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The Medicinal Chemistry of Dengue Fever

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Introduction

The dengue viruses (DVs[a]) are reported to infect 40−100 million people each year, and with more than half the world's population living in areas at risk of infection, the World Health Organization has estimated the true number of cases to exceed 50 million annually.[1,2] Transmitted through the *Aedes aegypti* mosquito, DVs account for 99% of all reported viral hemorrhagic fevers.[3] Along with other lethal human pathogens such as yellow fever (YFV), Japanese encephalitis (JEV), West Nile (WNV) viruses, and hepacivirus (hepatitis C virus), DVs belong to the family *Flaviviridae*.[3] There are four serotypes of dengue, DEN-1, DEN-2, DEN-3, and DEN-4,[4] and the infection severity ranges from the self-limiting dengue fever (DF) to the more serious dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), with reported ranges of 1−5% to 10−30% of cases resulting in death, respectively.[1,5] While infection with a single serotype of DV is believed to provide life-long immunity to that serotype, cross-protection to other serotypes only lasts a few weeks, and despite vaccines being developed for some other flaviviruses such as YFV, tick-borne encephalitis, and JEV, a vaccine for DV remains elusive.[6] In light of the difficulties associated with producing an effective vaccine for DV, the alternative strategy of chemotherapeutic development has become imperative.

Currently the medicinal chemistry of DV is in its infancy, with many targets and new lead compounds being identified with increasing frequency. This review examines the information currently available on DV inhibitors and potential targets for therapeutic invention. However, because of the diverse, scattered, and relatively limited information available, lead compounds possessing relatively weak activity have also been included here. In addition, although significant information can be reported in patents, they rarely specify exact structures of their best inhibitors and commonly describe a vast array of compounds utilizing a central skeleton. Many patents claiming molecules with activity against dengue do not actually present this inhibitory data with WNV inhibitory activities more commonly stated. Here we have confined ourselves to reporting data from patents that specifically state they are active against DV (cf. with the more general flavivirus or WNV activity) even if only general structure scaffolds are defined. With this approach, we aim to present a currently complete picture of the medicinal chemistry of DV to allow the rapid development of chemotherapeutics.

Targets for Therapeutic Intervention

Biology of the Dengue Viruses. The DVs contain a 10−11 kilobase, positive sense, RNA genome that encodes for three structural proteins and seven nonstructural (NS) proteins (Figure 1). Upon infection, a polyprotein is created from the viral RNA with the three structural proteins, capsid (C), premembrane (prM), and envelope (E), being synthesized first.[4] The seven NS proteins are then translated as NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 with the trypsin-like serine protease of NS3 responsible for processing of the polyprotein between NS2A-NS2B, NS2B-NS3, NS3-NS4A, NS4B-NS5 and possibly some processing of the capsid protein.[4,7] Other junctions between C protein-prM, prM-E protein, E-NS1, and NS4A-NS4B are believed to be cleaved by host cell signalase in the endoplasmic reticulum (ER) lumen.[8] Currently there are known inhibitors of E, NS3, and NS5 proteins (see section entitled Development of Chemotherapeutics against Dengue: Currently Investigated Viral Targets); however, all dengue proteins are potential future targets and a more detailed examination of their role is key to possible chemotherapeutic intervention.

The Structural Proteins. The C protein (Figure 2) contains 15 lysine and 9 arginine residues within its 115 residues, resulting in almost 25% of the protein consisting of basic residues.[4,9] It is believed that this enables the C protein to interact with the viral RNA by neutralizing the negative charge during encapsidation and is generally conserved among all DVs.[9,10] In addition, the hydrophobic residues 110−113 are believed to be involved as a signal for the translocation of prM across intracellular membranes.[10]

The membrane (M) protein, produced via cleavage of the prM protein, is membrane bound and consists of 75 amino acids, of which the N-terminal 40 residues form the ectodomain and the remaining 35 are involved in the C-terminal transdomain anchor.[12] It has been demonstrated that the C-terminal portion of the M protein forms ion channels that are permeable to a variety of ions including sodium, potassium, chloride, and calcium, though the last two are less permeable than the first

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[a] Abbreviations: DV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; C, capsid protein; E, envelope protein; M, membrane protein; NS, nonstructural; DCs, dendritic cells; NTPase, nucleotide triphosphatase; YFV, yellow fever virus; WNV, West Nile virus; JEV, Japanese encephalitis virus; ILs, interleukins; IFN, interferon; TNF, tumor necrosis factor; NO, nitric oxide; ADE, antibody-dependent enhancement; RTP, ribavirin triphosphate; IMPDH, inosine monophosphate dehydrogenase; GAG, glycosaminoglycan; ER, endoplasmic reticulum; EC_{50}, effective concentration required to reduce the population by 50%; ODCase, orotidine monophosphate decarboxylase; BBI, Bowman−Birk inhibitor; RNAi, RNA interference; PMO, phosphorodiamidate morpholino oligomer; NCI, National Cancer Institute; GTP, guanosine 5′-triphosphate; SAR, structure−activity relationship.

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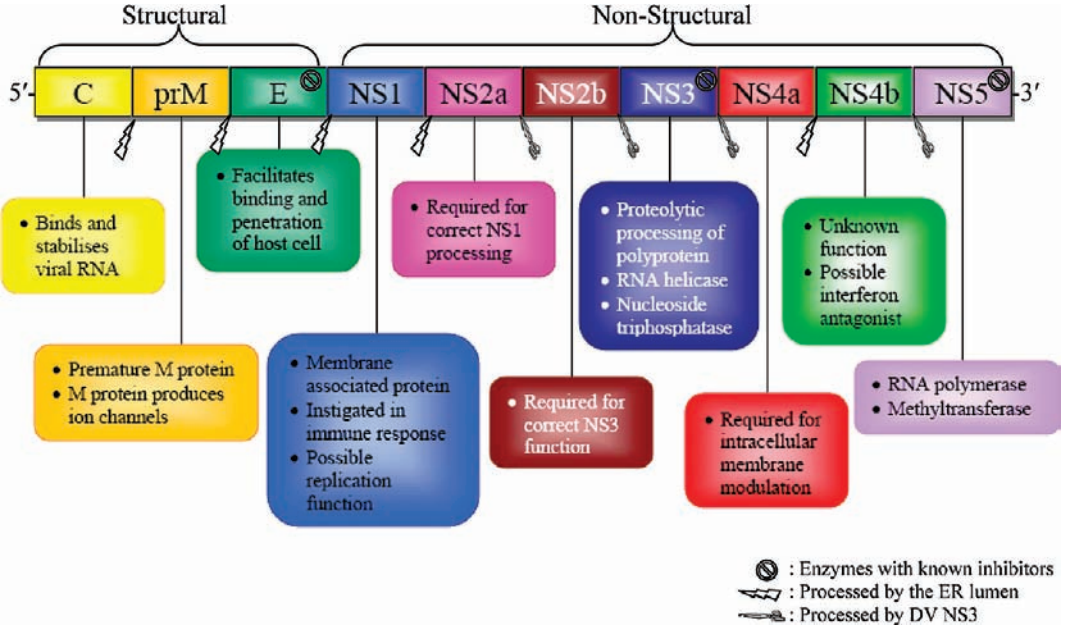


Figure 1. Proteins, and therefore potential targets, involved in the DV lifecycle.

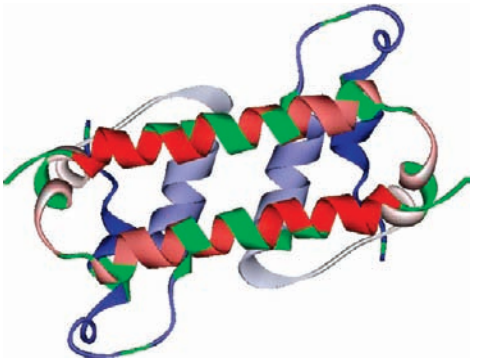


Figure 2. Ribbon representation of residues 20−100 of DV C protein as a homodimer. Blue fades to red, illustrating the protein from N-terminal to C-terminal. Basic residues are illustrated in green. Illustration is generated from PDB file 1R6R.[11]

two.[12] There is also evidence that the ectodomain contains an apoptosis promoting region.[13]

Proteolytic processing of the prM protein to yield the mature DV virions has been shown to occur at pH 6.0 after undergoing a reversible conformational change.[14] This suggests that the pr portion of the M protein is retained in the trans-golgi network and, with the prM-E heterodimer present, the pr peptide β-barrel structure covers E protein to prevent fusion with the host cell membranes during replication (Figure 3).[15]

The E protein (Figure 4) is used by the virus to facilitate binding and fusion to the host cell wall via insertion of a β barrel structure, allowing for injection of the viral genome.[16,17] The E protein exists as homodimers on the surface of the viral particles, with holes located between domain I and domain II of the E protein monomers, where the ectodomain of the M protein is located.[17]

Recently the presence of a "fusion peptide", in the form of a hair pin turn at residues 100−108 (Figure 4), facilitates the binding and penetration of the DV virions during the rearrangement of the E proteins from homodimers to homotrimers.[18]

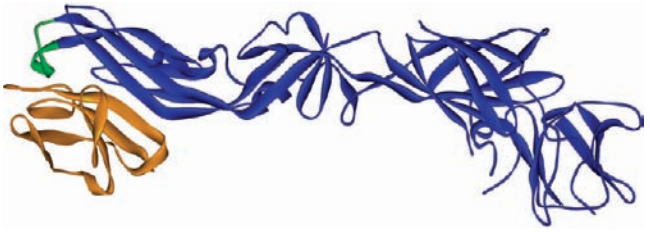


Figure 3. Ribbon representation of the prM-E heterodimer. The E protein is colored cyan (residues 100−108 highlighted in green), while the prM protein is illustrated in orange. Image generated from PDB file 3C6E.[15]

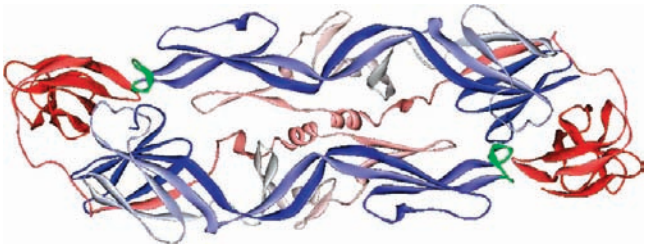


Figure 4. Ribbon representation of an E protein homodimer. Blue fades to red, illustrating the protein from N-terminal to C-terminal, while the fusion peptide (residues 100−108) is illustrated in green. Illustration is generated from PDB file 1OKE.[18]

Further to this, evidence suggests that the E protein fusion mechanism is driven by the irreversible conformational changes in E facilitated by this "fusion peptide" inserting into the lipid bilayer.[19] Through mutation studies Trp101 has been identified as a critical residue for internalization of DV due to its interactions with surrounding residues in the "fusion peptide".[20]

Nonstructural Proteins. The first of the nonstructural proteins, NS1, is synthesized in the rough ER as a hydrophilic, water-soluble, monomeric glycoprotein; however, shortly after its synthesis, it forms less hydrophilic membrane associated homodimers.[4,21] As such, NS1 has been implicated in the immune responses through DV challenges

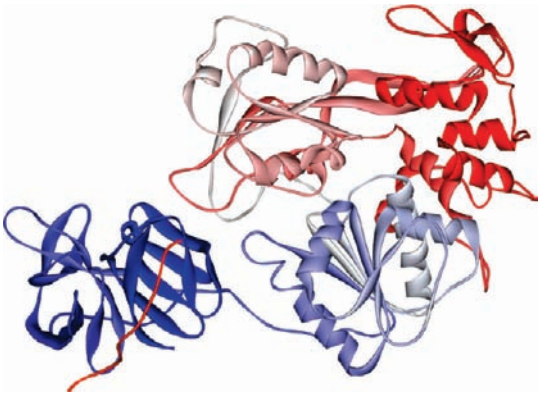


Figure 5. Crystal structure of the DV NS3 bound to an 18-residue NS2b cofactor. Blue fades to red, illustrating NS3 from N-terminal to C-terminal, while the orange illustrates the NS2b cofactor. Illustration is generated from PDB file 2VBC.[35]

following NS1 immunization, while more recently NS1 antibodies have been observed to have cross-reactivity with human platelets and endothelial cells, possibly causing inflammation that results in the pathogenesis of DF.[22] The NS1 C terminal domain, amino acids 271−352, has been investigated as the causative agent of the NS1 associated pathogenesis, illustrating this sequence as essential for NS1 antibody mediated platelet dysfunction.[23] In the related YFV, NS1 has been shown to possess a function in viral replication.[24] Although this is poorly understood, it has been observed that mutations in NS1 affect the initiation of minus RNA strand synthesis.[25]

NS2 consists of two proteins; NS2a contains several possible transmembrane domains and is implicated in the processing of C-terminal of NS1 from the polyprotein.[26] NS2b was initially thought to have no function in viral replication; however, later evidence demonstrated that it is required for the correct functioning of NS3.[27]

The hydrophilic protein, NS3 (Figure 5), was initially believed to be involved in postranslational processing of the polyprotein through protease activity or an RNA polymerase constituent or possibly a combination of both.[4,28] Later it was revealed that it forms a protease complex with NS2b and is responsible for proteolytic processing of some nonstructural protein junctions in the polyprotein.[29] Additionally it possesses RNA helicase activity with activity as a nucleotide triphosphatase (NTPase).[30] Until recently these activities had not been exclusively demonstrated in an unmodified protein; however, the helicase and NTPase were shown to be active when NS3 is complexed with NS5 in a dose-dependent manner.[31] WNV and YFV NS3 proteins also possess demonstrated NTPase activity.[32−34] Recently the NS3 protein was crystallized to a 3.15 Å resolution with a truncated NS2b cofactor,[35] while protein has been extensively reviewed for its potential as a antiviral drug target.[36]

Much like NS2, NS4 consists of two proteins, NS4a and NS4b. NS4a has been demonstrated to be tightly associated with intracellular membranes as well as being critical in the rearrangement of intracellular membranes,[37,38] while its C-terminal is believed to be involved in the translocation of NS4b to the lumen of the ER.[37] In contrast, NS4b has a undetermined function but is implicated in modulation of viral replication, through interactions with NS3 causing it to dissociate from viral RNA,[39] though recent evidence suggests that NS4B may be responsible for viral inhibition of interferon (IFN) via antagonistic effects.[40]

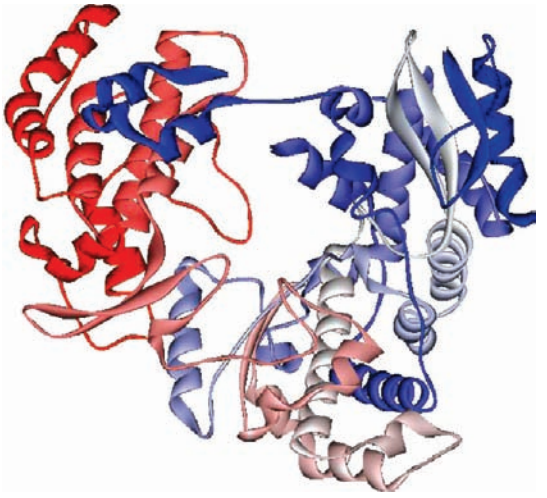


Figure 6. Crystal structure of DV NS5 RNA dependent RNA polymerase. Blue fades to red, illustrating the protein from N-terminal to C-terminal. Illustration is generated from PDB file 2J7U.[45]

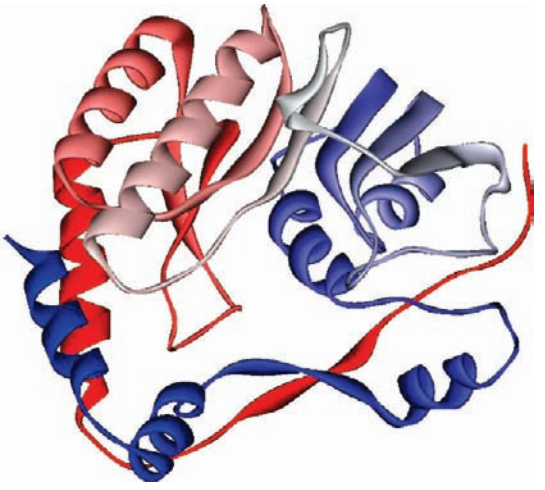


Figure 7. Crystal structure of DV NS5 methyltransferase. Blue fades to red, illustrating the protein from N-terminal to C-terminal. Illustration is generated from PDB file 2P41.[48]

The protein NS5, similar to NS3, has two functions: as an RNA dependent RNA polymerase and as a methyltransferase.[41] Its RNA polymerase activity was initially predicted from homology models but has since been demonstrated through cross species expression.[42,43] The methyltransferase activity has been predicted on the basis of the similarity of groups between dengue NS5 and methyltransferases from a wide variety of other species.[44]

The RNA polymerase active site of NS5, encompassing amino acids 270−900 of 900, has been crystallized to 1.85 Å resolution (Figure 6), illustrating two zinc ion binding motifs and nuclear localization sequences with the hope that new information will aid in the design of inhibitors targeting this RNA polymerase activity.[45]

The methyltransferase site of NS5, located at the N-terminal portion of the enzyme, has also been elucidated for DV at a resolution of 2.20 Å (Figure 7).[46] It has been shown that N-7 methylation activity is essential for WNV life cycle, justifying the methyltransferase activity of NS5 as a novel antiviral target.[47]

Clinical Manifestations

DF is typically characterized by a fever with chills, headache, retro-ocular pain, general malaise, myalgia, and arthralgia ("break-bone fever") and occurs abruptly 3−15 days after a bite from an infected mosquito and lasts 4−7 days.[1,4] DHF is usually indistinguishable from DF during the initial phase; however, 4−7 days later disordered hemostasis and increased vascular permeability occurs, resulting in vascular leakage, the key feature between DF and DHF.[1,4,49] DSS is characterized by cardiovascular collapse due to DHF associated capillary fragility, plasma leakage, and hemorrhage.[49]

DHF and DSS are more prevalent in regions where multiple serotypes of the DV are common, as patients in these regions possess pre-existing, but non-neutralizing, antibodies to the DV. These non-neutralizing antibodies bind to dengue virions, allowing for a bridge to be formed between the virion and an uninfected cell, thus facilitating infection.[50] The result is antibody-dependent enhancement (ADE), giving rise to an increased rate on infection.[50,51] ADE has also been noted to occur in young infants where maternal antibodies are inherited passively, resulting in circulating non-neutralizing antibodies in the infants.[52]

Pathogenesis of Dengue Fever

From studies of live patients, it has been observed that the cells infected by the DVs are typically dendritic cells (DCs), monocytes, macrophages, lymphocytes, and endothelial cells.[53] While the cause of the pathological changes resulting from infection are poorly understood, it has been observed that dengue infected cells often increase their production of interleukins (ILs), interferons (IFNs), tumor necrosis factors (TNFs), and nitric oxide (NO).[54] Therefore, it has been hypothesized that these factors elicit an inflammatory response and lead to vascular leakage.[54]

DV infection of human endothelial cells results in increased production and secretion of IL-6 and IL-8.[55] IL-8 has been observed to be responsible for the development of DHF/DSS from DF, resulting in the death of patients,[56] while IL-6 is known to mediate an increase endothelial cell permeability.[57] IL-10 and IL-12 have also been identified to have a role in the pathogenesis of DF and DHF, where increases in IL-10 concentration correlate directly with severity of DHF.[58] Conversely, decreases in the concentration of IL-12 result in increased severity of DF and DHF,[59] while IL-13 and IL-18 are also implicated in the pathogenesis of DHF.[60]

While TNF-α has been implicated in the pathogenesis of DF, its role is uncertain because of conflicting results.[61−64] The modulation of TNF-α concentration in dengue-infected patients also remains unclear, in many cases reported as being up-regulated and, with TNF-α known to be an inducer of IL-6, is likely a contributing factor in vascular leakage.[65]

IFNs have also been noted to have an increase in secretion in dengue infected patients; however, there is no significant increase in IFNγ concentrations between DF and DHF.[66] IFNγ is known to have indirect antiviral activity through the activation of particular genes that inhibit viral replication[67] while also inducing apoptosis mediating factors.[68] However, IFNγ has also been shown to increase plasma levels of IL-6 and IL-8 while not affecting the concentration of IL-4, IL-10, or TNF-α, which may contribute to vascular leakage.[68,69]

Management of Dengue Infections

As there are no commercially available vaccines or chemotherapeutics to target dengue infections, palliative treatments are employed to target and control the disease symptoms. Of key importance[3] is maintenance of the fluid balance by replacing losses incurred through diarrhea, vomiting, and plasma leakage to ensure patients do not develop worsening shock or acidosis. Management of dengue fever and the febrile phase of DHF also involves the use of the antipyretic paracetamol for temperature control. Further to this, progression toward severe DHF requires daily examination of fluid and electrolyte balance, vital signs, urine output, level of consciousness, packed cell volume, platelet counts, and liver enzymes. It may also be necessary to administer intravenous fluids. It is essential to ensure fluids are stopped when a patient has recovered from the leakage phase, as fluid overload can result in pleural effusions, respiratory compromise, pulmonary edema, or congestive heart failure.[3] If the disease progresses to DSS, additional intravenous fluids, including crystalloids and colloids, may be administered along with increased frequency of monitoring as detailed for DHF.[49] The current palliative treatments to target and control the disease symptoms for DV infections are tedious, laborious, and time intensive, adding impetus to the necessity of developing chemotherapeutics to target DF, DHF, and DSS.

Vaccine Development

Although vaccines have been one of the most important public health initiatives of the 21st century and are responsible for preventing millions of different infections each year, a dengue vaccine remains challenging. In 1956 the first report of clinical immunization with attenuated dengue was published;[70] however, despite continuing research in the intervening years, an immunogenic dengue vaccine still remains elusive. The challenge for a dengue vaccine is to induce broad, long-lasting, cross-protective immune responses against all four serotypes of DV. Previous attempts to develop vaccines have been hampered by vaccine-induced ADE of viral infections resulting in the development of DHF and DSS,[71] limited understanding of both the protective immune responses to, and molecular biology of, the DVs, and the lack of suitable animal models.[72,73] Various strategies have been employed to develop an effective DV vaccine including the use of live attenuated viruses, vector-based delivery systems including adenovirus and vaccinia, DNA vaccines, protein vaccines, chimeric YFV−DVs, and attenuated chimeric DVs.[74−76] Clinical trials have been undertaken to examine the safety and immunogenicity of various formulations of tetravalent live attenuated DV vaccines in adults and children with moderate levels of neutralizing antibodies reported; however, further work is required to improve immunogenicity.[77−82] Phase 1 clinical trials of a yellow fever−dengue 2 chimera (ChimeriVax-DEN2) has demonstrated that ChimeriVax-DEN2 is safe, immunization is not adversely affected by pre-existing immunity to YFV, and the vaccine can induce a long lasting and cross neutralizing antibody response to all four dengue serotypes. However, despite the evaluation of numerous candidates in preclinical and clinical studies, there is still no effective licensed tetravalent dengue vaccine available.[77,78,80]

Development of Chemotherapeutics against Dengue: Currently Investigated Targets

Previous attempts to develop effective antidengue chemotherapeutics have been hampered by inadequate rodent models capable of mimicking the human physiological responses to dengue infections[83] and an understanding of the molecular biology of the disease. More recently, an increased

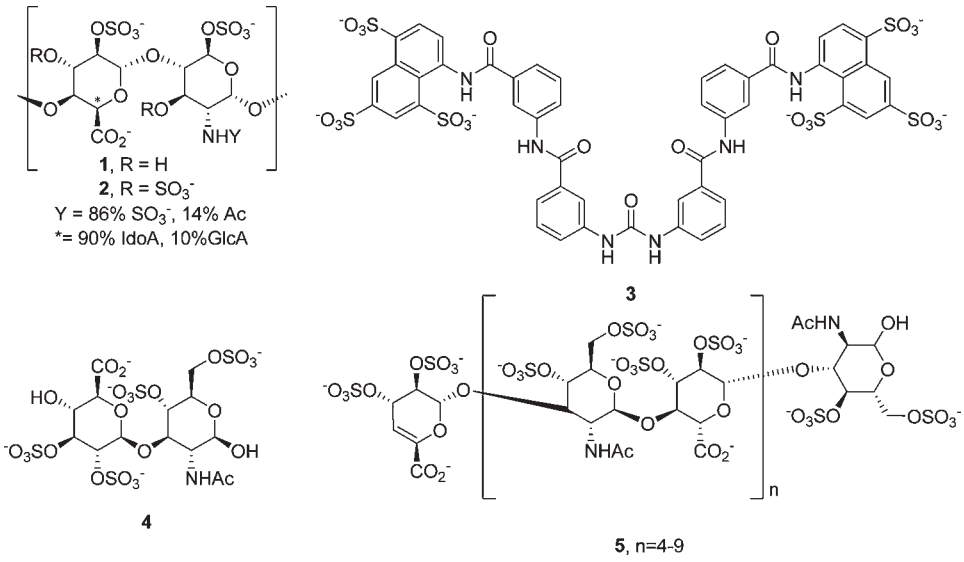


Figure 8. Selected viral entry binding inhibitors.

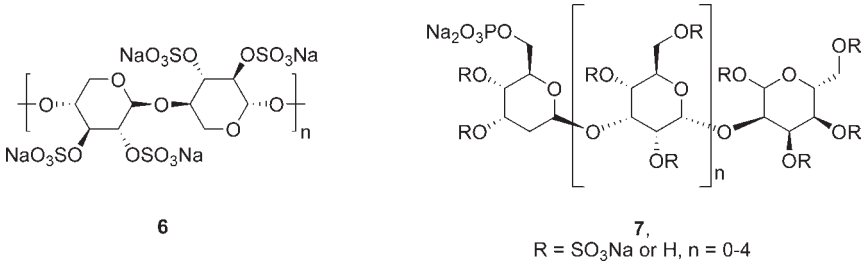


Figure 9. Two E protein binding inhibitors.

knowledge of the molecular mechanisms underlying the dengue replication cycle and each of the proteins' functions has enabled the identification of targets. In some cases this has led to the development of virtual (in silico) screening techniques capable of identifying leads and is increasing the rate of novel compounds for the treatment of dengue.[84] Targets currently being investigated include viral entry, viral RNA polymerase/methyltransferase, nucleotide synthesis, viral helicase/NTPase, viral serine protease, α-glucosidases, and kinases.

Inhibition of Viral Entry. The entry of viral particles into the host cell represents the first point of intervention available to chemotherapeutics. While it is known that the binding of DV particles to cells expressing antibody signals occurs and is believed to be responsible for ADE, it is not dependent upon this interaction for infection.[85] In the initial infections, the binding of the virus' E protein to the host cell membrane has been narrowed to interactions with a highly sulfated type of the glycosaminoglycan (GAG), heparan sulfate (**1**).[86]

The use of monoclonal antibodies and heparin has been shown to inhibit the binding and penetration of dengue viral particles.[87] The use of GAGs such as **1** by pathogens in achieving binding and penetration is well documented,[88−92] as well as the need for an additional receptor to facilitate binding.[93] DV particles are hypothesized to require the presence of GAGs and additional receptors to bind to the extracellular membrane, prior to endocytosis.

Targeting of viral entry with possible drugs has been attempted against both dengue and HIV-1. Naphthalene sulfonate polymers were observed to inhibit HIV-1 in vitro by binding to $CD4^+$ receptors, required for the infection of host cells.[94]

In this fashion, polyanion molecules, persulfated GAG-derived compounds, and oligosaccharides such as **1** (MW_{AV} = 16 000), over sulfated heparin (**2**), suramin (**3**), hyaluronic acid (**4**), and oligosaccharaides of hyaluronic acid (**5**), were tested for their ability to inhibit the binding of the E protein to immobilized heparin (Figure 8).[95] It was observed that despite the slow addition of these compounds to the binding domain, they had extremely slow release rates and bound to the E protein almost as strongly as heparin, based on competitive and direct binding assays.[95] Compounds **2** and **3** were effective inhibitors with IC_{50} values of 7.6×10^{-9} and 7.0×10^{-7} M, respectively. Both exceeded the inhibitory ability of **1** ($IC_{50} = 7.6 \times 10^{-7}$ M).[95]

In another study, **3** was analyzed in vivo in addition to pentosan polysulfate (**6**) (MW ≈ 5700) and PI-88 (**7**) (MW = 1400−3100) (Figure 9).[96] In vitro the effectiveness of **6**, **7** and **1** against DEN-2 produced EC_{50} values of ∼30, ∼200, and ∼60 μg/mL, respectively, with **1** able to inhibit the infectivity of dengue with an EC_{50} of 1 μg/mL. However, when tested in vivo, only **7** had any effect on survival rates when mice were infected with dengue fever and treated with **7**, prior to and following infection.[96]

Ono et al. have described the activity of two sulfated galactomannins extracted from the seeds of *Mimosa scabrella* and *Leucaena leucocephala*.[97] Both of these had in vitro and in vivo activity against DV and YFV, with the believed mechanism of action being inhibition of viral entry to host cells.[97] Sulfated polysaccharides have also been investigated demonstrating the ability to inhibit the entry of dengue virons into host cells,[98,99] with some differentiation between inhibitory activity and serotype being observed.[99] Despite

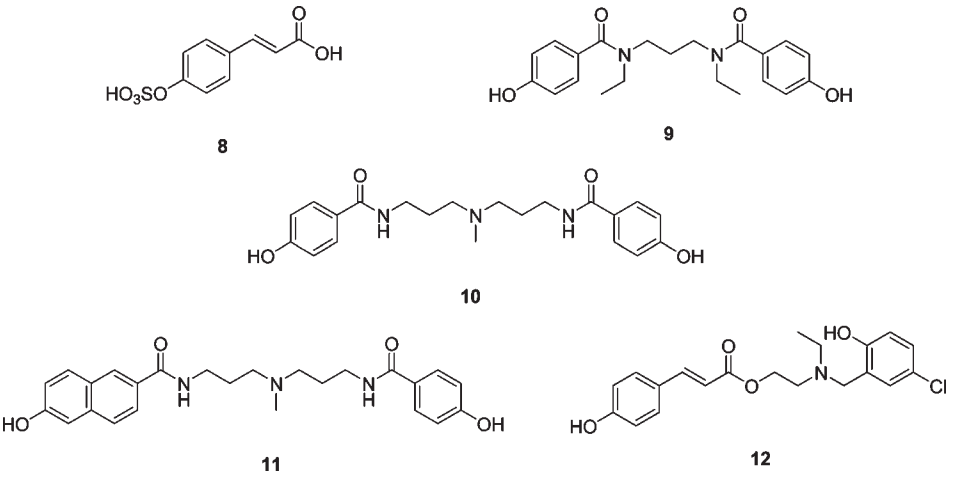


Figure 10. Various *p*-sulfoxycinnamic acid derived DV entry inhibitors.

the variations in inhibitory activity and the variations with respect to DV serotype, the inhibition of viral entry into cells could lead to promising new drug leads.

p-Sulfoxycinnamic acid (**8**), derived from the temperate marine eelgrass *Zostera marina*, has been shown to have a broad range of antiadhesion activities against algae, fungal spores, and bacteria. Because of these properties, coupled with its low toxicity and cost, a series of symmetrical and nonsymmetrical derivatives were synthesized. (Figure 10) Under the assumption that viruses may share common binding properties with algae, fungal spores, and bacteria, these were screened against the dengue viruses. Three compounds, **8**−**10**, showed modest IC_{50} values of 2 mM, while **11** and **12** were far more potent with IC_{50} values of 294 and 46 μM, respectively. Interestingly these compounds increase binding between the virus and host cell; however, disruption to viral entry results in inhibition of the virus.[100]

In silico screening has been a powerful tool in developing novel leads, structurally different but comparably active compounds. To this end, virtual screen targeting the E protein has been employed to identify inhibitors capable to locking the "fusion peptide" into a nonbinding conformation.[101] A small binding pocket identified on the E protein (Figure 11), believed to result from induced fit, has yielded results when the National Cancer Institute (NCI) library of 142 000 compounds were screened in silico.[101]

The resulting NCI compounds, **13**−**15** (Figure 12), were shown to have activity in the micromolar range.[101] These compounds were further refined to yield hybrid molecules that display improved in vitro activity against YFV.[102]

Further in silico studies have identified two more binding pockets believed to be capable of binding structurally unique inhibitors that will inhibit the structural modifications between pre- and postfusion E protein homodimers and homotrimers[103] (Figure 13A).

Database mining based on the gold binding pocket produced a novel inhibitor with an IC_{50} of approximately 4 μM (Figure 13B). This molecule was predicted to bind to the E protein; however, this has yet to be proven.[103]

Recently Novartis designed various antivirals (**16**−**18**) based around a thiophene−phenyl−chlorine core similar to that of the thiazole−phenyl−chlorine core of **13**−**15** [104] (Figure 14). These inhibitors are suggested to act against the DV via binding to the E protein, resulting in internalization of the DV virions; however, release from the endosome is prevented. These molecules have also been observed to prevent the production of the NS3 protein.[104]

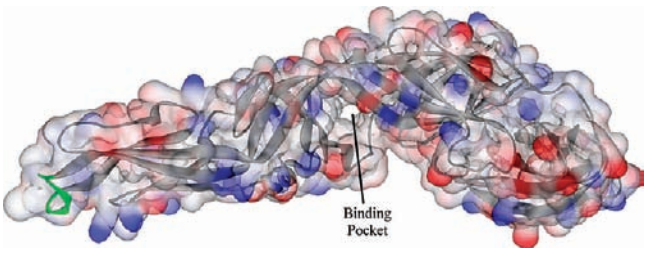


Figure 11. DV E protein monomer showing the surface of the protein and the binding pocket. The ribbon is colored gray, while the "fusion peptide" is illustrated in green. The surface is colored on the basis of electrostatic potential. Illustration is generated from PDB file 1OKE.[18]

Compound **17** shows submicromolar activity against all four serotypes of DV when tested in vitro; however, during in vivo testing in a mouse model the compound was observed to precipitate in the gastrointestinal tract of the mouse.[104]

Inhibition of Viral RNA Polymerase/Methyltransferase. Ribavirin (**19**) (Figure 15), a broad antiviral agent, has been shown to act as an inhibitor against a variety of DNA and RNA viruses.[105] Unfortunately it possesses weak activity against flaviviruses, with a EC_{50} of 49 μg/mL being reported against dengue,[106] though activity varies between DEN-1 and DEN-4.[107] Ribavirin triphosphate (RTP) has been shown to inhibit the methyltransferase activity of NS5 in addition to determining the RTP binding site as the guanosine 5′-triphosphate (GTP)/RNA binding cap.[108] While only having low activity, the identification of particular residues required for binding may allow for rational drug design.

Many other analogues of **19** have been produced in order to improve potency while conserving broad antiviral activity.[106] 5-Ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (**20**) (Figure 15) has been successful in conserving the broad antiviral activity and improving potency up to 100-fold.[109] A 2′,3′,5′-triacetate derivative has been identified as an effective lipophilic derivative capable of treating intracranial DV infections in a murine model.[110] Viramidine (**21**) (Figure 15), a prodrug of **19**, has also been shown to have greater liver targeting and less red blood cell accumulation in animal models.[111] This may also be able to prevent the hemolytic anemia commonly associated with ribavirin.[111]

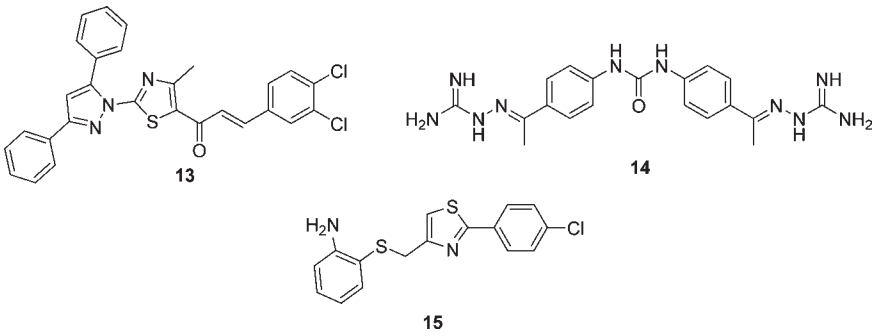


Figure 12. Selected NCI compounds developed on the basis of in silico screening.

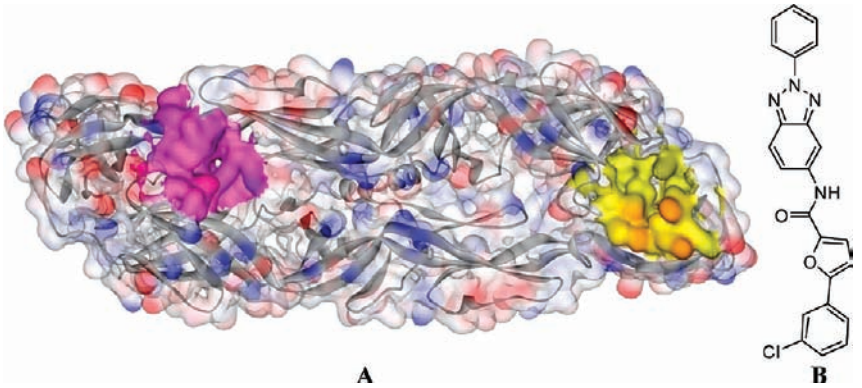


Figure 13. (A) DV E protein homodimer illustrating two unique binding pockets in purple and gold. Transparent surface is colored on the basis of electrostatic potential. Image is generated with PDB file 1OKE.[18] (**B**) Structurally unique inhibitor identified from database mining based on binding pocket illustrated in gold.

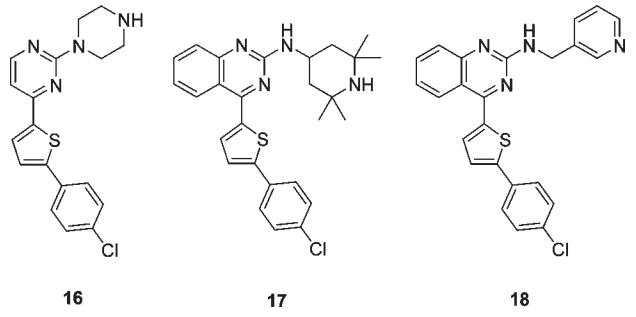


Figure 14. Selected Novartis DV entry inhibitors.

7-Deaza-2′-*C*-methyladenosine (**22**) (Figure 15), another nucleoside analogue, has also been shown to have inhibitory activity against dengue in cell cultures with a reported EC_{50} of 15 μM.[112] It has also been shown to inhibit NS5B of the hepatitis C virus, responsible for RNA-dependent RNA polymerase activity.[112] In addition, the in vivo activity has been shown in a murine dengue model resulting in decreased viremia and cytokine production.[83]

The use of barbituric and thiobarbituric acid analogues with the parent structure **23** (Figure 16) has been shown to inhibit the RNA polymerase activity of NS5 proteins in flaviviruses, in particular hepatitis C virus, though inhibitory data are not given.[113]

Patent WO 00/10573 describes a novel set of compounds **24** (Figure 16) that are capable of inhibiting flaviviral RNA polymerase activity in vitro, though no information is given on in vitro antiviral testing,[114] while the two nucleoside analogues **25a** and **25b** (Figure 16) are reported to inhibit the NS5B protein of hepatitis C virus.[115]

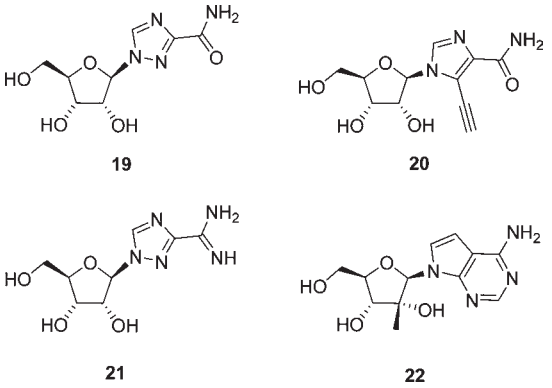


Figure 15. Ribavirin and some ribavirin analogues.

The nucleoside analogue **26** (Figure 17) has been found to inhibit RNA polymerase activity in hepatitis C virus NS5B and to inhibit hepatitis C virus replication through inhibition of NS5B.[116]

Aurintricarboxylic acid (**27**) (Figure 18) was determined to be a potent inhibitor of DV N7- and 2′*O*-methyltransferase activity exhibiting IC_{50} values of 2.3 and 127 μM, respectively.[117] On the basis of in silico docking studies, it was hypothesized that **27** bound tightly to Lys61 of the proposed RNA binding site,[117] a residue necessary for correct 2′O methylation of viral RNA.[47]

Inhibition of Nucleotide Synthesis. Inosine monophosphate dehydrogenase (IMPDH) is a key rate limiting enzyme that is required for de novo guanosine production for incorporation into DNA, RNA, or glycoproteins.[118]

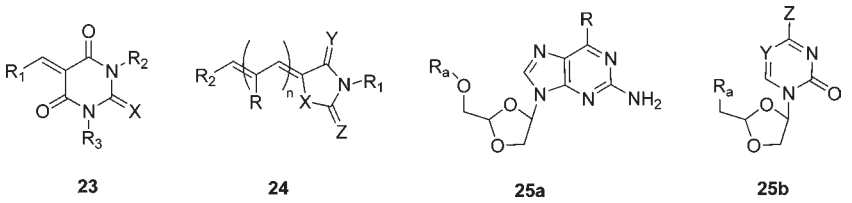


Figure 16. Examples of NS5 inhibitors. For further information on the R and X groups of 23, see ref 113. For further information on the R, X, Y, and Z groups of **24**, see ref 114, and similarly, for information on the R, Y, and Z groups of **25a** and **25b**, see ref 115.

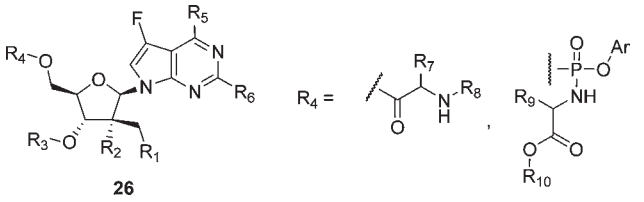


Figure 17. Generic structures of nucleoside analogues of DV. For further information on the R and Ar groups of **26**, see ref 116.

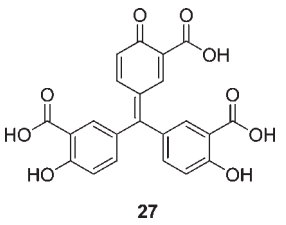


Figure 18. Structure of aurintricarboxylic acid, a potent methyltransferase inhibitor.

Mycophenolic acid (**28**) (Figure 19) is currently used clinically as an immunosuppressant to prevent rejection of transplanted organs by inhibiting IMPDH.[118] However, it has been noted that **28** has potent antiviral activity in concentrations well below those used in immunosuppression by inhibiting IMPDH.[119] The prodrug mycophenolate mofetil **28a** (Figure 19) is known to easily attain the plasma concentrations required for antiviral chemotherapy while avoiding immunosuppression.[120]

The binding of RTP to NS5 has been reported (see section entitled Inhibition of Viral RNA Polymerase/Methyltransferase); however, it also inhibits IMPDH, resulting in depletion of GTP pools and, consequently, inhibition of viral reproduction.[121]

Compounds **28**, 0.1 μg/mL, **19**, 1.6 μg/mL, and **20**, 38 μg/mL, have each demonstrated their ability to deplete cellular GTP pools in vitro.[121,122] Compound **19** has also been tested in vivo, although these results showed no improvement in DV viremia.[83]

The urea VX-497 (**29**) (Figure 20) is a structurally different, competitive IMPDH inhibitor with a broad range of activity across a variety of viruses while bearing a higher degree of potency than ribavirin, yielding an IC_{50} of 8 μM against DV.[123] The enzyme orotidine monophosphate decarboxylase (ODCase) catalyzes the production of uridine monophosphate from orotidine monophosphate in the final step of pyrimidine biosynthesis.[124] By inhibition of ODCase, depletion of intracellular pyrimidine nucleosides results in the inhibition of viral replication. 6-Azauridine (**30**) (Figure 21) has shown potent inhibitory activity on dengue in vitro with a reported EC_{50} of 0.1−0.5 $\mu g \cdot mL^{-1}$ depending on DV serotype; however, it

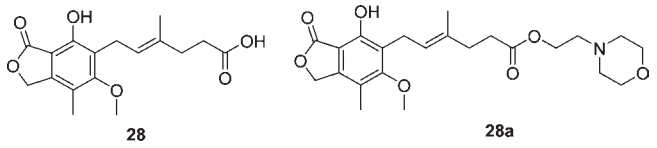


Figure 19. Structures of mycophenolic acid and its prodrug mycophenolate mofetil.

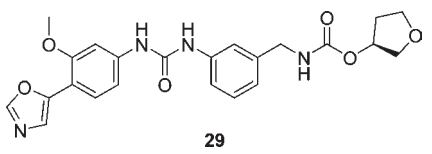


Figure 20. Structure of the IMPDH inhibitor VX-497.

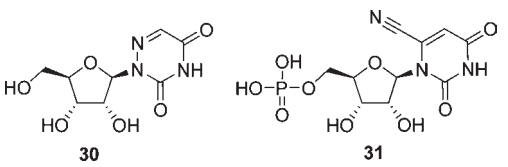


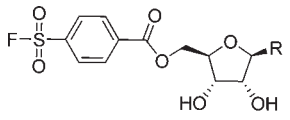
Figure 21. Structures of the known inhibitors of ODCase, 6-azauridine, and 6-cyanouridine monophosphate.

shows no selectivity between healthy and infected cells.[107] Analogues of **30**, such as 6-azauridine triacetate and 2-thio-6-azauridine, also possess activity against WNV.[125] 6-Cyanouridine monophophate (**31**) (Figure 21) has been noted to be a competitive inhibitor of yeast ODCase; however, no antiviral testing of this compound has been published.[126] Another uridine analogue, 4-thiouridine monophosphate, synthesized as a biological probe, showed inhibitory activity toward yeast ODCase; however, as above, no antiviral testing of this compound has been completed.[127]

Inhibition of Helicase/NTPase. Matusan and associates demonstrated that NS3 was required for the proper function and development of viruses, and if inhibited, growth was impeded.[128] Therefore, inhibition of this function is of interest in developing antiviral drugs.

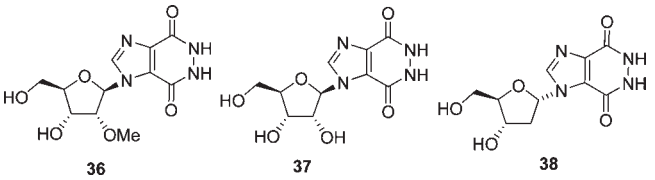
The specific testing of 5′-*O*-(4-fluorosulphonylbenzoyl) esters of ribavirin (**32**), adenosine (**33**), guanosine (**34**), and inosine (**35**) (Figure 22) for inhibitory activity against four flaviviruses showed little to no activity against the helicase or NTPase activities of flaviviral NS3s.[129]

Modulation of the helicase/NTPase activity in WNV NS3 protein was observed with the use of three compounds: 1-(2′-*O*-methyl-β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine-4,7(5*H*,6*H*)-dione (**36**), 1-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyridazine-4,7(5*H*,6*H*)-dione (**37**), and 1-(2′-deoxy-α-D-ribofuranosyl)-imidazo[4,5-*d*]pyridazine-4,7(5*H*,6*H*)-dione (**38**) (Figure 23). These nucleoside analogues produced interesting results when



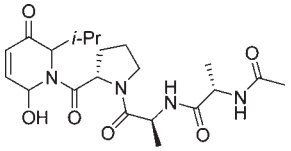
32, R = 1,2,4-triazole-3-carboxamide
33, R = adenine
34, R = guanine
35, R = hypoxanthine

Figure 22. Structures of four NTPase inhibitors of DV.



36 **37** **38**

Figure 23. Three modulators of NS3 helicase/NTPase activity in DV.



39

Figure 24. Example of a patented NS3 protease inhibitor of DV NS3.

tested in vitro, resulting in an IC_{50} of 30 μM for **36** while concentrations of approximately 100 μM only resulted in 30−35% inhibitory activity. It was shown that these compounds, when incubated with the DNA substrate, enhanced the helicase/NTPase activity of NS3.[130]

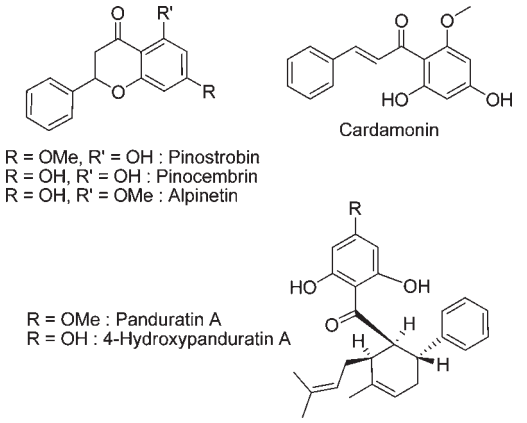
Inhibition of Serine Protease. The serine protease of NS3 is responsible for post-translational proteolytic processing of the polyprotein; therefore, it is an ideal target for drug design, as inhibition of serine protease would result in inhibition of viral replication.

Peptidomimetics, based on the cleavage sites of NS2A/NS2B, NS2B/NS3, NS3/NS4A, and NS4B/NS5, were tested for inhibitory activity of the serine protease. While not all the hexapeptides based on these cleavage junctions were active, it was found that the enzyme was susceptible to inhibition by dipeptides, resulting in the hypothesis that a small molecule could be developed to inhibit this proteolytic function.[131] Yin et al. have progressed this further by carrying out a detailed structure−activity relationship (SAR) study on the effectiveness of tetrapeptide based inhibitors.[132,133]

The use of a di- and tripeptide molecules to inhibit viral serine proteases has also been patented, with **39** (Figure 24) an example of these proposed serine protease inhibitors.[134]

A number of novel cyclohexenyl chalcone derivatives (Figure 25), isolated from *Boesenbergia rotunda*, a member of the ginger family, have shown good inhibition activity by both competitive binding and noncompetitive binding to DEN-2 NS3 protease.[135] These derivatives were being further tested for in vitro activity in addition to the development of a structure−activity relationship; however, these results are yet to be published.

The Bowman−Birk inhibitors (BBIs) are a well-studied group of naturally occurring serine protease inhibitors that



R = OMe, R' = OH : Pinostrobin
R = OH, R' = OH : Pinocembrin
R = OH, R' = OMe : Alpinetin

Cardamonin

R = OMe : Panduratin A
R = OH : 4-Hydroxypanduratin A

Figure 25. Structures of selected natural products exhibiting DV NS3 serine protease inhibition.

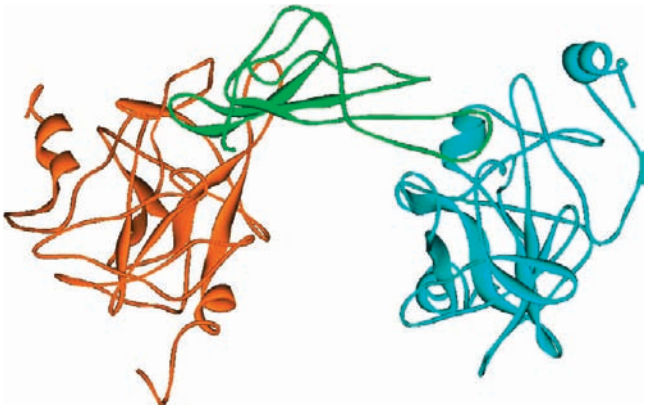


Figure 26. Crystal structure of two NS3 protease units (blue and orange) with bound mung bean BBI (green). Illustration is generated from PDB file 1DF9.[137]

are abundant in dicotyledonous and monocotyledonous plants and play a role in plant defense including resistance against pests and pathogens.[136] Analysis of the crystal structure of the DV NS3 protease complexed with the mung bean BBI demonstrated the effectiveness of the BBI inhibitors on NS3 (Figure 26).[137] These findings provide a starting point for the design of specific serine protease inhibitors.

In silico modeling and in vitro bioassays have been utilized to elucidate the mechanism of activation of NS3 by NS2b. These experiments have illustrated a dual mechanism of activation by NS2b involving the refolding of the NS3 protease domain and its necessity for correct substrate identification. In addition to this crucial residues required for the correct activation and functioning of the protease were identified that may aid in rational drug design and in silico screening.[138]

Inhibition of α-Glucosidase I. α-Glucosidase enzymes are required for the correct biosynthesis and processing of asparagine-linked oligosaccharides.[139] Both α-glucosidase I and α-glucosidase II are important proteins involved in protein folding by allowing glycoproteins and ER chaparones access to bind to the unfolded protein.[140] In dengue, α-glucosidase inhibitors have been reported to inhibit dengue viral budding and/or viral particle infectivity by interfering with the folding pathways, required for the correct formation of the prM and E proteins.[141]

Castanospermine (**40**) (Figure 27), a natural, water-soluble alkaloid derived from the black bean or Moreton Bay

chestnut tree (*Castanospermum australae*) is an easily obtained inhibitor of a variety of murine disaccharidases.[142] It has potent activity both in vitro (IC_{50} 1 μM) and in vivo. It prolonged survival against lethal DV challenge, with all serotypes, while showing significantly less potency against WNV and YFV.[143]

6-*O*-Butanoylcastanospermine (**41**) (Figure 27) improved the selectivity of **40** by reducing the inhibition of intestinal sucrases and reducing associated diarrhea.[144] It has been shown to have 30−50 times the activity of **40** in inhibition of HIV particle formation and/or infectivity.[145] Compound **41** has also been tested in vivo against dengue, demonstrating potent inhibitory activity,[83] and is currently being developed into an antiviral therapeutic against hepatitis C virus.[144]

It has been shown that 1-deoxynojirimycin (**42**) (Figure 28) is both an inhibitor of *Saccharomyces cerevisiae* α-glucosidase I and α-glucosidase II, showing 10 times better inhibition of α-glucosidase II than α-glucosidase I.[146] N-Alkylated derivatives of **42** have shown greater inhibition of α-glucosidases,[147] and interestingly these same derivatives, e.g., *N*-butyl-1-deoxynojirimycin (**43**) or *N*-nonyl-1-deoxynojirimycin (**44**), show selectivity toward α-glucosidase I (Figure 28).[139]

Compound **42** and the analogues **43** and **44** have been tested for antiviral activity against HIV,[148] illustrating that N-alkylation of **42** was required to substantially improve activity in culture.[148] Compound **44** has been tested against dengue in vivo showing a greater degree of potency than **41**, with 93% and 88% viremia reduction, respectively.[83] Separately, **44** has been shown to inhibit both dengue and JEV in vitro and JEV in vivo.[149] Other N-alkylated-1-deoxynojirimycin compounds, such as SP169 (**45**) (Figure 28), SP173 (**46**) (Figure 28), and OSL-95II (**47**) (Figure 28), have also been tested against flaviviruses including WNV and dengue.[150] These analogues lost some activity when compared with **44**; however, they had significantly reduced toxicity[150] while being noted that *N*-octadecyl-1-deoxynojirimycin is inactive against α-glucosidase I and II.[151] The SAR of these compounds has been further investigated through replacement or substitution of the N-substituted cyclohexyl ring and variation of the linker length, resulting in the development of compounds **48** and **49** with submicromolar EC_{50} values against DV (Figure 28).[152]

α-Homonojirimycin **50** (Figure 28), similar to **42**, has also had analogous compounds synthesized and tested for inhibitory activity against yeast glucosidase, where they showed activity similar to that of **40**.[153]

The glucopyranosides **51** and **52** (Figure 29) showed greater inhibition of yeast glucosidase than **42**, possibly giving a new class of inhibitors capable of antiviral activity.[154]

Further to these inhibitors, kojibiose-type pseudodisaccharides and a trisaccharide have been tested for inhibitory activity against yeast α-glucosidase and processing α-glucosidase, isolated from rat liver microsomes. While showing significant inhibition of the yeast enzyme, these compounds showed no inhibition of α-glucosidase isolated from rats, suggesting that these would be inactive against viral targets and that not all inhibitors of yeast α-glucosidase yield viral inhibitors.[155]

Kinase Inhibitors. Phosphorylation by protein kinases is known to be responsible for many processes including signal transduction responsible for improving cell survival during viral infection,[156,157] immune evasion,[158,159] and regulation of endocytosis in some viruses.[160] Phosphorylation has also been shown to regulate the location of NS5 and, presumably, its activity.[34,161] Assuming that it may be involved further in the regulation of viruses, kinases would be an attractive target for antivirals.

The Src family of kinases have been hypothesized to be important for the replication of the DV, as known Src kinase inhibitors were shown to inhibit dengue in vitro.[162] By use of selectivity c-Src, a subfamily of Src kinases has been more specifically identified as one of the kinases required for DV budding from the ER lumen.[162] The Src family has also been implicated in the replication processes of hepatitis B virus,[163] human immunodeficiency virus,[164] and herpes simplex virus 1,[165] while c-Yes, another belonging to the Src family, has been shown to inhibit trafficking of WNV particles via the host secretory system.[166] Further to this, two Src kinase inhibitors, dasatinib (**53**) and AZD0530 (**54**) (Figure 30) have been shown to inhibit the virion assembly against all four serotypes of dengue, validating the Src family as a therapeutic target and their inhibitors as viable dengue therapeutics.[162]

Antisense Oligonucleotides. The discovery of RNA interference (RNAi) has enabled the dissection of dengue virus−host cell interactions, has enabled identification of potential antiviral targets, and has provided the tools for the

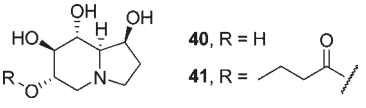


Figure 27. Structures of castanospermine and 6-*O*-butanoylcastanospermine.

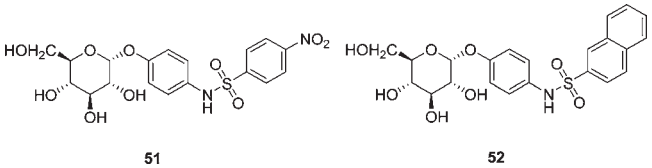


Figure 29. Two glucopranoside inhibitors of α-glucosidase I.

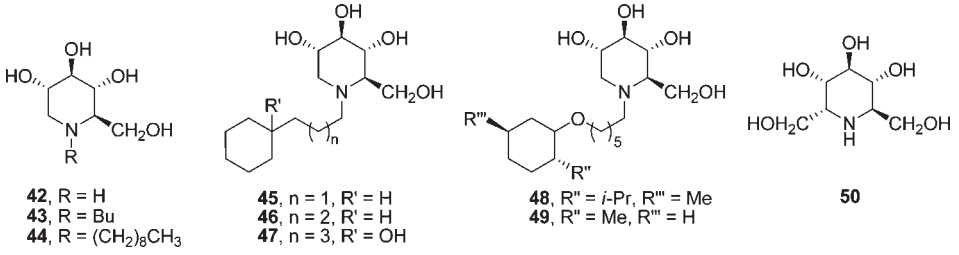
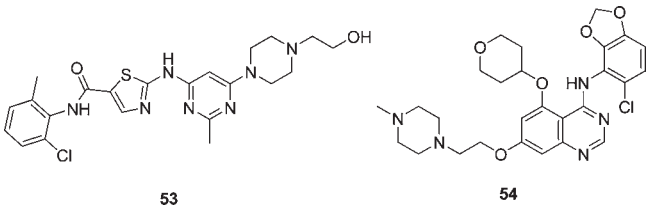
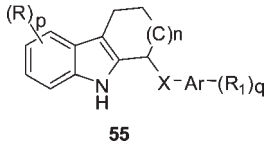


Figure 28. Structures of some nitrogen containing sugars with α-glucoside inhibitory activity.



53 54

Figure 30. Two Src kinase inhibitors capable of preventing dengue viral replication.



55

Figure 31. General structure of a potent inhibitor of DV replication with an unknown target. For further information on the nature of n, q, R and X groups, see the associated patent.[173]

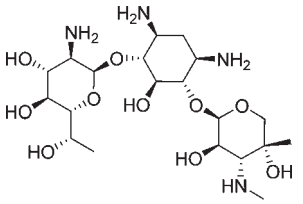
development of antisense-based approaches to address viral infections.[167] Antisense oligonucleotide compounds have been shown to affect gene expression in several viral pathogens of humans, including flaviviruses, with a number of oligonucleotide compounds in clinical trials.[168,169] Phosphorodiamidate morpholino oligomers (PMOs) are a class of nonionic antisense compounds, typically 20 base pairs in length, that contain purine or pyrimidine bases attached to a backbone of morpholine rings and phosphorodiamidate intersubunit linkages.[170] PMOs targeting the dengue virus 5′-proximal nucleotides or 3′-cyclization sequence have been shown to suppress the magnitude and duration of replication of all four DV serotypes during in vitro studies.[171] Peptide-conjugated PMO has also been shown to have antiviral efficacy against DENV-2 in vivo in the AG129 mouse model with treatment early in the infection extending the survival times of DENV-2-infected mice.[172]

Compounds with an Unknown Target. Some compounds, e.g., **55** (Figure 31), possess extremely potent activity in in vitro cell culture experiments despite having an unknown target. Compounds of the basic skeleton **55** are claimed to possess low nanomolar activity.[173]

Geneticin (**56**) (Figure 32), an aminoglycoside, has been shown to inhibit the replication of DV in in vitro studies by protecting against the cytopathic effect of DV (EC_{50} = 6 μM), reducing the viral yield (EC_{50} = 4 μM) and blocking DV RNA and protein synthesis.[174] The molecular mechanism of **56** is unknown, but activity is conserved against other RNA viruses in a variety of cell lines, suggesting that the antiviral activity is a direct result of interaction with viral targets.[174,175]

Concluding Remarks

Chemotherapeutics designed to combat the DVs, while in their infancy, cannot be discounted as reliable agents for prophylaxis and viral treatment. With the identification of abundant targets capable of eliciting complete inhibition of the virus, along with the recent advancements in the development of antivirals, it is foreseeable that a pharmaceutical formulation targeting one or more of these could be brought to market. Because of the vague understanding of many of the DV proteins, their functions, and their precise inhibitors, the development of medicinal chemistry through quantitative



56

Figure 32. Structure of Geneticin, an aminoglycoside inhibitor of dengue virus.

structure−activity relationship studies and random screening remains vital to the advancement of our knowledge. In addition, further understanding the molecular biology of the DVs will allow tailoring of lead compounds to achieve greater potency. With minimal progress in the development of an immunogenic DV vaccine over the past 50 years, it seems that chemotherapeutics remain our best hope for the treatment of DF, DHF, and DSS.

Acknowledgment. We thank the Australian Research Council and Biotron Ltd. for support.

Biographies

Andrew J. Stevens received his B.Med.Chem. (Hons) degree from the School of Chemistry of the University of Wollongong, Australia, in 2007. He is currently completing his Ph.D. focusing on the design and development of new anti-infective agents, particularly focused on new compounds exhibiting anti-HIV, antimalarial, and antidengue virus activities.

Michelle E. Gahan is a Research Fellow/Lecturer in the Virus and Inflammation Research Group at the Centre for Biomedical, Molecular and Chemical Sciences at the University of Canberra, Australia. She has previously worked in the field of vaccine research including optimizing oral vaccine delivery and transgenic plant-based vaccines for avian influenza. Her current research is focused on understanding mechanisms of disease processes triggered by viral infections and the interactions between viruses and infected hosts that lead to virus-induced inflammatory disease. Her research has implications for the development of treatment strategies to control viral infections including RNA interference.

Suresh Mahalingam is head of the Virus and Inflammation Research Group and Director of the Centre for Biomedical, Molecular and Chemical Sciences. He has an international reputation in the field of viral pathogenesis and has spent the past 13 years undertaking research to understand the interactions between viruses and the infected host that lead to virus-induced disease or to resolution of infection. His research has implications toward the design of vaccines that would modulate host immune responses to counteract an infection.

Paul A. Keller completed his B.Sc. (Hons) (1985) and Ph.D. at the University of New South Wales, Australia, before undertaking an Alexander von Humboldt funded postdoctoral fellowship at the University of Wuerzburg, Germany. Since 1994, he has worked at the University of Wollongong, Australia, and is currently Associate Professor in Organic and Medicinal Chemistry and Director of the Research Centre for Medicnal Chemistry and Pharmacology. His interests lie in the drug design and development of new generation anti-infectives and chiral ligand design for the synthesis of sterically hindered compounds.

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