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Novel Approaches to Flavivirus Drug Discovery

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Abstract

Introduction—The members of the family *Flaviviridae*, including West Nile virus, yellow fever virus, and dengue virus, are important human pathogens that are expanding their impact around the globe. The four serotypes of dengue infect 50 to 100 million people each year, yet the only clinical treatment is supportive care to reduce symptoms. Drugs that employ novel inhibition mechanisms and targets are urgently needed to combat the growing incidence of dengue worldwide.

Areas Covered—The authors discuss recently discovered flavivirus inhibitors with a focus on antivirals targeting non-enzymatic proteins of the dengue virus lifecycle. Specifically, the authors discuss the flaviviruses, the need for novel inhibitors, and the criteria for successful antiviral drug development. Current literature describing new advances in antiviral therapy at each stage of the flavivirus life cycle (entry, endosomal escape, viral RNA processing and replication, assembly, and immune evasion) are evaluated and summarized.

Expert Opinion—Overall, the prognosis of flavivirus antiviral drug development is positive: new effective compounds have been discovered and studied. However, repurposing existing compounds and a greater translation to the clinical setting are recommended in order to combat the growing threat of flaviviruses.

Keywords

antiviral; compound; dengue; inhibitor; peptide; small-molecule

1. Introduction

1.1 The flavivirus genus

The flavivirus genus is a member of the family *Flaviviridae*, which consists primarily of arthropod-borne human pathogens that infect millions of people each year. This genus includes dengue virus (DENV), West Nile virus (WNV), yellow fever virus (YFV), Japanese

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Declaration of Interest

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encephalitis virus (JEV), Kunjin virus (KUNV), and tick-borne encephalitis virus (TBEV) among others, and is closely related to the genus hepacivirus, whose sole member is hepatitis C virus (HCV). The four serotypes of DENV alone infect 50 to 100 million people each year, resulting in approximately 22,000 deaths annually. Upon DENV infection, patients first experience flu-like symptoms such as high fever, chills, nausea, joint pain, and dizziness. A few of the infections can progress to life-threatening dengue hemorrhagic fever and shock syndrome (DHF/DSS), characterized by rapid deterioration including rash, shock-like state, pinpoint dots of blood under the skin, drop in blood pressure, and amplification of primary symptoms (1). Over 100 countries are plagued by endemic DENV infection, mostly in the tropical and sub-tropical regions of the globe. The recent outbreaks of WNV in North America in particular, and the re-emergence of seemingly conquered YFV across the globe, have brought these viruses back to public awareness, if indeed they ever left (2).

Vaccines have been integral in combating YFV, JEV, and TBEV. However, even these strains have shown re-emergence in recent years most likely due to lax mosquito control, increased transportation of people and goods, unreachable populations, and the decline of vaccination in public opinion. For several members of the flavivirus genus, including DENV and WNV, vaccine development has stymied scientists despite many years of research. Although several clinical trials for vaccines are underway (1,3,4), the years that go into development require an interim substitute until such vaccines can be marketed.

Development of a vaccine for DENV presents a unique challenge. Not only would a vaccine need to address each of the four serotypes of DENV, but it would also have to overcome the potential for antibody-dependent enhancement (ADE). During a primary DENV infection, cross-reactive, non-neutralizing antibodies are produced. When an individual is infected with another DENV serotype, these antibodies can recognize, bind, but not neutralize the second infecting virus. The antibody-virus complexes can be internalized by Fc antibody receptors present on specific host cells. Thus these antibodies likely increase viremia by facilitating virus uptake through a novel mechanism and into a new subset of host cells (5,6).

Additionally, vaccination is only effective for those inoculated before infection with the virus. Those that lack such prevention and succumb to infection have no options for care beside treatment of symptoms. Countries that are already struggling to provide healthcare are stretched especially thin during DENV epidemics, and even such supportive treatment proves difficult (1). Clearly there is an urgent need for the development of antiviral drugs that will allow health professionals to cure, or at least diminish, these viruses after infection. Several studies have produced candidate drugs, such as ribavirin and its derivatives (7), that have passed the rigors of clinical trials to become at least rudimentary options for virus treatment. However, many of these treatments have serious limitations, and resistance is a constant concern (8). Not only are new drugs needed, but novel approaches to the development of effective antiviral drugs are crucial to control flavivirus infections.

1.2 The flavivirus proteins

Flaviviruses contain a single-stranded, positive-sense, 11KB RNA genome. The genomic RNA is translated into a single polyprotein that is cleaved by viral and cellular proteases to produce 10 viral proteins. Three proteins: capsid (C), pre-membrane (prM), and envelope

(E) are structural proteins that facilitate assembly and budding. Together with cellular lipids and genomic RNA, these are the proteins that constitute the viral particle. The remaining C-terminal 7 proteins (NS1, NS2A/B, NS3, NS4A/B, NS5) are required for replication of the genomic RNA. The C protein interacts with the genomic RNA to promote packaging into immature virions. The prM and E proteins are embedded in the cellular membrane prior to budding and form the projections of the immature virion. The prM protein functions as an M protein precursor that primarily prevents premature rearrangement of the E protein under the mildly acidic conditions of the trans-Golgi prior to virion release. In the trans-Golgi network, the prM protein is cleaved by cellular furin to allow M/E rearrangement to produce the mature virion (9,10). The E protein of the released mature virion subsequently recognizes an unknown receptor on target host cells to induce viral uptake. After uptake, it mediates low pH-mediated membrane fusion to release the genomic RNA into the cytoplasm for replication. (2, 11, 12).

The nonstructural proteins are not found in the virion, but rather are found primarily in the cytoplasm and consist of the protease, helicase, polymerase, and other necessary proteins of RNA replication. Nonstructural protein 1 (NS1) is a current subject of study. It has primarily been implicated in host immune response evasion through modulation of complement activation although other activities are suspected (13, 14, 15). Likewise, the function of the NS2A protein is poorly defined. It has been implicated in a variety of roles, including immune response evasion, genomic replication, and even assembly (16, 17, 18). NS2B functions as a cofactor protein in the protease function of NS3, which also doubles as a helicase (19). The function of NS4A is a matter of debate, but studies suggest that it has a role in induction of membrane rearrangement and/or autophagy response to viral infection of host cells (20, 21). The NS4B membrane protein is thought to anchor and target the replication complex to the Endoplasmic Reticulum (ER) membrane and has been connected to immune response antagonism (22, 23). NS5 is the largest of the nonstructural proteins and it contains a classic RNA-dependent RNA polymerase (RdRp) domain as well as methyltransferase and guanylyltransferase domains for mRNA capping necessary for a virus that replicates its mRNA in the cytoplasm (2, 24, 25, 26).

1.3 The need for novel antiviral drugs

Traditionally, antiviral strategies attempt to target the enzymatic replication proteins such as helicase (NS3), protease (NS2B/3), and polymerase (NS5). This is largely because loss of these specific proteins has proven lethal for virus replication, and therefore drugs targeting these enzymes can be particularly effective at reducing viral load (17, 23, 27). Additionally, host cells do not express RdRp's such as NS5, hence nonspecific detrimental effects of these drugs would theoretically be minimal. The same is not true for NS3, however, as molecules designed to target the WNV and DENV NS3 helicase would benefit from improved specificity (28). Furthermore, new viral targets are constantly in demand due to ready mutation of both NS3 and NS5 enzymes in the presence of inhibitors to regain function, as demonstrated in HCV (8, 24). It is of interest, therefore, to investigate new methods for targeting these infections, including pursuing novel targets in the viral lifecycle, such as entry, non-enzymatic replication proteins, membrane interaction, and others. Targeting non-enzymatic components of the virus may minimize both the appearance of resistance

mutations and poor specificity, as well as allow synergistic use with anti-enzymatic drugs to address infection more efficiently in the clinical setting (28).

Effective development of candidate drugs takes several factors into account. First, a certain level of inhibition is desired; ideally a drug will eliminate virus replication. In practice, 50–90% inhibition is optimal during drug design. Even a 50% reduction in virus replication can reduce the viral load to a manageable level so that the host immune system can readily clear it. Second, the drug should ideally be target specific. Binding to, or otherwise inhibiting, an unintended target in the host cell can lead to detrimental side-effects, even causing cell death. The drug should therefore only inhibit the host or viral proteins for which it is intended. In order to consider this during drug development, cell cytotoxicity (cell death) is monitored in addition to overall potency of the drug. Also, efforts are made to target unique proteins, essential for the virus lifecycle, such as the RNA-dependent RNA polymerase (RdRp) that is not present in an uninfected host cell. Third, an ideal drug would be able to act effectively on multiple viruses in the family (broad spectrum inhibition). In practice, however, even the evolutionarily related flaviviruses prove broad spectrum drugs are difficult to generate. Fourth, an ideal drug is readily able to enter cells, whether through active transport, diffusion, or as a prodrug. Fifth, an ideal drug would be fast acting. Patients infected with DENV often do not show symptoms for 4–10 days, and therefore drugs need to prevent viral spread relatively late in the infection cycle.

1.4 The flavivirus life cycle

Flaviviruses begin uptake into host cells through the interaction of E protein with an unknown cellular receptor. Contact between E protein and the receptor induces clathrin-mediated endocytosis that transports the virion into the cytoplasm (29–32). The endocytic vesicle containing the virion undergoes a change in pH that causes a rearrangement of the E protein required to fuse the viral and cellular membranes, thereby releasing the RNA genome into the cytoplasm (2, 33, 34). The genomic RNA is subsequently translated into a long polyprotein which is auto-catalytically cleaved by the NS2B/3 viral protease and host proteases into its constituent proteins (2). The released nonstructural proteins are targeted to the site of replication on ER-derived vesicle packets to initiate transcription (35, 36). Meanwhile, the prM and E proteins are embedded into the ER membrane and enclose the newly formed nucleocapsid as it buds into the ER lumen to form the immature particle. The particle is trafficked to the plasma membrane via the secretory pathway. The low pH of the trans-Golgi network causes substantial rearrangement of the prM/E proteins and permits furin cleavage of prM to M. With the release of the pr peptide, the virion is released from the cell and is able to infect another naïve host cell (37–46). The inhibitors discussed subsequently target various stages of the flavivirus life cycle (Figure 1).

2. Novel inhibitors of the flaviviruses

2.1 Entry

Entry of the virus into host cells is necessary for the virus to produce progeny since it has inadequate replication machinery on its own. An inhibitor that prevents entry into host cells is attractive because such a strategy potentially circumvents cytotoxicity and a variety of

negative effects on the cell cycle, as well as leaving the virion vulnerable to immune system clearance. Furthermore, since the E protein is responsible for both entry and membrane fusion, targeting the E protein has additional potential to inhibit downstream steps in the lifecycle such as endosomal release, should the virion evade entry inhibition.

The E protein's major conformational changes and well defined molecular structures, both pre- and post-fusion, present several targets amenable to inhibitor design (Figure 2). In particular, the crystal structure of the E protein displays a ligand-binding pocket that was occupied by a detergent molecule, n-octyl- β -D-glucoside (β -OG) (32). This discovery led to a veritable explosion of docking studies to identify and optimize potential inhibitors targeting this region of E (44-7).

One such computational study screened an NCI compound library to fit the β -OG pocket with an appropriate inhibitor. Using luciferase-tagged YFV constructs, the 23 top hits were narrowed to three top compounds that showed inhibition in the micromolar range (48). From these three compounds, several series of derivatives have been designed, including an inhibitor with a Selective Index (SI) of 256 (48-50). It is likely that these small molecule inhibitors prevent the E protein conformational change necessary for entry and uncoating and perhaps the late assembly maturation rearrangement. Further investigation is ongoing to confirm binding of these derivatives to the β -OG pocket, and to define their mechanism of action (49).

A similar study was carried using a docking program to screen 135,000 compounds for inhibition of the E protein via the β -OG pocket (51). The resultant top hits were tested for biological efficacy and cytotoxicity against DENV, KUNV, and YFV, proving that several inhibitors displayed micromolar inhibition across multiple flaviviruses. Cell-based fusion assays were used to prove that at least one compound, A5, directly inhibits E protein-mediated fusion. Further study of this group of inhibitors is needed, as optimization of these compounds could yield even more potent inhibitors (51). However, the results suggest that virtual screening of compound libraries is an effective approach to discovery of novel inhibitors. Additionally, this study confirms that targeting conserved protein regions has the potential to develop broad-spectrum inhibitors useful for treating a variety of flavivirus infections.

2.2 Endosomal escape

In addition to targeting the β -OG pocket, small peptides have been designed against the E protein stem region. The stem is a conserved region at the C-terminus of the protein, adjacent to the membrane-bound anchor region, and is essential for membrane fusion (52, 42). The peptide inhibitors were designed based on conserved sequence-specific binding interactions of the postfusion DENV E. DENV lends itself well to this approach since the E protein sequence of this region is well conserved among DENV 1-4 and other flaviviruses. These peptides are unique in that they inhibit the virus following entry into the cell, rather than prevention of entry itself. It was hypothesized that the peptides are able to bind to the virion nonspecifically as it is taken up by the cell. The exposure of the virus to the low pH of the late endosome, results in conformational rearrangements, inducing tight binding of the peptides, and inhibiting membrane fusion (53-5).

A comparable study performed by Costin, et al. used the pre-fusion DENV-2 E protein for rational design of peptide inhibitors via biologically validated computer modeling techniques (46). Particles treated with the peptidic inhibitors displayed rough outer surface morphology, contrary to the smooth outer surface of mature DENV, thus indicating that the E proteins were likely rearranged. Subsequent attempts to generate a structure for the treated virions suggested that the virion was no longer icosahedral, confirming that the virion structure was grossly affected (55). Furthermore, comparable peptides added before or after attachment to cells yielded similar inhibition and even proved to inhibit ADE *in vitro* (56). It is likely that binding of these peptides inhibit the interaction of the transmembrane regions and the fusion loop, which has been proposed in other studies (54). These studies not only validate fusion inhibitors as powerful potential antiviral drugs, but also verify the effectiveness of rational *de novo* small molecule design (55, 56). However, most peptide-based antiviral compounds are not readily absorbed when administered orally, requiring intravenous delivery. This means of treatment is impractical for global use, especially in areas where DENV is most prevalent (44). Internalization of these peptides may be increased through the use of protective liposomes able to deliver the drug directly to the cell. Liposome-based drug delivery can be used to target inhibitors to specific cells as well as deliver the drug in high concentration (57). Furthermore, these peptides should require testing in an *in vivo* model to evaluate their efficacy during genuine DENV infection.

An exciting new possibility to circumvent peptide instability is presented by self-assembling nanotubes. Such an inhibitor was originally discovered to target bacterial membranes and adenovirus, but has now been applied to HCV (58–61). In the case of HCV, a cyclic D, L- α -peptide library was screened for anti-HCV activity and nine amphiphilic peptides with promise were identified. These peptides self-assemble into inhibitory nanotubes that act after entry but before protein synthesis, and also control spread of the virus in culture. It is likely that they interact with a “specialized cellular membrane” to inhibit either membrane fusion or pH control (62). Although these nanotubes inhibit a cellular membrane, further study could apply them specifically to the virion membrane. Additionally, these proteins are chemically and proteolytically stable, thus they may be amenable to *in vivo* application. Clearly, more investigation is needed to determine exactly how these peptides are inhibiting HCV, and how to apply them to DENV and related flaviviruses.

2.3 Viral RNA processing

Directly targeting the viral RNA is a tempting approach for antiviral development. However, the flavivirus genome is a positive-sense ssRNA that closely resembles cellular mRNA. Although convenient for the virus, this makes targeting viral RNA (vRNA) without collateral inhibition of cellular mRNA challenging. However, a unique study has been recently published that is able to specifically target the flavivirus vRNA.

Short antisense peptide-conjugated oligomers, called phosphorodiamidate morpholino oligomers (P-PMOs) were designed with short nucleotide sequences able to form Watson-Crick pairs with a complementary target sequence in the DENV and WNV genomes, conjugated with arginine-rich peptides that facilitate uptake in culture (63, 64). These P-PMOs can form short duplexes that are able to inhibit RNA-RNA or RNA-protein

interactions in specific regions of the viral genome. Several P-PMOs were designed to target the initial 20 bases of the 5' UTR of DENV-2, a 3' cyclization sequence, and a 3' terminal stem-loop. It was shown that a 5' UTR targeted oligomer selectively inhibited translation of the viral transcripts, reducing virus production by 95 percent. Similarly, the 3' cyclization sequence oligomer specifically reduced RNA synthesis by a similar amount. The 3' stem-loop oligomer reduced both viral RNA synthesis and translation, resulting in an approximately 1000-fold reduction in virus replication. Furthermore, at low concentrations, all the P-PMOs were taken up into the cells and did not significantly affect cellular viability (63–5). These studies provide a novel mechanism of inhibition that neatly circumvents the non-specificity issues of targeting the viral RNA directly. However, these short oligomers are similar in design to siRNAs, and therefore may prove to have a short half-life in an *in vivo* model. A study investigating the long term effects of these P-PMOs needs to be conducted.

Another novel approach to inhibition of the vRNA involves small interfering RNA (siRNA) inhibition of flaviviruses. E protein targeted siRNAs proved to reduce TBEV particle production by 80 percent (66). Similarly, a study done in YFV targeted siRNAs to a variety of proteins including NS1, E, and NS5 (67). Cells treated with siRNA demonstrated up to 97 percent replication inhibition and even improved the infection outcome in a mouse model system. Although this study focused on YFV rather than DENV, targeting NS1 proves that the siRNA strategy can be applied to viral proteins not previously addressed by traditional approaches (67). Furthermore, the use of siRNA is an appealing strategy for antiviral drug design due to low cytotoxicity and sub-micromolar effective concentrations. However, these molecules have a short half-life in the host and often have difficulty entering the host cell (68). Although application of these powerful RNAs in DENV infection may prove fruitful, further development is needed.

2.4 Genomic Replication

2.4.1 NS4A/2K—Lycorine is a naturally occurring compound in daffodils (*Narcissus pseudonarcissus*) and bush lilies (*Clivia miniata*). This compound has activity against poliovirus, Severe Acute Respiratory Syndrome-associated coronavirus, herpes simplex-1 virus, and enterovirus, primarily reducing protein and RNA synthesis (69–72). In recent studies, it was revealed that this drug reduced DENV, WNV, and YFV titers by 10^2 to 10^4 fold at 1.2 μ M concentrations (73, 74). Further study selected for resistance in WNV and discovered a point mutation of Val9Met in the 2K peptide that confers resistance to the inhibitor (74). The 2K peptide is a 32 amino acid sequence at the C-terminus of NS4A that is important for targeting to the ER membrane. Upon insertion into the membrane, this 2K sequence is cleaved by virus and host proteases to produce mature NS4A and NS4B proteins (20, 75). It is unclear why interaction with 2K would confer such high susceptibility, especially since the function of 2K, beyond membrane targeting, is unknown. However, it has been hypothesized that 2K inhibition may interfere with membrane rearrangement or cleavage site recognition. Such a hypothesis implies that protease substrate targeting could also provide a wealth of new inhibitor strategies. Additionally, it seems logical that 2K could have another function in the replication complex besides protein targeting, since it localizes with the replication proteins. Therefore, inhibition of 2K requires further investigation for possible antiviral approaches.

2.4.2 NS4B—NS4B is an integral membrane protein that inserts into ER-derived membranes at the site of replication, and has proven vital for normal replication function (23, 76). Several inhibitors that interfere with NS4B's function have been identified, however it is unclear how these molecules specifically inhibit the protein, or even why such inhibition would be adequate to reduce viral load (77, 78). A high-throughput screen (HTS) was performed on YFV using pseudo infectious particles (PIPs) (77). YFV PIPs are constructed by transfecting the YFV luciferase-tagged replicon into cells, later followed by Sindbis virus structural proteins (C, prM, E). Such transfection produces particles able to enter cells and replicate normally but unable to produce progeny virus. Thus, the PIP replicon provides a means to study viral transcription and translation specifically, and is a powerful tool for antiviral drug discovery (Figure 3). The PIP HTS identified twenty potent YFV inhibitors from a compound library that reduced luciferase activity by 90%, indicating that virus production was likewise affected. The top two compounds were used to propagate resistant virus stocks, and the mutations responsible for the resistance mapped to NS4B (77). A comparable HTS using a DENV-2 luciferase-tagged replicon cell line also discovered inhibitors targeting NS4B (78). A compound library screened against the cell line identified a compound that caused 85 percent reduction in viral replication with limited cytotoxicity, and specificity for DENV alone. Furthermore, the compound proved to inhibit RNA synthesis rather than translation, and did not directly inhibit protease, NTPase, methyltransferase, or RdRp function. Passaging and cell sorting methods were also used to isolate resistant strains to identify compensatory mutations in the NS4B protein. Based on the location of the selected resistance mutation, it was hypothesized that the compound interrupts the NS3-NS4B complex formation discovered previously (78, 79).

2.5 Assembly

To date, there are no antiviral compounds that target C protein and its function in virion assembly and uncoating. However, inhibition of the C protein has been shown to severely reduce viral production (80, 81). Thus, it may prove to be an effective target in the future and should be investigated. Short hairpin RNAs (shRNAs) targeting capsid of WNV reduced viral production significantly (80). Fusion of C protein with a nuclease also severely reduced virus production (81). Theoretically, it could be possible to design an inhibitor able to block inter-capsid interactions or “freeze” the nucleocapsid core in a conformation that is not amenable to release of the genomic RNA after entry. Development of an *in vitro* assembly assay would be instrumental in discovery of such anti-C inhibitors. Initial steps toward assembly assay development have resulted in the production of DENV nucleocapsid-like particles, which may prove foundational for further study of particle assembly. However, the exact mechanism of assembly is still elusive, and inhibitor addition has not yet been attempted (82).

2.6 Immune Response Evasion

2.6.1 NS1—Celgosivir is a clinically approved prodrug of castanospermine, a product of the Moreton Bay chestnut tree (*Castanospermum australe*), that is converted to Cast upon diffusion into the cell (83, 84). It has been shown to affect the folding of proteins by inhibiting the loss of the terminal glucose of N-linked glycans in HIV (85). This drug has also been shown to significantly inhibit the production of all four DENV serotypes both *in*

vitro cell culture and *in vivo* mouse models (86). A combination of SDS-PAGE and Western blotting revealed that this drug prevents glycosylation of the NS1 protein, thus causing it to accumulate in the ER as shown through an immuno-fluorescence assay. Furthermore, it proved successful *in vivo* with an 80 percent survival rate for mice infected with DENV-2 and treated with Celgosivir within 1–2 days after infection (86). Although this inhibitor targets a cellular process, the efficiency of the inhibitor proves the vital role that NS1 plays in the replication of flavivirus infections, and also confirms that NS1 would be an effective novel target of antiviral design. More work is needed, however, with this particular natural compound. This study used DENV replicons to determine the efficacy and mechanism of the inhibitor. Yet both the prM and E proteins are also glycosylated during infection, and therefore this drug may be useful in combating various stages of the viral lifecycle (87). Since this compound is natural and has been previously studied, it could easily be used for new applications.

3. Conclusion

Collectively, these studies confirm that nontraditional protein targets have real promise for the development of new antiviral drugs. Additionally, such studies emphasize the need for further research into the functions of the individual nonstructural proteins. The greater the knowledge of viral protein functions the more opportunities arise to precisely target specific vital processes in the virus lifecycle. This development process is hampered by the fact that structures are often needed to guide the design of better inhibitors, however several replication proteins are integral membrane proteins and their protein structures have proven elusive. Thus it is difficult to design inhibitors based on structure-guided principles without known structures. Clearly, novel approaches are needed to address these proteins. HTS could be useful for further development of these proteins, not only to develop antiviral drugs for clinical use but also for determination of structure and function. Proteins such as NS2A and NS4A would greatly benefit from such studies as no direct antiviral approaches to these proteins have yet been developed.

3.1 Expert Opinion

Overall, there are many studies that have presented new options for DENV antiviral development and thus the field is clearly moving forward. It is especially interesting to note the studies involving new application of previously defined inhibitors. In particular, lycorine was developed to target other RNA viruses (69–72) and self-assembling nanotubes were originally discovered to inhibit bacteria (58), but these inhibitors have proved effective against *Flaviviridae* also (62, 74). Lycorine in particular demonstrates an exciting new application for a previously studied inhibitor, since 2K had not been previously targeted by antiviral drugs. Not only does lycorine present a new possible treatment option for flavivirus infection, it also encourages further investigation into the function of 2K. Furthermore, the efficacy of lycorine against a variety of viruses such as HSV-1 and SARS-CoV may even indicate shared function between 2K and viral proteins produced by viruses outside the flavivirus genus. Similarly, the transition of self-assembling nanotubes from bacterial to viral inhibition presents exciting new possibilities for antiviral treatment that may lead to further discovery about the nature of the membranes of the viral life cycle. Attention will not be lost

on the rapidly expanding list of FDA-approved HCV inhibitors, which primarily target the NS3 proteinase and NS5B polymerase. These have been traditional drug targets, and are now validated by a member of the Flaviviridae family. Indeed, analogous molecules and targets are currently in development for DENV, and are likely to be the first drugs to enter clinical trials.

Although this review focuses on viral proteins, existing drug application also applies to host system targets. Additional studies should be conducted to encompass this area even as DENV host cell requirements, such as membrane fatty acid composition, are elucidated (88, 89). New insights into host cell requirements for viral replication present promising new targets for antiviral therapeutics.

These studies emphasize the need for constant re-evaluation of existing strategies in parallel with discovery of new inhibitors. Although discovery of novel inhibitors is clearly beneficial and should be encouraged, the journey from compound discovery to marketable drug is a long and expensive process (Figure 3D). Therefore, further studies should focus on development of novel uses for existing treatments, in addition to discovering new antiviral compounds.

Additionally, more *in vivo* studies would greatly benefit the drug design process. Many antiviral compounds demonstrate great success *in vitro*, but either fail, or are not tested, *in vivo*. In particular, many of the studies reviewed involved potent E protein inhibitors of the β -OG pocket. Only a few of the reviewed E protein inhibitors have been developed beyond preliminary cell culture luciferase assays, and none have been tested *in vivo*. Although mouse models are not always indicative of all facets of DENV progression, they are more representative of genuine DENV infection than cell culture assays. Since the goal of antiviral research is to control human infections, compounds are desperately needed to move beyond the bench and into the clinic.

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Article Highlights

- The flaviviruses are a global health concern that lack effective treatment.
- Novel antiviral approaches are needed to treat flavivirus infection.
- Stem peptides, natural small molecules, peptide-conjugated oligomers, and self-assembling nanotubes are recent novel antiviral strategies.
- Clear progress has been made, however existing inhibitors and in vivo trials should be further developed in addition to new drug discovery.

Figure 1.

DENV lifecycle and drug targets. A. DENV enters via receptor-mediated endocytosis. Low pH E rearrangement releases the viral genome. The viral RNA is translated into a polyprotein which is proteolytically processed into the nonstructural proteins after translocation into the Endoplasmic Reticulum (ER). The replication complex is formed and transcription begins. The newly synthesized RNA associates with C and collects prM and E as it buds into the lumen of the ER to form the immature particle. The particle enters the secretory pathway. In the trans-golgi network, prM is clipped by furin but remains associated due to the low pH. In the neutral pH of the extracellular milieu, pr is released, and the now mature virion is capable of infecting a naïve host cell. B. Structure of DENV mature virion (above) and immature particle (below). Figure adapted from Perera et al. (43) copyright 2002 with permission from Elsevier. Also adapted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology Mukhopadhyay et al. (90), copyright 2005, and partially derived from Miller and Krijnse-Locker (91), copyright 2008. Amended with permission from American Society for Microbiology from Quinkert et al. 2005 (92). PDB identifiers 2BHR, 2J7W, 1L9K, 3C6E.

Figure 2.

Sites of DENV E protein Inhibition. The E protein domains are indicated by color; domain I in red, domain II in yellow, and domain III in blue. The relative position of the M protein is indicated by green shading. The β -OG pocket and stem region (boxed) are common sites of E protein targeting. Stem and M protein structure and location determined from secondary structural predictions and cryo-electron microscopy densities. Figure adapted from Modis et al. 2003 (32) copyright National Academy of Sciences, U.S.A. Adapted by permission from Macmillan Publishers Ltd: Nature Structural Biology, Zhang et al. (42), copyright 2003, and Nature Reviews Microbiology, Mukhopadhyay et al. (90), copyright 2005.

Figure 3.

PIP constructs and synthesis, drug design pipeline. A. YFV genome (top), YFV luciferase replicon construct (center), Sindbis virus replicon expressing YFV structural proteins for packaging (bottom). B. Schematic of pseudo-infectious particle production (PIPs) C. Schematic of PIP application for drug development D. Drug design pipeline. Figure adapted from Patkar et al. 2009 (77) with permission from American Society for Microbiology.