130, Pro132, Tyr150, and Tyr161. Furthermore, it was verified that the phthalazinone moiety seems to be inserted at

the hydrophobic pocket of NS3pro as well.

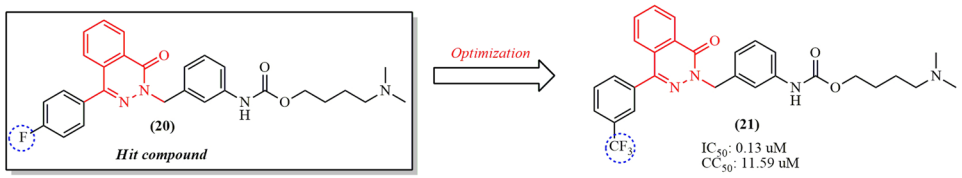


Figure 10: Pyridazinone analogs with activity against NS3 DENV2 protease.

Wu *et al.* [61] reported the synthesis of 2-aminobenzothiazole derivatives as selective and noncompetitive inhibitors of the DENV2 and 3 serotypes *in vitro* and in cells exhibiting IC_{50} values in the low-micromolar range. Additionally, the cytotoxic effects were determined in Vero cells. As result, compound (22) (**Figure 11**) was discovered as the most potent inhibitor in this series of the benzothiazole derivatives, it was able to inhibit the NS2B-NS3 proteases from DENV2 and 3 with values de IC50 of 4.2 and 0.99 µM, respectively. In cell-based assays, it was capable of inhibiting this enzyme with an IC50 value of 3.2 µM. Moreover, compound (22) demonstrated excellent antiviral activity in infected Vero cells (EC_{50} value of 0.8 µM), without cytotoxic effects up to 10 µM concentration.

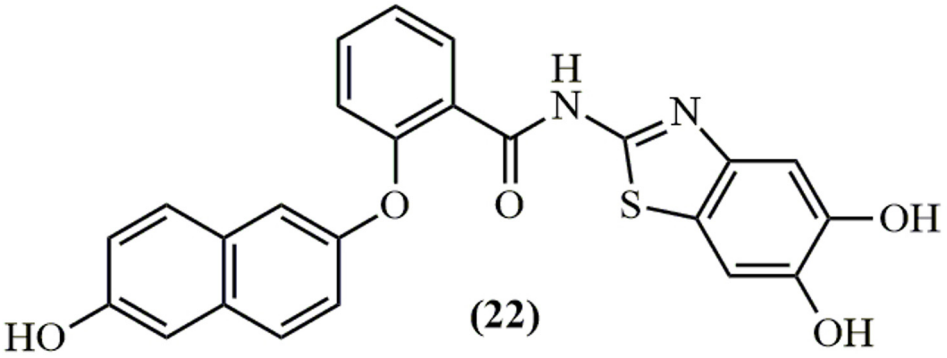


Figure 11: Potent 2-aminobenzothizole derivative against DENV2 and 3 serotypes.

Recently, Oliveira *et al*. [62] synthesized and biologically screened indan-1,3-dione derivatives against NS2B-NS3 protease from WNV. All compounds were synthesized employing $ZrOCl2\cdot(H_2O)_8$-catalyzed reactions, starting with different aromatic aldehydes, in water as the solvent. In total, 17 indan-1,3-dione analogs were obtained from this method, with yields ranging from 27 to 86%. Among these, two compounds (23 and 24) (**Figure 12**) presented better activity, with IC_{50} values of 11 and 3 µM, respectively. Additionally, it was observed that these both compounds inhibit the NS2B-NS3 WNV via a noncompetitive mechanism. Finally, molecular docking studies revealed that the para-hydroxyl group (23) interacts with Trp89 and Ile147 residues, while one of the carbonyl groups interacts with Gln167. In contrast, the *meta-hydroxyl* group (24) interacts with the Gly124 residue via hydrogen bonding. This fact suggests that this interaction is extremely significant for the activity, as well as the presence of a hydroxyl substituent at this position.

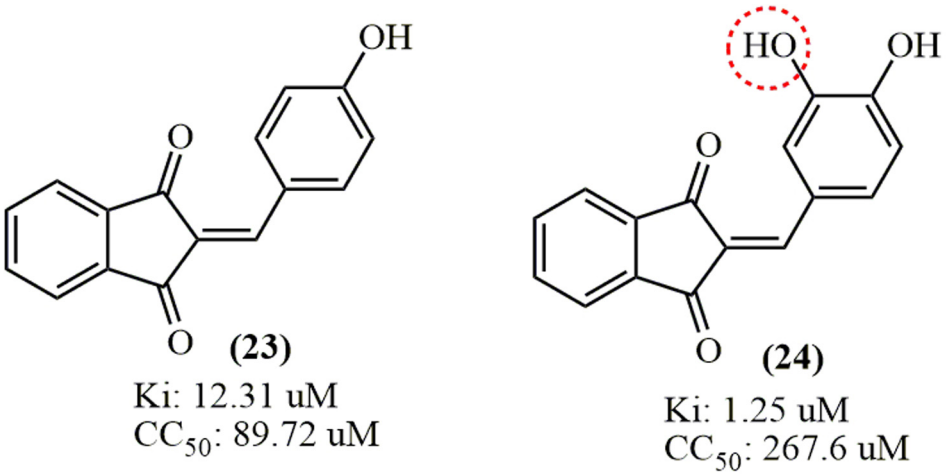


Figure 12: Indan-1,3-dione derivatives with activity against NS2B-NS3 WNV.

Li *et al.* [63] have investigated an FDA-approved food additive, erythrosine B (25) (**Figure 13**), a cherry-pink synthetic and potent flaviviral inhibitor. The authors found that this compound inhibits the NS2B-NS3 protease from ZIKV and DENV2, with IC_{50} values of 1.7 and 1.9 μM, respectively (*via* a noncompetitive mechanism). Additionally, it was verified that compound (25) can significantly reduce the activity of several arboviruses, such as DENV2 (EC50: 1.2 ± 0.2 μM), ZIKV (EC_{50}: 0.62 ± 0.12 μM), YFV (EC_{50}: 0.57 ± 0.06 μM), JEV (EC_{50}: 0.35 ± 0.06), and WNV (EC_{50}: 0.66 ± 0.13 μM). Moreover, compound (25) demonstrated to be well tolerated, with a CC_{50} value higher than 150 μM. Finally, it was capable of inhibiting ZIKV replication in ZIKV-relevant human placental and neural progenitor cells, suggesting that it is a pathway to minimize the risk of fetal acquired microcephaly resulting from ZIKV infection of pregnant women [64].

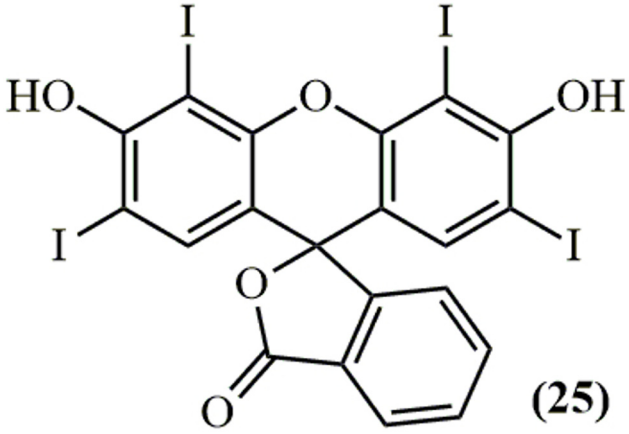


Figure 13: Chemical structure of erythrosine B.

Another similar recent study was performed in order to investigate a different compound, zafirlukast. It is an FDA approved drug employed for the treatment of asthma [65]. Moreover, it was reported to have antibacterial activity towards *Porphyromonas gingivalis* and *Streptococcus mutants*, two microorganisms responsible for oral infections [66].

The presence of a toluic acid-N-aryl sulfonamide and cyclopentyl carbamate in the zafirlukast structure (26) was considered as an interesting point to be investigated as an inhibitor

upon NS2B-NS3 WNV. In sense, Martinez *et al*. [67] initially found that compound (26) inhibits NS2B-NS3 WNV, with an IC_{50} value of at 32 μM. This fact was verified by a Michaelis-Menten plot, which also revealed that this compound acts through a mixed inhibition mechanism, in this case, as a noncompetitive inhibitor. Considering these facts, a series of zafirlukast analogs was synthesized and biologically evaluated against NS2B-NS3 WNV. Additionally, the SAR study suggested that the alkyl carbamate and N-toluic acid N-aryl sulfonamide portions are essential for the activity. Replacement of cyclopentyl with a phenyl ring improves the activity, leading to the best analog (27), with an IC50 value of 22 μM (**Figure 14**). Molecular docking studies revealed that (27) should present a similar binding interaction as compound (26). Finally, both carbamate and phenyl sulfonamide portions interact with Phe116, as exactly observed for the precursor drug (26).

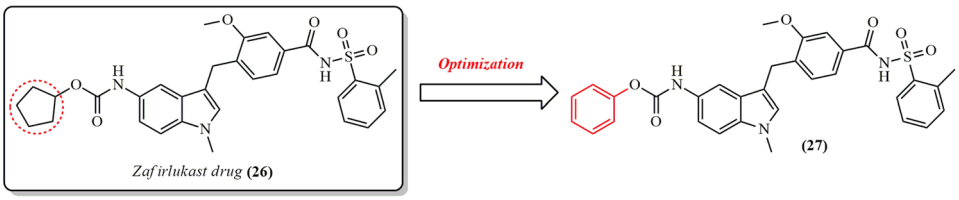


Figure 14: Zafirlukast and the most active analog against NS2B-NS3 WNV.

Gruba *et al*. [68] reported a combinatorial chemistry approach for the synthesis of internally quenched substrates of the ZIKV NS2B-NS3 protease that were optimized in prime positions of the peptide chain. They obtained the sequence ABZ-Val-Lys-Lys-Arg-Ala-Ala-Trp-Tyr ($3NO_2$)-NH_2 (28) as a final substrate (**Figure 15**). It displayed excellent kinetic parameter (k_{cat}/KM ~1.26 x 10^8 $M^{-1}S^{-1}$), which was 10-fold greater than previously reported (7.7 x 106 M-1S-1). In addition, it was verified that this novel substrate is more selective towards WNV protease. By molecular docking (PDB ID: 5LCO) it was proposed that this substrate interacts at the catalytic site in the NS2B-NS3 protease. Finally, the authors state that its kinetic parameters along with the low detection limit make this substrate a sensitive probe to detect low quantities of catalytic activity of NS2B-NS3 WNV.

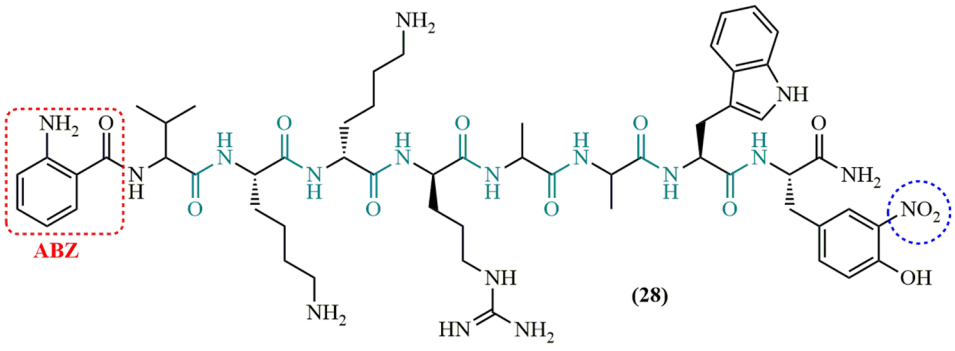


Figure 15: A new substrate promising as a probe to detect flavivirus NS2B-NS3 protease.

Therapies incorporating substrate-mimetic inhibitors have been considered a successful and alternative strategy for the treatment of HCV and HIV infections [69,70]. In addition, the introduction of metals (such as copper, iron, cobalt, and nickel) in peptidomimetic inhibitors have provided metallopeptides with activity as antiviral and antimicrobial agents [71–74].

Moreover, these compounds have shown catalytic inactivation toward some proteins by the generation of metal associated reactive oxygen species (ROS) [71–75]. Considering all these advances, Pinkham *et al.* [76] developed catalytic metallopeptides that incorporate the copper-binding motif into either the *N*- or *C*-terminus of known NS2B-NS3 WNV targeting peptides. In sense, it was verified that these Cu-peptide inhibitors demonstrated an affinity to the residues Ser135, Thr132 and 134, as well as residues located into the S2 binding pocket. In DNPH-based assay was observed that these new metallopeptides are responsible to cause oxidative damage, showing the formation of carbonyls in NS2B-NS3 WNV treated with them. Based on this, it was suggested that these metallopeptides are attenuating NS2B-NS3 WNV agents, which act by irreversible oxidation of amino acids essential to the catalytic activity. Finally, the most promising Cu-peptides (29-32) are shown below (**Figure 16**), with their corresponding K_i values ranging from 13 to 8 µM concentrations.

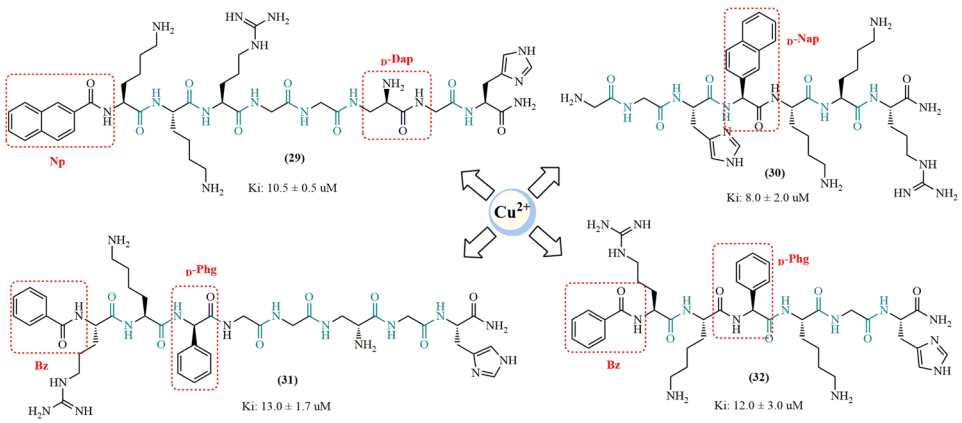


Figure 16: Cu-metallopeptides active against NS2B-NS3 WNV. Sequence: Cu[Np-Lys-Lys-Arg-Gly-Gly-(D-Dap)-Gly-His-NH2] (29); Cu[Gly-Gly-His-D-Nap-Lys-Lys-Arg-NH2] (30); Cu[Bz-Arg-Lys-D-Phg-Gly-Gly-(D-Dap)-Gly-His-NH2] (31); Cu[Bz-Arg-Lys-D-Phg-Lys-Gly-His-NH2] (32).

3.3. Virtual Approaches Applied to the Development of Ns2b-N3 Inhibitors

In general, drug design is a complex and very expensive process, which frequently results in failures. In an attempt to reduce the failures and high costs to bring a new drug to market, several computational techniques have been employed that can predict molecular properties *via* quantitative structure-activity relationship (QSAR), pharmacophore modeling, structure- and fragment-based drug design [77]. Basically, these computational-based methods may provide the fast and safe response in order to identify the most promising compounds for drug development [23].

Considering this, Deng *et al.* [22] performed a study involving 600,000 compounds in a structure-based virtual screening, using NS2B-NS3 protease as a target (PDB ID: 2FOM). From this screening, 27 putative hit compounds were selected and evaluated in enzymatic assays upon NS2B-NS3 DENV-2. Subsequently, compound (33), a benzimidazole derivative, was found to be the lead molecule, with an IC_{50} value of 13.1 ± 1.03 µM and a CC_{50} of 12.6 µM. Additionally, binding mode studies revealed that the benzimidazole nitrogen forms a hy-

drogen bonding interaction with Asn167 in the positive region P1; while the phenol oxygen and one of the nitrogens of the hydrazine moiety interact with Lys74. Moreover, the benzimidazole moiety hydrophobically interacts with Trp69 and Trp83 (at P3). Finally, the ethyl ester group has both hydrophobic and electrostatic interactions with the hydrophobic region P2, including the residues Leu85 and 149. Starting from all these interactions observed by docking, it is possible to suggest that this compound acts by interaction with an allosteric binding site in the NS2B-NS3 protease surface. Considering these results, several analogs were synthesized and screened toward NS2B-NS3, resulting in three best analogs (34-36), with IC_{50} values of 7.83 ± 0.94, 7.46 ± 1.15, and 9.45 ± 0.78 µM, respectively (**Figure 17**).

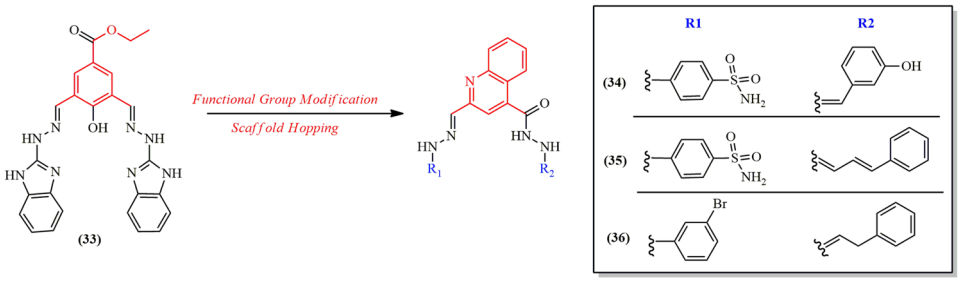


Figure 17: Benzimidazole derivatives with activity against NS2B-NS3 DENV-2.

Currently, studies focus on targeting essential viral enzymes in the infection process by the direct or indirect inhibition of their viral replication. For example, it has been demonstrated that the NS2B-NS3 inhibition decreases 80% of DENV in host cells [78]. Based on this, Cabarcas-Montalvo *et al*. [79] applied *in silico* methods to investigate a chemolibrary of compounds from the NCBI® PubChem Database. In total, 40 molecules were evaluated by a molecular docking protocol (PDB ID: 2FOM), including active and inactive reported inhibitors against DENV2. In general, only compounds with IC_{50} values less than 15 µM were selected. All 3D-structures were drawn by the Gaussian® 09 package program and they were geometrically optimized using Density Functional Theory (DFT) at the B3LYP/6-31G the level of theory. In sequence, the web-based software tool FAFDrugs3 was used to filter for Pan-Assay Interference Scaffolds (PAINS). Finally, *in vitro* studies were performed involving fluorometric enzyme activity assays, cell and virus, cell proliferation, and virus yield reduction bioassays. Finally, two compounds (CID54692801 (37) and CID54715399 (38)) were found to be most promising as anti-DENV2 agents (**Figure 18**), with moderated selectivity indexes (SI). Compound (CID54715399 (38)) hydrophobically interacts with the residues Trp69, Leu76, Thr118 and 120, Ile123, Val154, Ala164 and 166 and via electrostatic interactions with the residues Ans52, Lys74, and Ala164. In contrast, compound (CID54692801 (37)) forms some hydrogen bonding with Leu74, Gly87, and Leu149 residues. Furthermore, interactions with any residue from the catalytic site were not observed.

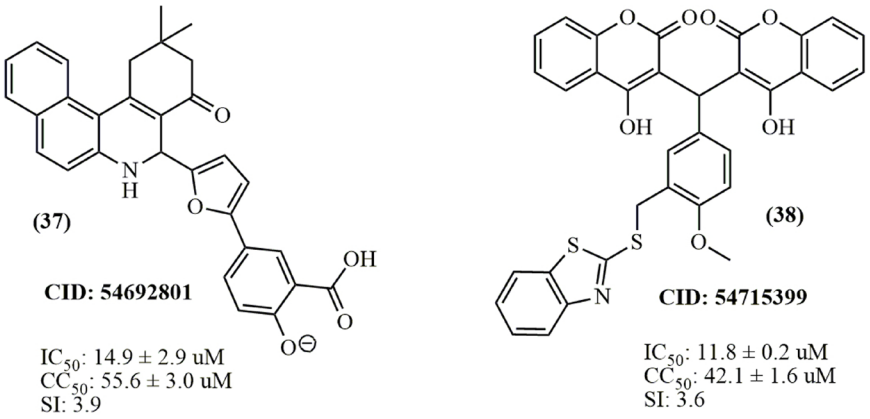


Figure 18: Piperidine and flavanone analogs synthesized by Cabarcas-Montalvo *et al.*

In a study performed by Mirza *et al*. [80], approximately eighteen million compounds from the ZINC® drug bank were virtually screened toward NS2B-NS3 from DENV4 (PDB ID: 2VBC). Subsequently, five top-ranked compounds (39-43) (**Figure 19**) were chosen, based on their predicted binding energy values with the catalytic triad residues. The authors have utilized different docking algorithms and virtual approaches [81–83] to predict their energy binding (E_b) upon that target, suggesting that these compounds are the most promising for drug development as anti-DENV agents, due to their affinity toward the catalytic triad.

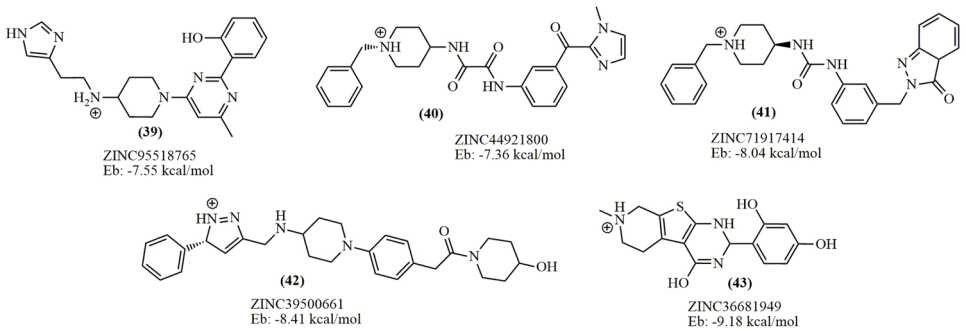


Figure 19: Cationic heterocycle compounds selected based on virtual screening performed by Mirza et al.

Similarly, Byler *et al*. [7] developed a study involving protein-ligand docking studies based on homology-modeled structures of the ZIKV NS2B-NS3 protease, obtained from the crystal structure of Murray Valley encephalitis virus (PDB ID: 2WV9); ZIKV NS5 methyltransferase (MTase) from the JEV methyltransferase (PDB ID: 4K6M); and ZIKV NS5 RNA-dependent RNA polymerase (RdRp), based on the WNV RdRp (PDB ID: 2HFZ). The authors investigated a total number of 2263 phytochemical compounds. Among these, the terpenoids were found to be the poorest docking ligands, in particular, monoterpenoids. Finally, several natural compounds (44-48) (**Figure 20**) presented good selective docking.

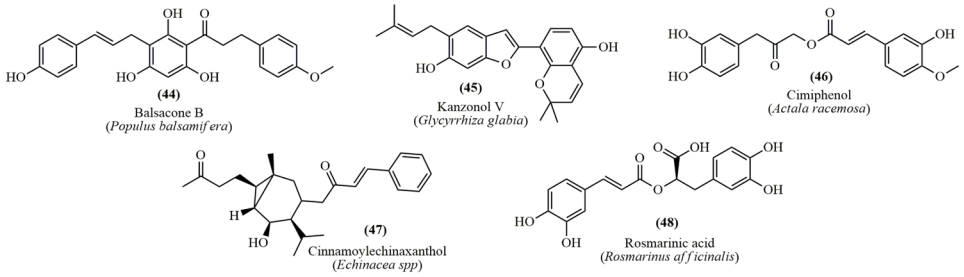


Figure 20: Promising phytochemical compounds against ZIKV.

Applying a fragment-based screening with 429 fragments from the Ro5 Maybridge® library, in combination with Saturation Transfer Difference NMR Spectroscopy (STD-NMR) as a read-out, Schöne *et al*. [41] performed an investigation in order to identify promising fragments to design novel antiviral agents against NS2B-NS3 WNV. After the STD-NMR competition assay, it was observed that 14 fragments are able to interact at the active site and other 22 fragments have more affinity for a second binding site. In **Figure 21**, the most promising molecular fragments (49-57) are shown.

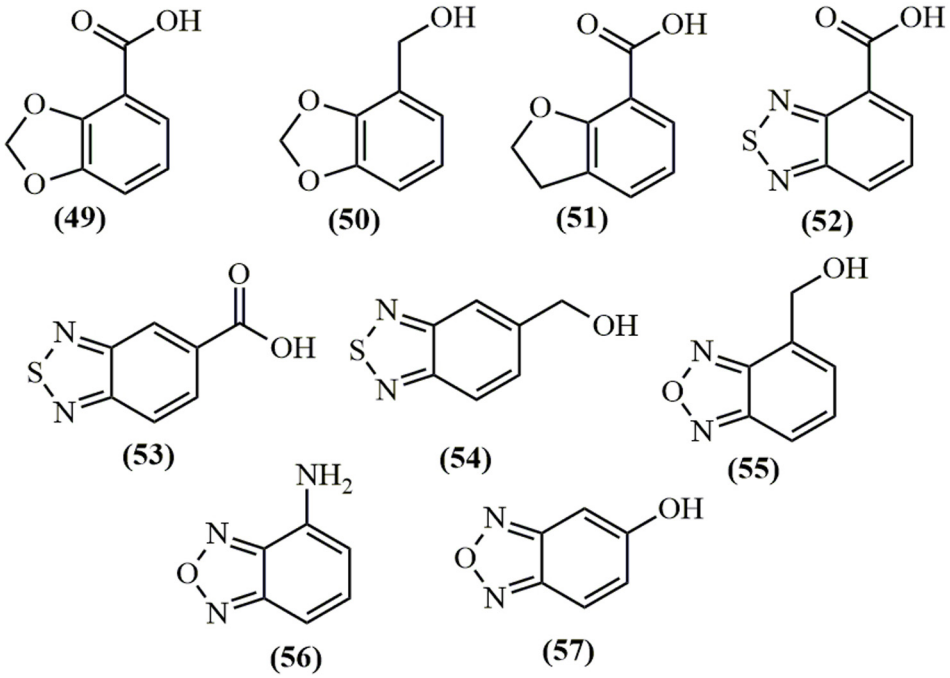


Figure 21: Promising phytochemical compounds against ZIKV.

Mirza *et al*. [84] performed a second study involving approximately eighteen million drug-like compounds from the ZINC® library, molecular dynamics (MD) simulations, molecular mechanics/Generalized Bor Surface Area (MM/GBSA) calculations, Quantum-Polarized Ligand Docking (QPLD), and Structure-based Pharmacophore Modeling (E-Pharmacophore) in order to identify promising hit compounds toward the DENV2 NGC strain. Additionally, cytotoxic effects and cell proliferation were evaluated in Huh-7 cells treated with the compounds pre and post infection. From these steps, four selective hit compounds were identified (**Figure 22**). However, these compounds only demonstrated significant results in post-treated cells. Compounds ZINC36681949 (58), ZINC44921800 (59), and ZINC39500661 (60) presented approximated inhibition values of 45, 100, and 100% at 100 μM concentration. In addition,

ZINC95518765 (61) was found to be the most active compound, with inhibition of 100% at 10 μM concentration. Furthermore, it is proposed to interact with two residues from the catalytic triad of the NS2B-NS3 DNV2, His51 and Ser135.

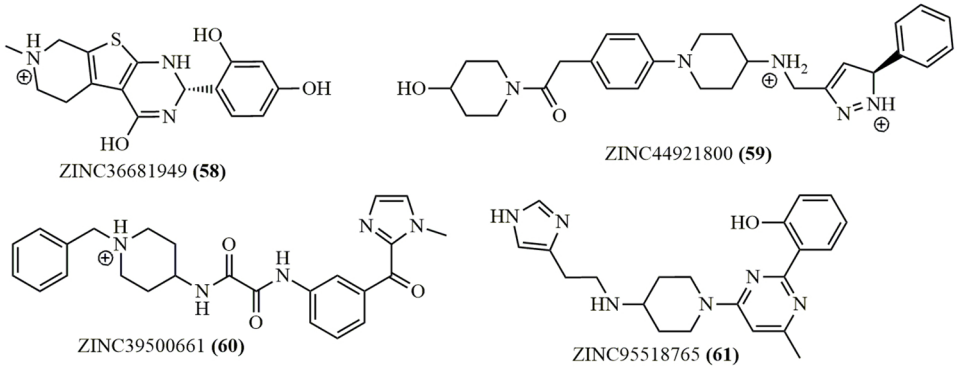


Figure 22: Drug-like compounds with promising activities against NS2B-NS3 DENV2.

Using similar techniques, such as molecular docking, dynamic simulations, and ligand-based pharmacophore modeling, Chen *et al*. [85] developed a virtual study to provide insights into DENV NS2B-NS3 protease inhibition. During this, a total of 55 DENV inhibitors were collected from different studies found in the literature [22,40,79,86–88], where 44 known active inhibitors and 11 inactives were used to compose the test set of compounds. From this, it was observed that two aromatic rings and hydrogen bond donor/acceptor groups are essentially necessary for binding into the catalytic cleft (**Figure 23**). Additionally, the hydrogen bonding is more important for site A, while hydrophobic and aromatic features are needed for both sites, B and C.

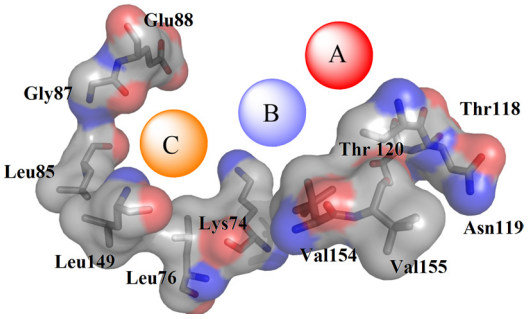


Figure 23: Molecular features essential for the DENV NS2B-NS3 inhibitors. Code: A - site for H-bond donor/acceptor groups; B and C - site for hydrophobic and aromatic substituents.

4. Conclusion

In general, arboviruses represent a significant health problem worldwide, with over 2 million people living in the risk zones, and considering the climate changes and the presence of *Aedes spp* mosquitoes. TheNS2-NS3 proteases from flaviviruses are considered as a valuable targets in drug development, due to their involvement in the replication process, encoding the viral structural and nonstructural proteins. Significant advances have been reported and studied by several different research groups. In sense, the compounds obtained from natural product's sources can be considered as the most moderate inhibitors. Although, flavanones represent an interesting alternative as NS2-NS3 inhibitors. With respect to the synthetic com-

pounds, peptidomimetic, cyclopeptide, and dipeptide compounds are the most potent inhibitors toward NS2B-NS3 proteases. Additionally, one peptidomimetic compound associated with the boronic acid warhead, is the most active compound found in the literature. It has excellent activities against DENV, ZIKV, and WNV NS2B-NS3 protease, acting as a pan-flaviviral agent. Considering this, it is possible to suggest that boronic acids represent a great alternative to improve the anti-flaviviral activity. On the other hand, the synthesis of substrates can be used as an interesting alternative to the development of high-sensitive probes to detect NS2B-NS3 WNV. Finally, it is possible to verify that the application of different virtual approaches in drug development is the broadest method for the identification of new promising compounds. Furthermore, expressive advances have been made which provided insights into the molecular structure of NS2B-NS3 protease, as well as information about allosteric sites. In sense, beside the catalytic triad to be considered as essential to NS2B-NS3 protease, it is possible to suggest that a compound can act as a highly potent NS2B-NS3 inhibitor via a noncompetitive mechanism.

5. Acknowledgment

This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brazil (CAPES) - Finance Code 001. Also, the authors would like to give thanks to the National Council for Scientific and Technological Development (CNPq) - Brazil.

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