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Chemical Biology
LETTERS

Recent advances in development of Inhibitors of Dengue infection

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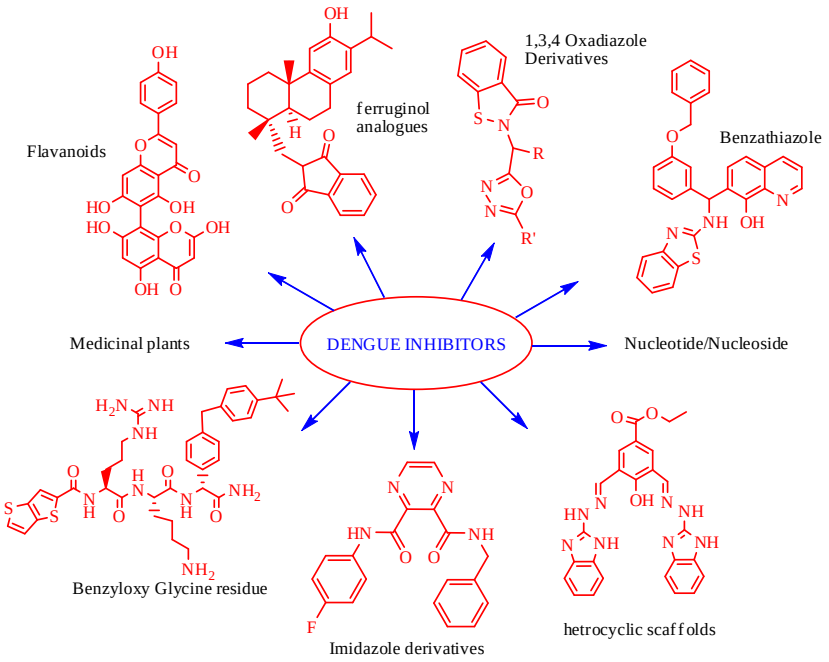
ABSTRACT



Dengue virus infection is a serious threat to global health since an estimated 2.5 billion people worldwide are at risk for epidemic transmission. Dengue virus (DENV) is mosquito-borne arboviruses responsible for causing acute systemic diseases and grievous health conditions in humans. Currently, there is no vaccine available to combat dengue infection therefore; there is an urgent need to develop an efficient, specific, safe and low-cost vaccine or drug that can act against dengue virus infection. This short review discusses current and newly discovered peptidyl and non-peptidyl inhibitor scaffolds that target the DENV protease effectively.

Keywords: DENV virus, Anti-dengue, Viral diseases, Protease, Inhibitors

INTRODUCTION

We are now auscultating a sudden increase in the number of autochthonous infections from all over the world. Dengue infection is one of the critical viral infections that cause a disease whose multiform nature makes it very difficult to treat. According to the World Health Organization, the infection affects over a 100 million people annually and dengue is

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considered one of the most severe arthropod-borne disease and a substantial public health problem. DENVs are composed of five serologically distinct viruses, designated DENV1, DENV2, DENV3 and DENV4, in addition to an emerging fifth serotype.[1] DENV is transmitted by *Aedes* mosquitoes (*Aedes aegypti* and *Aedes albopictus*) present in tropical and subtropical areas in the world, where at least 2.5 billion people live.[2] Thus the development of novel therapeutic agents is essential for combating the increasing number of cases of dengue fever in endemic countries and among a large number of travelers from non-endemic countries.

Dengue virus (DENV) is a member of the family *Flaviviridae*, genus flavivirus. The family contains more than 70 members organized into three genera including the flaviviruses (arthropodborne viruses), the pestiviruses, and the hepaciviruses. The family contains serious pathogens such as West Nile virus (WNV), tick-borne-encephalitis virus (TBEV), yellow fever virus (YFV), Japanese encephalitis viruses (JEVs), hepatitis C virus (HCV) etc.[3] The flavivirus genome consists of a positive sense RNA virus of approximately 50nm in diameter which is translated into a precursor polyprotein.[4] The dengue virus genome contains about 11,000 nucleotide bases, which code for the three different types of protein molecules (C, prM and E) that form the virus particle and seven other types of protein molecules (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) that are found in infected host cells only and are required for replication of the virus. The latter is then cleaved co- and post translationally, resulting in a small number of structural and non-structural (NS) proteins. The NS3 protein contains a serine protease domain,whose activity depends on the formation of a non-covalent complex with the NS2B protein as cofactor.[5-6] The NS2B–NS3 complex is responsible for the proteolytic processing of the viral polyprotein. It is hence crucial for the virus replication and represents a promising target for development of antiviral drugs.

A small fraction of dengue-infected patients develops variety of symptoms from the most common dengue fever (DF), to more serious conditions, in particular dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS).[7] Each year, dengue infections result in ~25,000 deaths, primarily in children. Dengue fever (DF), the most common form of the disease, is most often characterized by typical flu-like symptoms, including fever, fatigue, rash, severe headache, nausea, vomiting, diarrhea, myalgia, arthralgia, retro-orbital pain, and minor hemorrhagic manifestations such as epistaxis, petechiae, and gingival bleeding.[8] Early symptoms of DHF, which most often occurs in children, are the same as those for dengue fever. Hemorrhagic symptoms, however, are much more severe in DHF than in typical DF with the primary pathophysiologic contributor being plasma leakage resulting in hemoconcentration, thrombocytopenia, pleural effusions, hypoproteinemia, and/or hypoalbuminemia.[9]

When a mosquito carrying dengue virus bites a person, the virus enters the skin together with the mosquito's saliva. The virus infects nearby skin cells called keratinocytes, the most common cell type in the skin. The dengue virus also infects and replicates inside a specialized immune cell located in the skin, a type of dendritic cell called a Langerhans cell. It binds to and enters white blood cells, and reproduces inside the cells while they move throughout the body. The white blood cells respond by producing a number of signaling proteins, such as cytokines and interferons, which are responsible for many of the symptoms, such as the fever, the flu-like symptoms and the severe pains. In severe infection, the virus production inside the body is greatly increased, and many more organs (such as the liver and the bone marrow) can be affected. Fluid from the bloodstream leaks through the wall of small blood vessels into body cavities due to capillary permeability. As a result, less blood circulates in the blood vessels, and the blood pressure becomes so low that it cannot supply sufficient blood to vital organs. Furthermore, dysfunction of the bone marrow due to infection of the stromal cells leads to reduced numbers of platelets, which are necessary for effective blood clotting; this increases the risk of bleeding, the other major complication of dengue infection.[10] Consequently, if dengue fever is diagnosed at an early stage through identification of the viral genome by RT-PCR or the presence of circulating NS1 protein in the blood, then antiviral treatment could be useful in limiting the viral load, thereby reducing the severity of the disease.[11]

Currently there is no approved vaccine or a targeted drug therapy available for treatment of dengue infection.[12] A dengue vaccine has proven difficult to develop, in part because of genome's limited coding capacity and their lifecycle's complexity. There are four major closely related, but antigenically distinct, serotypes of the virus, each with slightly different viral proteins. Infection by one serotype does not ensure protection of the patient from infection by the other three serotypes. Therefore, if vaccine were produced for only one or two serotypes, the other serotypes would increase the risk of more serious illness. Many researchers currently believe that the deadly dengue hemorrhagic disease is caused when a person is infected with one subtype, and then infected later by a second subtype. The antibodies, and immunity, gained from the first infection appear to assist with the infection by the second subtype, instead of providing a general immunity to all subtypes. This means that an effective vaccine will have to stimulate protective antibodies against all four types at once, a feat that has not yet been achieved. Accordingly, development of a safe, effective therapeutic agent for dengue viruses is urgently required. In this mini review, our focus is mainly on the recently developed peptidyl and non-peptidyl inhibitors of dengue and the understanding of their action as the molecular viral targets for the development of potential drugs.

Replication Cycle of DENV Virus

In the viral infection process, initially the virus binds to a cell-surface receptor. This process is mediate by E protein, which acts as a viral attachment protein for DENV, directed to virus penetration into the host cell.[13,14] prM also act as a chaperone-like protein which is responsible for the control of the fusion activity and the correct folding of protein.[15] After the fusion, the translation of the genomic RNA into a polyprotein is

the first process in DENV-infected cells. A small polypeptide is synthesized, followed by the RNA– ribosomes–nascent protein complex docking at the endoplasmic reticulum (ER), and translation and processing of the viral polyprotein continue in association with the ER. Cellular and viral proteases carry out the processing of the polyprotein. NS1–NS2A cleavage occurs shortly after synthesis by a still-unknown host protease of the ER[16], while host-cell signal peptidase occupant in the ER cleaves the C–prM, prM–E, E–NS1 and NS4A–NS4B joints, and the cleavages at NS2A–NS2B, NS2B–NS3, NS3–NS4A and NS4B–NS5 junctions (Figure 1) are performed by the viral serine protease, NS3.[17] Different functions of the replicative cycle are performed by NS proteins. NS1 glycoprotein is present on the cell surface. Its coexistence with doublestranded RNA, together with other evidence, proposes the major role of intracellular NS1 protein in the replication of viral RNA.[18] NS2A, NS2B, NS4A and NS4B are small hydrophobic proteins that are linked with the membranes. In particular, NS2B is connected with the NS3 protease to form an active serine protease complex.[19] NS3 is implicated in the polyprotein processing and RNA replication. The NS3 protein is a multifunctional protein with an N-terminal protease region (NS3pro), an RNA triphosphatase, an RNA helicase and an RNA stimulated NTPase domain in the C-terminal region.[20] The protease and NTPase enzymatic functions share an overlapping region between residues 160 and 180 of the NS3 protein.[21] The RNA triphosphatase may contribute to RNA capping,[22] whereas the NTP⁄ helicase activity may separate nascent RNA strands from the template.[23] The NS3 viral protease is absolutely essential (along with the viral-encoded cofactor NS2B) for viral replication. In addition, the viral serine protease is also responsible for internal cleavages in the C, NS2A, NS3 and NS4A proteins.[24] The most important flavivirus protein is NS5. It is characterized by a methyltransferase motif in the N-terminal region and by an RNA-dependent RNA polymerase located at its C-terminal region.[25] After processing of the viral proteins, most of the NS proteins associate with the 3'-UTR of viral RNA to form a replication complex for RNA synthesis.[26] Initially in the virion assembly protein C is associated with the genomic RNA on the cytosolic face of the ER membrane and the particles are secretarially transported to the cell surface for release.

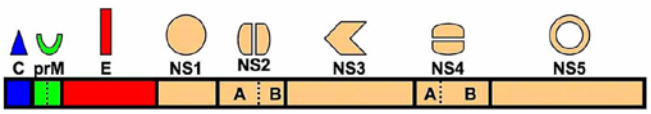


Figure 1. Structural elements of the DENV polyprotein.

Possible mechanisms and pathways in the treatment of dengue

Presently, there are no drugs against treatments for dengue fever. Only regular procedures such as nursing care, fluid balance, electrolytes and blood clotting parameters for the treatment of fever are followed. Patients with dengue fever are treated symptomatically for example, sponging, acetaminophen (paracetamol), bed rest and oral rehydration therapy, and if signs of dehydration or bleeding occur the patients are usually hospitalized. Platelet count and Hematocrit should be measured daily from the suspected day of illness until 1–2 days after defervescence. Aspirin should be avoided as it may cause bleeding. In addition, mosquito control programs are necessary for prevention but difficult to implement and maintain. A validated target is only derived by analogy to other viruses for which drugs have proven to be effective. Another validated potential target involved in DENV life cycle for antiviral therapy is cellular protein. The host cell is actively involved at many levels during DENV infection, providing co-factors and template for promoting replication of the virus, thus cellular protein will have to be inhibited or this promoting activity should be inactivated. Cellular proteins involved in pathogenesis and not viral replication directly also represent interesting targets. Proteases and glucosidases are two important cellular proteins and pathways that exert an anti-dengue effect when affected or inhibited, whereas other cellular proteins (kinases, cholesterol synthesis enzymes, proteins involved in immune response.) are progressively discovered and validated through siRNA studies.

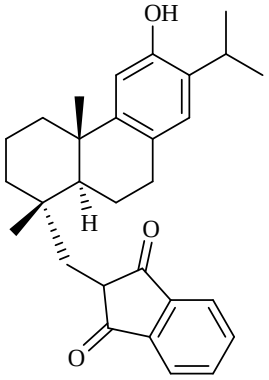
Cellular proteases are mainly of two types: furin and signal peptidase. Furin is involved in the maturation of the M protein from its precursor prM encoded in the dengue polyprotein. The consensus sequence cleaved by furin is well defined and could theoretically serve to design inhibitors. However, the delicate specificity balance between host cell targets of furin and dengue prM has not been fully investigated. Another are the signal peptidases, located in the ER membranes, which initiate further dengue polyprotein processing before NS2B/NS3 protease takes over and maturates the whole NS enzymes.

From last decade, crucial studies of Glucosidases have been made regarding their inhibition, and lots of carbohydrates and carbohydrate imitators have been synthesized and prove to be potent glucosidase inhibitors *in vitro* and *in vivo*. In dengue replication cycle, a number of DENV proteins (prM, E, and NS1) are decorated by glycosylation upon travelling through the ER. They are further maturated upon de-glycosylation by cellular glucosidases I and II, which leaves a single carbohydrate unit at their surface. It has been shown that inhibition of these enzymes has a potent antiviral effect, since these maturation events are required for proper folding of the viral proteins. Castanospermin and deoxynojirimycin derivatives have shown interesting antiviral effects against dengue.

Medicinal Plants act as Effective Inhibitors of Dengue

The use of herbal-based medicine and medicinal plants to treat many diseases is growing worldwide as they have few or no adverse effects. *Alternanthera philoxeroides* (Commonly called Alligator Weed), *Andrographis paniculata* belonging to family Acanthaceae, Azidarachta indica (neem), Boesenbergia rotunda (Chinese ginger), Carica papaya, *Castanospermum austral* (Black Bean), *Cladogynos orientalis (*white-stellate-hairy shrub), *Cladosiphon okamuranus*, *Cryptonemia crenulata*,

*Cymbopogon citrates (*lemon grass), *Euphorbia hirta, Flagellaria indic, Gastrodia elat, Gymnogongrus griffithsiae, Hippophae rhamnoides, Houttuynia cordata, Hippophae rhamnoides, Kaempferia parviflora, Leucaena leucocephala, Lippia alba* and *Lippia citriodora, Meristiella gelidium, Mimosa scabrella, Ocimum sanctum, Phyllanthus urinaria, Piper retrofractum, Psidium guajav, Quercus lusitanica, Tephrosia crassifolia, Zostera marina(*eelgrass), *Uncaria tomentosa.* These plant species of medicinal plants from various families exhibited inhibitory activity on DENV-1, DENV-2, DENV-2 NS3 protease, DENV-3 and DENV-4 viruses and proved effective for anti-dengue activity.[27] Recently Vicky C. Roa-Linares et al. have discovered[28] the analogues of abietane diterpenoid ferruginol as important inhibitors of Human Herpesvirus (HHV-1 and HHV-2) and Dengue virus (DENV-2). Ferruginol is a bioactive compound isolated from various plants and it is known for its unique pharmacological properties. They have synthesized different analogues of ferruginol and performed antiviral activity at postinfective stages. The compound **1** showed remarkable reduction in the viral plaque-size of HHV strains and reduction of cytopathic effect during DENV-2 infection at a concentration of 29.0 µM (Figure 2).



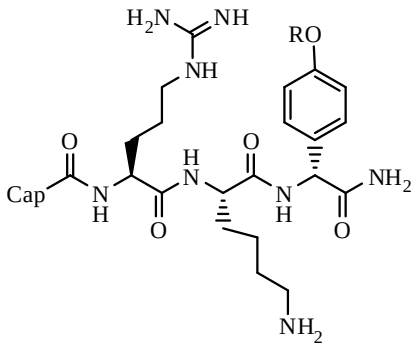
1 IC50 (µM)= 29.0

Figure 2. Abietane ferruginol analogues as anti dengue

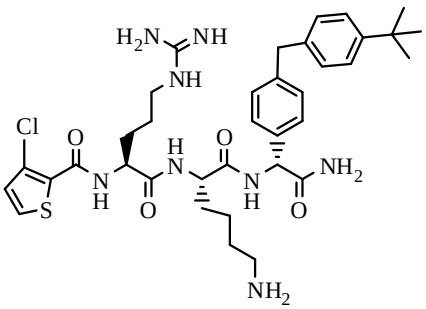
PEPTIDYL INHIBITORS

Protease inhibitors containing a 4-benzyloxyphenylglycine residue

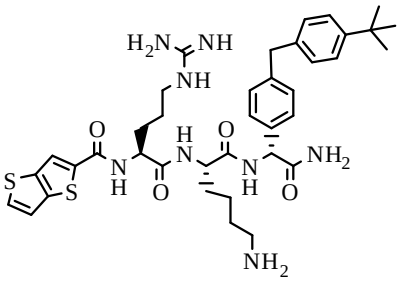
Mira A. M. Behnam discovered novel protease inhibitors containing a 4-benzyloxyphenylglycine residues with enhanced inhibitory action against the DENV-2 and WNV proteases in cell-based assays of virus replication, with an EC50 value of 3.4 μM at DENV-2 for the most active analogue.[29] The benzoyl-capped benzyl ether analogue **2** (Bz–Arg–Lys–(4-benzyloxy)-D-Phg–NH_2 sequence) showed a promising initial improvement of potency against both target proteases that was achieved by fragment growth from the previously described phenylglycine derivative (Bz–Arg–Lys–L-Phg–NH_2 sequence). The sequence was then optimized through simultaneous C-terminal and N-terminal modifications. Based on SAR analysis, selected activity-enhancing fragments were merged to provide a series of highly affine inhibitors. Compounds **3** (3-Cl-thiophene cap, 4-*tert*-butyl benzyl ether), **4** (thienothiophene cap, 4-*tert*-butyl



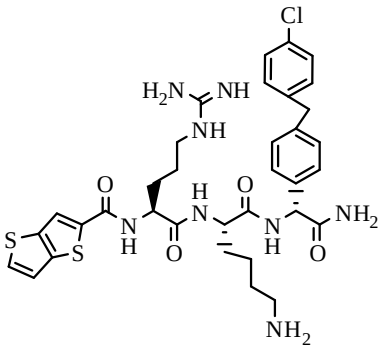
2 IC_{50} (µM)= 0.069



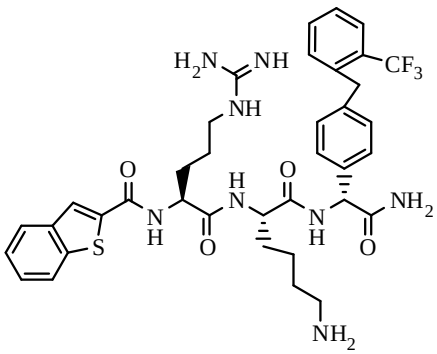
3 IC_{50} (µM)= 0.121



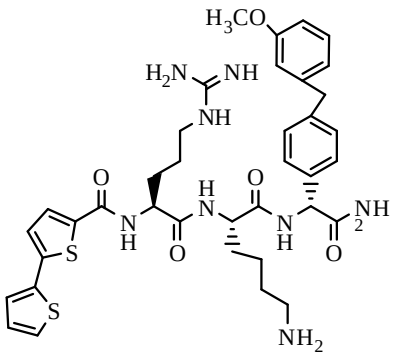
4 IC50 (µM)= 0.157



5 IC50 µM)= 0.170



6 IC50 (µM)= 0.0.243



7 IC50 (µM)= 0.176

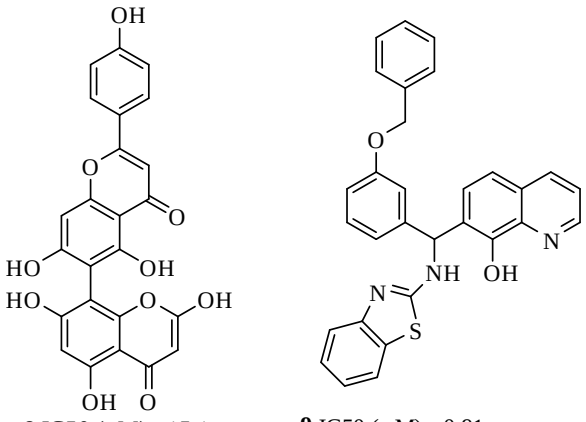
Figure 3. Peptidyl Dengue Inhibitors

benzyl ether), **5** (thienothiophene cap, 4-Clbenzyl ether) and 6 (benzothiophene cap, 2-CF_3-benzyl ether) showed more selectivity towards dengue protease. The effect of the protease inhibitors on viral replication was validated in cellular assays, which showed reduction of dengue and West Nile virus titers and favorable selectivity indices. The derivative **7** (bithiophene cap, 3-OCH_3-benzyl ether) possessed the highest antiviral activity against DENV-2 and WNV, and displayed an improved permeability and metabolic stability[30] (Figure 3).

NON-PEPTIDYL INHIBITORS

Flavanoids as dengue inhibitors

A large number of small non-peptidyl inhibitor scaffolds for DENV and WNV proteases have been described by various reserchers. In search for the novel targets of dengue, flavanols and biflavnols was found to be effectual inhibitors of NS2B-NS3 proteases of the Dengue virus serotypes 2 and 3 with IC50 values ranging from 15 to 44 µM. Among all the compounds, biflavanoids **8** was the most active with IC_{50} value 15.1 ± 2.2 and 17.5 ± 1.4 µM against DENV2 and DENV3 protease, respectively (Figure 4). These compounds showed non competitive mode of inhibition. The mechanism of inhibition was understand by dockings studies using LeadIT- flex with the recently solved crystal structure of DENV3 protease in complex with the aldehyde inhibitor Bz-nKRR-H (pdb: 3U1I), which is the only available structure of a DENV protease in complex with a low-molecular weight inhibitor.[31]



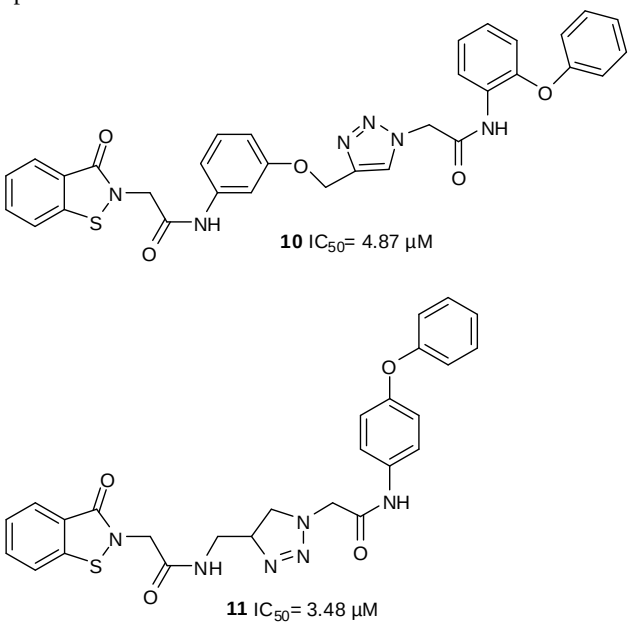
8 IC50 (µM)= 15.1 **9** IC50 (µM)= 0.91

Figure 4. Flavanoids and 8-hydroxy quinolone hybrid derivatives as potent non peptidyl dengue inhibitors

Benzathiazole derivatives as a dengue inhibitors

Huiguo Lai et.al. synthesized[32] various 8-hydroxy quinolone derivative coupled with aminothiazole and aminobenzothiazole moiety and studied its inhibition against DENV2 NS2BNS3pro along with the molecular docking. These compounds responded well against WNV protease. The invitro study was done using the tetrapeptide substrate Bz-Nle-Lys-Arg-Arg-AMC. The rational SAR studies revealed that benzothiazole moiety contributed to better inhibition against the protease than the thiazole moiety. Compound **9** showed maximum inhibition (IC50 = 0.91 ± 0.02 µM) among all the four compounds (Figure 4). The molecular docking studies supported the invitro studies.

Freitas de Sousa et.al. synthesized[33] benzisothiazole derivatives by click chemsitry and studied its inhibitory activity toward Dengue virus NS2B-NS3 protease. These compounds were tested at 25 µM and protease inhibitor concentration was 25nM. Compound **10** and **11** showed noteworthy inhibitory effect against DENV2 protease (Figure 5). This class of compounds provide a reasonable basis for a hit-to-lead optimization studies.

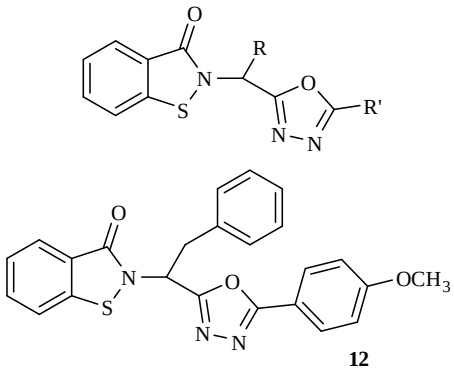


10 IC_{50}= 4.87 µM

11 IC_{50}= 3.48 µM

Figure 5. Benzisothiazole derivatives as a non-peptidyl dengue inhibitors

Further Huigoi Lai et.al.[34] studied a series of novel 1,2-benzathiazol-3(2H)-one and 1,3,4-oxadiazole hybrid derivatives, as potential competitive inhibitors of DENV2 and WNV NS2B/NS3 proteases. In this two heterocyclic scaffold provides multiple sites of hydrogen bond donor and acceptor for interaction between protease and inhibitor compound **12**. The highest inhibitory activity was notarized where R= phenyl group (65 % at 10 µM) and lowest inhibition was manifested where R=H (5 % at 10 µM). The biochemical studies was well corroborated with the docking studies. (Figure 6)

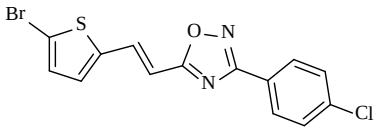


12

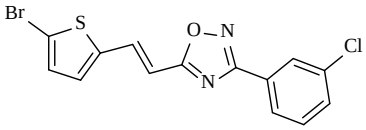
Inhibition %= 65 at 10 µM

Figure 6. 1,2-benzisothiazol- 3(2H)-one and 1,3,4-oxadiazole hybrid derivatives as a non-peptidyl dengue inhibitors

Recently, Fatiha et.al. synthesized novel series of non nucleoside 1,2,4 and 1,3,4 oxadiazole derivatives. Its inhibitory effect against dengue virus targeting NS5 polymerase was also studied. Brominated thiophhene exhibited potent inhibitory effect against the four dengue virus series DENV 1-4. Compound **13** and **14** were the most potent inhibitors against four serotypes.[35] Also, the studies revealed that the 1,2,4 oxadiazole derivatives found to be more effective against DENV virus (Figure 7).



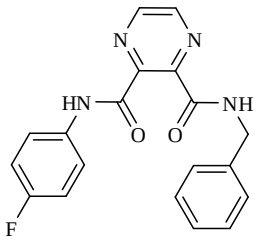
14 IC_{50} (μM)= 3.0- 8.5



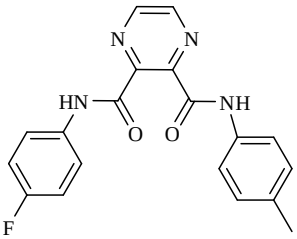
15 IC_{50} (μM)= 3.0

Figure 7. 1,2,4 and 1,3,4 substituted oxadizole derivatives of Brominated/phenylated thiophene as a DENV inhibitors (DENV 1-4)

M. Saudi et al. worked on a series of tetracyclic imidazole 4,5-dicarboxamide and pyrazine 2,3-dicarboxamides scaffolds. The structural analog of imidazole 4,5-dicarboxamide showed potent DENV inhibitory properties with EC_{50} = 1.93 μM and the derivatives of pyrazine 2,3-dicarboxamides **15** and **16** were found to possess effective inhibitory activity with EC_{50} = 1.93 μM against dengue virus (Figure 8). The five-membered imidazole ring is a chief entity present in various chemotherapeutic agents and the drug having imidazole core always attract medicinal chemists to further explore and synthesize variety of structural analogues comprising this chief moiety.[36]



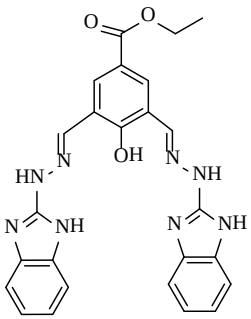
16IC_{50} (μM)= 0.94



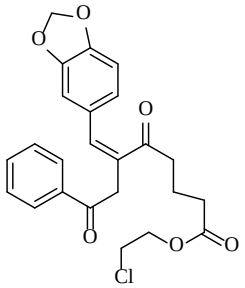
17 IC_{50} (μM)= 0.94

Figure 8. Imidazole derivatives as an antidengue

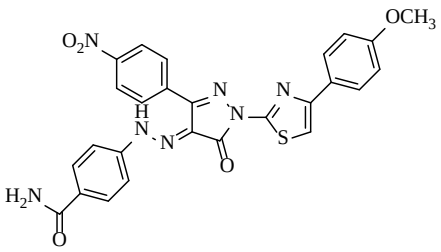
J. Deng et. al. reported docking-based virtual screening of approx. 600 000 compounds in the ACD database out of which 27 predicted hits were bought and tested for NS2B-NS3 protease inhibitor activity, leading to the discovery of three active small molecule compounds (**17**−**19**) with different scaffolds (Figure 9). Keeping in mind inhibitory activity, structural variability, and synthetic accessibility, further structural optimization by functional group modification and scaffold hopping approaches was started by using Compound **17**. They discovered 17 compounds (**17**, **20**-**35**) showing moderate dengue viral NS2B-NS3 protease inhibition in vitro, and eight compounds (**17**, **21**, **25**, **30**-**35**) are active to some extent against the replication of the dengue viral replicon in vitro.[37] These compounds could be used as leads for further search for small molecule dengue viral NS2B-NS3 protease inhibitors with enhanced potency. (Table 1)



19 IC_{50} = 13.1μM



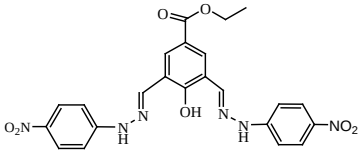
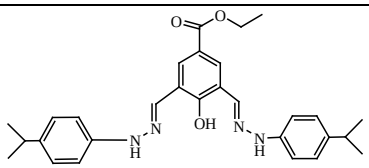
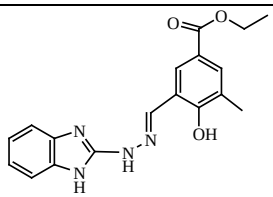
20 IC50 = 242.4

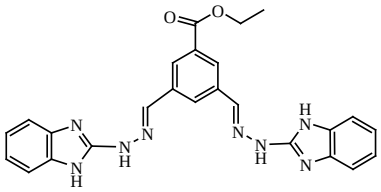
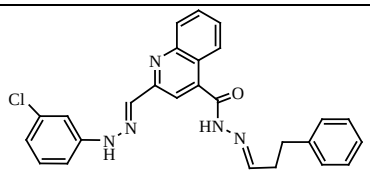
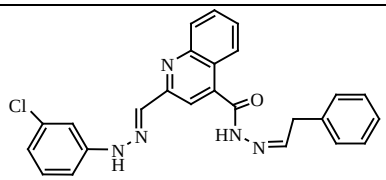
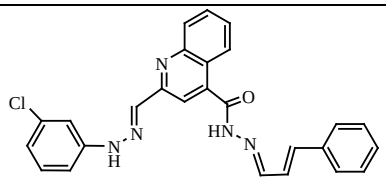
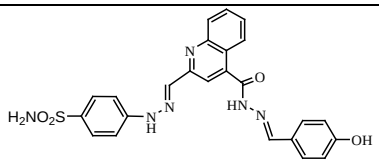
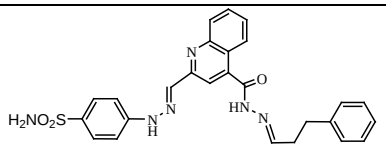
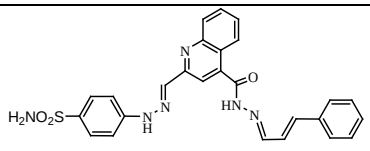
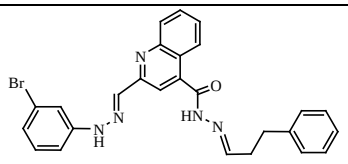
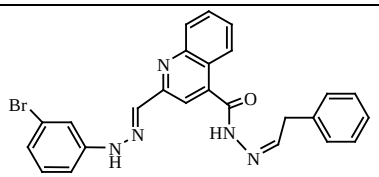
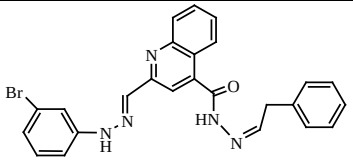
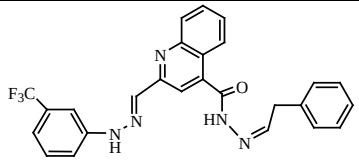
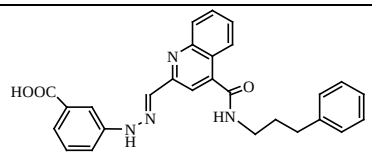
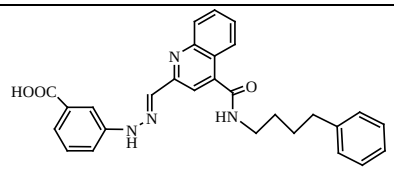


21 IC50 = 219.9

Figure 9. Various heterocyclic scaffolds as DENV NS2B-NS3 protease inhibitor

Table 1. Chemical structure of compounds **20**-**35** and their bio activity against dengue virus. IC_{50} (Inhibitory rate at 100 μM)

Sr. No.	Compound	IC_{50}(μM)
1	 **20**	14.58 ± 2.06
2	 **21**	39.46 ± 1.43
3	 **22**	48.59 ± 3.46

4	 **23**	29.53 2.15	±
5	 **24**	29.04 1.78	±
6	 **25**	28.12 1.96	±
7	 **26**	14.32 2.49	±
8	 **27**	7.83 0.94	±
9	 **28**	36.02 2.05	±
10	 **29**	7.45 1.15	±
11	 **30**	19.93 0.98	±
12	 **31**	9.45 0.78	±
13	 **32**	9.45 0.78	±
14	 **33**	19.8 1.15	±
15	 **34**	41.24 5.53	±
16	 **35**	35.28 4.36	±

CONCLUSION

Significant progress has been made over the past few years in the development of dengue virus inhibitors. A variety of improved and influential methods for peptidyl and non-peptidyl inhibitor have been developed. There is no suitable animal model available so far for the dengue virus diseases. The initial testing of potential antiviral compounds are mainly facilitated by mammalian cell culture systems which are useful to monitor the effects of anti-dengue inhibitors on the growth of the virus. Complications mainly arise because of the presence of four related dengue serotypes which show marked differences in their inhibition profiles. Drug designing against related human pathogens of the flavivirus complex such as yellow fever virus, Japanese encephalitis virus and West Nile virus would be of large benefit to know the progress for inhibitors against the dengue virus protease. Intensive efforts and sustained multidisciplinary approach is required in the future to cope up with the challenging task of a contributory treatment for dengue virus diseases. However, in order to bring an effective anti-dengue drug, researchers should primarily concentrate on the problem of selectivity against pharmacologically relevant human proteases, evaluation of prognostic markers for disease severity and the pathobiochemistry of dengue haemorrhagic fever and the risk of adverse effects.

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