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ARTICLE in EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · OCTOBER 2014

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Mini-review

A perspective on targeting non-structural proteins to combat neglected tropical diseases: Dengue, West Nile and Chikungunya viruses



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ARTICLE INFO

Article history:

Received 13 September 2014

Received in revised form

29 September 2014

Accepted 4 October 2014

Available online 6 October 2014

Keywords:

Dengue virus

West Nile virus

Chikungunya virus

Non-structural proteins

Protease inhibitors

ABSTRACT

Neglected tropical diseases are major causes of fatality in poverty stricken regions across Africa, Asia and some part of America. The combined potential health risk associated with arthropod-borne viruses (arboviruses); Dengue virus (DENV), West Nile Virus (WNV) and Chikungunya Virus (CHIKV) is immense. These arboviruses are either emerging or re-emerging in many regions with recent documented outbreaks in the United States. Despite several recent evidences of emergence, currently there are no approved drugs or vaccines available to counter these diseases. Non-structural proteins encoded by these RNA viruses are essential for their replication and maturation and thus may offer ideal targets for developing antiviral drugs. In recent years, several protease inhibitors have been sourced from plant extract, synthesis, computer aided drug design and high throughput screening as well as through drug reposition based approaches to target the non-structural proteins. The protease inhibitors have shown different levels of inhibition and may thus provide template to develop selective and potent drugs against these devastating arboviruses. This review seeks to shed light on the design and development of antiviral drugs against DENV, WNV and CHIKV to date. To the best of our knowledge, this review provides the first comprehensive update on the development of protease inhibitors targeting non-structural proteins of three most devastating arboviruses, DENV, WNV and CHIKV.

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1. Introduction

Neglected tropical diseases (NTDs) are a group of infectious diseases, which though manageable, continue to be the leading cause of morbidity and mortality among the world's poorest populations [1–3]. These infectious diseases can be termed as promoters of both poverty and long-lasting health injustice especially to the poor; this certainly places arboviruses as NTDs. Dengue virus (DENV), West Nile virus (WNV) and Chikungunya virus (CHIKV) are re-emerging mosquito-borne viruses that are predominantly found in the tropics [4,5]. However, the rapidly evolving climatic conditions coupled to the worldwide distribution of their competent mosquito vectors have increased the potential of these vectors spreading to cause outbreaks in previously unaffected areas [6]. These positive sense RNA viruses belong to alphaviruses

(Chikungunya) and flaviviruses (Dengue and West Nile viruses) families and have life cycles that requires a vector and a definitive host, usually mammal [7]. Despite recent outbreaks in several parts of the world and the potential risk of spreading to new areas, there are currently no effective drugs or vaccines against these diseases and thus they pose a significant public health risk [8–10].

Annually, there are an estimated 50 million cases and 25,000 deaths of DENV with a further 2.5 billion people at risk of infection [4]. There are no effective vaccines or drugs available against any of the four DENV serotypes (DENV1-4) [11]. The 10.7 kb single-stranded DENV RNA genome encodes a single precursor polyprotein which is processed by viral and cellular proteases into three structural proteins: the capsid, pre-membrane, and envelope proteins and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) [11]. Similar to DENV, West Nile virus is a single stranded, positive-sense 11–12 kb RNA genome that encodes five non-structural proteins and three structural proteins [12]. Chikungunya virus is a positive sense single-stranded RNA genome of approximately 11.6 kb that is predominantly found in the tropics

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of Africa, Asia and Indian Ocean islands [13]. However, the rapidly evolving climatic conditions have widened the geographical distribution of CHIKV where it has acquired competent mosquito(6) placing it as a potential public health concern even in the temperate regions. This warranted the US National Institute of Allergy and Infectious Diseases (NIAID) to place CHIKV as a category C priority virus [14]. The majority of drug discovery efforts towards these three arboviruses target the non-structural proteins Table 1.

Due to a dearth of new chemical entities in the pipeline of pharmaceutical giants and the fact that these neglected tropical diseases are concentrated among world's poor, there is an urgent need to develop novel drug molecules against these diseases [15]. Open source drug discovery foundations, academic research, public-private partnerships, crowd collaboration projects have come together to foster a global effort to develop novel drug-like molecules targeting these neglected tropical diseases specifically against DENV, WNV and CHIKV [5,16].

Non-structural proteins are critical targets in the inhibition of viral replication and maturation. Recent success in designing novel drug-like molecules targeting hepatitis C [17] (Hepacivirus, family: Flaviviridae) has rejuvenated efforts to develop novel drug-like molecules (Fig. 1) targeting non-structural proteins in DENV, WNV and CHIKV.

Modern drug discovery approaches mainly rely on five major approaches; namely high throughput screening (HTS), natural products, drug repositioning, synthesis and computer-aided drug design in order to identify and develop potential lead molecules [18]. Recently, drug repositioning approach was applied to identify dasatinib and AZD0530 tyrosine kinase inhibitors as potential drug leads against DENV (Fig. 2) [19]. Thus, these approaches augment current efforts geared towards identifying novel chemical scaffolds against these NTDs.

The use of high throughput screening, natural plant products, synthetic products and computer-aided drug design to speed up identification of novel drug compounds has been well documented after the successful application of these methods in the development of maraviroc [20], artemisinin [21], HIV-protease inhibitors and tyrosine kinase inhibitors [22] (Fig. 3).

The availability of the resolved crystal structures of key DENV, WNV and CHIKV non-structural proteins as well as the identification of inhibitors targeting these non-structural proteins using structure-based drug design approach is a significant leap forward towards developing more potent and selective inhibitors(11, 23, 24). Moreover, an array of biological activity assay techniques for these inhibitors is an impetus in the development of novel inhibitors for these arboviruses. This review seeks to provide a comprehensive account of protease inhibitors targeting Dengue, West Nile and Chikungunya viruses' non-structural proteins, current status of drug discovery targeting these three arboviral diseases and the future prospective.

2. Non-structural proteins as emerging targets

Targeting viral proteases is a well established strategy for designing and developing effective drug molecules and novel leads. As previously highlighted [17], the huge success of protease inhibitors to combat two of the deadliest viral diseases, HIV and HCV, has triggered a global effort to target different viral encoded proteases across several viruses. The beginning of the past decade experienced a revamped effort in the scientific community aimed at developing potential drug-like compounds targeting neglected tropical diseases and viral proteases emerged as a promising drug candidates. The prominent role of the non-structural proteins in the life cycles of DENV, WNV and CHIKV as well as their structural similarity further informs the common approaches in combating these reemerging arboviruses.

2.1. Non-structural proteins of DENV

The DENV genome is a 10–11 kb positive-sense, single-stranded RNA genome that encodes three structural and five non-structural proteins. The non-structural proteins are involved in the post-processing of structural proteins and have emerged as potential targets for development of novel antiviral agents [11,25].

2.1.1. DENV non-structural protein 1 (NS1)

DENV NS1, the first of the five non-structural proteins of DENV is a hydrophilic membrane-associated homodimer synthesised in rough endoplasmic reticulum. The C-terminal residues of NS1 ranging from amino acids 271 to 352 are believed to be involved in the NS1 associated pathogenesis [11]. Interestingly, mutation in NS1 protein disrupts RNA synthesis RNA. Thus, NS1 may be vital in synthesis of viral RNA and ultimately in viral replication. Currently there is no crystal structure for NS1 protein as well as that of viral NS1-NS2A catalytic domain, which is a challenge in establishing the proper 3-D conformation of NS1 subunit and its implication in viral pathogenesis.

2.1.2. DENV non-structural protein 2 (NS2)

DENV NS2 consists of two subunits, NS2A and NS2B. NS2A contains several transmembrane domains and is an activator for correct processing of NS1 subunit. The NS2B subunit is an activator of NS3 for its proper functioning and together they form the active serine–protease complex [11]. NS2B behaves like a chaperone which helps in the folding of NS3 subunit in its active conformation, thereby playing the key role in regulating substrate–enzyme interaction as well as in membrane association (Fig. 4).

2.1.3. DENV non-structural protein 3 (NS3)

The 69 kDa multifunctional NS3 protein has protease, helicase, and nucleoside 5'-triphosphatase (NTPase) activities. In

Table 1

An overview of viral genome, encoded proteins and crystal structures of DENV, WNV and CHIKV.

Virus	Genome	Encoded proteins	PDB IDs of non-structural proteins
DENV (serotypes 1–4)	10.7 kb single-stranded positive RNA	Structural proteins: Capsid, Envelope, Fusion peptide Non-structural (NS): NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5	<ul style="list-style-type: none"> • NS2B-NS3: 4M9T [99], 4M9K [99], 4M9M [99], 4M9F [99], 4M9I [99], 4HHJ [100], 3P8Z [101] • NS3: 2VBC [29], 2WHX [102], 2M9P [103], 2M9Q [103], • NS5: 2J7W [31], 1L9K [104], 2XBM [105], • NS1: 4OIE [32], 4OII [32], 4O6C [106], 4O6D [106], • NS2B-NS3: 2YOL [33], 3E90 [107], 2IJO [108], 2GGV [108], 2FP7 [26], 2HCN [35], 2HCS [35], 2HFZ [35], • NS3: 2QEQ [109], • NS2: 3TRK [110], • NS3: 4TUO [111]
WNV	11–12 kb single stranded, positive-sense RNA	Structural proteins: Capsid, Envelope, Fusion peptide Non-structural (NS): NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5	
CHIKV	11.6 kb positive sense, single-stranded RNA	Structural proteins: Capsid, Envelope, Fusion peptide Non-structural (NS): NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5	

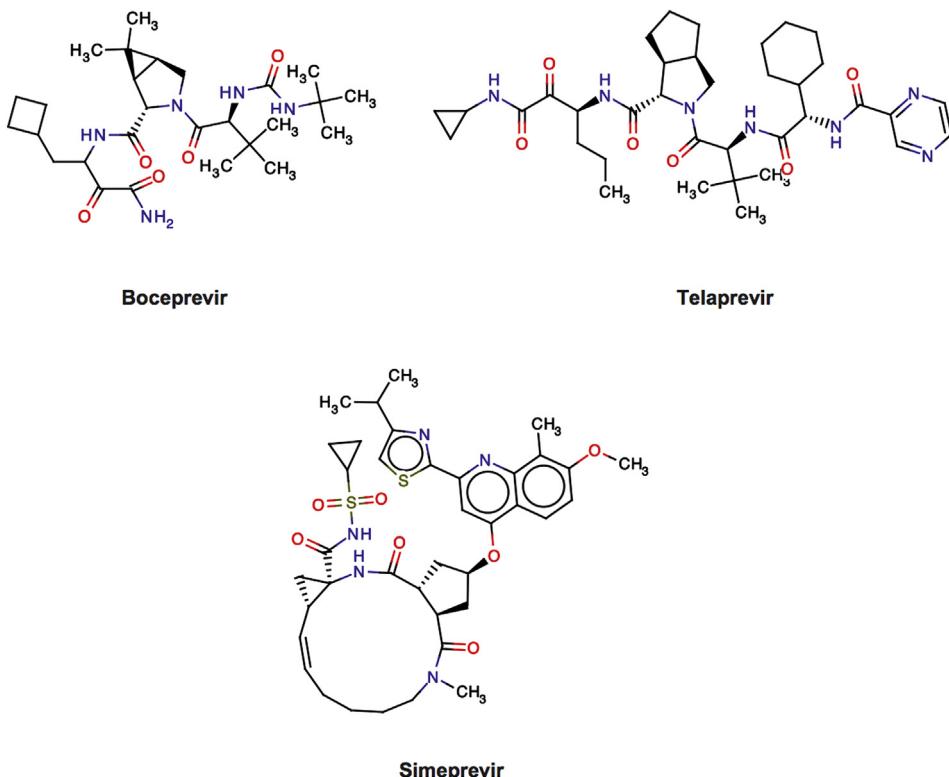


Fig. 1. Currently approved non-structural protease inhibitors against hepatitis-C virus.

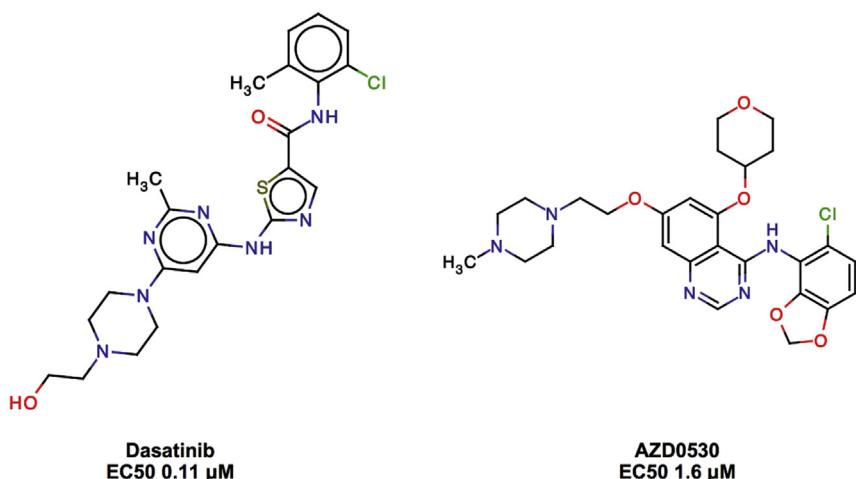


Fig. 2. The repositioned Src kinase inhibitors showing promise in inhibiting dengue virus (DENV) RNA replication.

combination with its activator NS2B subunit, NS3 is essential in the proteolytic cleavage of the non-structural proteins junctions namely, NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 [11]. Recently, two crystal structures of NS2B-NS3 complex, representing the folded and unfolded arrangement were resolved. The “open” (PDB:2FOM) [26] and the “closed” conformation (PDB:3U1I) [27] of serine protease provides the research community with a structural template for developing drug-like compounds against DENV. The substrate bound closed conformation of NS2B-NS3 subunit (PDB: 3U1I) is a practical representation of the folded ligand bound conformation and thus it is the preferred template for drug design [28]. Further analysis of the crystal structure of NS2B-NS3 protease

showed a catalytic triad consists of His51, Asp75 and Ser135 necessary for its proteolytic activity (Fig. 4). Additionally, several crystal structures of NS3 have been resolved from different DENV serotypes and as such may form the basis for developing serotype specific inhibitors. Recently, the resolved crystal structure of DENV NS3 helicase bound with NS2b cofactor [29] provides crucial details regarding the ligand binding domain of NS3 helicase which is also a potential hotspot for further drug discovery (Fig. 5).

2.1.4. DENV non-structural protein 4 (NS4)

Similar to NS2, NS4 also consists of two subunits, NS4A and NS4B. NS4A participates in intracellular membrane modulation

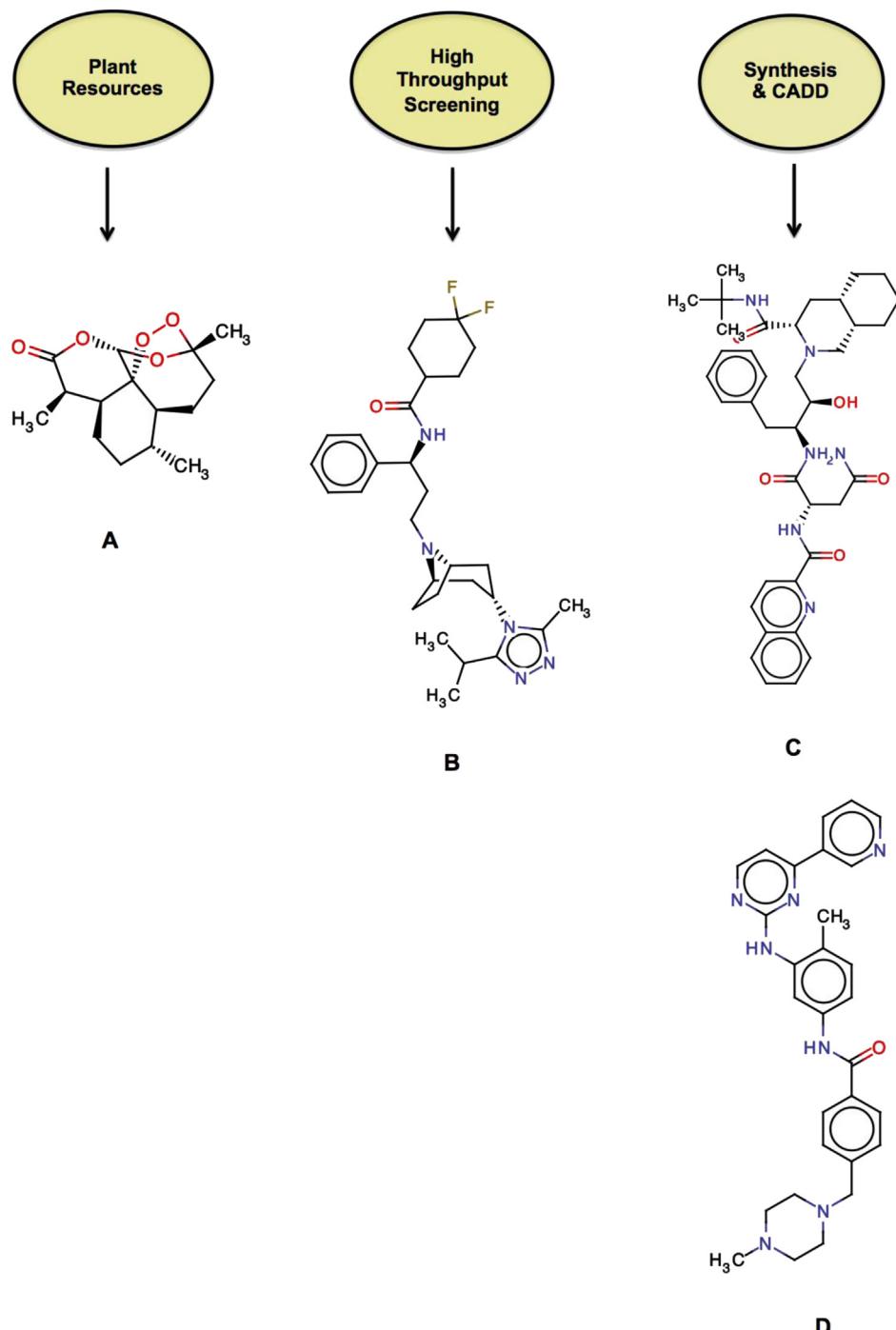


Fig. 3. Applications of different drug discovery approaches in drug development. Drug molecules such as artemisinin (A), maraviroc (B), saquinavir (C) and imitanib (D) from different sources such as plant extracts, high throughput screening, synthesis and computer aided drug design, respectively.

while its C-terminal end is involved in the translocation of NS4B subunit. The mechanism of NS4B subunit is poorly understood though recent evidence suggests that it might act as an interferon antagonist [11].

2.1.5. DENV non-structural protein 5 (NS5)

Similar to NS3, NS5 possess two major activities, an RNA dependent RNA polymerase and methyltransferase. NS5 methyltransferase (MTase) is a surface polyprotein which is essential in viral attachment to the host cell. Thus, the ligand bound crystal structure of N-terminal domain of NS5 methyltransferase [30] has

emerged as a crucial drug target for current and future drug discovery efforts (Fig. 6). The crystal structure of the RNA polymerase shows an active site with two zinc ion binding motifs [31] which are ideal target for designing novel RNA dependent RNA polymerase inhibitors (Fig. 7).

2.2. Non-structural proteins of WNV

As a *flavivirus*, the West Nile virus non-structural proteins are structurally and functionally similar to their DENV counterpart. The five non-structural proteins of WNV include NS1, NS2, NS3, NS4 and

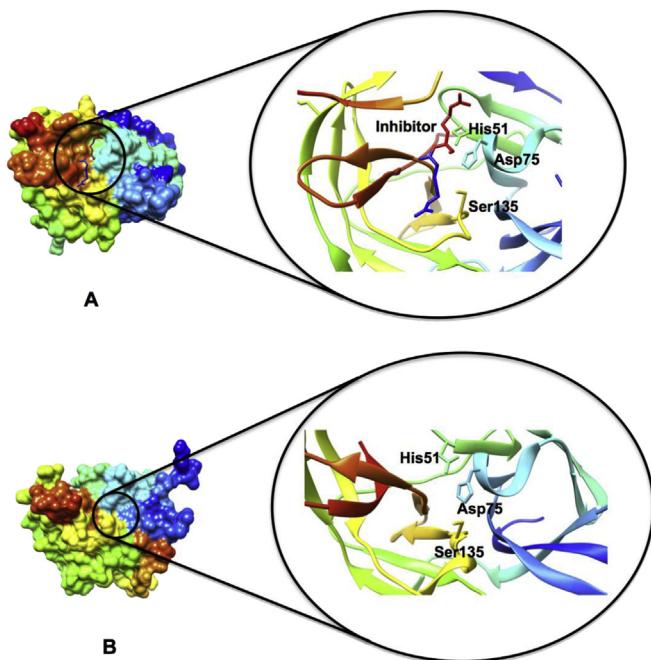


Fig. 4. The NS2B-NS3 complex in its closed (A) and open (B) conformation. The closed conformation (PDB: 3U11) [27] gives an overview of ligand bound conformation of DENV NS2B-NS3 complex and position of catalytic triad (His51, Asp75 and Ser135) in respect to inhibitor. The open conformation (PDB: 2FOM) [26] showed a slightly different arrangement of DENV NS2B-NS3 serine protease complex and corresponding active site residues. The ribbons were presented using “rainbow” representation of residues build in with Chimera.

NS5 and are important in the formation of protein complex necessary for RNA synthesis and viral replication [23].

2.2.1. WNV non-structural protein 1 (NS1)

West Nile Virus, NS1, is a glycoprotein that demonstrates the neuroinvasiveness nature of WNV though its precise role is poorly understood. However, this role may be unraveled following the recent resolution of its crystal structure (PDB: 4OIE) [32]. The crystal structure provides the first structural evidence which may the foundation for designing potent protease inhibitors targeting WNV NS1 (Fig. 8).

2.2.2. WNV non-structural protein 2 (NS2)

The WNV NS2 is composed of NS2A and NS2B subunits. The role of NS2A in the lifecycle of WNV has not been established, however, future efforts to resolve the structure may form the basis for understanding its role as well as targeting this NSP with potential

inhibitors. NS2B, a membrane associated co-factor, is important in the replication of the virus where it activates NS3 which then cleaves junctions between viral proteins. The availability of the crystal structure of ligand bound conformation of WNV NS2B-NS3 serine–protease complex (PDB:2YOL) [33] further increases the scope for designing potent antivirals against WNV. The WNV NS2B-NS3 (Fig. 9) ranks as the most ideal target for developing novel drug candidates due to its special role of cleaving the precursor polypeptide into all the viral non-structural proteins [23].

2.2.3. WNV non-structural protein 3 (NS3)

West Nile virus' NS3 is a multifunctional protein composed of two distinct domains. The N-terminal combines with NS2b subunit to form the serine–protease complex while C-terminal of NS3 bears helicase, NTPase and RNA triphosphatase activities. Despite the lack of crystal structures of WNV NS3 C-terminal domain, its structural similarity with DENV and *kunjin* virus NS3 protease [23] may provide an insight into WNV NS3 C-terminal binding landscape. This may entail using approaches such as homology modelling which will further assist in designing novel inhibitors targeting WNV C-terminal NS3 domain.

2.2.4. WNV non-structural protein 4 (NS4)

The hydrophobic core non-structural protein, NS4 is composed of two subunits, NS4A and NS4B. The function of NS4A in the viral lifecycle is poorly understood. However, based on mutational studies, NS4B subunit is believed to be important in the RNA replication where it is believed to associate with NS3 at the N-terminal and thus may influence helicase activity. This indicate NS4 may serve as an attractive target for structure based drug design against WNV [23].

2.2.5. Non-structural protease 5 (NS5)

NS5 is the most critical member of non-structural proteins across all flaviviruses. It comprises of N-terminal methyl transferase and C-terminal RNA dependent RNA polymerase. Currently, there are several crystal structures of N-terminal and C-terminal domains of WNV NS5 resolved at different resolution. The N-terminal methyl transferase (MTase) (PDB: 2OYO) [34] is located on viral surface and acts similarly to that of its DENV counterpart and thus it is regarded as an important target in novel antiviral discovery (Fig. 10). The C-terminal RNA dependent RNA polymerase (PDB: 2HCS) [35] is also a major target for novel viral polymerase inhibitors given its essential role in RNA synthesis and viral replication (Fig. 11). Recently, there has been significant success in the development of potent polymerase inhibitors targeting HCV [36]. These RNA polymerase inhibitors mainly belong to different classes of nucleoside analogs and may provide ligand templates for developing novel RNA dependent RNA polymerase inhibitors to counter WNV and other flaviviruses such as dengue and thereby boost the fight against neglected tropical diseases.

3. CHIKV non-structural proteins

The genome of CHIKV, an alphavirus, consists of an 11.8 kb positive-sense, single-stranded RNA. Similar to *flavivirus* genomes, it also encodes non-structural proteins as well as structural proteins. The non-structural proteins, NS1–4, are encoded on the 5'end of CHIKV RNA [24].

3.1. CHIKV non-structural protein 1 (NS1)

CHIKV NS1 is a palmitolated protein consists of 535 amino acids. The N-terminal region of NS1 is responsible for the methyltransferase (MTase) and guanylyltransferase activities. Recently, this

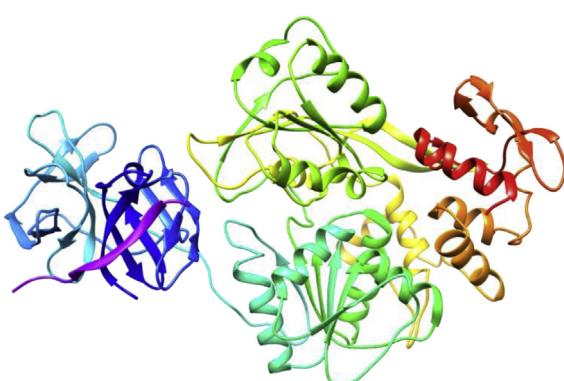


Fig. 5. Crystal structure of DENV NS3 helicase (PDB: 2VBC) [29] bound to NS2b cofactor (highlighted in “pink”).

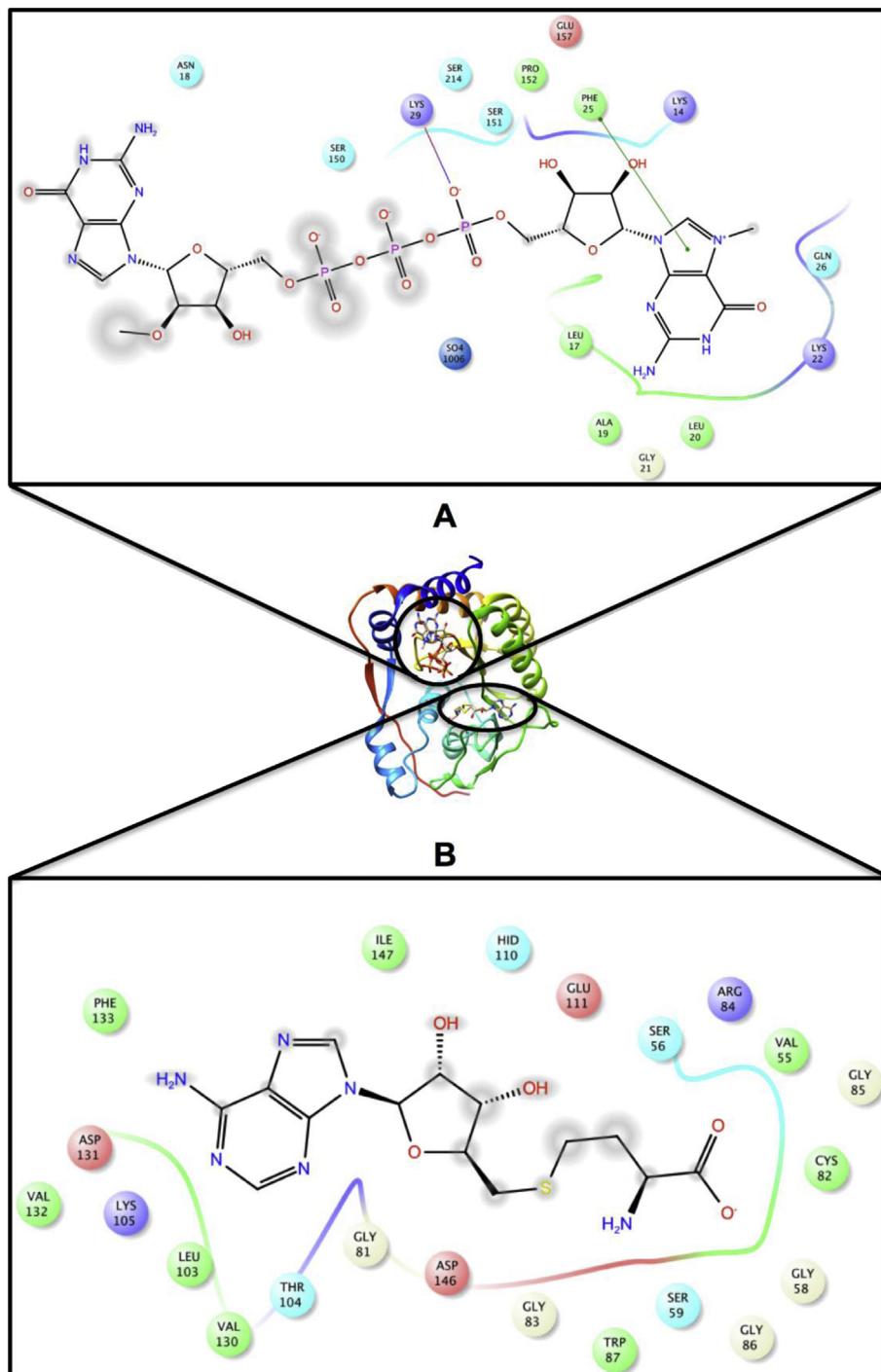


Fig. 6. Crystal structure of NS5 methyltransferase (PDB:2P41) [30] bound to 7MeGpppG2'OMe (A) and S-Adenosyl-L-homocysteine (B). Active site residue interactions with 7MeGpppG2'OMe and S-Adenosyl-L-homocysteine were represented using Maestro's 2-D ligand interaction map.

protein was reported to play a crucial role in the down-regulation of bone marrow stromal antigen-2 (BST-2). This finding places NS1 as potential target for development of BST-2 mediated therapeutics targeting CHIKV [24].

3.2. CHIKV non-structural protein 2 (NS2)

Similar to DENV and WNV, Chikungunya NS2, cleaves viral polyprotein into four non-structural proteins via thiol protease complex at the C-terminal region. Whereas, the RNA

triphosphatase activity essential for RNA capping is carried at the N-terminal region of NS2. Recent resolved crystal structure of CHIKV NS2 consisting of both C-terminal and N-terminal region might act as a crucial starting point to develop novel antivirals targeting CHIKV [24,37] (Fig. 12).

3.3. CHIKV non-structural protein 3 (NS3)

The CHIKV NS3 is constructed by two major domains. The N-terminal macrodomain of NS3 binds to ADP-ribose derivative and

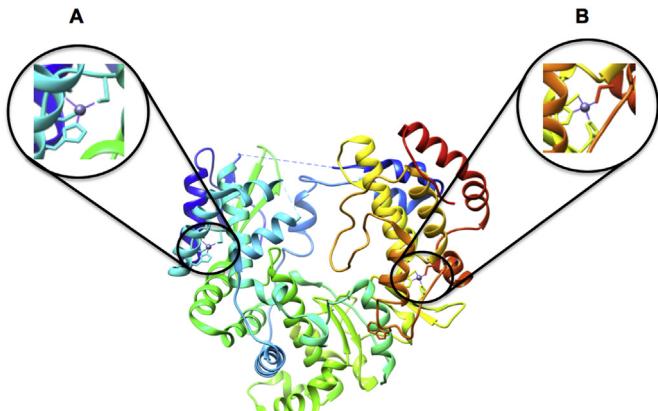


Fig. 7. Crystal structure of DENV RNA dependent RNA polymerase (PDB: 2J7U) [31] with two zinc binding domains (A and B).

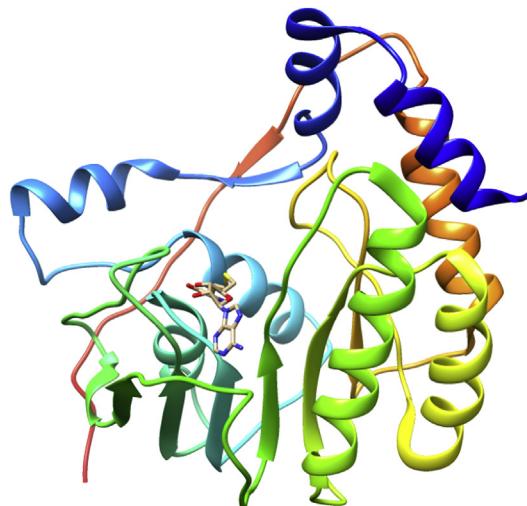


Fig. 10. The crystal structure of WNV NS5 methyltransferase (PDB: 2OYO) [34] bound with S- Adenosyl-L-Homocysteine.

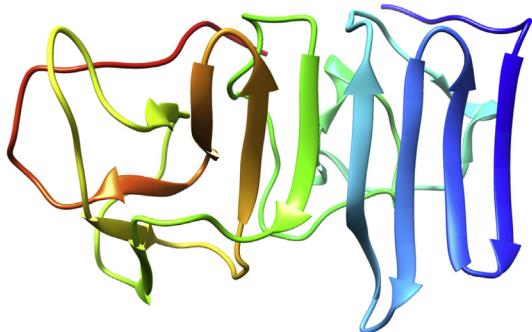


Fig. 8. Crystal structure of west nile virus non-structural protein, NS1 (PDB: 4OIE) [32].

believes to play regulatory function in the cell. Very recently the crystal structure for CHIKV macrodomain was resolved (PDB: 3GPO) [38]. The ADP-ribose binding region played the role of active site and act as a potential hotspot towards development of novel antivirals (Fig. 13). Whereas, the C-terminal domain is comparatively less conserved than the N-terminal domain and consisted of several phosphorylated residues necessary for RNA synthesis. Recent findings report that the NS3 domain of CHIKV interacts with the SH3 domain of amphiphysin 1 and 2 proteins of host cell and thus affect several cellular functions [39]. The interaction of the conserved SH3 domain with host proteins may indicate that this domain participates in viral replication and thus may constitute an important target for antiviral development [24].

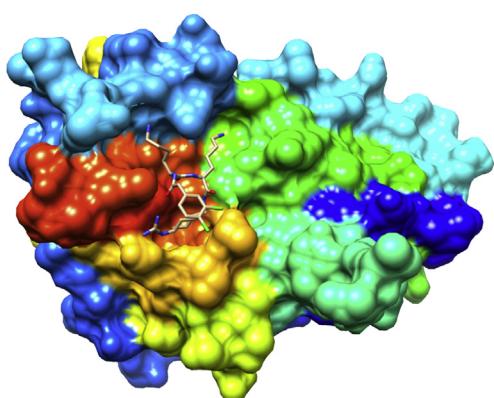


Fig. 9. The crystal structure of WNV NS2b-NS3 serine protease complex with a peptidomimetic inhibitor (PDB: 2YOL) [33].

3.4. CHIKV non-structural protein 4 (NS4)

CHIKV NS4 is an RNA-dependent-RNA-polymerase (RdRp) believed to participate in protein unfolding in host cell, which ultimately leads to translation of viral protein by reducing phosphorylation of eIF2 α [24]. The crystal structure of CHIKV NS4 have not been resolved so as to understand its conformational and binding landscape. However, further studies on this NSP may provide details on its role in viral replication and thus make it potential target for future drug discovery.

4. Chemotherapeutics targeting viral non-structural proteins: medicinal chemistry perspectives

4.1. Targeting DENV

The recent advances in medicinal chemistry and computer-aided drug discovery as well the availability of crystal structures of several DENV proteins has led to an increased focus in developing potent inhibitors targeting key DENV non-structural proteins

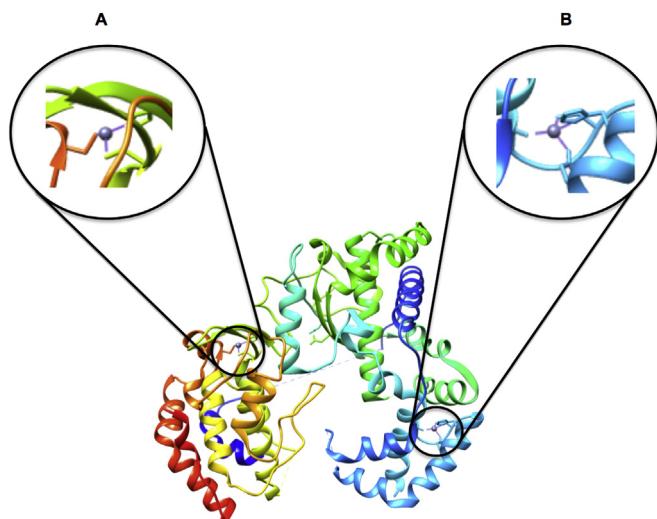


Fig. 11. The RNA-dependent-RNA-polymerase (RdRp) domain (PDB: 2HCS) [35] of WNV NS5 A and B highlights the zinc binding domains of RdRp.

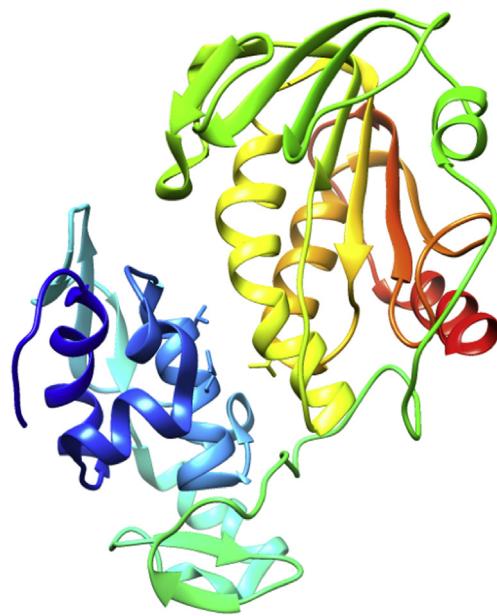


Fig. 12. The crystal structure of CHIKV non-structural protein 2 (PDB: 3TRK) [110].

[7,36]. These efforts have been concentrated on NS2B-NS3 protease, NS3 helicase, NS5 methyltransferase and RNA-dependent-RNA-polymerase, which are key enzymes in the DENV lifecycle. In recent years, there has been regained efforts at identifying novel inhibitors for these key proteins through experimental as well as computational approaches. Herein, we summarised novel inhibitors targeting Dengue virus non-structural proteins from a medicinal chemistry perspective.

4.1.1. Inhibition of DENV NS2B-NS3 and NS3

DENV NS2B-NS3 is essential in the cleavage of viral polypeptide and as such may serve as target for designing and developing novel chemotherapeutic agents against Dengue [40]. Similarly, the NS3 domain possess NTPase and helicase activities and thus emerged as a promising target [11]. Several small organic compounds, peptide

analogs and non-peptidomimetic agents have been reported as inhibitors of these key non-structural proteins important in the replication and maturation of dengue. High Throughput Screening (HTS) has been successful in the identification of potential lead compounds that may form the basis for development of protease inhibitors targeting key enzymes in the viral lifecycle. Yang et al. [41] discovered three potent small molecule inhibitors targeting DENV serine protease from a high throughput screening campaign. These small molecules exhibited moderate activity on DENV-2 NS2b-NS3 serine protease. One of the compound, BP2109 had an IC₅₀ of 15.43 μm in the enzyme based protease assay (Fig. 14). The novel compounds identified from this HTS screening possessed a quaternary nitrogen atom and were believed to act by hindering the interaction between the central hydrophilic portion of NS2B and NS3 protease [41]. Another compound, BP13944, mined from a set of 60,000 compounds possessed an IC₅₀ of 1.03 μm and can be a possible structural template to develop more potent antivirals targeting DENV [42] (Fig. 14). This novel aliphatic compound with a quaternary nitrogen atom inhibited DENV replication by interfering with viral NS3 protein. Despite their potency against DENV serine protease these compounds containing tertiary nitrogens may possess both pharmacokinetics and ADME challenges due to the presence of long aliphatic chains and quaternary nitrogen atoms. Thus they have a huge potential to act as starting points for developing and designing novel small molecule inhibitors targeting DENV serine protease.

In recent years, computational tools have been applied to design novel inhibitors targeting a variety of enzymes. Majority of the computational studies uses an ensemble of virtual screening, molecular docking, fragment based drug design, structure based drug design approaches to identify potential lead compounds [25]. Recently, fragment based drug design approach was used to identify 23 hit molecules from the ZINC repository. Two lead compounds from this exploration were found to possess structural similarity and inhibited DENV-2 protease with IC₅₀ values 7.7 μM and 37.9 μM and K_i of 2.0 μM and 31.1 μM, respectively (Fig. 15) [43].

Two potential hits code-named, SK-12 and ARDP0006 identified via structure based drug design approach were also found to inhibit all four DENV serotypes [44,45]. SK-12 was the most potent with an EC₅₀ ranging from 0.74 to 2.43 μm whereas ARDP0006 exhibited a slightly higher value ranging from 0.88 to 5.96 μm. SK-12 acts as a

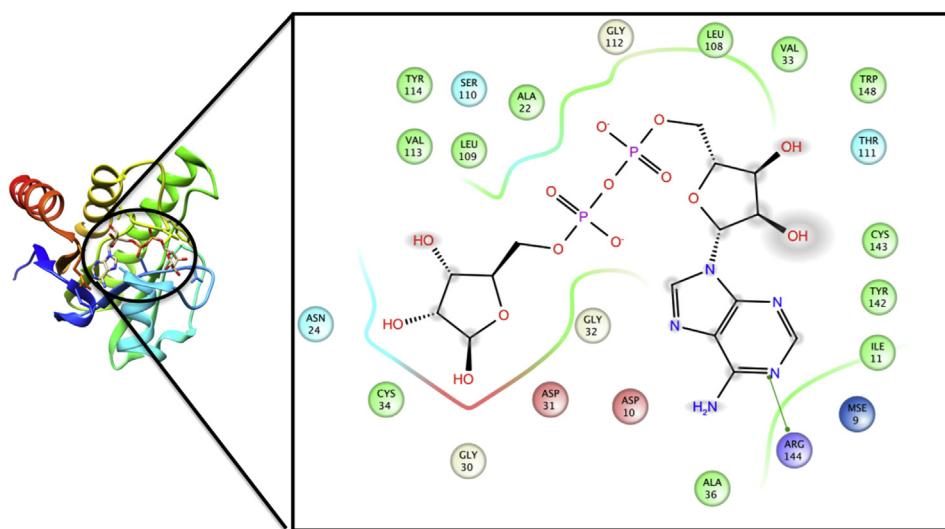


Fig. 13. Crystal structure of CHIKV NS3 N-terminal macrodomain (PDB: 3GPO) [38] bound with ADP ribose. The 2-D ligand interaction diagram showing crucial ligand protein interactions of ADP ribose in the active site of CHIKV NS3 macrodomain.

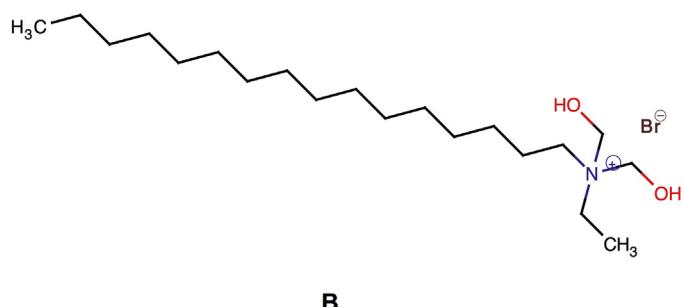
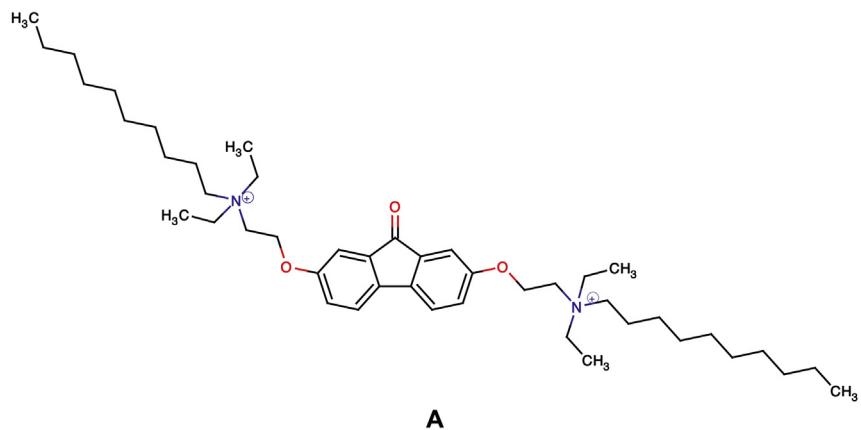


Fig. 14. Two potent DENV NS2b-NS3 protease inhibitors discovered from High Throughput Screening (HTS). Compound A and B codenamed as BP2109 and BP13944 respectively found to possess IC₅₀ values of 15.43 μM and 1.03 μM, respectively.

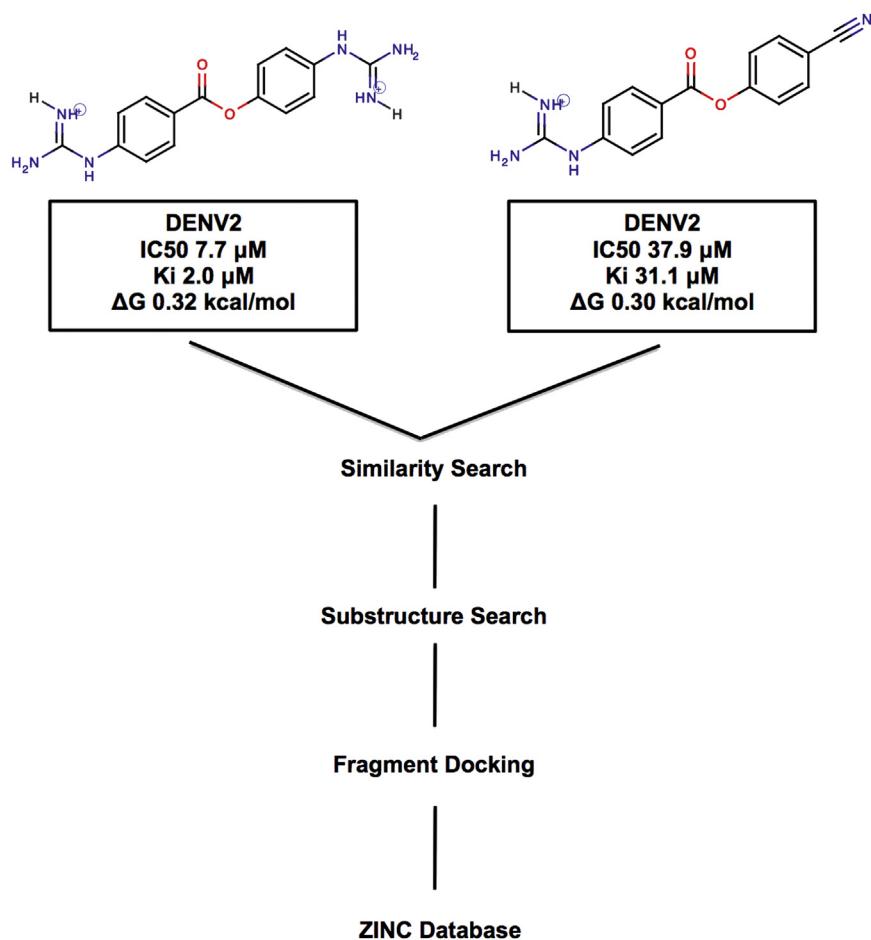


Fig. 15. The NS2B-NS3 inhibitory activity of two top hit compounds identified using a fragment based drug design approach.

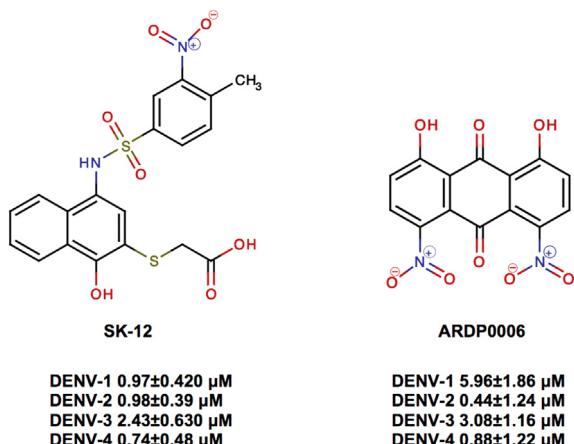


Fig. 16. The compounds code-named, SK-12 and ARDP0006 identified using a structure based drug design approach with their EC₅₀ values against 4 serotypes of dengue as determined by the cell-based viral replication assay.

non-competitive inhibitor of NS2B-NS3 protease that blocks the interaction of NS2B subunit with viral NS3 (Fig. 16).

Further, Deng et al. developed a chemical library using virtual screening and scaffold hopping approach and identified twelve compounds with quinoline scaffold as inhibitors of DENV NS2B-

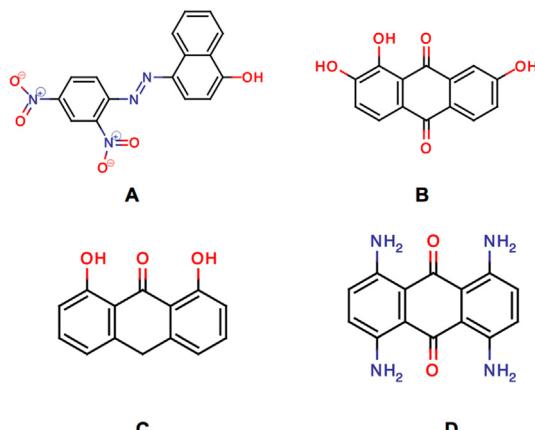


Fig. 19. Anthracene based potent inhibitors targeting DENV serine protease.

NS3. Only compound B emerged as a promising inhibitor with an IC₅₀ value of $9.45 \mu\text{M}$ [46] (Fig. 17).

Due to its huge potency in identifying novel lead in a more rational manner, virtual screening approach was further used to identify seven compounds with IC₅₀ values ranging from 3.9 to $86.7 \mu\text{M}$. Among the seven hits, three novel compounds with diverse scaffolds (A, B & C) were confirmed as a competitive

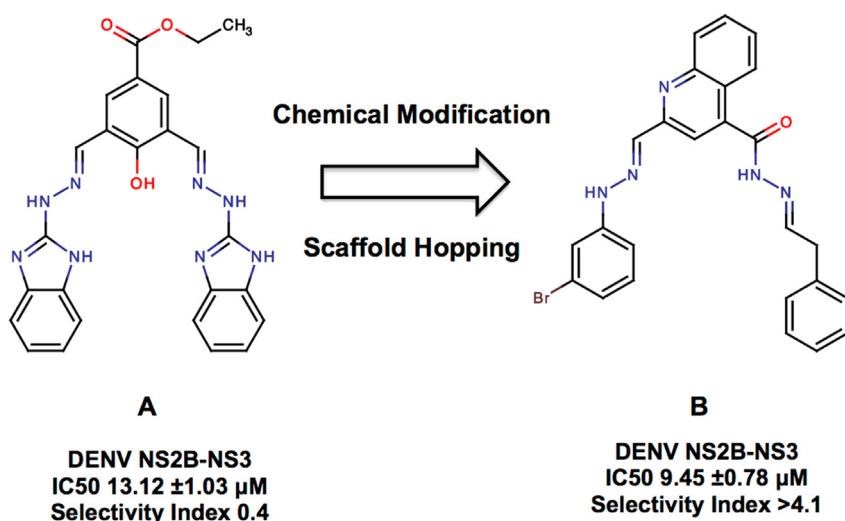


Fig. 17. The use of scaffold hopping approach to identify compound B. Compound A acts as a strating structure identified using structure based virtual screening.

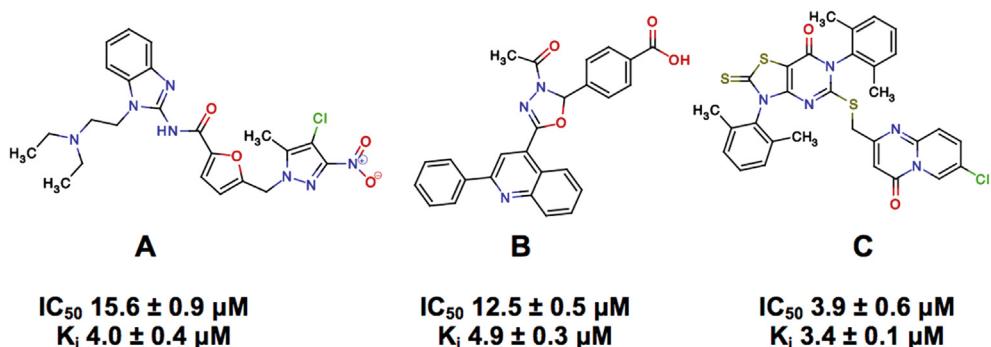


Fig. 18. 2-D chemical structure and activity of three novel DENV NS2B-NS3 inhibitors.

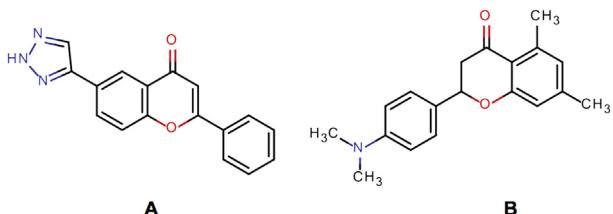


Fig. 20. Structural representation of two potent flavones identified using virtual screening approach.

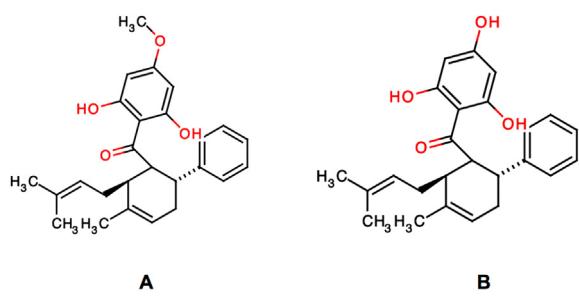


Fig. 21. The structure and K_i values of 4-hydroxypanduratin A (A) and panduratin A (B) against DENV NS2b-NS3 protease.

inhibitors of NS2b-NS3 with K_i values of 4.0 μM , 4.9 μM , and 3.4 μM , respectively [47] (Fig. 18).

Anthracene based scaffolds were also found to inhibit DENV serine protease complex. These anthracene based scaffolds may act as cornerstone for designing new chemical entities with an improved enzyme specificity (Fig. 19) [44]. Virtual screening approach was used to screen compounds from the ZINC database having structural features of chalcones, flavanones and flavones [48]. This exploration led to the identification of two novel hits with K_i values of 69 μM (A) and 121 μM (B) respectively [48] (Fig. 20).

Chalcone derivatives extracted from *Boesenbergia rotunda*, 4-hydroxypanduratin A and panduratin A, were found to possess inhibitory activity against DENV NS2b-NS3 by a competitive mechanism. 4-hydroxypanduratin A and panduratin A showed K_i values of 21 μM and 25 μM respectively and thus can be used as a potential starting candidate for further exploration [49] (Fig. 21).

Aminobenzothiazole containing 8-hydroxyquinoline analogs identified through synthesis and structure activity relationship

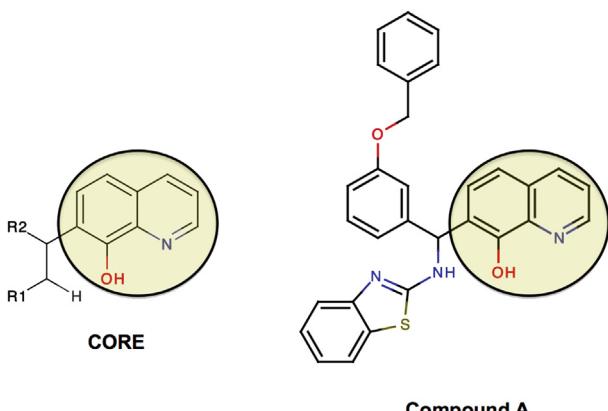


Fig. 22. The discovery of compound A, an analog having the 8-hydroxyquinoline as its core moiety.

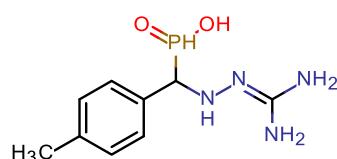


Fig. 23. The most potent non-substrate based small molecules inhibitor against DENV serine protease.

(SAR) study showed inhibitory activity against DENV NS2B-NS3 [50]. Compound A found to be most potent with an IC_{50} value at lower micromolar scale where it inhibits DENV2 NS2b-NS3 competitively (Fig. 22).

A list of non-substrate based compounds were also identified which inhibits dengue NS2B-NS3 serine protease. Among these, is a small molecule possessing a phosphorous group and guanidine moiety as a side chain which was found to be most potent with a K_i value of 14 μM (Fig. 23). This phosphorous group, may be essential in improving the activity and the compound can act as a template to design novel potent and specific inhibitors targeting DENV NS2B-NS3.

Alpha-ketoamide based pharmacophore have been investigated for their activity towards DENV protease. Among the compounds screened, one of the compound containing b,c-unsaturated alpha-ketoamide was observed to inhibit DENV replication in cell-cultural assay which resulted in 1000-fold reduction in virus titers [51] (Fig. 24). Structure activity relationship (SAR) study predicts that further modification at the b-position and at the amide nitrogen while keeping the core indole moiety intact may improve the selectivity of the generated compounds to inhibit DENV NS3 protease [51].

Click chemistry based synthesis gained much interest because of its ability to tailor substances rapidly and reliably by joining small fragments. Tiew et al. employed the modern click-chemistry approach to generate potential new inhibitors from the scaffold of benz [d] isothiazol-3 (2H)-one resulting in the discovery of at least six potent compounds with IC_{50} values ranging from 3.48 to 13.36 μM against DENV serine protease [52] (Fig. 25).

Retrocyclin-1 (RC-1) synthesized in *Escherichia coli* was previously found to inhibit HIV-1. Recent investigation unveiled that RC-

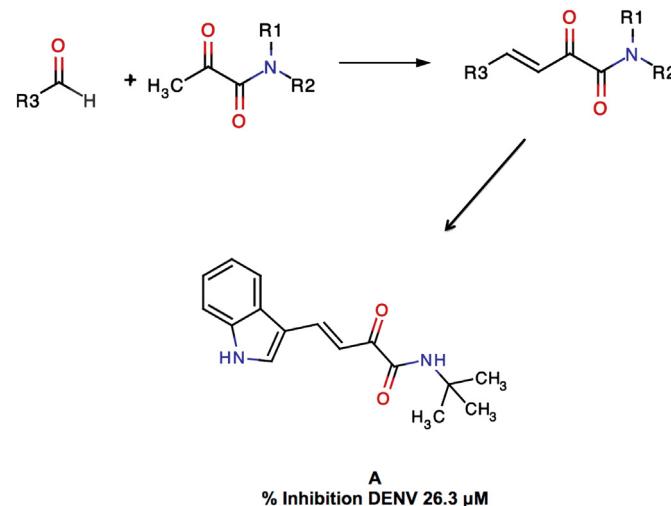


Fig. 24. 2-D chemical structure of potent alpha-ketoamide based lead compound (A) contained indole as core moiety. The schematic synthetic scheme to design compound A may be provide useful to develop more structural analogs.

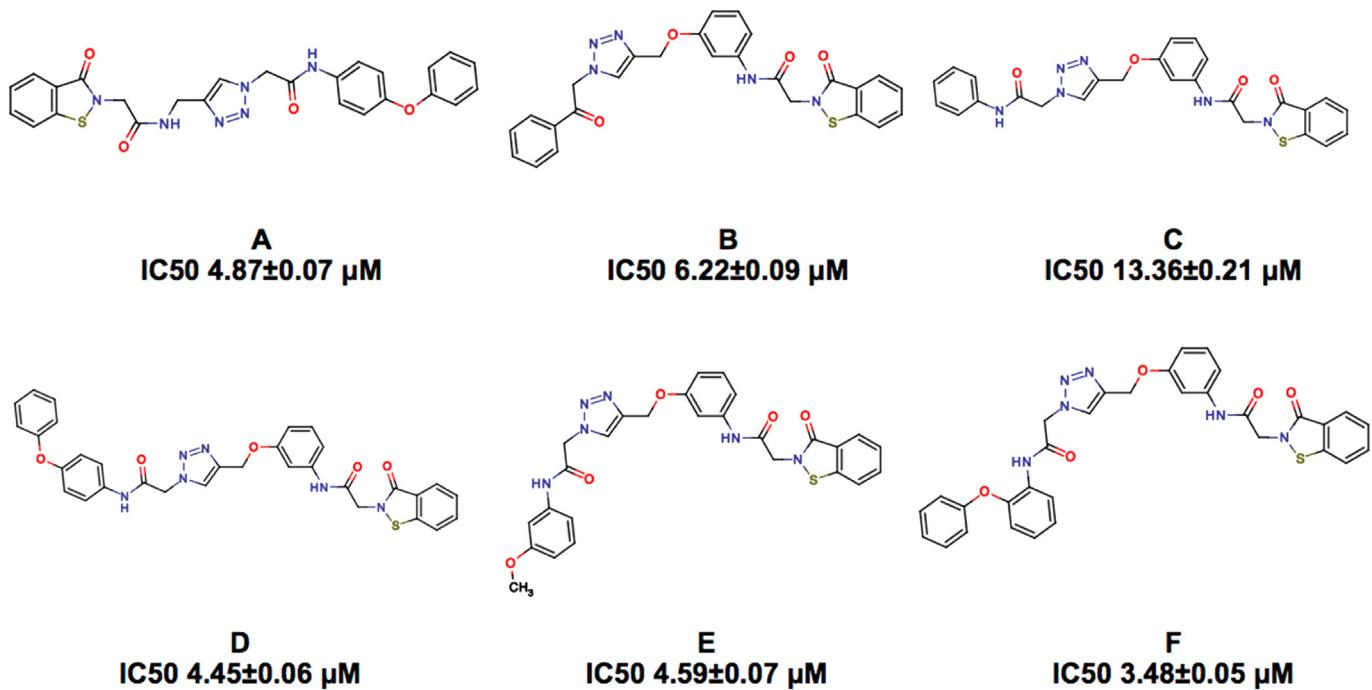


Fig. 25. The 6 potent serine protease inhibitors developed using click chemistry based approach using the core scaffold of benz [d] isothiazol-3 (2H)-one.

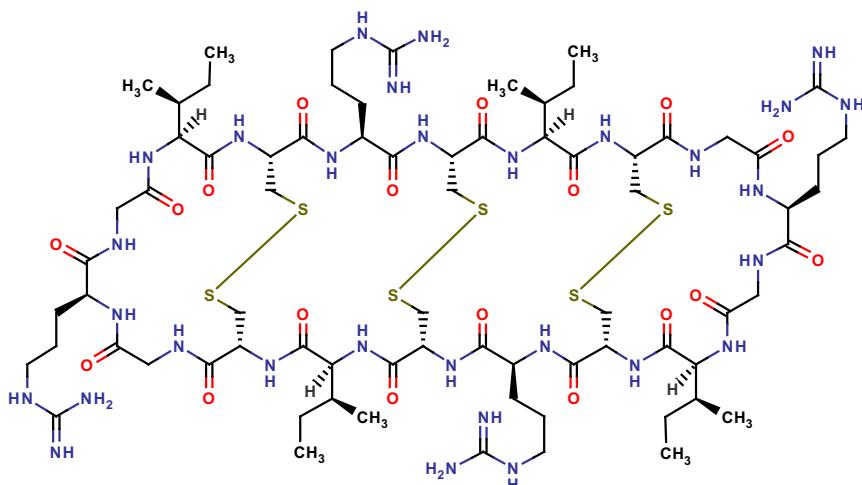


Fig. 26. The chemical structure of retrocyclin-1 (RC-1) showing sulphur bridge.

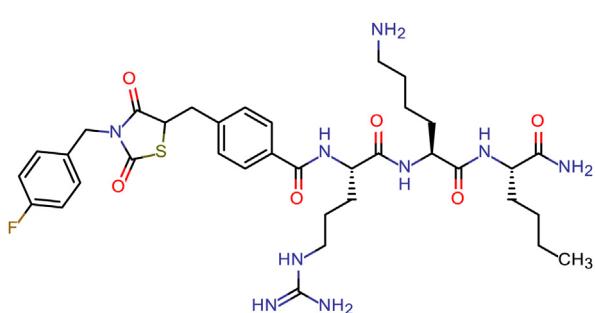


Fig. 27. 2-D structural representation of compound 1, a potent inhibitor of DENV NS2B-N3pro with a K_i value of 1.5 μM .

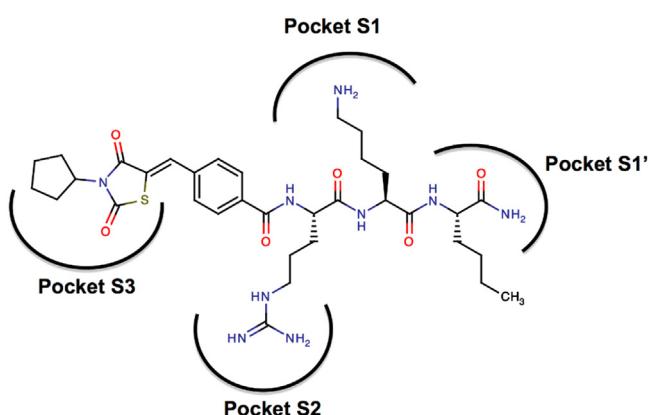


Fig. 28. The binding mode of compound 2 in the active site of DENV NS2B-NS3 protease.

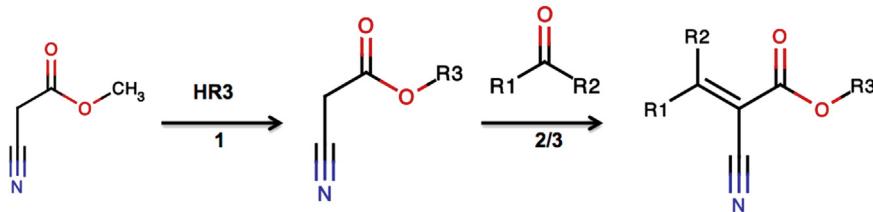
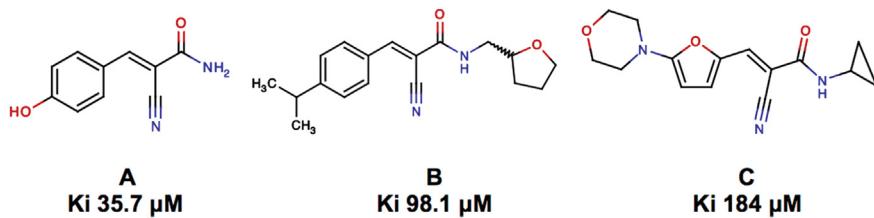


Fig. 29. A Representative synthetic scheme to develop cyanoacrylamide analogs.

Fig. 30. Three most potent post aryl cyanoacrylamide analogs with their K_i values against DENV NS2b-NS3pro. The para-hydroxy substituted compound (A) found to possess the best K_i value against viral protease.

1 has inhibits the replication of DENV by targeting NS2B-NS3 serine protease complex (Fig. 26). Peptide PG-1, which is usually synthesised by solid-phase peptide synthesis was also observed to inhibit dengue NS2B-NS3pro at an IC_{50} of 11.7 μM [53].

Recently, a series of N-substituted 5-arylidenethiazolidinone based peptide hybrids targeting DENV NS2B-NS3 protease have been reported [54]. Sulphur or an oxygen atom at position 2 of the heterocyclic moiety contributed significantly to the inhibitory activity of these compounds. Promising *in vitro* affinities were observed for thiazolidinedione-based peptide hybrids containing hydrophobic groups with K_i values between 1.5 μM (compound 1)

and 1.8 μM (compound 2) with a competitive inhibitory mechanism (Figs. 27 and 28).

Further, modification of 3-aryl-2-cyanoacrylamide scaffold led to synthesis of some novel DENV serine protease inhibitors (Figs. 29 and 30). Out of the eighty-six analogs synthesized, the para-hydroxy substituted analog was found to be the most potent inhibitor of DENV NS2b-NS3 with K_i -value of 35.7 μM [55].

To combat DENV, synthetic peptides have also emerged as a key strategy to target DENV NS2B-NS3 protease. The most potent

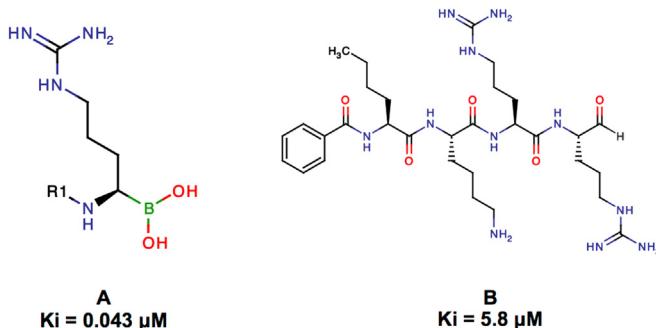


Fig. 31. The 2-D chemical structures boronic acid containing peptide inhibitor (compound A) and one of the potent peptidomimetic inhibitor (compound B).

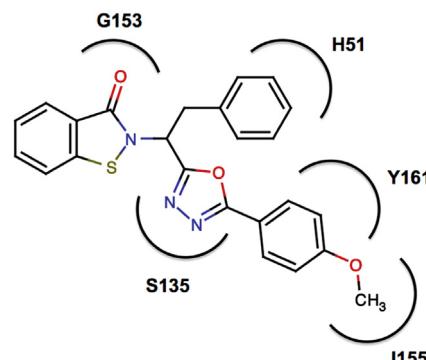
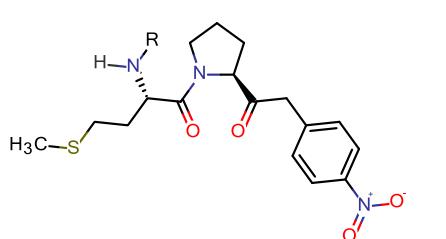
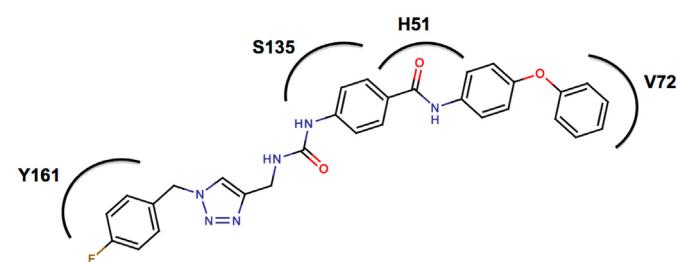
Fig. 33. 2-D chemical representation of a hybrid oxadiazole entity ($\text{IC}_{50} = 3.75 \mu\text{M}$) interacting with the prominent active site residues of DENV NS2B-NS3pro.Fig. 32. The common structural feature of methionine–proline anilide ($R = H$, Boc compound 1 and compound 2, respectively).

Fig. 34. The potent aminobenzamide scaffold containing molecule and its interaction with prominent active site residues.

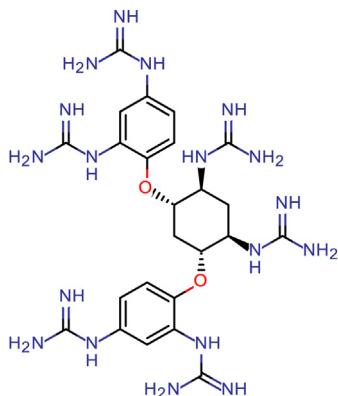


Fig. 35. The identified guanidinylated 2, 5-dideoxystreptamine based compound found to inhibit viral NS3 protease.

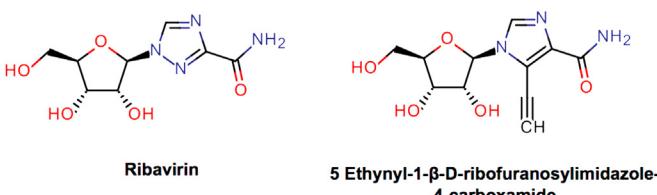


Fig. 36. The chemical structures of Ribavirin and one of its most potent analog reported to inhibit DENV NS5 MTase activity.

synthetic peptide, Ac-RTSKKR-CONH₂ found to inhibit DENV NS2B-NS3pro with a *K*_i value of 12.14 μM. Yin et al. in a two part communication reported a series of peptide inhibitors targeting DENV NS3 protease [56]. In the first part they report a series of tetrapeptide inhibitors with electrophilic warheads. They found that boronic acid containing peptide inhibitor has the highest affinity with a *K*_i of 43 nM. In the subsequent study they reported some tetrapeptide aldehydes based on a lead structure of Bz-Nle-Lys-Arg-Arg-H. A number of analogs found to be effective with compound B emerged as the possible lead candidate for further exploration (Fig. 31).

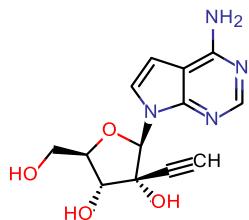


Fig. 37. The chemical structure of nucleoside analog codenamed as NITD008.

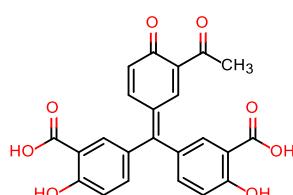


Fig. 38. 2-D chemical representation of aurintricarboxylic acid.

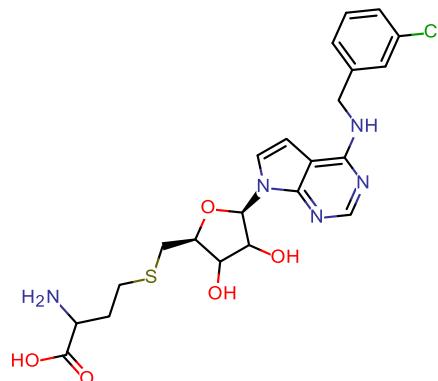


Fig. 39. One of the potent SAH analog that selectively inhibits DENV methyltransferase activity with *K*_i values of 0.82 μM and 0.17 μM against DENV-3 N7 and 2'-O methyltransferase activities.

A series of methionine–proline dipeptide derivatives have been reported as potential DENV NS2B-NS3 inhibitors. Zhou et al. identified two potent compounds with *K*_i values of 4.9 [57] and 10.5 μM [58], respectively [59] (Fig. 32). Further SAR analysis and

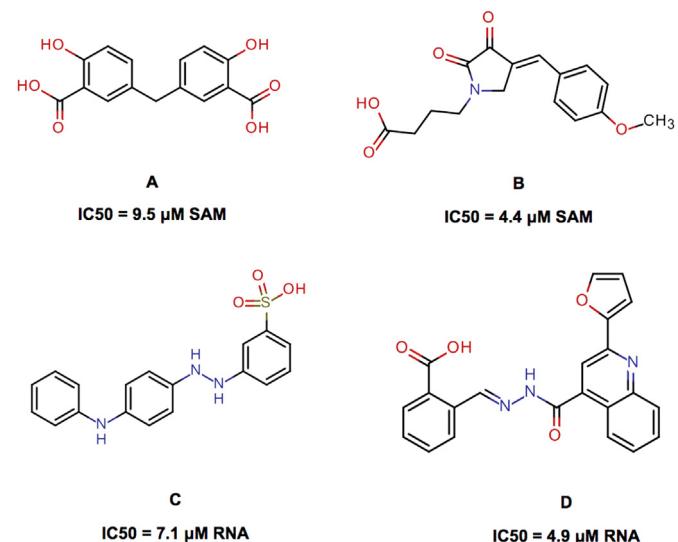


Fig. 40. The top two DENV methyltransferase (MTase) and RdRp inhibitors identified using a structure based virtual screening approach. The DENV methyltransferase inhibitors A and B target the SAM (S-adenosylmethionine) binding region of MTase whereas RdRp inhibitors (C and D) target the RNA site.

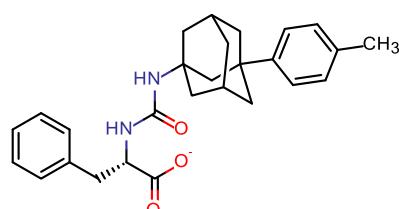


Fig. 41. An inhibitor of DENV methyltransferase with a previously unreported scaffold.

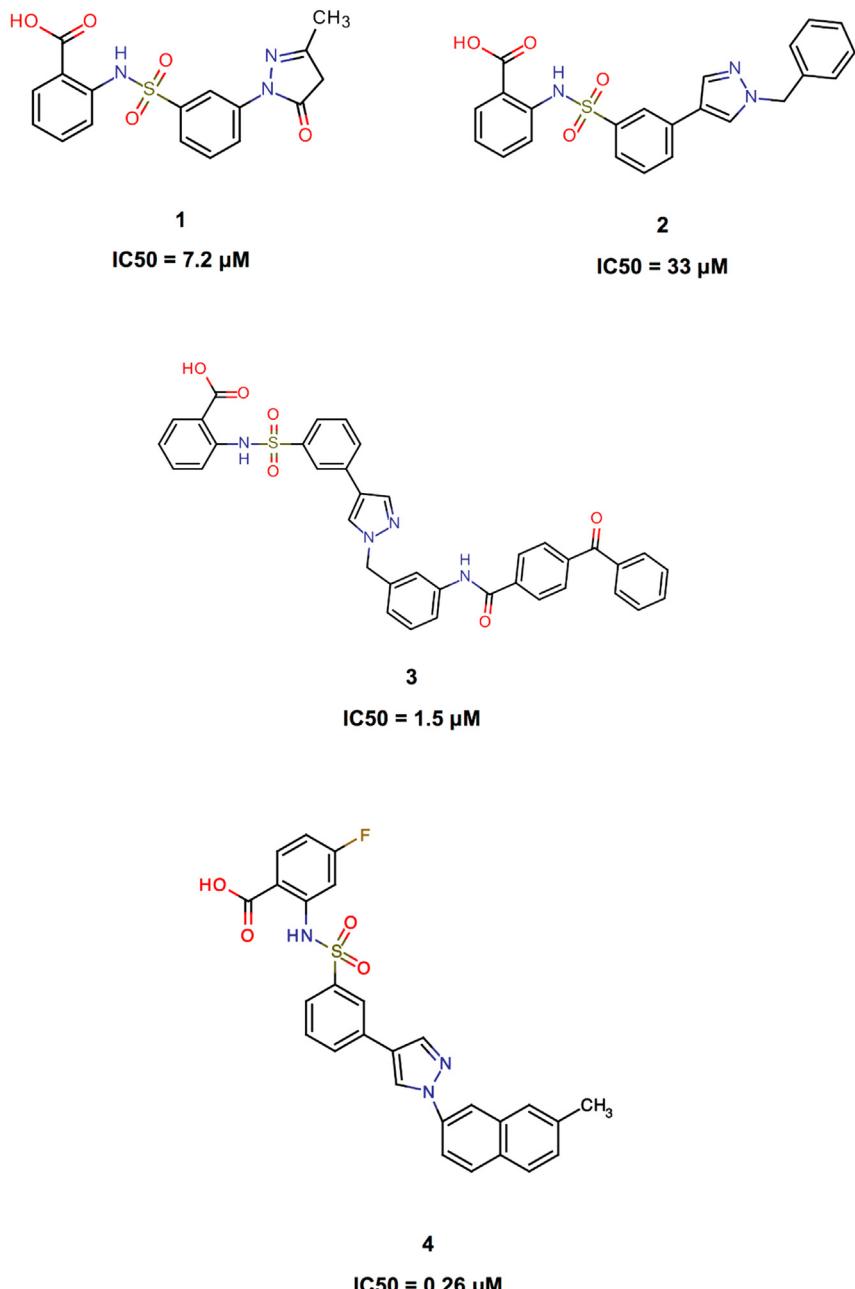


Fig. 42. The *N*-sulfonylanthranilic acid containing scaffolds showing promising activity as DENV polymerase (RdRp) inhibitor.

molecular docking study revealed that chemical part of L-proline, L-methionine and p-nitroaniline where highly responsible for blocking the active site of NS2b-NS3 leads to DENV inhibitory activity.

Apart from the dipeptide analogs, a novel uncharged tetrapeptide, WYCW-NH₂ was found to exhibit inhibitory effect with K_i values of 4.2, 4.8, 24.4, and 11.2 μM against the four different serotypes of DENV proteases [60]. Further, application of molecular hybridization approach based on 1, 2-benzisothiazol-3-(2H)-one and 1, 3, 4-oxadiazole scaffolds leads to development of a novel oxadiazole entity, with an IC_{50} of 3.75 μM . This chemical entity inhibits the DENV NS2B-NS3pro and thus can be carried forward in future hit to lead campaigns (Fig. 33).

Aravapalli et al. [28] identified some potent DENV serine protease inhibitors based on aminobenzamide scaffold. The inhibition constant (K_i) for the most potent compound against DENV protease was found to be 8.77 μM (Fig. 34). The kinetics data suggested a competitive inhibitory mechanism.

Another class of compounds, guanidinylated 2, 5-dideoxystreptamine [61], inhibited the NS3 protease from four serotypes of DENV with 50% inhibitory concentration values in the 1–70 μM range (Fig. 35).

Gao et al. [62] derived a series of kalata B1 analogues as potent inhibitor against DENV NS2B-NS3 protease. The oxidized derived from these cyclopeptides forms substrate-competitive inhibitors of the dengue viral NS2B-NS3 protease. Two oxidized isoforms

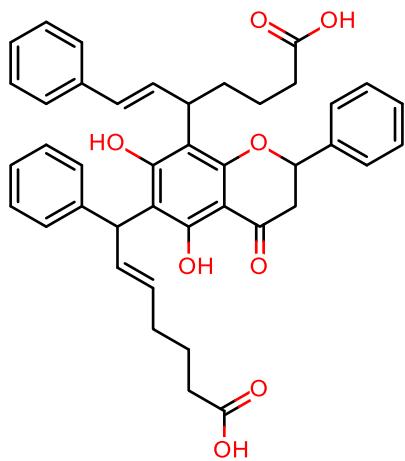


Fig. 43. The chemical structure of one of the most potent chartaceones reported to have an IC_{50} value of $1.8 \mu M$.

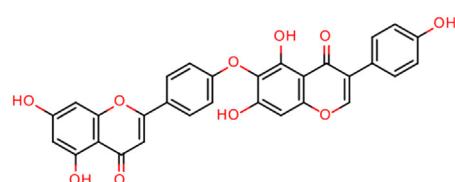
showed potent inhibition with K_i of $1.39 \mu M$ and $3.03 \mu M$, respectively.

4.1.2. Inhibition of viral RNA-dependent-RNA-polymerase (RdRp), Methyltransferase (MTase) and helicase

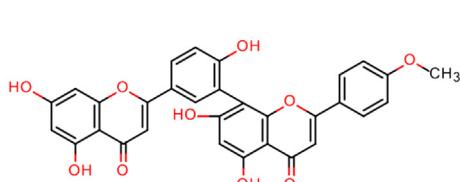
Ribavirin, is a broad spectrum antiviral agent found to possess weak activity against DENV with a EC_{50} of $49 \mu M$. Ribavirin 5'-triphosphate inhibits the activity of the methyltransferase activity of DENV NS5. Benarroch et al. revealed the complete molecular level mechanism of ribavirin 5'-triphosphate inside DENV NS5 methyltransferase (MTase) by providing crystal structure of a ternary complex consisting of NS5 MTase of DENV, ribavirin 5'-triphosphate, and S-adenosyl-l-homocysteine at a resolution of 2.6 \AA [63]. Several analogs of ribavirin were designed in order to improve its potency against DENV. One derivative, 5 Ethynyl-1- β -D-ribofuranosylimidazole-4-carboxamide improved the potency by 100 fold [64] (Fig. 36).

NITD008 [65], another nucleoside analog reported to have direct inhibition on RNA-dependent RNA-polymerase (RdRp) activity of DENV and can act as an effective structural template for future ligand based and structure based drug discovery (Fig. 37).

Aurintricarboxylic acid [11] (Fig. 38) has emerged as a potent inhibitor of DENV N7- and 2' O-methyltransferase activity with IC_{50} values of 2.3 and $127 \mu M$, respectively. Molecular docking study revealed a major role of that active site residue Lys61 in the process of inhibition. This residue is essential for the correct 2' O methylation of viral RNA.



Compound A
 $IC_{50} = 0.26 \mu M$



Compound B
 $IC_{50} = 0.75 \mu M$

Fig. 44. Two of the most potent bioflavonoids extracted from *Dacrydium balansae* showing promising inhibitory activity against DENV polymerase.

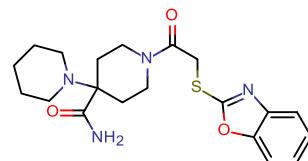


Fig. 45. The novel DENV NS3 helicase inhibitor, ST-610 identified from an HTS campaign.

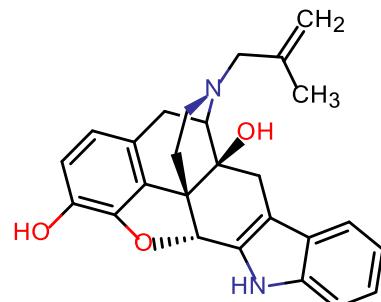


Fig. 46. Chemical structure of SDM25N, a potent DENV NS4B inhibitor.

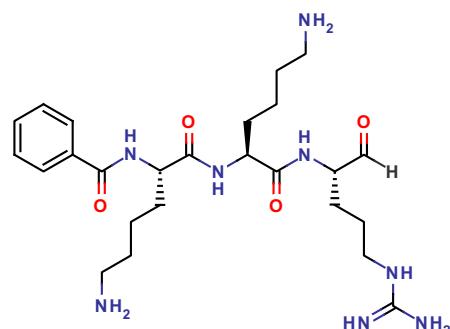


Fig. 47. Potent WNV NS2B-NS3 protease tripeptide inhibitor with nonpeptidic caps at the N-terminus and aldehyde at the C-terminus.

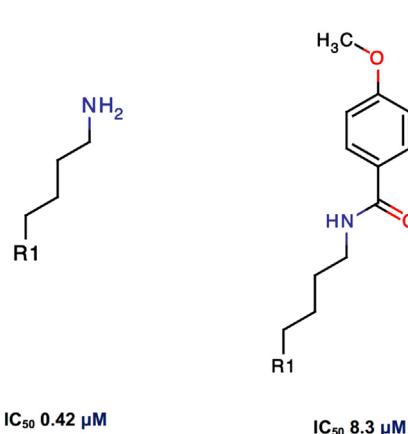
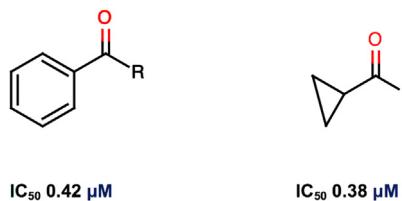


Fig. 48. Some potent tripeptide WNV NS2b-NS3 analogs and their respective IC_{50} values. R and R1 stands for KKR-H and Bz-K-K-R-H, respectively.

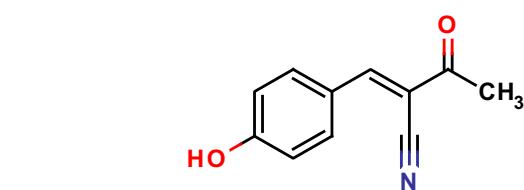


Fig. 50. The para hydroxy substituted molecule having a cyanoacrylamide scaffold.

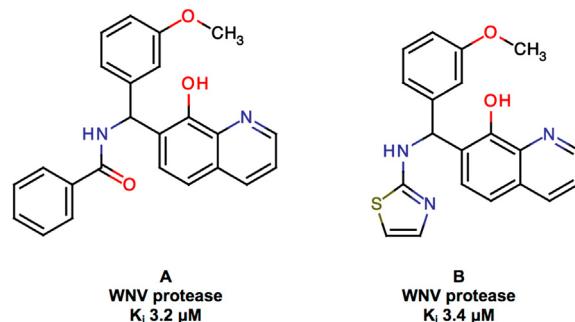


Fig. 51. The two novel hits identified from high throughput screening targeting WNV NS2b-NS3.

The modification of S-adenosyl-homocysteine (SAH) with various substituents generated inhibitors with enhanced and selective activity against DENV methyltransferase (MTase). Crystal structure of dengue virus MTase bound with the most potent SAH derivative (Fig. 39) further revealed the necessary structural

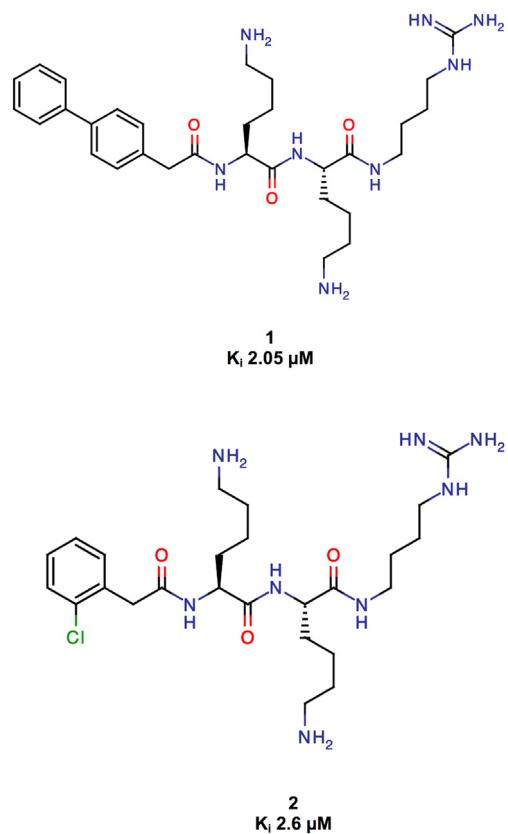


Fig. 49. Chemical structures of two potent dipeptide inhibitors with their respective K_i values against WNV NS2b-NS3 protease.

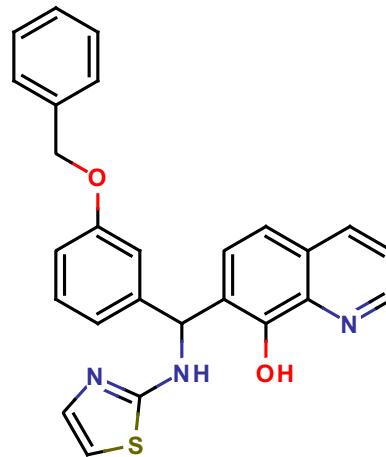


Fig. 52. The most potent benzoxy substituted 8-HQ analog active against WNV NS2b-NS3 protease.

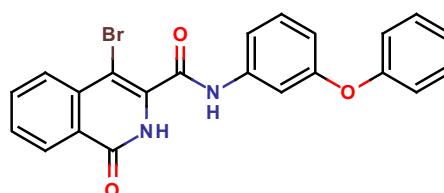


Fig. 53. 4-bromo-1, 2-dihydroisoquinolin-1-one containing WNV serine protease inhibitor.

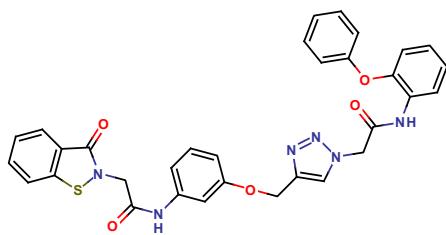


Fig. 54. Structural representation of one of the most potent WNV NS2B-NS3 protease inhibitor synthesised using click chemistry based approach containing benz[d] isothiazol-3(2H)-one scaffold targeting.

features required for selective and potent inhibition of methyltransferase. These findings provide clues to overcome the major hurdles for the development of selective MTase therapy [11].

Novel compounds targeting DENV RNA dependent (RdRp) and methyltransferase (MTase) from virtual screening of a database of five million commercially available compounds. Two novel inhibitors in each category with IC_{50} values lower than 10 μM were identified [66] (Fig. 40).

Lately an ensemble of similarity search, pharmacophore filtering and molecular docking approach by Luzhkov et al. have been used to identify novel inhibitors targeting DENV 2' O-methyltransferase. This study identified a novel molecule with a previously unknown scaffold with an IC_{50} value of 60 μM [67] (Fig. 41).

A novel class of compounds containing N-sulfonylanthranilic acid moiety [112] (Fig. 42) specifically inhibits dengue viral polymerase (RdRp) with the most potent compound (compound 4) having an IC_{50} value at lower micromolar range (Fig. 42). Flavanones are privileged scaffold that act on several disease conditions. A series of new mono and di alkylated flavanones named chartaceones along with pinocembrin were isolated from chemical investigation of *Cryptocarya chartacea* [68]. The di alkylated chartaceones exhibited the most significant NS5 RdRp inhibiting activity, with IC_{50} ranging from 1.8 to 4.2 μM (Fig. 43). These compounds may represent a new class of non-nucleoside inhibitors that will prove useful in targeting DENV NS5 RdRp.

Biflavonoids, are another class of compounds extracted from *Dacrydium balansae* which showed potency towards DENV NS5 RdRp with IC_{50} values of 0.26 μM and 0.75 μM for the two most potent inhibitors [69] (Fig. 44).

Avicularin, quercitrin, hyperoside, betulinic acid, spiraeoside, quercetin-3, 4'-di-O-glucoside and rutin are a group of compounds isolated from *Carpolepis laurifolia*. These compounds have been observed to interfere with DENV replication with IC_{50} ranging from 1.7 to 2.1 μM where they are believed to inhibit RdRp activity [70]. High-throughput screening of a library of compound using a

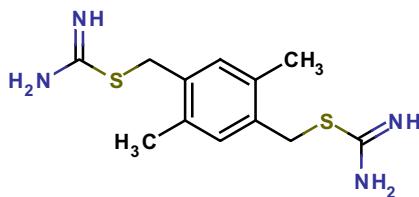


Fig. 56. Chemical structure of a non-peptidic inhibitor of WNV NS3 protease by high throughput docking.[113].

whole-virus assay identified a novel small molecule inhibitor of DENV, ST-610, which showed potent and selective *in vitro* activity against all four DENV serotypes (Fig. 45). Molecular-beacon-based helicase assay unveiled that ST-610 worked via inhibiting DENV NS3 helicase RNA unwinding activity [70].

Interestingly, a δ opioid receptor antagonist, SDM25N (Fig. 46) showed antiviral activity against subtype-2 of DENV [71]. Further biological testing and assay results suggest it inhibits the DENV replication via targeting viral NS4b subunit. This is the first molecule which inhibits DENV by attacking viral NS4b subunit and thus can be explored as a structural template for future structure based drug design efforts.

4.2. Targeting West Nile Virus (WNV)

Similar to DENV, tetrapeptide and aldehyde based benzoyl-norleucine-lysine-arginine (Bz-nKRR) analogs act as inhibitors of West Nile Virus NS3 protease. Knox et al. reported three novel tetrapeptide analogs reported to have an IC_{50} ranging from 0.7 μM to 1.9 μM [72]. In another study investigators found, a potent WNV NS2B-NS3 protease tripeptide inhibitor with an IC_{50} of 1.6 μM (Fig. 47) [73]. This tripeptide inhibitor had crucial properties such as serum stability, cell permeability and showed no trace of toxicity in initial study.

Another series of tripeptide aldehyde inhibitors for WNV NS3 protease were identified via structural modification of a common peptide skeleton with compounds showing submicromolar range activity [74] (Fig. 48). These analogs and substitutions could be a possible cornerstone for further SAR based drug discovery.

In two subsequent studies Lim et al. synthesized and tested the inhibitory activities of novel non-covalent peptidomimetic inhibitors against WNV NS2B/NS3 protease. These inhibitors contained a decarboxylated P1 arginine (agmatine; 4-aminobutylguanidine) and related analogues [75,76]. One agmatine peptidomimetic (4-phenyl-phenacyl-Lys-Lys-agmatine; compound 1 of Fig. 49) was shown to be a competitive inhibitor with a binding affinity (K_i) of 2.05 μM [75]. The results seem to further suggest the presence of agmatine at position Pi of the peptidomimetics could potentially act as the basis for designing of non-covalent competitive protease inhibitors due to their relative stability and ease of chemical synthesis compared to inhibitors containing reactive electrophilic warheads [76]. Modification of compound 1 (Fig. 49) yielded a novel agmatine dipeptide inhibitor [76] (compound 2 of Fig. 49) which could be an exciting compound in exploring different scaffold based substitutions.

Compounds containing cyanoacrylamide scaffold with a *para*-hydroxy substitution (Fig. 50) were found to be the potent inhibitors of WNV NS2B-NS3 protease with a K_i value of 44.6 μM [55]. This discovery could lead to future efforts in targeting both DENV and WNV NS2B-NS3 protease using multi-targeted small molecules.

High throughput screening based exploration also led to identification of two novel 8-hydroxyquinoline (8-HQ) analogs [77] as potent WNV NS2b-NS3 protease inhibitors (Fig. 51).

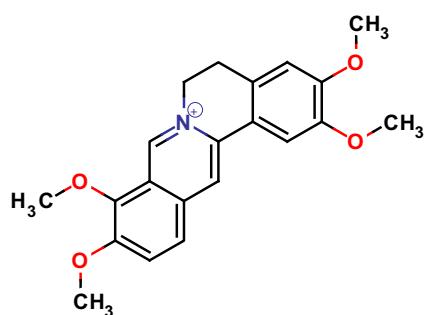


Fig. 55. 2-D structural representation of palmatine.

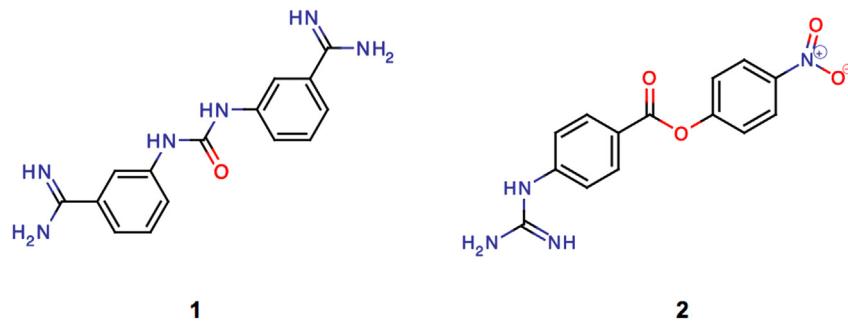


Fig. 57. Chemical structure of a non-peptidic inhibitor of WNV NS3 protease identified by Molecular dynamic simulations.

Structural modification of these analogs led to the identification of a benzoxy substituted potent 8-HQ analog with an IC_{50} value of 2.01 μM [50] (Fig. 52). This moiety is the most potent 8-HQ analog discovered up to date and is an ideal target for developing broad spectrum serine protease inhibitor...

The 4-bromo-1, 2-dihydroisoquinolin-1-one scaffold can form as the basis for developing hit-to-lead optimization as it showed inhibitory activity against WNV serine protease (Fig. 53) [78].

Akin to their observed potency against DENV NS2B-NS3pro, benz[d]isothiazol-3(2H)-one [52] scaffold derived from click chemistry were found to possess inhibitory activity against WNV NS2B-NS3 protease (Fig. 54) thereby demonstrating ideal properties that it may constitute a structural template for designing multi-target NS2B-NS3pro inhibitors against both DENV and WNV.

Jia et al. [79] investigated the specific inhibition of West Nile virus (WNV) NS2B-NS3 protease and viral propagation by

palmatine, a chemical compound from *Coptis chinensis*. Palmatine inhibited WNV NS2B-NS3 protease activity in a non-competitive manner, with a 50% inhibitory concentration (IC_{50}) of 96 μM . Palmatine suppressed WNV without detectable cytotoxicity (EC_{50}) of 3.6 μM and a 50% cytotoxicity concentration [CC_{50}] of 1031 μM (Fig. 55).

Modern state of the art computational methodologies namely automated high throughput docking and molecular dynamics simulation were used in the identification of novel inhibitors targeting WNV NS3 protease (Figs. 56 and 57).



Fig. 60. The potent allosteric inhibitor of WNV NS2b-NS3 protease.

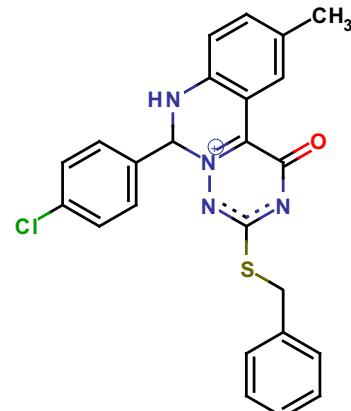


Fig. 61. A novel uncompetitive inhibitor of WNV NS2b-NS3 protease.

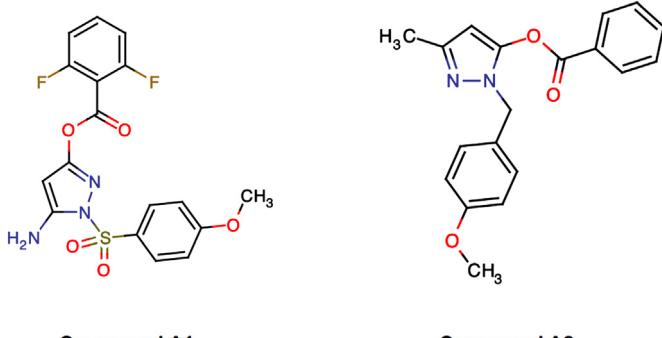


Fig. 59. The pyrazole containing chemical moieties as WNV NS2b-NS3 protease inhibitor.

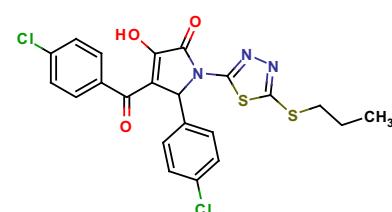


Fig. 62. Novel WNV NS2b-NS3 protease inhibitor with 1, 3, 4, 5-tetrasubstituted 1H-pyrrrol-2(5H)-one scaffold.

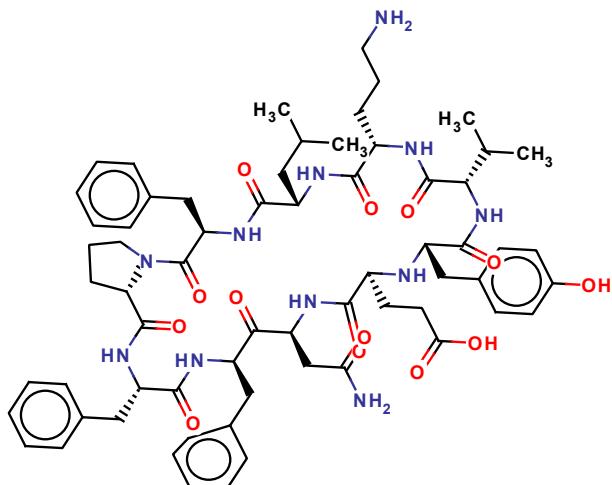


Fig. 63. Identified novel peptidomimetic inhibitor identified from a parallel validated HTS.

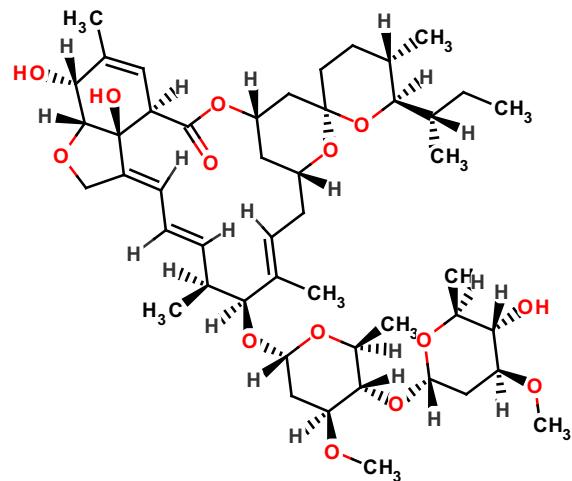


Fig. 67. Chemical structure of Ivermectin, a potent antiparasitic agent reported to possess WNV NS3 helicase inhibitory activity.

In HTS two novel non-competitive inhibitors containing pyrazol-5-amine scaffold were identified [80]. These compounds interfered with the productive interactions of the NS3pro with its cofactor NS2B (Fig. 58) [80].

Further modification of chemical scaffolds identified using HTS approach lead to identification of two new protease inhibitors targeting WNV serine protease (Fig. 59) [81].

Modification of scaffold containing 2-{6-[2-(5-phenyl-4H-1,2,4-triazol-3-ylsulfanyl) acetylaminobenzothiazol-2-ylsulfanyl} acetamide moiety and optimization of this initial hit by synthesis and biological screening led to the identification of a novel non-competitive inhibitor (Fig. 60, IC₅₀ = 3.4 μM) of the WNV NS2B-NS3 protease. Molecular docking study suggested that this novel hit inhibits the WNV protease by allosterically inhibiting the interaction of NS2b and NS3 subunit [82].

Further, solution-phase synthesis and screening of a focused library of compounds identified a novel, non-competitive inhibitor (Fig. 61, IC₅₀ = 5.41 μM) of WNV NS2B-NS3 protease. Molecular docking of this chiral compound onto the WNV protease indicated that the S enantiomer of the identified hit interfered with the proper interactions of the NS2B cofactor and the NS3 protease domain thus inhibiting viral replication [78].

Another scaffold having 1,3, 4, 5-tetra substituted 1H-pyrrol-2(5H)-one moiety was identified by screening of a small library of nonpeptidic compounds [83]. Optimization of this initial hit by synthesis and screening of a focused library of compounds led to the discovery of a novel non-competitive WNV NS2b-NS3 protease inhibitor with an IC₅₀ value of 2.2 μM (Fig. 62). Contrary, to the compound mentioned above (Fig. 61) molecular docking of this chiral compound onto the WNV protease indicated that the R

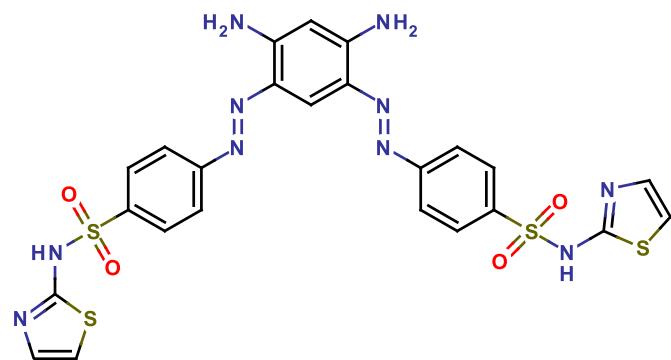


Fig. 64. One of the potent allosteric inhibitor of WNV serine protease discovered from a virtual screening campaign.

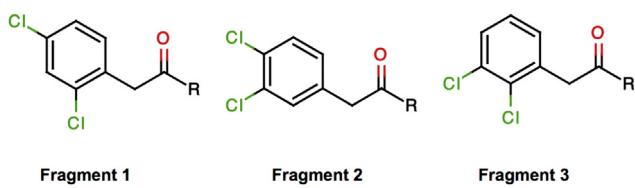
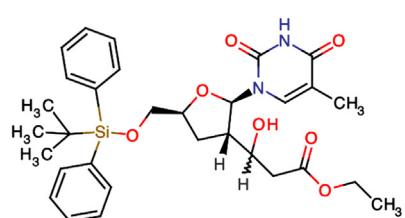
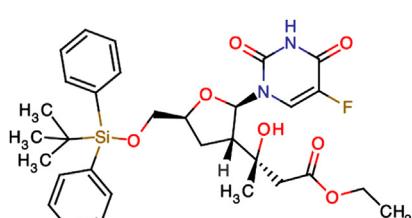


Fig. 65. Top three fragments containing di-chloro analogs of GCMA as potential WNV NS2b-NS3 protease inhibitor.

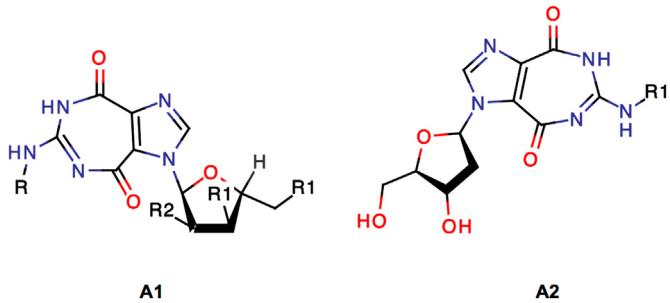


Inhibitor A



Inhibitor B

Fig. 66. Two potent nucleoside analogs as competitive inhibitors of WNV methyltransferase. The K_i value of inhibitor A and B found to respectively against WNV MTase.

**Fig. 68.** 2-D structural representation of two most potent REN's.

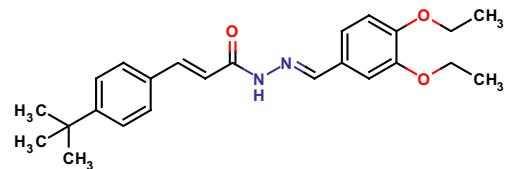
enantiomer of this novel hit interfered with the correct interactions between the NS2B cofactor and the NS3 protease domain.

Furthermore, a parallel validated HTS was used to identify one of the potent WNV NS2B-NS3 protease inhibitor with a K_i value of $2 \pm 0.2 \mu\text{M}$ (Fig. 63) [84].

In another study, a series of allosteric inhibitors were identified through virtual screening approach to screen a library of an estimated 275,000 compounds library [85]. The allosteric inhibitors were capable of targeting the NS2B-NS3pro interface rather than the NS3pro active site. The selectivity of the inhibitors was confirmed using the *in vitro* cleavage assays with human serine proteinase, which has similar substrate preference with WNV NS2B-NS3pro. One of the potent allosteric inhibitor showed lower micromolar activity against WNV and thus could be a possible lead for future drug development (Fig. 64). Conceptually, similar *in silico* drug discovery strategy may be extended to the identification of selective inhibitors targeting other flaviviruses.

A series of new decarboxylated arginine mimetics were found to inhibit WNV NS2B-NS3 protease at lower micromolar range [33]. In combination with dichloro-substituted phenylacetyl groups at the P4 position, three inhibitors with inhibition constants of $<0.2 \mu\text{M}$ were discovered. These inhibitors possessed a better selectivity profile and emerged as a potential template to develop novel drug candidates (Fig. 65). Crystal structure of 3, 4-dichlorophenylacetyl-Lys-Lys-GCMA ($K_i = 0.13 \mu\text{M}$) in complex with WNV NS2B-NS3 revealed a horseshoe-like conformation of the inhibitor in the active site, most likely due to a hydrophobic contact between the P4 phenyl ring and the P1 cyclohexyl group, which is further stabilized by an intramolecular hydrogen bond between the P1 guanidino group and the P4 carbonyl oxygen atom. These inhibitors are stable, readily accessible, and have a non-covalent binding mode. Therefore, they may serve as suitable lead structures for further drug development [33].

Nucleoside analogs have been less studied for their methyltransferase inhibitory activity. Two of the nucleoside analogs (inhibitor A and B of Fig. 66) can effectively and competitively inhibit the WNV MTase with IC_{50} values in micromolar range and, more importantly, do not inhibit human MTase. These compounds also

**Fig. 70.** The most potent CHIKV NS2 inhibitor developed using a combination of computational and synthetic approach.

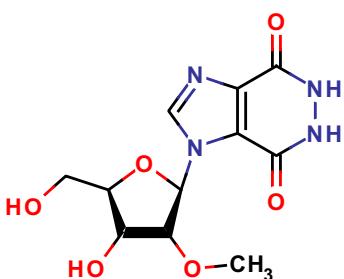
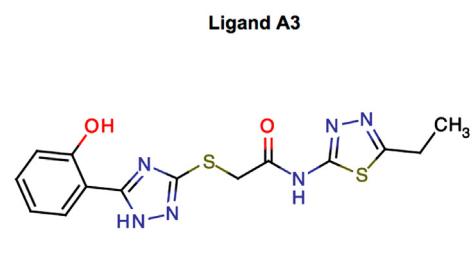
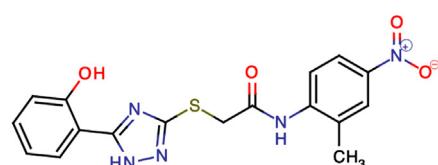
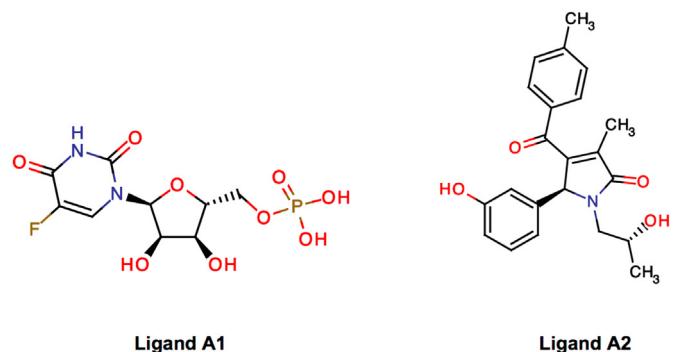
suppress the WNV replication in cell culture [86]. The selectivity of these compounds towards WNV MTase qualifies them as promising future drug candidates to explore.

Interestingly, ivermectin a broad spectrum antiparasitic agent has also been reported to inhibit WNV NS3 helicase (Fig. 67) [87].

Recently a series of ring-expanded nucleoside analogues (RENs) containing the 6-aminoimidazo[4,5-e][1,3]diazepine-4,8-dione ring system have been synthesized and screened for inhibition of NTPase/helicase of the West Nile Virus (WNV) (Fig. 68) [88]. Another nucleoside analog having imidazo[4,5-d]pyridazine ring [89] also found to possess inhibit WNV replication by acting as NTPase/helicase inhibitor (Fig. 69). The common pharmacophoric features of these nucleoside analogs may prove effective in designing potent and selective NTPase/helicase inhibitors against WNV and other flaviviruses.

4.3. Targeting Chikungunya Virus (CHIKV)

Parallel to DENV and WNV the research focusing on identification of novel non-structural protein inhibitors targeting CHIKV

**Fig. 69.** Chemical structure of a novel nucleoside analog having imidazo[4,5-d]pyridazine ring (HMC-HO4).**Fig. 71.** Novel *in silico* hits targeting CHIKV NS2.

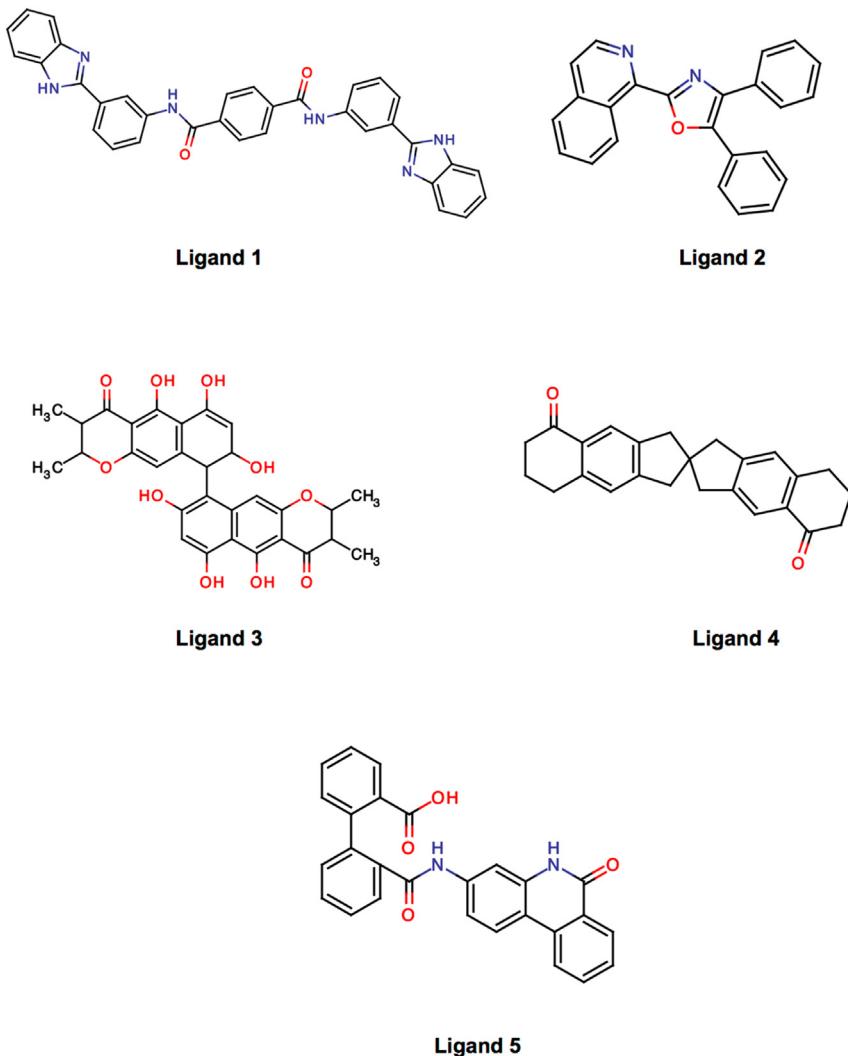


Fig. 72. *In silico* hits targeting NS3 macrodomain of CHIKV.

continue to attract less attention due to the lack of crystallographic evidence and enzyme based assay techniques. Very recently, a series of CHIKV NS2 inhibitors were identified by using a combination of computational and synthetic approaches (Fig. 70). One of the potent inhibitor interfered with the CHIKV viral replication at a 50% effective concentration of 3.2 μM and may thus act as structural template for further lead development [90].

Combination of homology modelling, molecular dynamics and pharmacophore mapping approach also has led to identification of some potent *in silico* hits as potential CHIKV NS2 inhibitors (Fig. 71) [91].

A combination of virtual screening and molecular dynamics simulations was applied in the identification of a variety of *in silico* hits targeting the NS3 macrodomain of CHIKV (Fig. 72) [92]. Though these hits are exciting leads for designing novel protease inhibitors against these Chikungunya, further validation of their inhibitory activity through wet lab experiments need to be performed.

4.4. Crowd-computing and collaborative research: an alternative perspective

Recent advances in computational tools have reenergized existing strategies aimed at designing and identifying potent drugs

in a relatively a rapid and cost effective manner. Crowd computing encompasses crowd sourcing, automation, idea sharing and machine learning to deliver the best computational outcome possible. The success of FightAIDS@Home project provides researchers with immense opportunities to initiate crowd computing projects targeting Neglected Tropical Diseases. For instance, “Discover Drugs against Dengue (DDD)” is one of the major crowd computing projects which uses client computers and engages volunteers globally in identifying novel antiviral drug candidates against DENV, WNV and related flaviviruses [93]. Under the banner of “World Community Grid” DDD project launched on August 21, 2007 the Phase I aim is to identify novel antiviral candidates targeting NS3 protease of flavivirus using AutoDock. In phase I, a plethora of compounds was screened downstream refining Phase II. The second phase entails the use of molecular dynamics simulations based binding free energy calculations in the CHARMM molecular dynamics package [94] to identify potential hits for further biological screening (Fig. 73).

Using viral non-structural proteases as targets, the crowd computing based structure-based drug design approach will arguably be amenable in the identification of novel small molecule inhibitors targeting these Neglected Tropical Diseases. Moreover, the availability of more crystal structures revealing the binding site conformation of DENV, WNV and CHIKV non-structural proteins

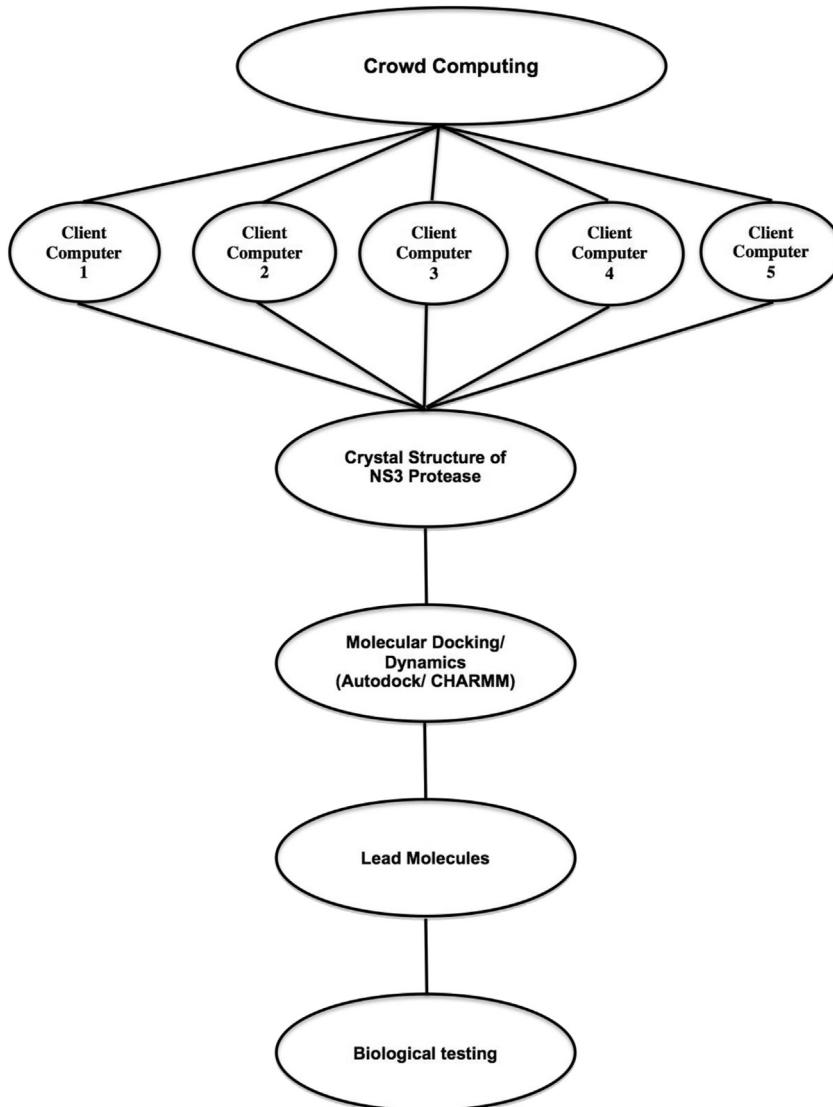


Fig. 73. Flowchart illustrating the drug discovery approach used in the DDD crowd-computing project.

will make it possible to use crowd computing based screening approaches to identify novel broad spectrum antivirals.

Akin to crowd computing, collaborative efforts in drug discovery also attracts considerably huge attention since the genome repertoire annotations coupled to proper identification of crystallographic structure is regarded as the foundation of any modern era rational drug discovery. "VIZIER" is one such collaborative research effort whose goal is to characterize all core proteins involved in viral replication [95]. VIZIER is a collaborative research project, with networks in prominent research institutions within the European Union. Genome sequencing, gene annotations, crystallization, structure determination and target validation performed under the VIZIER project unveiled crystal structures of two main non-structural proteins of viral replicative cycle, NS3 and NS5 [96]. Elucidation of these crystal structures for both DENV and WNV further led to the founding of another collaborative project named "SILVER" with the aim of identifying small molecule inhibitors targeting these emerging tropical RNA viruses [97,98]. Using an integrated structure based drug design; a library of compound library is screened with the objective of identifying novel small molecule leads with a favourable *in vitro* ADME and toxicity profile.

Despite the success of these global collaborative efforts, there is need for sustained research towards developing novel and diverse small molecule inhibitors. The emergence of drug resistant viruses may in future present a challenge in the development of effective small molecule inhibitors targeting these NTDs. However, a more rationally designed open source approach may prove to be the turning point in a rapid and effective strategy for developing effective drugs against these NTDs.

5. Concluding remarks and what's in the future?

To date, a variety of chemical entities and novel scaffolds have been explored for their role as drug molecules targeting three major NTDs in inhibiting. The increased crystallographic information publicly available detailing the structural and the functionality of key viral proteins in DENV, WNV, CHIKV and other NTDs eases the rational developing novel inhibitors targeting these proteins. Further the reported success of new chemical entities targeting one of the flavivirus, Hepatitis C gives a strategically vital starting point to design and develop selective and potent drug-like molecules targeting other flaviviruses. Despite significant efforts in

discovering novel entities targeting NTDs the dearth of clinically approved drug molecules is a challenge in countering sudden disease outbreaks. As stated previously, global pharmaceutical giants lag behind in the development of drugs to fight neglected tropical diseases. This may be informed by their negligible contributions towards such efforts and the low profit such ventures may attract. However, recent collaborations between some of the major pharmaceutical companies raises hope to find the first possible drug molecules targeting three major NTDs namely Dengue, West Nile and Chikungunya viruses. Also exciting is the outcome of DDD, which is currently in its phase II stage. In conclusion, we believe that future NTDs drug discovery efforts should take into account both public and private ownership and adapt an “open source” model to foster unrestricted innovation. Hopefully with the growing efforts of public-private ownership and emergence of new open source drug discovery initiatives it will be within reach humanity to get effective drugs for different NTDs.

Conflicts of interests

Authors declare no conflicts of interest.

Acknowledgement

The authors like to acknowledge the School of Health Sciences, University of KwaZulu-Natal, Westville for financial support.

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