Archives of Virology (2018) 163:575–586
https://doi.org/10.1007/s00705-017-3641-5

REVIEW

Pharmacophoric characteristics of dengue virus NS2B/NS3pro inhibitors: a systematic review of the most promising compounds

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Received: 8 August 2017 / Accepted: 29 September 2017 / Published online: 16 November 2017

Abstract
Dengue virus (DENV) infection can lead to a wide range of clinical manifestations, including fatal hemorrhagic complications. There is a need to find effective pharmacotherapies to treat this disease due to the lack of specific immunotherapies and antiviral drugs. That said, the DENV NS2B/NS3pro protease complex is essential in both the viral multiplication cycle and in disease pathogenesis, and is considered a promising target for new antiviral therapies. Here, we performed a systematic review to evaluate the pharmacophoric characteristics of promising compounds against NS2B/NS3pro reported in the past 10 years. Online searches in the PUBMED/MEDLINE and SCOPUS databases resulted in 165 articles. Eight studies, which evaluated 3,384,268 molecules exhibiting protease inhibition activity, were included in this review. These studies evaluated anti-dengue activity *in vitro* and the IC_{50} and EC_{50} values were provided. Most compounds exhibited non-competitive inhibition. Cytotoxicity was evaluated in BHK-21, Vero, and LLC-MK2 cells, and the CC_{50} values obtained ranged from < 1.0 to 780.5 µM. Several groups were associated with biological activity against dengue, including nitro, catechol, halogen and ammonium quaternaries. Thus, these groups seem to be potential pharmacophores that can be further investigated to treat dengue infections.

Introduction

Emergent and re-emergent viral diseases represent a critical problem for public health [1]. The four dengue virus serotypes (DENV 1-4), which are transmitted by *Aedes* mosquitoes, are considered some of the most important re-emergent arboviruses with regard to geographic distribution and infection incidence [2]. In the last few decades, there has been a dramatic epidemiological increase in dengue virus serotype and vector distribution worldwide [3]. Approximately 400 million cases of this infection are reported every year. Severe cases affect about 500,000 patients, of which more than 20,000 patients die due to hemorrhagic complications [2, 3].

Currently, there has been extensive focus on vector control. Studies with novel biotechnological interventions, such as sterile insects, paratransgenesis (e.g. symbiotic bacteria *Wolbachia pipientis*), and production of genetically modified vectors have shown good results, however, they are still preliminary and limited in scope [4]. On the other hand, despite advances achieved through immunotherapy, there is no effective and safe multivalent vaccine against all four serotypes [5, 6]. Specific licensed drugs to treat the disease are also inexistent, and the measures currently used are only

Handling Editor: Tim Skern.

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palliative, based on patients' overall health and comfort [7]. Therefore, there is an urgent need to search for new molecules to reduce viral load and prevent disease progression to more severe forms [8, 9].

Dengue viruses have small particles (40-60 nm in diameter) comprised of positive single-stranded RNA (ssRNA+) containing 10,173 nucleotides. Their genome contains a single open reading frame (ORF) that is translated into a unique polyprotein (Fig. 1A) [10]. Host and viral proteases cleave this polyprotein, producing seven non-structural (NS) (i.e. NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) and three structural (i.e. C, prM, and E) proteins [10–13]. Among the non-structural proteins, NS3 (69 KDa) is a multifunctional protein complex (Fig. 1B), and its C-terminal region (Fig. 1C) exhibits helicase (NS3Hel), nucleotide-triphosphatase (NTPase) and RNA-triphosphatase (RTPase) activities, which are essential during viral genome replication and transcription [14]. The *N*-terminal region of NS3 is very unstable and its active enzymatic conformation is dependent on the NS2B accessory protein [15–17]. The NS2B/NS3pro complex processes polyproteins in regions of the infected cell where the cellular proteases furin and signalase may not have access, and this activity is essential during viral replication and maturation [15, 18]. The protease activity of NS2B-NS3 is essential for virus proliferation: NS3 mutant viruses containing mutations in the active site of the enzyme are non-infectious [19]. Therefore, the interaction between NS2B and NS3pro is a promising molecular target for the development of new therapeutic agents against DENV infection. However, one of the major obstacles in drug development targeting the NS2-NS3 interaction is that the active site of the protease is flat and it would require a substantial conformational change in NS2B to enable the inhibitor to bind [20].

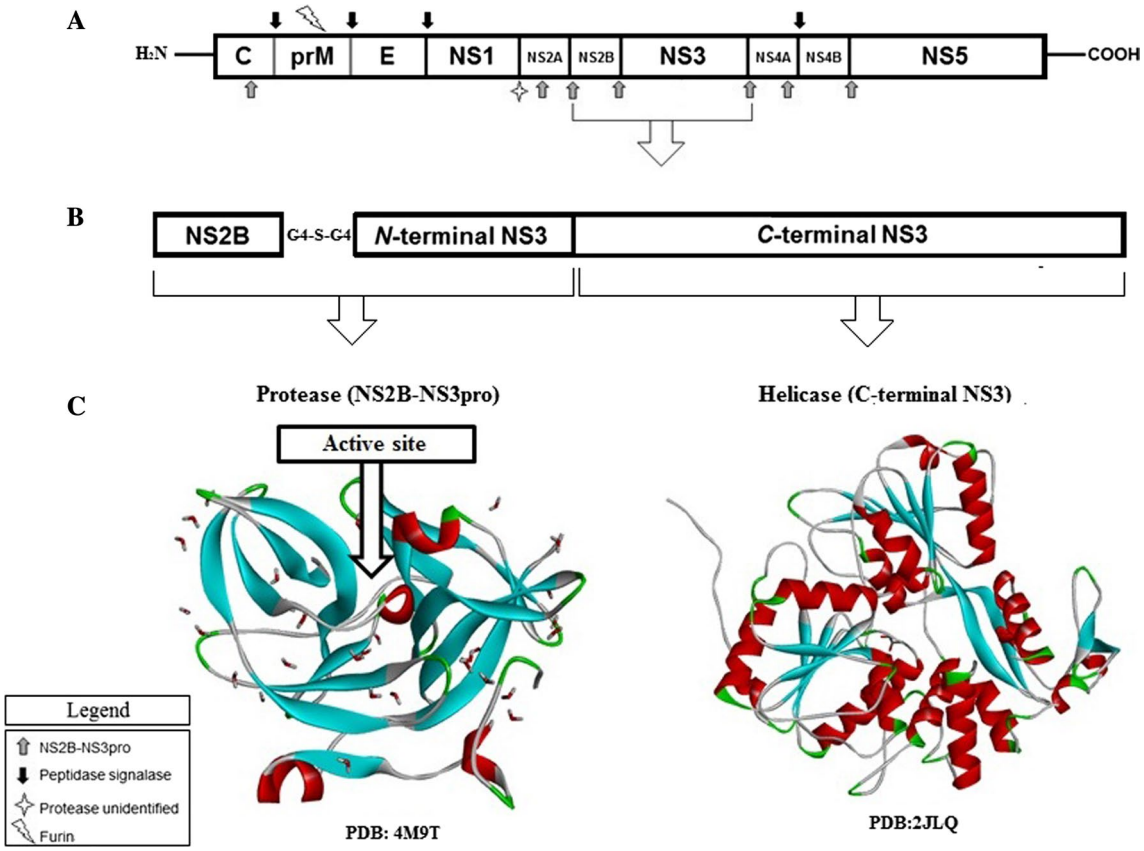


Fig. 1 Schematic representation of DENV polyprotein organization and processing, highlighting NS3. **A**) The viral genome is comprised of a single-stranded positive sense RNA, containing one single open reading frame (ORF). The DENV genome encodes a precursor polyprotein, which is cleaved by cellular (furin and secretase) and viral (NS2B/NS3pro) proteases, generating three structural (C, E, and prM) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. **B**) The NS3 protein is considered multifunctional and essential to DENV multiplication and maturation, showing distinct enzymatic mechanisms. The *N*-terminal region associates with NS2B to form the protease complex NS2B/NS3pro, which is essential for the cleavage of the polyprotein at several points (indicated by grey arrows). The *C*-terminal region is highlighted and has a helicase activity, which is important to separate double-stranded RNA that is formed during viral replication. **C**) Crystal structure of NS2B/NS3pro complex and helicase obtained from Protein Data Bank (PDB)

NS2B/NS3pro is a chymotrypsin-like protease that has a catalytic triad formed by His51-Asp75-Ser135, which is essential for its biological activity [21, 22]. Upon binding of the substrate, the amino group of His51 binds to the carboxylic group of Asp75 via a hydrogen bond, leading the hydroxyl group of Ser135 to attack the peptide bond by acting as a nucleophile [10, 19]. Subsequently, a tetrahedral acyl-enzyme is formed, in which the negative charges of the carbonyl oxygen are stabilized through hydrogen bonds into the oxyanion hole, formed by Gly151, Gly153, and Ser135 (Fig. 2) [21]. The instability of the negatively charged oxygen induces the spontaneous collapse of the tetrahedral, forming a double bond and breaking the peptide bond [22]. The NS2B protein, functions as the cofactor for NS3pro and residues 35–48 in the central region of the protein are required for the protease activity *in vitro*, while its *N*- and *C*-terminal flanking hydrophobic regions are predicted to anchor the NS2B/NS3pro complex into the membrane of the endoplasmic reticulum (ER) [21]. Expression of recombinant NS2-NS3pro containing only the central hydrophilic region of NS2B is sufficient to yield a soluble and active protease. This recombinant $NS2B_{Hydrophilic}$/NS3pro has greatly facilitated early *in vitro* inhibition screening tests and provides a fast and cost-effective platform [23]. Thus, an inhibitor that would be able to bind to any amino acid residues that form catalytic triads, the oxyanion hole or the hydrophilic region of NS2B would be a promising candidate for inhibition of DENV NS2B/NS3pro [24].

Another important target for anti-DENV drug development is the NS5 protein, which is the largest protein encoded by the flavivirus genome and also the most highly conserved, with approximately 67–82% amino-acid sequence identity between the four serotypes of DENV [25]. It bears the RNA-dependent RNA polymerase (RdRp) and methyltransferase (MTase) activities, which are essential for viral genome replication. In addition, NS5 interacts with different host proteins to counteract the IFN-α mediated-antiviral response [26]. Since host cells lack RdRp, NS5 represents a promising antiviral target for the design of specific inhibitors with low toxicity [27].

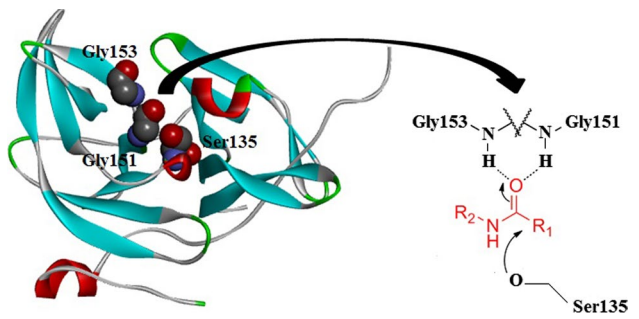


Fig. 2 Crystal structure of NS2B-NS3pro complex (PDB: 2FOM) highlighting the oxyanion hole. In the reaction catalyzed by the protease, Gly151 and Gly153 residues stabilize the negative charge of the oxygen. The Ser135 residue interacts with the carbonyl carbon to ensure stability of the intermediate. These three residues together form the oxyanion hole

Of note, the inhibition of viral proteases has been one of the most successful approaches for discovery of new antiviral therapies. For example, inhibitors of human immunodeficiency virus (HIV) proteases such as tazanavir, darunavir, ritonavir, and saquinavir are currently available, and are effective in the control of viral load in HIV-infected patients [28]. Therefore, the discovery of bioactive compounds that target NS2B/NS3pro protease complex may contribute to new therapies against dengue. Also, the NS2B/NS3pro sequence is highly conserved among other viruses classified within the *Flavivirus* genus, such as West Nile virus (WNV), Yellow fever virus (YFV), Japanese encephalitis virus (JEV), and Zika virus (ZIKV), making this protein complex a multi-viral target [17, 29]. Despite numerous efforts to identify anti-DENV drugs, no NS2B/NS3pro inhibitor has advanced, to date, into clinical trials [30]. Here we conducted a systematic literature review to summarize data published in the past 10 years regarding the pharmacophoric characteristics of compounds active against DENV NS2B/NS3pro. In addition, this study describes how the pharmacodynamics of these hits influences their *in vitro* biological activity, and how that may contribute to an antiviral effect.

Materials and methods

Search strategies

A systematic search of PUBMED/MEDLINE and SCOPUS databases was performed by three independent researchers (C.L.A., M.S., and A.C.F.) in September of 2016. Medical Subject Heading (MeSH) terms were used to define describers. The keyword "dengue" was used in combination with the describers "antiviral", "NS3", and "inhibitors", using the connector "AND" between them. The search was limited to articles published between 2006 and 2016.

Inclusion and exclusion criteria

Researchers evaluated the eligibility of the papers found, in which titles, abstracts, and keywords were analyzed according to the following inclusion criteria: (1) evaluation of antiviral activity against DENV NS2B/NS3pro; (2) original results; (3) *in vitro* studies against DENV; and (4) enzymatic inhibition assays. Exclusion criteria were as follows: (1) review papers, notes, e-mails, editorials, letters, and papers that did not present original material; (2) evaluation of raw plant extracts exhibiting anti-dengue activity; and (3) studies of only *in silico* or *in vitro* antiviral activity

without enzymatic inhibition assays. A full text evaluation was done for studies that met the inclusion criteria. In case of any disagreement, a fourth researcher (W.G.L.) was consulted to reach a consensus regarding the inclusion/exclusion criteria of the study.

Data analysis

Articles that met the inclusion criteria were submitted for an analytical full text reading to identify variables of interest, as it follows: authorship, name and structure of the compound, cytotoxicity (cell line used, 50% cytotoxic concentration - CC_{50}, and selectivity index - SI), *in vitro* antiviral activity (50% effective concentration - CE_{50} and serotype being tested), enzymatic inhibition (50% inhibitory concentration - IC_{50}, and inhibition type), and intermolecular interaction. Data are summarized in Table 1 and authors made a critical analysis and interpretation from the information found.

Results

Literature search

In the first electronic search, 165 articles were identified after removal of duplicates (116 duplicates). These publications were then evaluated based on the eligibility criteria, specifically regarding their title and abstract. After the screening process, 46 relevant studies were identified and full text reading evaluations were performed. Finally, eight studies that met the eligibility criteria were selected for analysis and discussion. The remaining 38 studies did not perform analysis of *in vitro* antiviral activity against DENV, and they were considered as containing only enzymatic inhibition assays against NS2B/NS3pro. The search flowchart is shown in Fig. 3.

Study characteristics *in vitro*

The variables identified in the selected studies are summarized in Table 1. Results are grouped based on the antiviral activity of compounds against DENV-2 serotype (n = 8) [1, 23–28, 31]. Only one paper (Pambudi and co-workers) showed activity against all four serotypes [32]. In total, 3,384,268 molecules were evaluated in these studies. Compounds were screened based on their ability to inhibit the protease complex NS2B/NS3pro in studies by fluorescent (n = 6; 15 compounds) [1, 32, 33, 35–37] and colorimetric methods (n = 2; 2 compounds) [34, 38], molecular docking (n = 5; 12 compounds) [1, 32, 33, 35, 36], ELISA binding assay (n = 1; 7 compounds) [35], and other methods that investigate physical-chemical characteristics of inhibitor-enzyme complexes (n = 2; 8 compounds) [1, 36]. The IC_{50} values for inhibition of the NS2B/NS3pro of DENV ranged from 1.0 to 120.0 µM. Most compounds showed compatible kinetics with a non-competitive inhibitor (n = 4) [32, 34, 36, 38]; two compounds were characterized as competitive inhibitors [1, 37], with an EC_{50} ranging from 0.1 to 10.4 µM. The renal cell lines BHK-21 (n = 4) [1, 34, 37, 38], Vero (n = 2) [32, 36], and LLC-MK2 (n = 1) [33] were the most frequently used cell lines to perform antiviral activity and cytotoxicity assays; one study also used the hepatic cell line HepG2 (n = 1) [35]. The CC_{50} values reported were widespread, ranging from <1.0 to 780.5 µM. However, the SI values of the molecules were within an acceptable therapeutic range, varying from 7.7 to 172.0, except for compound 8 [1], which showed a narrow safety margin (SI <1).

In silico studies

Intermolecular interactions that favor the association between ligand-receptor, which are essential for biological activities, were evaluated using theoretical methods (n = 5) [1, 32, 33, 35, 36] and/or by site-directed mutagenesis (n = 3) [34, 37, 38].

Intermolecular interactions through hydrogen bonds (HB), and hydrophobic interactions (HP), were the most frequently reported. However, four reports did not describe intermolecular interactions [33, 34, 37, 38]. Only molecules described by Tomlinson and co-workers [33] formed interactions with amino acids of the catalytic triad (His51 and Ser135). However, other molecules formed interactions with important residues found in the NS2B/NS3pro catalytic region, such as Gln27, Glu66, Glu80, Gln106, and Arg133 (n = 4) [1, 32, 34, 38].

Discussion

Dengue is the arbovirus with the greatest potential for global dissemination and the risk of a pandemic is a real possibility [3]. Because there are no effective drugs to treat the different stages of the disease, it is important to search for novel biomolecules to develop antiviral drugs against dengue [7]. These compounds must be able to reduce the viral load and prevent the progression to more severe stages of disease, which can lead to lethal hemorrhagic complications [39].

Studies using *in vitro* and *in silico* methods can help to identify compounds that exhibit affinity to likely targets already characterized for DENV [40, 41]. As such, NS2B/NS3pro has an essential role in the replication of flaviviruses, and is considered an important target based on the potential antiviral effects against DENV [18]. Here, we investigated the pharmacological characteristics of *hits* against NS2B/NS3pro based on studies published in the last

Table 1 Summary of the chemical structures and biological proprieties of the main compounds that exhibit inhibitory activity against DENV NS2B/NS3pro

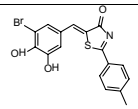
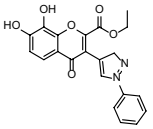
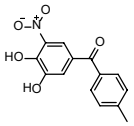
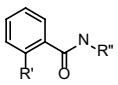
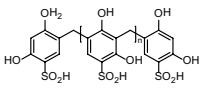
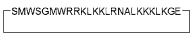
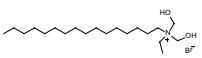
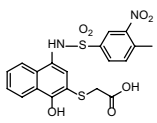
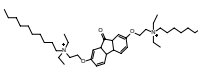
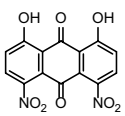
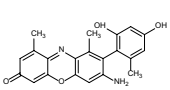
	Compound	Anti-Dengue activity		Cytotoxicity activity			Anti-NS3 Activity			Inhibition type	References
		Serotype	EC_{50} (μM)	Cell Line	CC50 (μM)	IS	Method	IC_{50} (μM)	Interations		
	Compound C	DENV-2	6.9 ± 0.6	BHK-21	C: 76.11	11.02	FPA	4.05 ± 0.18 [a]	ND	Competitive	[37]
	Compound E	DENV-2	10.4 ± 4.2	BHK-21	80.01	7.69	FPA	4.84 ± 0.60 [a]	ND	Competitive	[37]
	Compound F	DENV-2	2.29 ± 0.3	BHK-21	29.16	12.73	FPA	0.98 ± 0.06 [a]	ND	Competitive	[37]
	**Figure 6**	DENV-2	1: 3.5±0.3 2: ND 3: 0.1±0.0 4: 0.3±0.1 5: 0.9±0.1 6: 0.8±0.2 8:>3	Vero	1: 30 [b] 2: <1 [b] 3: 1 [b] 4: 3 [b] 5: 10 [b] 6: 10 [b] 8: 3 [b]	1: 8.6 2: ND 3: 10 4: 10 5: 11 6: 12.5 8: <1	FPA, HPLC-EA, MT-BA, and MD	1:98±4; 31.8±4.5 [c] 2: 34±5; 5.4±2.9 [c] 3: 22±1; 21±4 [c] 4: 26±1; ND [c] 5: 66±3;12.3±2.2 [c] 6: 4.2±0.4;0.9±0.1 8: 3.6±0.1;9.1±1.0 [c]	3: HB with Asn152 6: HB with Thr120, Gln167, Asn152, Lys73. [d] 8: HB with Asn152, K73, Lys74.	Non competitive	[36]
	Policresulen	DENV-2	8.48	BHK-21	780.58	92.07	FPA, SPR, DSC, UV spectral analysis and MD	0.82	HB: Gln106, Gln114, Thr132, Arg133 [e] HP: Ile109, Ile115, Val131 E: His130	Competitive	[1]
	Ltc1	DENV-2	24 hrs: 8.3±1.2 48 hrs:7.6 ± 2.7 72 hrs: 6.8 ±2.5	HepG2	52.51±3.6	7.72 (72hrs)	FPA, ELISA-BA and MD	40°C: 6,58±4,1 37°C: 12.68±3.2	Hydrophobic interaction [f]	ND	[35]
	BP13944	DENV-2	1.03 ± 0.09	BHK-21	72.40±0.95	69,94	CPA	22.63 ± 0.74	Important interactions with Glu66 [g]	Non competitive	[38]
	SK-12	DENV-1 DENV-2 DENV-3 DENV-4	0,97 ± 0,42 0,98 ± 0,39 2,43 ± 0,63 0,74 ± 0,48	Vero	67.26±7.00	69,34 68,63 27,67 90,76	FPA and MD	120	HB: Lys26, Gln27 [h], Arg24, Met59, His60. HP: Leu58, Met59, Ile25.	Non competitive	[32]
	BP2109	DENV-2	0,17 ± 0,01	BHK-21	29.28±0.43	172	CPA	15,43 ± 2,12	Important interactions with Arg55 and Glu80 of NS2B [h]	Non competitive	[34]
	ARDP0006	DENV-2	4.2±1.9	LLC-MK2	69±4	16,6	FPA and MD	ND	Interaction involving Gln35, His51, Ser135, Gly151, Gly153	ND	[33]
	ARDP0009	DENV-2	35 ± 8	LLC-MK2	>300	>8.6	FPA and MD	ND	Interaction involving Gln35, His51, Ser135, Tyr150, Gly151, and Gly153	ND	[33]

Table 1 (continued)

FPA: Fluorescent peptide substrate assay; CPA: Colorimetric peptide substrate assay; MD: Molecular docking; ELISA-BA: ELISA binding assay; HPLC-EA: High Performance Liquid Chromatograph based enzyme assay; MT-BA: Binding assay based on microscale thermophoresis; SPR: Surface plasmon resonance (SPR) technology-based assay; DSC: Differential scanning calorimetry assay. HB: Hydrophilic interactions; HP: Hydrophobic interactions. E: Electrostatic. ND: Non-determined

[a]Values refer to the IC50 for the NS2B-NS3 protease complex of DENV-2. The study also carried out protease activity assays for DENV-1 (IC50: C- 4.06±0.21, E- 6.20±1.20, F- 1.15±0.1), DENV-3 (IC50: C- 2.94±0.18, E- 5.0±1.40, F- 0.91±0.06) and DENV-4 (IC50: C- 3.40±0.11, E- 5.32±0.60, F- 0.64±0.03). Another compound that was able to inhibit NS2B/NS3pro but did not exhibit anti-DENV-2 activity was not included in this review

[b]This value refers to the concentration at which no influence on cellular metabolism was detected

[c]The first value of average ± error refers to the inhibition of proteases of DENV-2 serotypes and the second value refers to the inhibition of proteases of DENV-3 serotypes

[d]The compound 6 also makes hydrophobic interactions involving K73, K74, T120, T168, T118, Q167, Q88, N169 and I123 residues

[e]The single mutations Q106G and R133G, and the double mutation Q106G/R133G significantly increase the IC_{50} of policresulen from 0.48 μM to 4.99 μM, 4.3 μM and 60.8 μM, respectively. These results showed the importance of Q106 and R133 residues for the pharmacodynamics of this compound

[f]The study did not report which residues were targeted, but it was shown that four leucine and two tryptophan residues of the peptide inhibitor (ltc1) participate in hydrophobic interactions with amino acid residues of NS2B-NS3pro

[g]The E66G substitution in the NS3 protease region reduced its sensitivity to BP13944 at 22.63 ± 0.74 μM to 71.10 ± 0.79 μM

[h]Q27A mutation significantly reduced the sensitivity of DENV-4 to SK-12 (EC50: 10.77 ± 2.53μM against DENV-4 Q27A mutant vs. 3.79 ± 2.54 μM against wild-type DENV-4)

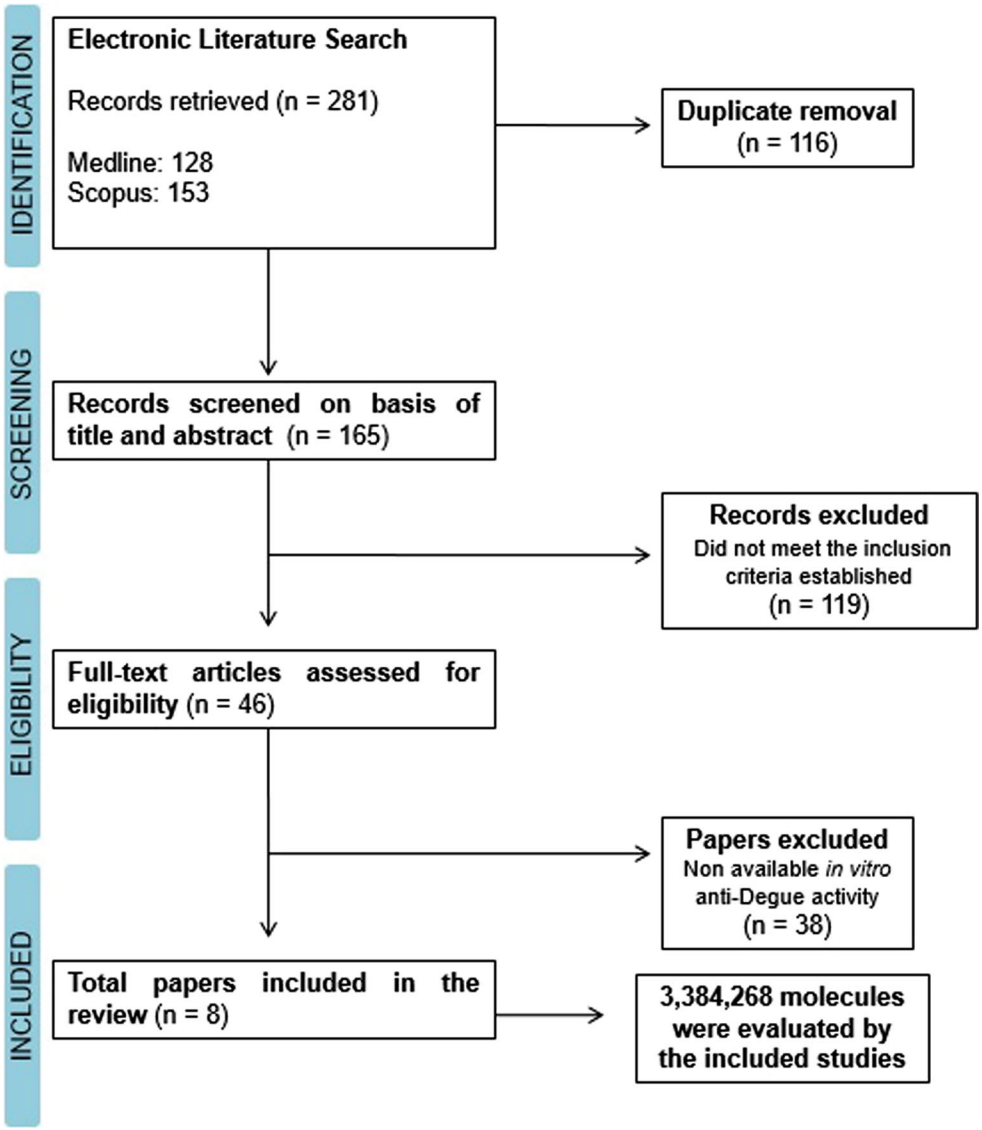
[i]The double mutation R55K and E80K increased the IC50 for NS2B/NS3pro from 15,43 ± 2,12 μM in the wild type virus to 158.44 ±1.20 μM in the mutated virus. Additionally, the EC50 for DENV-2 containing this double mutation (R55K/E80K) was 73.8-fold higher than that of the wild type virus

10 years, focusing on pharmacophoric analysis and the possible effectiveness of these compounds as antivirals.

Several studies have reported the therapeutic potential of compounds containing the catechol group against several pathogens [42–47]. This group of compounds, characterized as dihydroxybenzene, when combined into the structure of a cephalosporin, forms an adduct compound. This significantly increases the activity against various species of bacteria, including multi-resistant strains [42, 44, 47]. The antiviral effect of catechol compounds has also been reported for retroviruses [43, 46] and influenza virus [45]. Balasubramanian and co-workers [37] reported that compounds generically named C, E, and F showed effective inhibitory effects against DENV NS2B/NS3pro (IC_{50} 1.0-4.8 μM), suggesting that the catechol group is related to the pharmacologic activity [37]. These compounds showed high anti-dengue effects (EC_{50} 2.3-10.4 μM) and wide selective indexes (7.7-12.7). The binding efficiency of inhibitors against the NS2B/NS3pro active site measured by PatchDock score (compound C: 3784, compound F: 3340) and by atomic energy (ACE) (compound C: -236.1, compound F: -182.0), indicated a stable association with the catalytic region of the molecular target, suggesting a competitive inhibition [28, 48]. In fact, kinetic studies have shown that these inhibitors are competitive (Fig. 4A).

The presence of the catechol moiety also promoted the biological activity of benzothiazole derivatives [36] (Fig. 5). Methylation of the hydroxyl group in the catechol ring reduced the antiviral biological activity of compound 7 (10% inhibition at 50 μM), compared to compound 8 (100% inhibition), in which the catechol group was maintained (Fig. 5) [36]. On the contrary, Balasubramanian and co-workers [37], found that the molecules with catechol groups associated with non-competitive inhibition, which was confirmed by kinetic analysis. In the benzothiazole, the hydroxyl group favors hydrogen bonding with two major residues (Asn152 and Lys73) localized in a region external to the enzyme active site.

In addition, nitro-substituted benzothiazole is a likely DENV NS2B/NS3pro inhibitor [36]. The number of nitro groups was an important predictor of the pharmacological effect. Compound 1 (EC_{50} 3.5 ± 0.3 μM), which is mononitrated, has lower antiviral activity compared to bi-nitrated substances, such as compound 4 (EC_{50} 0.3 ± 0.1 μM). The presence of halogens also enhanced the antiviral activity of nitro-substituted compounds, reducing the effective concentration up to 35-fold (compound 1 EC_{50} 3.5 ± 0.3 μM vs. compound 4 EC_{50} 0.3 ± 0.1 μM) (Fig. 5) [36]. Previous reports confirm the importance of the nitro group and halogens in the determination of antimicrobial activity [49, 50]. Hydrazides that have nitro and chloride substituents have high antibacterial, antifungal, and antiviral activities [49]. Vesicular stomatitis virus (VSV) was particularly sensitive to the antiviral activity of these compounds, and like DENV, is an enveloped RNA virus [49]. Nevertheless, the presence of the nitro group increased the cytotoxic effects of the compounds, although SI was still satisfactory in the majority of cases [36]. The nitro group, as well as the catechol group, effectively formed hydrogen bonds with the Asn152 residue, suggesting that it is essential for antiviral activity (Fig. 4B).



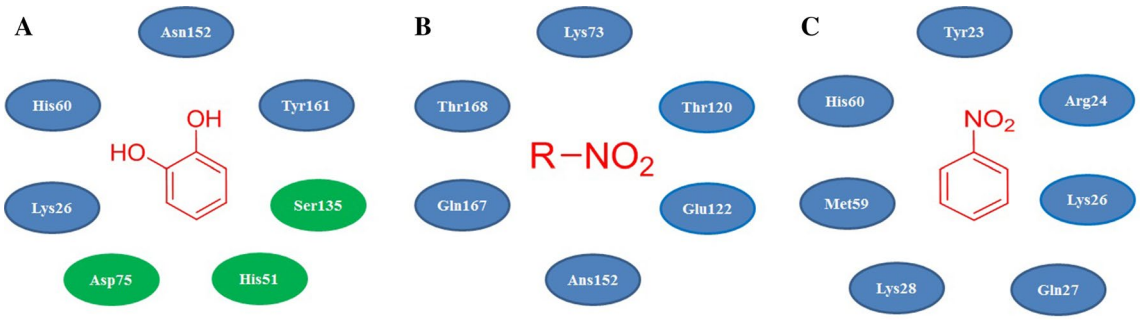


Fig. 3 Search strategy for the systematic review

Fig. 4 Interaction between the pharmacophoric groups: nitro, catechol, and nitrobenzene, with DENV NS2B/NS3pro. (**A**) Schematic representation of the interaction between compound C with DENV NS2B/NS3pro. The catechol group interacts with two residues of the catalytic triad (Ser135 and His51) [37]. (**B**) Schematic representation of a nitro group showing the hydrogen bonds and hydrophobic interactions with the Lys73, Thr120, Asn152, and Gln167 residues. The hydrogen bond between the Asn152 residue and the nitro group seems to be essential for antiviral activity [36]. (**C**) Schematic representation of the interaction between the nitrobenzene rings from compound SK-12 with DENV NS2B/NS3pro. This compound was predicted to interact with the Lys26, Gln27, Met59. and His60 residues [32]. The residues of the catalytic triad are shown in green

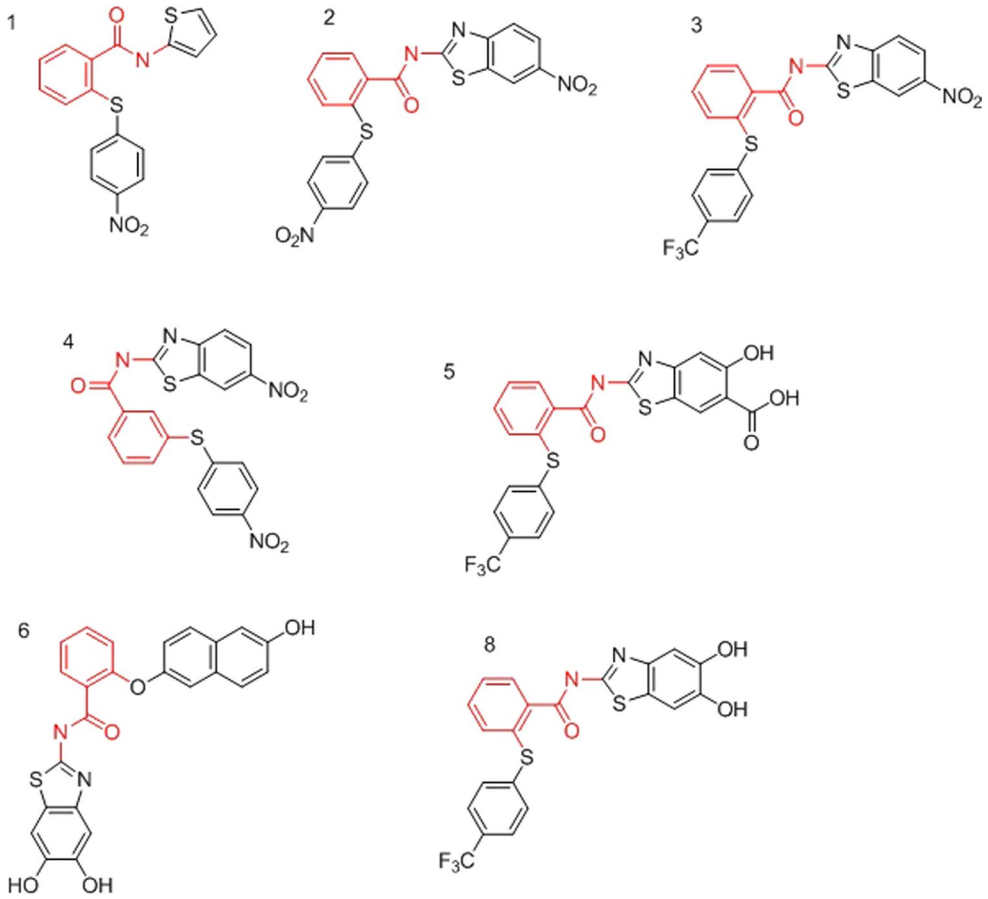


Fig. 5 Chemical structures of compounds tested in the study by Wu and co-workers [36]. The generic names of these compounds were maintained with the same Arabic numerals used by the authors (included in Table 1). Functional groups are identified in red

Furthermore, amino acid sequence alignments of NS2B/NS3pro showed that Asn152 is a conserved residue among DENV serotypes [51]. In fact, compounds 3, 6, and 8, all of which showed interaction with the Asn152 residue, showed similar activity towards the two serotypes tested (DENV-2 and DENV-3) [36]. The binding site for the benzothiazoles is a hydrophobic pocket localized in a deep region to the right and below the enzyme active site, being formed by the side chains of the Lys73, Lys74, Thr120, Thr168, Thr118, Gln167, Gln88, Asn169, and Ile123 residues [36].

Other studies also reported the importance of the nitro group for anti-dengue activity. The nitrobenzene ring was essential to the interaction of the compound SK-12 with DENV NSB/NS3pro. The nitrobenzene ring acts as a hydrogen bond acceptor for Gln27, which when swapped for an alanine through site-directed mutation, significantly reduced the activity against DENV-4 (Fig. 4C) [32]. Gln27 is localized in a region outside the binding site; kinetic assays confirm its non-competitive inhibition. Therefore, an increase in the concentration of the enzyme's natural substrate, which is the viral polyprotein, in response to virus proliferation, will not compromise the antiviral activity of the compound [32]. A similar antiviral effect was found against DENV-1, DENV-2, and DENV-4 whereas for DENV-3, it was lower. The Gln27 residue is conserved among these four serotypes, suggesting a broad anti-dengue effect [51]. Even though many studies describe the toxic effect of nitro groups [52, 53], the compound SK-12 showed a high SI (27-90) [32].

Compared to SK-12, the nitro-compound known as ARDP0006 showed competitive inhibition. Although kinetic studies did not confirm this behavior, the interaction mediated by nitro groups of ARDP0006 with His51 and Ser135 residues, which comprise the NS2B/NS3pro catalytic triad, corroborate this conclusion. In addition, both ARDP0006 and ARDP0009 interact with Gly151 and Gly153 residues in the oxyanion hole. If the interaction with these two glycine residues is inhibited by compounds, NS2B/NS3pro enzymatic activity is highly compromised [24, 54]. ARDP0006 was significantly more effective against DENV-2 (4.2 ± 1.9 μM) than its analogue ARDP0009 (35.0 ± 8.0 μM). ARDP0006 has nitro-groups to form hydrogen bonds with amino acid residues, confirming the importance of this group as a pharmacophore to anti-dengue activity [21, 45].

Ammonium quaternary compounds have the potential to contribute to the development of antiviral drugs against DENV [23, 31]. Several studies have demonstrated the effective antimicrobial effect of substances containing a quaternized nitrogen, especially against *Staphylococcus aureus*

[55, 56] and *Mycobacterium spp* [57] species. Additionally, an antiviral effect has been shown against influenza virus A (H1N1; EC_{50} 6.9 μM) [58, 59], herpes simplex virus 1 (HSV-1; EC_{50} 48.0 μM, as effective as acyclovir) [60], Nipah virus (pNiV; IC_{50} 218.0-525.0 μM) [61], and Hendra virus (HeV; IC_{50} 778.0-2,679.0 μM) [61]. Noteworthy, Baron and co-workers [62] demonstrated that urine exhibits significant biological activity against numerous species of viruses in culture (e.g., vaccinia virus, vesicular stomatitis virus, herpes simplex 1 virus). This effect is attributed to the quaternary ammonium salts present in this biological fluid, supporting the assertion that these compounds likely have antiviral effects [62]. These findings suggest that quaternary ammonium compounds have the potential to be further investigated in the development of anti-dengue compounds. Two quaternary ammonium compounds (BP13944 and BP2109) showed effective activity against DENV-2 [23, 31] as NS2B/NS3pro non-competitive inhibitors. Studies involving site-directed mutagenesis, in DENV, revealed that mutations in the Glu66 residue are associated with low efficiency of the compound BP13944 [38] whereas changes in the Arg55 and Glu80 residues, were predicted to be ineffective (BP2109) [34]. Additionally, the cytotoxic concentration was found to be lower than the effective concentration, showing that these compounds are selective regarding their pharmacological target. However, the clinical pharmacokinetic properties of ammonium quaternary compounds do not make them feasible to use [63, 64]. Oral administration is not an option; in fact there is a need for parenteral administration, which requires trained professionals to administer these drugs to patients [65]. Moreover, the neuromuscular blockage caused by many ammonium quaternary compounds raises safety questions as to their clinical use [66, 67].

The use of peptides as protease inhibitors has been indicated as an effective therapeutic approach, independent of viral disease [68]. Peptidomimetic compounds showed effective antiviral effects against the NS3/4A protease in hepatitis C virus, taxonomically a member of the *Hepacivirus* genus and *Flaviviridae* family [69]. In addition, peptide inhibitors have been shown to be active against a variety of flaviviruses, such as WNV [70], JEV [71], and YFV [72]. In this context, Rothan and co-workers [35] described the antiviral effect of the peptide latarcin (LTC-1) against NS2B/NS3pro. The activity against DENV-2 was satisfactory, reaching its maximum effect after 72 hours; the concentration of the protein NS1 was considerably reduced in treated cells [35]. Molecular docking assays investigated interactions in the active site of the viral protease, involving the Ser135 residue [21]. The antiviral activity was greater when treatment was performed during (viral load of 0.7 ± 0.3 pfu mL^{-1}) and after (viral load of 1.8 ± 0.7 pfu mL^{-1}) cells were infected with DENV; the antiviral activity was lower when the virus was exposed to the compound prior to infection (viral load of 4.5 ± 0.6 pfu mL^{-1}). This indicates that the peptide does not have virucidal activity. Moreover, inhibition of the NS2B/NS3pro enzyme was almost two-fold greater at 40 °C (IC_{50} 6.6 ± 4.1 μM) compared to 37 °C (IC_{50} 12.7 ± 3.2 μM), suggesting that the pharmacological effect of the peptide is expected to be greater in symptomatic disease patients infected with dengue virus [35].

Like peptides, polymeric structures have been shown to have antiviral effects. Nucleic acid polymers, for instance, show significant activity against HIV-1, hepatitis B, and hepatitis delta virus [73]. In addition, cationic peptides, constituted from polyethyleneimine, have been shown to be inhibitors of human papillomavirus and cytomegalovirus replication, in concentrations lower than the cytotoxic dose [74]. Wu and co-workers [1] revealed that policresulen, a sulfonated polymer, was effective against DENV-2 NS2B/NS3pro activity (IC_{50} 0.4 μM). Policresulen bound to the active site of the enzyme, characteristic of competitive inhibition [1]. This polymer interacted with catalytic residues, such as Gln106 and Arg133. Site-directed mutagenesis involving the substitutions Gln106Gly and Arg133Gly, as well as the double mutation Gln106Gly/Arg133Gly, increased the IC_{50} values of policresulen from 0.5 μM to 5.0 μM, and 4.3 μM to 60.8 μM, respectively. Additionally, analysis of the ultraviolet–visible (UV) spectrum of the protein, and evaluation of its thermal stability, revealed that policresulen can also reduce the structural stability of NS2B/NS3pro [1], suggesting the overall physical-chemical structure of the protein is compromised.

Conclusions

This review reports the main pharmacophoric groups of dengue virus NS2B/NS3pro inhibitors evaluated in the last 10 years. Selected studies investigated nitro, catechol, halogen, and nitrogen quaternary compounds as well as peptides and sulfonated polymers. Potential targets were further investigated for antiviral activity against DENV. In addition, these compounds and chemical groups have low toxicity, suggesting that they could be used clinically. However, there is little evidence of the pharmacokinetic characteristics of these compounds. One of the main barriers in the development of antiviral agents is the optimization of their absorption, distribution, metabolism, and excretion (ADME) properties [75]. The effective inhibitory activity found in kinetic studies *in vitro* with NS2B/NS3pro should not solely be used in determining the pharmaceutical potential of compounds. Thus, further studies should be conducted to characterize the kinetics and efficacy of these potential antivirals *in vivo*.

Acknowledgements W.G.L and C.A.L are grateful to FAPEMIG for master's degree fellowship.

Compliance with ethical standards

Funding This study was funded by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (UNIVERSAL 446997/2014-5 and 449984/2014-1) and Fundação de Amparo a Pesquisa de Minas Gerais (FAPEMIG) (EDITAL APQ-00557-14).

Conflict of interest All authors report that they do not have any conflicts of interest.

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

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