Current Approaches in Antiviral Drug Discovery Against the Flaviviridae Family

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Abstract: Viruses belonging to the Flaviviridae family primarily spread through arthropod vectors, and are the major causes of illness and death around the globe. The Flaviviridae family consists of 3 genera which include the Flavivirus genus (type species, yellow fever virus) as the largest genus, the Hepacivirus (type species, hepatitis C virus) and the Pestivirus (type species, bovine virus diarrhea). The flaviviruses (Flavivirus genus) are small RNA viruses transmitted by mosquitoes and ticks that take over host cell machinery in order to propagate. However, hepaciviruses and pestiviruses are not antropod-borne. Despite the extensive research and public health concern associated with flavivirus diseases, to date, there is no specific treatment available for any flavivirus infections, though commercially available vaccines for yellow fever, Japanese encephalitis and tick-born encephalitis exist. Due to the global threat of viral pandemics, there is an urgent need for new drugs. In many countries, patients with severe cases of flavivirus infections are treated only by supportive care, which includes intravenous fluids, hospitalization, respiratory support, and prevention of secondary infections. This review discusses the strategies used towards the discovery of antiviral drugs, focusing on rational drug design against Dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Yellow Fever virus (YFV) and Hepatitis C virus (HCV). Only modified peptidic, non-peptidic, natural compounds and fragment-based inhibitors (typically of mass less than 300 Da) against structural and non-structural proteins are discussed.

Keywords: Dengue virus (DENV), West Nile virus (WNV), Japanese encephalitis virus (JEV), Yellow Fever virus (YFV), Hepatitis C virus (HCV), drug discovery, antiviral.

1. INTRODUCTION

The genus Flavivirus is a group of aboviruses that belongs to the Flaviviridae family. It consists of over 70 viruses mostly transmitted by mosquitoes or ticks [1]. West Nile virus (WNV), Japanese encephalitis virus (JEV), Dengue virus (DENV), Tick-borne encephalitis virus (TBEV), Yellow Fever virus (YFV), Murray Valley encephalitis virus (MVLEV) and St Louis encephalitis virus (SLEV) are considered important global flavivirus pathogens which are responsible for human and animal diseases and deaths. Most of these viruses cause severe hemorrhaging fever while others cause neuroinvasive diseases ranging from mild febrile illness to fatal encephalitis. DENV, JEV, YFV and TBEV are the most dangerous flaviviruses that have mortality rates up to 30% [2]. The viruses are endemic in many areas of the world and thousands of lives are lost during recurring outbreaks. Although extensive researches have been conducted, currently there is no specific treatment available for any flavivirus infection, though commercially available vaccines for yellow fever, Japanese encephalitis and tick-born encephalitis exist [3]. Available license drug such as ribavirin is known to suppress the replication of some flaviviruses in vitro, however, in vivo studies have been limited to few rodent models [4]. Furthermore, the management of mosquito and tick-borne populations has shown to be problematic [5]. In addition, due to the globalization and gradual climate changes, the viruses are emerging in new geographic areas and populations, thus there is an urgent need to find new drugs to fight these emerging threats [3]. For example, in the USA, the first case of WNV was reported in New York in the year 1999. Since then, more than 30,000 clinical cases of WNV and neuroinvasive diseases were reported by the Centers for Disease Control and Prevention (CDC), with the largest outbreaks in the summer of 2002, 2003 and 2012 (ref). Currently, the most important mosquito borne disease is the dengue fever with an estimated 50 million cases of febrile illness each year, and an increasing number of cases for hemorrhagic fever [6].

The flavivirus genome is a positive single-stranded RNA with a size of ~11 kb, which includes the 5’ and 3’ noncoding regions of approximately 100 to 600 nucleotides, respectively (Fig. 1). In the infected cells, the messenger RNA (mRNA) of the RNA genome encodes three structural proteins (C, capsid protein; precursor membrane protein; and envelope E protein) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3 [protease, helicase], NS4A, NS4B and NS5 [methyltransferase, RNA-dependent RNA polymerase]) that form the apparatus involved in viral synthesis and replication [7, 8]. Virus-encoded serine protease, NS2B-NS3, and host-encoded proteases, including signalase and furin, are responsible for the processing of the three structural and seven NS proteins encoded by the viral mRNA [9]. Currently, researchers are targeting the structural and non-structural viral proteins for drug development [10-14]. According to previous knowledge of drugs that are now in clinical trials, RNA dependent RNA polymerase (RdRp) has shown to be the most promising target. This enzyme plays a critical role in viral replication by forming part of a multimeric complex, and since there is no host cellular protein equivalent to flavivirus RdRp, no toxicity issues exist [14]. Like anti-HIV protease inhibitors that are already in the market for clinical use, flavivirus protease is another attractive target for drug development. However, since cellular serine protease such as furin also recognizes two basic amino acid residues at P1 and P2 positions [15], its toxicity issues remain to be determined. Another attractive choice for antiviral therapeutics is the virus entry inhibitors. For example, virus entry inhibitors have been developed and approved for treatment of HIV due to the emergence of drug resistance and cellular toxicity towards inhibitors of DNA and RNA polymerases and proteases [16-18]. Another novel target for flaviviruses is the NS3
RNA helicase that forms a multimeric complex with NS5, which is required for viral replication, and apparently needed for unwinding the double-stranded RNA intermediate [12]. In addition to these approaches where the virus-coded function is blocked, another approach is to block the cellular functions needed for the viral multiplication. In the later approach, knowledge of the virus-host protein interactions is fundamental.

Development of drugs through trial-and-error is time-consuming and may be costly. With the advancement of science in drug discovery, scientists are focusing more on structure-based rational drug design techniques that link NMR and crystallography, computational advances in docking and virtual screening, and high-throughput screening (HTS) with combinatorial chemistry to increase the speed and efficiency of drug discovery. On another note, structure-based drug design may improve initial discovery, but it has yet to significantly improve the effectiveness or reduce the cost of clinical trials. In rational drug design, the process is more streamlined and requires detail knowledge of the target of the drug, as well as the drug itself. It usually involves the study of the three-dimensional structure of a drug target, followed by the search of a compound that can interact with the target. By aiming at well-characterized specific target of the virus, it is hoped that the selected compound would be specific and potent in its activity, desirably with less side effects. Rational drug design requires sound knowledge of chemistry as well as biology because the bioactivities of drugs depend on the chemical interactions between the drugs and their targets. Two possible rational approaches that could be used in the design of antiviral compounds are by modification of the known antiviral drugs or inhibitors, and design of new antiviral compounds directed at specific steps in the replication cycle of particular viruses. Several other strategies for discovery of chemotherapeutic agents for flaviviruses include reverse genetic system-based screening, macromolecular inhibitors like antibodies and interferon, nucleic acid-based agents (for example antisense oligomers and siRNA) [19] and vaccine development [20]. However, in this review article, only strategies for discovery of modified peptidic, non-peptidic, natural compounds and fragment-based inhibitors (typically of mass less than 300 Da) against DENV, WNV, JEV, YFV and HCV structural and non-structural proteins are discussed.
DENV type 2 NS3 protease showed that the tripeptide aldehyde docking study of phenylacetyl-KRR-H to a homology model of protein especially towards its co-factor NS2B. On the other hand, DENV NS3 protease due to the marked structural flexibility of the Nevertheless, the peptide inhibitor gave poor inhibition towards protease compared to reference peptide inhibitor, benzoyl-nKRR-H. 

Results revealed that the tripeptide aldehyde phenylacetyl- and their inhibitory effects against DENV type 2 protease were tested. Schuller [22] found a few tripeptide aldehydes that were successfully synthesized, structure modification and optimization. Recently, Deng et al [21] reported a compound, obtained through virtual screening, to be active against NS2B-NS3 protease. This compound was selected as the lead structure after considering its biological activity, structural variability and synthetic accessibility. In fact, modification on the functional group on R1-R4 substituents in this compound has resulted in four new inhibitors with moderate biological activity, structural variability and synthetic accessibility. In fact, modification on the functional group on R1-R4 substituents in this compound has resulted in four new inhibitors with moderate biological activity, structural variability and synthetic accessibility.

The development of new retro di- and tripeptide hybrids that did not require highly electrrophilic warheads (serine-trap) such as aldehydes, keto-amides, and boronic acids to combine with macrocyclic fragments to achieve affinities in the low micromolar range have been described by Nitsche et al [24]. Retro peptides were found to give the best activities for both DENV and WNV proteases. Retro and retro-inverse peptide sequences of target are usually used for protein inhibition study and to understand the intermolecular recognition. For retro peptides, only the C- and N-termini of a given peptide sequence are interchangeable. And as for retro-inverse peptides, the streocenters are also interchangeable. An inversion of the sequence from R-Arg-Lys-His to the non-retro sequence R-Lys-Arg-NH₂ resulted in loss of DENV activity and invariant activity for WNV protease. The DENV protease was found to be more sensitive to molecular changes in retro-peptide inhibitors compared to WNV protease. This study summarized that retro peptides were the best choice for creation of potent and selective DENV protease inhibitors, and were superior to natural sequences or retroinverse peptides.

A guanidinilated 2,5-dideoxystreptamine was identified as a competitive inhibitor of NS3 protease for all four DENV serotypes and WNV with IC₅₀ values in the range of 1-70 μM [25]. This compound was discovered through screening of about ~12 000 compound library using FRET 96-well plate based assay. The concentration of the tested compound was fixed at 25 μM. Hits were identified if the compounds inhibited the proteolytic activity more than 40% and were characterized as follows: determination of the IC₅₀ against both DENV (all four serotypes) and WNV NS2B-NS3 pro-

### Table 1. X-ray crystal structures available in PDB² for DENV protease.

<table>
<thead>
<tr>
<th>DENV serotypes</th>
<th>PDB²</th>
<th>Resolution (Å)</th>
<th>Comment</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENV1</td>
<td>3L6P</td>
<td>2.20</td>
<td>NS2B-NS3pro²</td>
<td>[175]</td>
</tr>
<tr>
<td>DENV1</td>
<td>3LK W</td>
<td>2.00</td>
<td>NS2B-NS3pro² (active site mutant)</td>
<td>[175]</td>
</tr>
<tr>
<td>DENV1</td>
<td>2WZQ</td>
<td>2.80</td>
<td>NS2B-NS3 full length</td>
<td>[176]</td>
</tr>
<tr>
<td>DENV2</td>
<td>2FOM</td>
<td>1.50</td>
<td>NS2B-NS3pro²</td>
<td>[126]</td>
</tr>
<tr>
<td>DENV3</td>
<td>3U1H</td>
<td>2.30</td>
<td>NS2B-NS3pro² complexed with peptide [BEZ][NLE][KR][OAR]</td>
<td>[177]</td>
</tr>
<tr>
<td>DENV3</td>
<td>3U1J</td>
<td>1.80</td>
<td>NS2B-NS3pro complexed with aprotinin</td>
<td>[177]</td>
</tr>
<tr>
<td>DENV4</td>
<td>2VBC</td>
<td>3.15</td>
<td>NS2B-NS3 full length</td>
<td>[178]</td>
</tr>
<tr>
<td>DENV4</td>
<td>2WHX</td>
<td>2.20</td>
<td>NS2B-NS3 full length</td>
<td>[176]</td>
</tr>
</tbody>
</table>

²Protein data bank (PDB)
³NS2-NS3protease (NS2-NS3pro)
teases, identification of potential promiscuous behavior and the mechanism of inhibition. Generally, inhibition of DENV type 4 NS2B-NS3 protease activities is reduced by 2 to 5 fold compared to the other serotypes. The highly positively charged nature of the compound suggested that they acted as substrate mimetics and interacted with the highly negatively charged non-prime portion of the substrate-binding pocket. Inhibition kinetics study by colorimetric pNA assay demonstrates competitive behavior for DENV type 2 and WNV NS2B-NS3 proteases. The compounds only interacted with the non-prime side and catalytic machinery within the active site, indicating that the compounds exclusively bind only to the non-prime side. The Vero cell cytotoxicity was assessed using an MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide)-based viability assay and the viral growth was measured by ELISA detection of virally produced envelope protein. The compound at 100 μM showed low cytotoxicity nor measurable reduction of DENV type 2 growths in Vero cells, which could be due to their highly cationic nature.

Knehans et al [26] demonstrated the use of in silico fragment-based drug design approach to identify small molecule inhibitors against DENV NS2B-NS3 protease. This approach was believed to be less time consuming compared to the normal high-throughput docking. Instead of docking millions of compounds, only a small set of compounds is required to be docked. The DENV protease model was then subjected to fragment-based drug design approach. Molecular fragments were then generated by retrosynthetically cleaving 14 million compounds of the ZINC screening database. Fragments that displayed high binding affinity towards DENV protease binding site were then identified by high throughput docking with Autodock Vina. Two sets of high-scoring fragments were later chosen based on the S1 and S2 binding pocket scores. High-scoring fragments were then linked to generate a potential DENV protease...
inhibitor. Molecular docking has predicted the binding mode of this potential inhibitor and found that this compound interacted with Asp129 of the S1 pocket via hydrogen bonding and electrostatic interactions. A total of 23 compounds were then tested experimentally and only two compounds were discovered to inhibit dengue protease \((IC_{50}=7.7 \, \mu M \text{ and } 37.9 \, \mu M, \text{ respectively})\). Both compounds exhibited almost similar \(IC_{50}\) values in protease assay and may act as covalent inhibitors. The ester group may also react with the nucleophilic groups outside the active site, opening the possibility for unspecific or allosteric binding. However, enzyme kinetic analysis of both compounds indicated a competitive mode of inhibition, as expected by active site directed inhibitors. In this analysis, the first and second compound exhibited an inhibition constant \(K_i\) of 2.0 \(\mu M\) and 31.1 \(\mu M\) for DENV type 2, respectively.

Frimayanti et al. [27] used fragment-based approach based on natural product extracts (4-hydroxypanduratin A and panduratin A) and synthesized compound, 246 DA. Our previous work indicated that 4-hydroxypanduratin A and panduratin A, isolated from finger root (Boesenbergia rotunda) exhibited inhibitory activities against DENV type 2 NS3pro with \(K_i\) value of 21 and 25 \(\mu M\), respectively. These components of finger root were known to be competitively inhibiting the protease activity [28]. Using fragment-based approach, the ligands were divided into separate portions or fragments: hydrophilic, hydrogen bonding and van der Waals fragments, followed with docking of each fragment into the active site of DENV type 2 NS2B-NS3pro (PDB code 2FOM) using the MOE software package (Chemical Computing Group). All fragments were minimized before docking. CHARMM27 force field was used for docking purposes. Fragments that formed critical interactions with the binding site of the serine protease NS3 would serve as a starting point, and by linking each fragment, new ligands were constructed. The new ligands were further docked against NS3pro in a similar manner. The predicted \(K_i\) of the fragments and the new ligands were significantly lower when compared to the initial docked ligands, thus indicating tighter binding. The new ligands were able to bind well into the NS3pro active site, suggesting a greater potential as competitive dengue inhibitors. Indeed, as was reported, fragment-based drug design have several advantages such as it is a very fast method, and a large variety of molecules could be generated due to the possibility of enormous combinations of fragments to be connected [29].

Major conformational changes and well-defined molecular structures of pre- and post-fusion E protein present several targets for the design of inhibitors (Fig. 3). The crystal structure of the E-protein indicates a ligand-binding pocket that is filled with a detergent molecule, \(\eta\)-octyl-\(\beta\)-D-glucoside (\(\beta\)-OG) [30]. This finding led to a number of docking studies to identify and optimize possible inhibitors targeting at this position of the E protein [31-33]. One such computational study used a docking program to screen 135,000 compounds for inhibition of E protein via the \(\beta\)-OG pocket [34]. Several inhibitors obtained from the docking study displayed micromolar inhibition against DENV, YFV and Kunjin virus. A compound named A5 directly inhibited E protein-mediated fusion as shown by the cell-based fusion assays. Further study and optimization of these compounds could lead to the discovery of potent inhibitors. Besides targeting the \(\beta\)-OG pocket, small peptides have been designed against the E protein stem region. The C-terminus of the stem protein is essential for membrane fusion and is adjacent to the membrane-bound anchor region [35, 36]. The conserved sequence-specific binding interactions of the post-fusion DENV E were exploited for the design of peptides inhibitors. The E protein sequence of the stem region is conserved among all the four DENV serotypes and other flaviviruses. Compared to the inhibitors targeting at the \(\beta\)-OG pocket, these inhibitors were unique in that they inhibited the virus following entry into the cell, rather than prevention of entry itself. It was speculated that these peptides were
able to bind to the virion in a non-specific manner as they were taken up by the cells. In the late endosome, the virus is exposed to the low pH causing conformational rearrangements, thus inducing the tight binding of the peptides, and inhibition of membrane fusion [37-39]. A similar study done by Costin et al [39] used the pre-fusion DENV type 2 E protein and computer modeling techniques to discover peptide inhibitors. Rough outer surface morphology was observed on particles treated with the peptide inhibitors, compared to the untreated smooth outer surface of mature DENV, which indicates that the E proteins were likely rearranged. The virion structure was grossly affected as confirmed by the treated virion structures that were no longer icosahedral [39]. Other studies showed that these inhibitors were likely to inhibit the interaction of the transmembrane regions and the fusion loop [40].

3. WEST NILE VIRUS – NEEDS AN INTRO PARAGRAPH JUST LIKE DV AND JE.

West Nile virus (WNV) infections first appeared in the United States in 1999 (Centers for Disease Control and Prevention, June 2013). This virus causes epidemics of febrile illness, meningitis, encephalitis, and flaccid paralysis. Infected mosquitoes spread the virus that causes it. WNV has a spherical, enveloped capsid with a icosahedral symmetry. This virus has 2 genetic lineages; lineage 1 strains are found in North America, Europe, Africa, Asia and Australia; lineage 2 strains have been reported to have antiviral activity against WNV [44]. Among these inhibitors, mycophenolic acid was the most promising inhibitors since it exhibited potency at therapeutic concentration and inhibited other flaviviruses [47]. The inhibitory mechanism of mycophenolic acid was by directly binding to IMPDH [48] thus decreasing guanosine level to a point where viral RNA synthesis was interrupted [49]. A patent described mycophenolic acid as a carbamate prodrug, which was metabolized in the liver to produce an active moiety of mycophenolic acid but with undesirable pharmacological properties [50]. However, this prodrug could provide a lead for flavivirus inhibition and could be improved by chemical modification.

3.1. West Nile Virus Inhibitors

Several flavivirus inhibitors have been identified with high efficacy and low toxicity. Among these, ribavirin, mycophenolic acid, and 6-azauridine acetate, pyrazofurin, 2-thio-azauridine have been reported to have antiviral activity against WNV [44]. Among these inhibitors, mycophenolic acid was the most promising inhibitors since it exhibited potency at therapeutic concentration and inhibited other flaviviruses [47]. The inhibitory mechanism of mycophenolic acid was by directly binding to IMPDH [48] thus decreasing guanosine level to a point where viral RNA synthesis was interrupted [49]. A patent described mycophenolic acid as a carbamate prodrug, which was metabolized in the liver to produce an active moiety of mycophenolic acid but with undesirable pharmacological properties [50]. However, this prodrug could provide a lead for flavivirus inhibition and could be improved by chemical modification. Drugs like ribavirin and mycophenolate are nucleoside inhibitors that were developed against the host factors. Recent in vitro and in vivo studies showed that mycophenolic acid inhibited potential therapeutic activity against JEV with an IC50 of 3.1 μg/ml, and a therapeutic index of 16 [51]. Borowski et al showed that imidazo[4,5-d]pyridine-4,7(5H, 6H)-dione inhibited the helicase activity of WNV NS3 with an IC50 of 30 μM. This compound also inhibited WNV replication[52]. Nucleoside analog inhibitors developed for HCV, 2′-C-methyl-adenosine and 2′-C-methyl-guanosine also showed inhibition towards WNV with EC50 of 5.1 μM and 30 μM, respectively [53].

To date, there are three crystal structures of WNV NS2B-NS3pro in complex with inhibitors and one apo crystal structure of WNV NS2-NS3pro (Table 2). Like DENV protease, the catalytic triad (His51-Asp75-Ser135) is located at the cleft between the two β-barrels with arginine preferably located at the non-prime site P1 position of the protease active site, while arginine or lysine at the P2 position, thus highlighting the importance of electrostatic interactions with the negative S1 and S2 pockets [54]. Subsequently, Steomer et al reported potent and small molecule inhibitors, catonic tripeptides with non-peptidic at the N-terminus and aldehyde at the C-terminus for WNV protease [55]. One of the compounds with Ki of 9 nM was stable in serum (more than 90% intact after 3 hrs at 37°C), cell permeable and exhibits antiviral activity (IC50=1.6 μM) without cytotoxicity (IC50>400 μM). Recently, Shiller et al synthesized and analyzed a series of 17 tripeptides with the sequence X-KRR-H and X-KKR-H, where X is a varying N-terminal cap group [56]. The nature of the cap groups was selected to cover a large range of physicochemical properties such as length, polarity, flexibility and bulkiness. Bulk syntheses of tripeptide aldehydes were done according to standard solution-phase chemistry [57]. The peptides showed strong inhibition against WNV NS3 protease compared to DENV type 2 protease where the IC50 values were in sub-micromolar and micromolar ranges, respectively.

A non-peptidic inhibitor (4-(carbamimidoylsulfonylmethyl)-2,5-dimethylphenoxy)methylsulfonyl-methanimidamide was identified to be a lead compound against WNV NS3 protease from automated fragment-based docking study [58]. The compound showed a good ratio of binding affinity versus molecular weight, with ligand efficiency of 0.33 kcal/mol per non-hydrogen atom. This compound was discovered through screening of approximately 12,000 compounds from the iResearch database (ChemNavigator Inc. 2006) using Filter v2.0.1 (OpenEye Scientific Software, Inc). Selections of compounds were based on the structural information of the S1 to S3 pockets of the active site which contained 19 hydrogen bond acceptors. The binding free energy was calculated using the LIECE

### Table 2. X-ray crystal structures available in PDB for WNV protease.

<table>
<thead>
<tr>
<th>PDB</th>
<th>Resolution (Å)</th>
<th>Comment</th>
<th>Reference</th>
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<tr>
<td>2FP7</td>
<td>1.68</td>
<td>NS2B-NS3pro complexed with Bz-NKRR-H</td>
<td>[126]</td>
</tr>
<tr>
<td>2GGV</td>
<td>1.80</td>
<td>NS2B-NS3pro</td>
<td>[179]</td>
</tr>
<tr>
<td>2IJO</td>
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<td>[179]</td>
</tr>
<tr>
<td>3E90</td>
<td>2.40</td>
<td>NS2B-NS3pro complexed with Naph-KK-H</td>
<td>[180]</td>
</tr>
</tbody>
</table>

*Protein data bank (PDB)

**NS2-NS3protease (NS2-NS3pro)
A novel ligand with potential inhibitory activity against the NS3 protease of WNV, as well as for DENV, JEV, Murray Valley encephalitis virus (MVEV), and Usutu virus (USUV), has been identified via in silico techniques [62]. In this study, homology models for WNV, DENV, JEV, and USUV were constructed using MODELLER 9v7 based on the available NS3 protein structure of MVEV in the PDB (PDBid 2WV9) as template. The protease catalytic triad consisting of His51, Asp75, and Ser175 were determined based on a previous study [63]. The lead compound or the ‘seed molecule’ was revealed based on the previous available inhibitor studies for NS3 protein [64]. Following the ‘Lipinski’s Rule of Five’, fragment 1H-1,2,4-triazole was identified as the ‘seed molecule’ and docked into the MVEV NS3 protein. The resulting ‘seed molecule’ conformation was used for building 100 novel ligands for the inhibitory activity against NS3 protease of flavivirus family since the NS3 protein of WNV, DENV, JEV, MVEV and USUV exhibit similar structural features.

A series of meta and para aminobenzamide scaffold derivatives were synthesized, followed by in vitro screening against WNV and DENV proteases [65]. The $K_i$ values of the most potent compound, namely 7n, against WNV and DENV proteases were 5.55 and 8.77 μM, respectively. According to the kinetic data, compound 7n exhibited competitive binding mode against both proteases. The competitive binding mode was further supported by the prediction of bound $K_i$ value of 7n docked at the active site. This article provides an example on how iterative medicinal chemistry/structure-activity relationship studies and in vitro screening might develop new chemical scaffolds to yield potent inhibitors. A cinnamyl moiety combined with the ketoamide electrophiles has been identified to be potent inhibitors for WNV protease as well as for DENV protease [66]. Sufficient potency and selectivity were achieved with this covalent mode of inhibition. In another study [67], the promising cinnamyl moiety was combined with an alternative electrophile, the nitrile group, resulting in 3-aryl-2-cyanoacrylamide formation. A total of 86 analogs were synthesized based on the 3-aryl-2-cyanoacrylamide scaffolds to study the structure-activity relationships in detail. The most potent inhibitor for WNV protease was the para-hydroxy substituted analog with $K_i$ value of 44.6 μM. This analog also inhibited DENV protease with $K_i$ value of 35.7 μM. The discovery of chimeric peptide inhibitors, which inhibited the activation of the NS3 protease of a virus from the Flaviviridae family has been described in a patent invented by Glay et al [68]. However, these chimeric peptides were not capable of penetrating the target cells, thus, did not inhibit the viral infection in cell lines. The inhibition of the viral infection was achieved by combining the chimeric peptides with a cell-penetrating segment.

The present WNV NS2B-NS3 inhibitors are mainly peptide-based that target directly at the active site of NS3pro domain with $K_i$ values in a nanomolar range [56, 69]. However, in vivo practical use of these peptide inhibitors is limited. Several modestly successful high-throughput screening (HTS) assays to identify allosteric inhibitor against WNV NS2B-NS3pro have been developed. Johnston et al [70] identified a noncompetitive inhibitor of WNV protease, a common 5-aminof-1-(phenyl)sulfonyl-pyrazol-3-yl scaffold, by HTS of a small library (screened 65,000 compounds) through the National Institutes of Health (NIH) Molecular Libraries Initiative (MLI). These allosteric inhibitor interacted with the NS2B-binding cavity in the NS3pro domain, and the interaction intervened with the productive conformation of the active NS2B-NS3pro proteins complex [71]. The discovery of allosteric inhibitors could be a more superior drug discovery strategy since the active site of the human and viral serine protease is largely conserved.

Shiryaev et al [72] applied a focused structure-based approach to identify de novo allosteric small molecule against NS2B-NS3pro using virtual ligand screening (VLS) technology. In this study, about 275,000 molecules, openly available from NCI compound library of the Developmental Therapeutics Program NCI/NID (http://dtp.nci.nih.gov) have been used for high throughput in silico docking. The crystal structure of NS2B-NS3pro protein (PDB code 2IJO) was used in the docking simulations with the NS2B removed from the protein complex. A binary space partitioning tree-like structure was applied in order to increase the VLS performance. The Q-MOL L.L.C., San Diego, CA molecular modeling package with the Optimized Potential for Liquid Simulations (OPLS) force field [73] was used for protein-ligand complex optimization. Extensive experimental in vitro and cell based tests were conducted which led to the identification of three most promising novel inhibitory scaffolds. In vitro experiments indicated that these inhibitory scaffolds exhibited nanomolar level of inhibitions towards WNV NS2B-NS3pro. Furthermore no cross-reactivity was observed when these inhibitors were tested against human furin, and they moderately inhibited the highly homologous DENV NS2B-NS3pro. Cell based experiments confirmed the specificity of the inhibitors since flaviviral replication was inhibited. Recent study by Samanta et al [74] discovered a non-peptidic zwiterion-type inhibitor, a 9,10-dihydro-3H,4aH-1,3,9,10a-tetraazaphenanthren-4-one scaffold via screening a small library of 110 compounds with various scaffolds. The compounds were screened at 100 μM to filter potential inhibitors in the preliminary WNV NS2B-NS3 protease inhibition assay. Focused optimized libraries of 69 compounds were synthesis based on 9,10-dihydro-3H,4aH-1,3,9,10a-tetraazaphenanthren-4-one scaffold and thus led to the discovery of a novel uncompetitive inhibitor molecule with $IC_{50}$ of 45.4 ± 1.05 μM. The binding mode of compound 1a40 was identified using molecular docking simulation with the AutoDock4 program.

A recent report by Jia et al [75] showed palmatine, a chemical compound from Coptis chinensis Franch could specifically inhibit WNV NS2B-NS3 protease. Palmatine was demonstrated to non-competitively inhibit protease activity with $IC_{50}$ of 96 μM. No detectable cytotoxicity was detected (EC$_{50}$=3.6 μM and CC$_{50}$ [a 50% cytotoxicity concentration]=1,031 μM). Dengue virus and yellow fever virus were also suppressed by palmatine in a dose dependent manner.

The envelope glycoprotein (E) of flaviviruses belongs to class II and comprises mainly of beta sheets. Since viral fusion proteins mediate cell entry by undergoing a series of conformational changes, peptides that mimic portions of these beta sheets might...
inhibit structural rearrangements of the fusion proteins, thus prevent viral infection. Hrobowskiet al [76] used physiochemical algorithm, the Wilmley-White interfacial hydrophobic scale (WWHS) [77] in combination with known structural data to identify potential peptide inhibitors of WNV as well as DENV infectivity that targeted the viral E protein. The peptides were designed based on the known secondary structures of several subdomains of E protein, which included the fusion peptide domain, a portion of sub-domain Ib, the pre-ancher stem region following domain III, and the transmembrane domain. The selected peptides were synthesized via solid-phase conventional N-α-9-fluorenylmethyl-oxycarbonyl chemistry (Genemed Synthesis, San Francisco, CA), purified by reverse-phase high performance liquid chromatography and confirmed by amino acid analysis and electrospray mass spectrometry. Unlike the inhibition of flavivirus by neutralizing antibodies that appeared to be involved in receptor blocking by binding to domain III, peptides designed based on domain Iib and the pre-ancher stem region showed to be involved in structural rearrangements during fusion, rather than making direct contact with cellular receptors. The viral entry inhibitions by the peptides were confirmed by viral inhibition assays with IC50 in the 10 μM range. Cell viability assayed with an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay indicated that these inhibitory peptides were noncytotoxic and acted in a sequence specific manner.

4. JAPANESE ENCEPHALITIS AND YELLOW FEVER VIRUSES

Japanese encephalitis (JE) causing acute infection and inflammation of the brain is mainly a pediatric disease with 30,000-50,000 cases reported annually [78]. Around half of the JE survivors have severe neurological sequel with JE causing high fatality rate of 30%. Despite the catastrophes it causes, JE has remained a tropical disease uncommon in the West [79, 80]. Like other flavivirus infections, currently there is no antiviral therapy to treat the disease and to date, the main strategies to control JE infections are by preventive methods such as vaccination and mosquito control [81-83]. Although there are five genotypes (I-V) of the envelope gene (E), the current approved vaccines are designed only for genotype III virus [84]. Although commercial vaccines are available which have reduced the number of JE cases, about 55,000 (81%) of the total cases still exist in the areas even with well-established JE vaccination program [85]. The drawback of the current available JE vaccines, although they are relatively safe and effective, is that multiple doses are required [86, 87]. In addition, the delivery cost to the poor communities still remains a challenge[88]. Thus, at present antiviral therapy remains a useful alternative when the vaccination system does not cover the whole population at risk of JEV infection.

Unlike DENV and WNV proteases, which are extensively characterized as potential drug targets [89, 90], JEV protease is comparatively less studied with regards to its structure-activity relationship. To date, there is no x-ray structure available for JEV protease (NS3) complexed with NS2B. Currently, only one x-ray crystal structure of the catalytic domain of JEV NS3 helicase/nucleoside triphosphate is available at a resolution of 1.8 Å [91]. The crystal structure of JEV NS3 consists of three domains, with an asymmetric distribution of charges on its surface, and has a tunnel large enough to fit single-stranded RNA. The NTP-binding pocket is made of three motifs; namely motif I (Walker A), II (Walker B) and VI. Based on mutagenesis study, all residues in Walker A motif (Gly199, Lys200 and Thr201), polar residues within the NTP-binding pocket (Gln457, Arg461 and Arg464), and also Arg458 (outside of the pocket in motif VI) were important for NS3 helicase and helicase activities in viral replication. Consequently, NS3 helicase/NTPase seems to be a promising antiviral drug target.

Borowski et al [92] reported that the helicase activities could be inhibited by the peptides containing Arg-rich conserved motif (motif VI) of the NS3 helicase/NTPase. The inhibition of the viral enzyme activity may result from blockade of the intramolecular interactions of the NTPase/helicase domains. The JEV peptides (1959 to 1975 amino acids) that were homologous to the 17 peptides located in motif IV within domain 2 of HCV NS3 helicase/NTPase (1484 to1500 amino acids) were synthesized and evaluated for NS3 helicase/NTPase inhibition activity. Shortened versions of the 17 peptides (5-, 8-, 11-, 14-peptide) were synthesized and tested for inhibition of the unwinding activity of the enzyme. JEV peptides moderately inhibited JEV helicase activity with an IC50 of 196 ± 9.4 μM. Kinetic analysis showed that the binding of the peptides did not interfere with the NTPase activity of the enzyme, but could be optimized and serve as lead compounds to reduce virus propagation.

Recently, Kaezor and Matosiuk [93] used structure-based virtual screening to identify novel inhibitors against NS3 helicase/NTPase. This study reported, for the first time, new potential competitive JEV NS3 helicase/NTPase inhibitors that are structurally different from nucleosides and their analogs. A natural ligand, ATP, and two known JEV NS3 helicase/NTPase inhibitors, namely compounds 1 and 2 [94] were docked into the binding pocket using the flexible docking method of Surflex [95] incorporated in SY-BYLS8.0. Ligand-enzyme complex obtained for ATP and inhibitors (compounds 1 and 2) of NS3 helicase/NTPase were further optimized with Yamber3 force field energy in YASARA Dynamics [96]. This step was performed for optimization of the residue conformations located at the binding pocket, thus provided the final enzyme structure that was possible for use in virtual screening. The reported mutagenesis study and literature data regarding the mechanism of ATP hydrolysis by helicase/NTPase [97] agreed with the obtained binding mode of ATP interactions with the enzyme. The optimized ligand-enzyme complexes were then utilized to generate structure-based pharmacophore model, using Interaction Generation module of DISCOVERY STUDIO 2.1, which contained 15 hydrogen bond donors, 3 hydrogen acceptors and no lipophilic moieties. The pharmacophore model was verified positively as it recognized the known inhibitors 1-2 as hits and sensitive to false positive as none of the noncompetitive inhibitors 3-4 and inactive compounds 5-7 was interacting with the ATP-binding site in the virtual screening of a database of 10,000 ZINC drug-like compounds. In the subsequent screening, 15 hits with the highest scores were selected from about 1,161,000 lead-like compounds freely available from ZINC database [98]. The screening was performed using the Screen Library module of DISCOVERY STUDIO 2.1, based on consensus scoring procedure, which combined the screening and docking results [99]. Based on the calculated Preadmet server (preadmet.bmdrc.org), most of the compounds were simulated to not cross the blood-brain barrier easily, except for 5 compounds which could be good candidates for further ADMET optimization. Nevertheless, the antiviral activities of these compounds warrant experimental validation. Despite this, reliability of the reported computational results were enhanced by three factors: first, a refined crystal structure of the catalytic domain of JEV NS3 helicase/NTPase was used to construct the pharmacophore model; second, residues contributing to the ATP-binding site were discovered in the mutational studies; and third, usage of the consensus screening procedure improved the hit list.

Currently there are two available structure of envelope protein domain III of JEV solved by NMR (PDB code 1PJW) [100] and x-ray crystallography (PDB code 3P54) [101]. Li et al [102] showed that the envelope glycoprotein domain III (EDIII) and the loop3 peptide derived from EDIII exhibited antiviral activities against JEV (NJ 2008 strain). The EDIII protein at a concentration of 25 μg/ml and loop3 peptide at a concentration of 10 ± 1.4 μM exhibited the ability to 50% inhibit JEV infection in BHK-21 cells. The
EDIII of JEV consists of six antiparallel strands namely β1, β2, β3, β4, β5, and β6, which correspond to strand A, B, C, D, E, F and G as in WNV and YFV. The antiviral activity of EDIII domain was determined by using plaque assay, quantitative PCR and western blot at 48 hours post JEV infection (multiplicity of infection/MOI=0.02). According to the NMR structure, four of the exposed peptide loops before or between the β-barrel loops of EDIII, namely loop1 (amino acids 302-309), loop2 (amino acids 329-337), loop3 (amino acids 362-370) and loop4 (amino acids 385-391), were chosen for antiviral screening against JEV. The idea of synthesizing these exposed peptide loops located on the surface of EDIII was based on two information: that the EDIII protein can inhibit virus entry as shown in DENV type 1 and 2 as well as in WNV [103-105], and several studies showed that some peptides located on the envelope protein exhibited the ability to inhibit virus infection as in DENV type 2 and WNV [106-108]. Even at 200 μM, the loop peptides did not exhibit significant cytotoxicity in BHK-21 cells. In order to address whether loop3 inhibition was sequence-specific, a scrambled loop3 was synthesized. A plaque assay showed that the scrambled loop3 did not inhibit JEV infection. The binding of virus after incubation with peptide was detected with ELISA (Enzyme-linked immunosorbent assay). Significant decrease in virus concentration in the different concentration of the peptide loop3 was detected especially at 100 μM (with viral load of p < 0.02). However no significant changes in the JEV titer was observed when the peptide loop3 was added after virus infection, demonstrating that peptide loop3 exerted its inhibitory ability via interfering with virus attachment. In vivo study showed that, when administered before lethal JEV challenge, peptide loop3 could significantly increase the survival rate in mice. This study indicated that other peptides of the envelope protein might also be important for the post-binding process of JEV entry since JEV DEIII and the loop3 peptide could not inhibit JEV infection completely in BHK-21 and in mice. Nevertheless due to the conservation between envelope protein domain III and the loop3 peptides, the inhibitory loop3 could serve as a lead to develop new therapeutics for the JEV of different genotypes or other flavivirus infections.

Yellow fever virus (YFV) is one of the most lethal and feared diseases. It originates from Africa and spread to the western hemisphere during the slave trade era, with the first epidemic reported in 1648 in the Yucatan [109]. However for unknown reason, yellow fever has not spread to Asia yet. The illness caused by YFV ranges from a self-limited febrile illness to severe liver disease with bleeding. Like other flaviviruses, in most cases, patients with the most clinically severe cases of YF are treated by supportive care. As for JEV, vaccines too have been an integral part in fighting YFV. The first attenuated vaccine against YFV namely 17D strain was discovered by Theiler and coworker. This work was awarded the Nobel Prize in 1951. The development of live-attenuated 17D vaccines in the 1930s was a turning point in the history of the disease. The 17D vaccine has been considered to be one of the safest and most effective live virus vaccines ever developed [110, 111]. Around the same time, another life-attenuated vaccine was designed, called the French neutralic vaccine (FNV) and was used widely in the 1960s in francophone Africa, and led to the disappearance of the disease [110]. However recent re-emergence has been reported in South America and Africa and has brought this virus back to the public attention [112]. This could be most likely due to the decline of vaccination, unreachable populations, lack of mosquito control and increased transportation of people and goods. According to Khasnatinov et al [113], currently there is a major gap of knowledge of how to manage and treat sick patients infected with JEV or manage the serious vaccine-associated adverse events. To date, no effective specific antiviral therapy for yellow fever has been identified. So there is a clear need for safe and effective drugs to treat patients in each steps of the disease progression. Currently there are eleven JEV protein structures (apo and ligand) consist of envelope (E), NS3 helicase, and NS5 methyltransferase available in Protein Data Bank solved by NMR and X-ray crystallography [114-116]. The ligand structure of helicase consists of complex with ADP (PDB code 1YMF) and ligand structure of methyltransferase consists of complexes with S-adenosyl-L-homocysteine (PDB code 3EVA, 3EVB), GTP (PDB code 3EVD), GpppA (PDB code 3EVE), and Me7-GpppA (PDB code 3EVF).

Although NS3 protease is an attractive potential target for search in inhibitors in JEV, DENV and WNV, this is not the case for YFV. Since DENV, WNV, and YFV NS2B-NS3 proteases share similar cleavage recognition sites [117, 118], Löhrs et al [119] postulated that peptidic inhibitors designed against DENV [120, 121] and WNV [25, 69] could potentially work on the YFV protease. A construct of YFV protease namely YFV CF40-gly-SNSpre190 was designed with 47 core amino acids of NS2B [122] and the N-terminal 190 amino acids NS3 protease domain via a linker Gly4-Ser-Gly4. Active site-titration with the competitive, tight-binding inhibitor aprotinin (K_i=17.6±0.56 nM) and curve fitting based on the Morrison equation [123] indicates that the total amount of active protein in the purified was 100%. Proteolytic activity was tested with two different substrates; the tripeptide Boc-GGR-AMC [124, 125] and the tetrapeptide Bz-nKR-AMC [126]. As in DENV [124], the enzyme showed nearly 10-fold higher affinity and processed it for 34-fold greater efficiency for tetrapeptide Bz-nKRR-AMC substrate while the catalytic efficiency for Boc-GGR-AMC was comparable to the work done by Bessaud et al [125]. The increased catalytic efficiency in YFV and DENV for tetrapeptide Bz-nKRR-AMC could be due to the additional P4 residue of norleucine in the tetrapeptide sequence. Based on this result, further YFV protease enzyme characterization and inhibitor studies were explored using tetrapeptide Bz-nKR-AMC substrate. The K_i value of substrate-based peptide Bz-nKRR-H for DENV is 5.8 μM [120, 121] and 2.1 μM for WNV [69]. Thirteen peptide-based inhibitors were synthesized according to the aldehyde derivative of the substrate-based peptide Bz-nKR-H. Evaluation of the importance of the P4-P3-P2-P1 positions was carried out. The potency of Bz-nKRR-H against YFV was nearly 15-fold more potent than DENV and 5-fold better than WNV. According to the enzyme-inhibitor constant values, replacement with electrophilic boronic acid group improved the potency to almost 8-fold while complete loss and significant loss of activities were observed when P1 (Arg) and P2 (Arg) were replaced with either Ala, Phe, or Lys. Replacement of P3 (Lys) and P4 (Nle) with either Ala or Phe and shortening to tri- and dipeptides did not significantly affect the inhibitory activity. These results showed that the major determinants of the peptidic binding were S1 and S2 subsites. In order to understand the binding mode of the inhibitors, a homology model approach was taken where the crystal structure of WNV NS2B-NS3 protease in complex with Bz-nKR-H (PDB code 2FP7) was used as the template. Prime 1.2 (Schrodinger) was used for performing the homology modeling, and the docking of individual peptidic aldehydes was done using SYBYL 6.9 and further optimized with Macromodel 9.0. The YFV homolog model agreed with the kinetic analysis data where the S1 and S2 pockets were the dominant interaction sites for the peptidic inhibitor which resembled the WNV crystal structure [126]. As in YFV protease inhibition study, WNV protease shows that the replacement of the Arg with Lys in P2 position was tolerated but not in P1 [69, 126]. However this was not the case for DENV protease where Lys replacement in P1 and P2 positions were not tolerated [121], suggesting that the DENV S1 pocket might be different. The knowledge obtained from this paper indicates that it may be possible to design pan-flavivirus drugs against NS2B-NS3 protease and the peptidic inhibitors can be used as a starting point for rational drug design.

Recently Mastrangelo group [127] discovered that anti-helminthic drug ivermectin as a highly potent inhibitor of YFV replication (sub-nanomolar EC50 values). This drug also selectively inhibited cell culture of JEV, TBEV and DENV type 2 (in sub-
micromolar \(EC_{50}\) values). Furthermore in vitro analysis showed that ivermectin inhibited the YFV, WNV and DENV type 2 helicase domains at sub-micromolar concentrations. Ivermectin has been licensed for more than 20 years for the treatment of parasitic infections in man. The idea was to target specifically against the RNA binding and unwinding mechanisms mediated by NS3 helicase [128, 129] and not against the ATP-binding pocket. This was due to the ATP-mimetic molecules designed as inhibitors against NS3 helicase may create adverse effects on the host cell since ATP is the key nucleotide of host cell metabolism. The group used in silico docking to search for possible inhibitors targeting at ssRNA access site of NS3 helicase. A modelled of WNV NS3 helicase complex with RNA was built by superimposition of the WNV NS3 helicase (PDB code 2QQE) [129] with the DNA-bound bacterial helicase PcrA (PDB code 3P1R) [130] since when this work was initiated, no DENV or YFV helicase in complex with RNA structure was available. The modeled of WNV NS3 helicase complex with RNA was used to locate the putative ssRNA access within the WNV NS3 helicase domain. Later, a crystal structure of DENV in complex with ssRNA was solved (PDB code 2JLU, 2JLW) [131] and confirmed the model-based prediction. The predicted putative ssRNA binding site was then explored for inhibitors binding via in silico docking low molecular weight of 1280 commercially compounds from the virtual Library of Pharmaceutically Active Compounds (LOPAC) accessed from Sigma-Aldrich. The docking was performed with the AutoDock4 software package. Three compounds with free binding energy (\(AG\)) between -11.5 to -9.5 kcal/mol for WNV NS3 helicase were identified from the docking search were ivermectin, ouabain (a poisonous cardiac glycoside) and paromomycin sulphate (an aminoglycoside antibiotic). These commercially available drugs were obtained from Sigma-Aldrich and chosen for further biochemical evaluation. Two different types of helicase assays were performed in order to show that the selected drugs inhibit the NS3 helicase unwinding activity were; an assay using radiolabelled double-stranded RNA substrate in the presence of Mg\(^{2+}\) and ATP [132] and FRET-based helicase assay [133]. The ATPase activity of NS3 helicase was not affected by ivermectin at a concentration of 1 \(\mu M\) as indicated by NS3 ATPase assay, showing that this compound did not restrict the helicase ATP binding site. The ivermectin was predicted to bind at a distance of ~25 Å from the ATP binding site. The NS5 RdRp activity assay showed that ivermectin at concentration of 1 \(\mu M\) did not inhibit NS5 RdRp activity of RdRp domains and the NS5 full-length proteins from DENV and WNV. Furthermore, thermodrhamometric assays indicated that the inhibition of helicase activity did not result from ligand-induced protein aggregation or denaturation. Specificity of ivermectin for the flaviviruses helicase (WNV, DENV, JEV, TBEV) was confirmed since activity assays of HCV helicase was not inhibited by ivermectin (up to 100 \(\mu M\)). HCV was chosen for comparison since the virus belongs to the family Flaviviridae and is the closest to flaviviruses helicase. Due to the failure in obtaining crystal structure of the protein-ligand complex, in silico approached was used to simulate the conformations of ivermectin inside the ssRNA access site in order to characterize the part of protein involved in ivermectin binding. Based on virtual docking, two amino acid namely Thr408-Asp409 of DENV, Thr409-Asp410 of WNV and Thr413-Asp414 were found to frequently interacts with ivermectin. Mutation of Thr to Ile and Asp to Glu preserved the double-stranded RNA unwinding activity but were not inhibited by ivermectin. This result indicated that ivermectin closely interact with these residues thus support the predicted structural analysis of ivermectin bind at the single-stranded RNA access site. Kinetic assays showed that the mechanism of ivermectin is via noncompetitive inhibition by binding to NS3 helicase/RNA complex to inhibit the unwinding activity. According to a time-of-drug-addition assay, ivermectin is only active during the first 14 hours after virus enters the cells. This meant that the compound is only effective in certain phase of the viral life cycle where the NS3 helicase is functionally active [134]. However other mechanisms besides helicase inhibition may contribute to the action of ivermectin inhibition in cell culture since the \(EC_{50}\) value in YFV cell culture is 0.5 nM while the \(IC_{50}\) value of NS3 helicase of YFV is 120 nM. Since ivermectin has been used in man for the treatment of a variety of parasitic disease for more than 20 years, the potential of ivermectin to go through clinical trials for the treatment of life-threatening flaviviruses may not require much effort. Currently, ivermectin has been patented for the treatment, prevention or amelioration of flavivirus infections [135].

5. HEPATITIS C VIRUS

HCV infection exists throughout the world, and believed to be transmitted only by blood. Until year 1990/91 where anti-HCV screening tests for blood donors were introduced in Europe and the United States, HCV remain the major cause of transfusion-associated hepatitis [136]. Acute infection is generally asymptomatic thus the incidence of HCV on a global scale is not well known [137]. To date there are no vaccines against HCV. According to World Health Organization (WHO) there are more than 170 million chronic carriers who are at risk of developing liver cancer and/or liver cirrhosis with about 3% of the world’s population infected with HCV [138]. HCV is currently classified into six major genotypes [139-144], which differ from each other by more than 30% at the nucleotides level. Although HCV share some similarities with other members of the Flavivirus genus, it does exhibit differences in the genome organization as shown in (Fig. 4) where the HCV 5’UTR is not capped. The HCV NS5B RdRp was extensively studied in the hope to find a novel drug to treat HCV since this protein possesses pivotal role in the synthesis and replication of viral RNA [143]. Several inhibitors of the NS5B RdRp have been developed and currently are in preclinical and clinical development (for example TMC649128, R-7128, PSI-7977, PSI-938, INX-189, IDX184) [145]. Another traditional antiviral target besides NS5B is the NS3 protease. Despite the catalytic site being a shallow and largely hydrophobic groove as observed in DENV and WNV NS3 protease thus making it a difficult target, several compounds inhibitors of the NS3 protease of HCV have either been approved for use in humans (for example telaprevir (VX-950) and boceprevir (SCH503034)) or currently in preclinical and clinical trials (such as BI12202, MK-7009, TMC435, ITMN-191) [146]. Current standard of care for HCV-infected patients consists of combined therapy of PEG IFN-\(\alpha\) and ribavirin. However this therapy has its drawback as we previously described in the WNV section. The crystal structure of HCV NS5B has right hand architecture with fingers, thumb and palm domains. The active site is located within the palm domain circumscribed by the fingers and thumb polymere subdomains [147, 148]. A spherical hydrophobic pocket-allosteric binding site proximal to the active site has been identified, which comprises of Pro197, Arg200, Leu384, Met414 and Tyr448 [148].

In a study by Patel et al [149], 3D QSAR (quantitative structure-activity relationship) was used to interrogate the inhibitory activity of benzimidazol and its derivatives toward HCV NS5B RdRp. They revealed that for NS5B inhibitors to have substantial inhibitory activity, it requires hydrogen bond donor and receptor groups at the 5-position of the indole ring, and an R substituent at the chiral carbon. This conclusion was made based on the results generated form the comparative molecular field analysis (CoMFA) and comparative molecular similarity indices analysis (CoMSIA) of 67 benzimidazole derivatives as HCV NS5 inhibitor. In this respect, acquisition of a high-resolution NS5B protein structure into the structure-activity analysis (SAR), followed by NMR and modeling studies established the improved structure configuration of benzimidazol 5-carboxamide derivatives. The incorporation of the \(\alpha\)-disubstituted amino acid at the designated site of the derivatives enhanced the inhibition of the enzymatic activity up to \(IC_{50} = 40\) nM [150]. However, despite their potencies, further development of these derivatives as potential drug candidates were hampered by the...
high EC\textsubscript{50} values [151]. This has resulted in the replacement of the benzimidole core of truncated carboxylic acid residues with a greater lipophilic N-methyindole isoesters. This replacement demonstrated significant improvement in the \textit{in vitro} study with an EC\textsubscript{50} value less then 100 nM [152].

HCV NS3-NS4A is another attractive drug target since it is also essential for the replication of HCV. The crystal structure of HCV NS3-NS4A possesses a catalytic triad consisting of a protease domain and a helicase domain that are segregated and connected by a single strand [153]. A competitive HCV NS3-NS4A serine protease inhibitor, VX-950 (telaprevir) developed by Vertex and Johnson & Johnson had been recently released into the clinical practice [154]. Telaprevir is a potent peptidomimetic inhibitor of HCV serine protease with IC\textsubscript{50} of 280 nM. It functions by forming a reversible covalent enzyme-inhibitor complex with the viral protease, especially with the genotype 1a HCV, at a steady-state inhibition constant (K\textsubscript{i}) of 7 nM. Noteworthy, Li and co-workers [155] discovered new series of acyclic P4-benzoxaborole-based HCV NS3 protease inhibitors, which has a smaller molecular weight than the first generation drug such as telaprevir. A systematic SAR analyses was performed by exploring various linkers and substitutions around the benzoxaborole moiety as well as the P2\textsuperscript{a} groups in order to achieve better \textit{in vivo} absorption and bioavailability. The inhibitory activities of the synthesized compounds were evaluated by FRET NS3-NS4A protease domain assays, while \textit{in vitro} assay was determined by acquiring 1a and 1b HCV replicon. Two compounds were identified with low nanomolar potency. In the FRET NS3-NS4A protease assay, compound 5g and 17 exhibited IC\textsubscript{50} of 1.7 nM and 3.1 nM, respectively. The linker-truncated compound 5j exhibited improved \textit{in vivo} absorption by 4.9% and oral bioavailability by 4.3%. These findings suggested that further reduction of molecular weight and polar surface area in benzoxaborole-based compounds could provide an effective strategy towards the discovery of drug-like boron-containing HCV protease inhibitors.

In order to overcome the rapid emergence of the HCV variance that showed resistance towards the first generation HCV protease inhibitors [156], researchers had developed two strategies: screening compound with the clinical HCV protease mutants, and generating improved method in assisting the search for novel HCV inhibitors [157-159]. A new virtual screening method was recently published providing another alternative approach in conducting structure-based drug design [157]. In this method, a new induced-fit docking system termed GENIUS was introduced. As a result, 97 out of 166,206 compounds were identified against HCV NS3-NS4A protease (PDB: 1DXW). Subsequent protease inhibition and cell-based assays identified two potential compounds with similar potency of 10 \textmu M and EC\textsubscript{50} values of 13 and 23 \textmu M. Compound 10, that exhibited a potency of 10 \textmu M with an IC\textsubscript{50} value of 8.58 \textmu M and an EC\textsubscript{50} value of 12 \textmu M was identified as a lead compound for future synthetic development. This compound, unlike the first generation compounds that were on clinical trials [154], lacks an asymmetric carbon and does not have a macrocyclic structure. Novel urea-based HCV protease inhibitors against protease resistant mutants of HCV have been discovered via collaborative effort of GlaxoSmithKline.

Fig. (4). HCV genome organization and translation. The HCV genome consists of a positive single-stranded RNA genome (9.6 kb) with a single open reading frame codes for structural and non-structural proteins including the 5’ untranslated region (UTR) with four highly structured domains (I, II, III and IV) and the internal ribosom entry site (IRES), and the 3’ UTR consists of stable stem-loop structures and an internal poly(U)/polyprimidine tract. The single open reading frame codes for polyprotein of slightly more than 3000 amino acids depending on the HCV genotype. The polyprotein enzymatic processing of the 10 HCV proteins are shown. Cleavage site for host-cell signal peptidase are represented by the diamond shape (colored in purple), autocatalytic cleavage by the NS2-NS3 zinc-dependent metalloprotease of the NS2-NS3 junction is represented by the triangle shape (colored in yellow) and NS3-NS4 serine protease complex cleavage sites are represented by the clear triangle shape [182-185].
and Ancor Pharmaceuticals [159]. The macrocyclic urea2, a by-product in the synthesis of benzoxaborole [191], which showed inhibitory activity against HCV NS3-NS4A offered substantial opportunity for lead compound optimization. Thus, by setting macrocyclic urea 2 as a lead compound, 24 urea analogues (namely compounds 3-26) were synthesized and evaluated in panel laboratories against a variety of clinical HCV protease mutants Ala156Ser, Ala156Thr, Ala156Val, Asp168Ala, Asp168Val, and Arg155Lys. Several compounds, for example compound 12, demonstrated higher potency in HCV replicon assay with an IC50 of 0.01 nM, as compared to those leading inhibitors such as cipivir, telaprevir and danoprevir that had IC50 > 1.99 nM. In addition, following oral administration in vivo, compound 12 exhibited a half-life of 8.1 hour in the rat’s liver [159].

Kota and co-workers [161] reported that short peptides derived from the HCV core structural protein possessed antiviral properties. These inhibitors prevented viral core dimerization, thus perturbing viral release. This rational design was based on the idea that protein-protein interactions could be inhibited by peptides derived from one of the interacting partner by small molecules [162]. The NIH AIDS Research and Reference Program (ht: www.aidsreagent.org) provided 14 synthetic peptides designed to span the first 109 residues of the HCV core structural protein of the HCV H77 1a genotype. The peptides consisting of 18 residues, with 11 residues apart, were resynthesized with 95% purity or more as verified by HPLC. These peptides were then tested for their inhibitory activities using in-house developed Amplified Luminescent Proximity Homogeneous Assay (ALPHA). As a result, two peptides demonstrated appreciable inhibition of core dimerization, namely SL173 that showed 68% inhibition and SL174 with 63% inhibition. SL175 peptide, identical to SL174 but with deleted 3 amino acid residues at the C terminal, showed a 50% inhibition activity with an IC50 of 21.9 μM. A fluorescent polarization assay revealed that SL175 peptide interacted with the hydrophilic core protein region, comprising 106 residues with a Kd of 1.9 μM. When measured by surface plasmon resonance, peptide SL175 was shown to bind to core protein of 169 residues with a Kd of 7.2 μM [160]. However, these three peptides did not inhibit viral replication, but blocked the release of infectious virus when added to HCV-infected cells. Nevertheless these core-derived peptides would be useful as a tool to study the role of core dimerization in the virus life cycle and interaction of core protein with cellular protein such as Dicer [163]. These core-peptides could also act as peptidomimetic drugs against HCV infection with improved affinity and serum stability.

A short peptide derived from the N-terminus of host claudin-1 (CLDN1) protein, which inhibited virus entry at the post binding stage had been identified [64]. This approach was feasible due to the sufficient knowledge of the HCV entry-processing field. It is known that HCV uses at least four cellular membrane proteins for entry: CD81, scavenger receptor B1 (SR-B1), claudin-1 (CLDN1) and occluding (OCLN) [165-168]. The rational design was based on the postulation that HCV entry might be blocked due to the competing peptides derived from host entry factors, located on the cell surface and incoming virion. A library consisting of 121 overlapping 18-mer peptides with 13 amino acids offset spanning the entire protein sequences of human CD81, SR-B1, CLDN1 and OCLN were designed to inhibit HCV pseudoviral particle (HCVpp) infection of HuH7.5.1 cells. Due to solubility problem, thirty-two peptides were abandoned. Out of 89 peptides, only 2 peptides derived from the first transmembrane region of CLDN1, namely CL58 and CL59, were identified to inhibit HCVpp entry by more than 80% at a concentration of 50 μM. CL58 was more potent in inhibiting HCV entry compared to CL59. To determine the maximal inhibition of CL58, eight additional 18-mer peptides (namely CL58.1 until CL58. 8) were synthesized with different length and sequence. The 18-mer peptides were synthesized with a-3-amino acid offset, thus extended further into the first transmembrane and extracellular loop region of CLDN1. However none of these peptides showed inhibition to the extent as that shown by the parental CL58. In the following optimization, the length of the peptides were altered by adding or deleting residues from the CL58 C terminus. Shortening the peptide by 2, 4, or 6 amino acids caused the IC50 to drastically increase, while extending the peptide by 2, 4, or 6 amino acids only slightly increased the IC50. Slightly lower IC50 was observed with a D-isomer of CL58, thus parental CL58 length and sequence seemed to be the optimum for inhibiting HCV entry. In order to understand how CL58 interacted with HCV envelope (E1 and E2) proteins, a communoprecipitation experiments using Flag-tagged CL58 were performed. According to the communoprecipitated HCV envelope, CL58 interacted with HCV glycoproteins, since the E1 and E2 envelope proteins of HCV and HCVpp co-precipitated with CL58 tagged with Flag peptide at the C terminus. However, it was unclear how CL58 mediated the inhibition. Cell culture experiments indicated that CL58 did not affect the cellular distribution of CLDN1 and was not cytotoxic even at a concentration 100-fold higher from its IC50 determined using MTT assay. These finding indicated that a new class of inhibitors that blocked virus-host interactions could be developed. Furthermore the strategy described in this study may offer advantages since the inhibition target is the cellular protein thus reduce the likelihood of developing resistance.

An exciting study was performed to possibly overcome the peptide instability using self-assembling nanotubes. Montero and coworkers [169] presented a detailed study on using peptide nanotubes to effectively block the entry of HCV into target cells. Originally such inhibitors were discovered against bacterial membranes and adenovirus [170-172]. A cyclic D,L-α-peptide library was screened for anti-HCV activity and nine promising amphiphilic peptides were identified. The peptides exerted the anti-HCV activities through self-assembly into inhibitory nanotubes which acted by interacting with HCV glycoproteins, since the E1 and E2 envelope proteins of HCV and HCVpp co-precipitated with CL58 tagged with Flag peptide at the C terminus. However, it was unclear how CL58 mediated the inhibition. Cell culture experiments indicated that CL58 did not affect the cellular distribution of CLDN1 and was not cytotoxic even at a concentration 100-fold higher from its IC50 determined using MTT assay. These finding indicated that a new class of inhibitors that blocked virus-host interactions could be developed. Furthermore the strategy described in this study may offer advantages since the inhibition target is the cellular protein thus reduce the likelihood of developing resistance.

High-throughput screening of a library of more than 1 million small molecules against HCVpp with genotype 1b clinical isolate 432-4 on modified hepatic cell line had led to the discovery of a group of compounds, which inhibited viral entry with the best IC50 of 0.016 μM [173]. Synthetic chemistry efforts were used to interrogate the SAR of the triazine series (EI-2 - EI-12) using N'-4-(nitrophenyl)-N'-pentylen-6-(2,2,2-trifluoroethoxy)-1,3,5-triazine-2,4-diamine as a lead compound, and were assessed against HCVpp 1a and 1b. As a result, it was found that the para- and meta-methyl analogs delivered a 4-fold greater potency when compared to the nitro analogs. Introduction of para- substituents that closely mimicked the polarity and shape of the nitro analogs afforded sub-100 nM EC50 values in both HCVpp subtypes. A patent recently invented by Gary et al. [174] described the isolation of peptide fusion inhibitors for flavivirus infection. The idea of this invention was based on the knowledge that in order to deliver the viral genome into the cell cytoplasm, the virion envelope of flaviviruses and membranes of the target cell had to fuse. It was hoped that by employing peptides or peptide derivatives that blocked the fusion, the entry of the viral genome into the target cell could be blocked. The peptides or peptide derivatives as fusion inhibitors for flavivirus family are identical (at least 70% identity) to the amino acid sequences of HCV fusion inhibitory peptides. A non-peptide drug can be developed, once an effective inhibitor is identified. The discovery of these peptides could serve as lead target for development of peptide drugs to treat flavivirus infections.
6. CONCLUSION

The helicase (NS3), protease (NS2B/NS3) and polymerase (NS5) are enzymes that are traditionally targeted for antiviral drug discovery. This is largely because loss of these specific proteins has proven lethal for virus replication, thus drugs targeted at these enzymes may reduce viral load. According to the discussed antiviral discovery against flaviviruses (DENV, WNV, JEV, YFV), the complex of NS3 proteases with inhibitors are mostly characterized for DENV, while allosteric inhibitors against NS3 protease have been identified and characterized mainly for WNV. The NS3 helicase inhibitors have been immensely studied especially in JEV and YFV. Inhibitors developed for NS5 polymerases are mostly characterized and studied for HCV where several NS5 polymerases inhibitors (nucleoside and non-nucleoside polymerase) have reached preclinical and clinical phases of drug development, and others are commercially available. Limited research publications on small molecule inhibitors against JEV and YFV NS3 serine proteases and NS5 polymerases compared to the other flaviviruses may probably be due to the decline in the infection caused by these viruses because of the available vaccines. However, current increase in the JEV and YFV infections in the area where vaccination are available creates a demand to find new antiviral drugs. Due to the existing mutation in the presence of the inhibitors of both NS3 (protease/helicase) and NS5 polymerase, new viral targets are constantly in demand. Currently, the E protein is one of the attractive novel targets for designing entry inhibitors for flaviviruses. The development of inhibitors against E protein becomes feasible due to the availability of E protein X-ray crystal structures and sufficient information of its function. The E protein as a drug target provides several strategies for designing potent peptide inhibitors for entry and also endosomal escape via inhibiting membrane fusion. The discovery of nanotube inhibitors that target the cellular membrane as shown in HCV provides new strategies for developing fusion inhibitors for other flaviviruses. Another promising strategy for the discovery of new inhibitors against flaviviruses is by targeting the host proteins that interact with the flavivirus structural and nonstructural proteins. However, the drawback of targeting the host proteins is that it could provide serious side effects to human. On the other hand, targeting cellular proteins will overcome the viral mutations as seen in clinical development of NS3 protease and NS5 polymerase of HCV. Advances in computational techniques and HTS have increased the speed of flaviviral drug development in terms of function and structure determination. In order to use rational drug design approach, sufficient information of the viral protein functions is essential. The possibility to precisely target specific viral processes in the virus life cycle is higher if greater understanding of the viral protein functions is acquired. This development is sometimes hampered by the difficulty in getting X-ray structures to guide the design of better inhibitors because it is often difficult to design inhibitors based on structure-guided principles without knowing the actual structure. Fortunately other complementary techniques such as NMR as well as cryo-electron microscopy might provide some clues about the structure as well as the function. Besides, traditionally drug targets such as NS3 protease/helicase and NS5 polymerase, and novel targets like NS1, NS2A and NS4A/B may pose as new targets for inhibitor design, since currently, no approaches have been attempted on these proteins.

7. OUTLOOK

The field of flavivirus antiviral development is clearly moving forward as indicated by new options discussed in many studies. It is quite interesting to see the study of known drug-like anti-helminthic drug, ivermectin, that was originally developed for the treatment of parasitic infections in man, proved effective against the YFV, WNV and DENV type 2 helicase domain, as well as inhibited cell culture of YFV, WNV and DENV type 2. Similarly the discovery of self-assembling nanotubes that originally inhibited bacteria had proven to have antiviral activities against HCV, thus may lead to the understanding of the nature of the membranes of the viral lifecycle. Furthermore the rapidly expanding list of Food and Drug Administration (FDA)-approved HCV inhibitors that mostly targets the NS3 protease and NS5B polymerase is now validated by other members of the Flaviviridae family. Although the journey from compound discovery to marketable drug is a long and expensive process, novel drug discovery is clearly beneficial and must be encouraged. Therefore, further studies should focus on novel drug development as well as with existing drug with novel uses. Many antiviral compounds showed great success in vitro but either not tested or failed in vivo, thus more in vivo studies will greatly benefit drug design process. For example, many of the literature studies described only the potency of the E protein inhibitors of the β-OG, but few continued the study in cell culture luciferase assays and none have been tested in vivo. Proper animal models of infection for DENV is more representative of the genuine DENV infection compared to cell culture. The goal of antiviral research is to control the human infections thus compounds discovered are desperately needed to move beyond the bench and into the clinical trials. Indeed the future bears hope for a breakthrough in flaviviral drug discovery with the advance technologies of biomedical at hand. However it is the responsibility of the government of different countries and WHO to extend the fruit of this research so it can be delivered to the large populations at an affordable cost.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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