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Review

Ten years of dengue drug discovery: Progress and prospects

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ABSTRACT

To combat neglected diseases, the Novartis Institute of Tropical Diseases (NITD) was founded in 2002 through private–public funding from Novartis and the Singapore Economic Development Board. One of NITD's missions is to develop antivirals for dengue virus (DENV), the most prevalent mosquito-borne viral pathogen. Neither vaccine nor antiviral is currently available for DENV. Here we review the progress in dengue drug discovery made at NITD as well as the major discoveries made by academia and other companies. Four strategies have been pursued to identify inhibitors of DENV through targeting both viral and host proteins: (i) HTS (high-throughput screening) using virus replication assays; (ii) HTS using viral enzyme assays; (iii) structure-based *in silico* docking and rational design; (iv) repurposing hepatitis C virus inhibitors for DENV. Along the developmental process from hit finding to clinical candidate, many inhibitors did not advance beyond the stage of hit-to-lead optimization, due to their poor selectivity, physiochemical or pharmacokinetic properties. Only a few compounds showed efficacy in the AG129 DENV mouse model. Two nucleoside analogs, NITD-008 and Balapiravir, entered preclinical animal safety study and clinic trial, but both were terminated due to toxicity and lack of potency, respectively. Celgosivir, a host alpha-glucosidase inhibitor, is currently under clinical trial; its clinical efficacy remains to be determined. The knowledge accumulated during the past decade has provided a better rationale for ongoing dengue drug discovery. Though challenging, we are optimistic that this continuous, concerted effort will lead to an effective dengue therapy.

Contents

Abbreviations: DENV, dengue virus; WNV, West Nile virus; YFV, yellow fever virus; TBEV, tick-borne encephalitis virus; HCV, hepatitis C virus; WEEV, Western equine encephalitis virus; VSV, vesicular stomatitis virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus; DAA, direct antiviral agent; PI, protease inhibitors; HTS, high-throughput screening; MTase, methyltransferase; RdRp, RNA-dependent RNA polymerase; NI, nucleoside analog inhibitors; NNI, non-nucleoside inhibitor; PoC, proof-of-concept; TI, therapeutic index; SAR, structure–activity relationship; ER, endoplasmic reticulum; DHF/DSS, dengue hemorrhagic fever/dengue shock syndrome; SPA, scintillation proximity assay; FP, fluorescence polarization.

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1. An unmet medical need: dengue

Dengue fever (DF), also known as 'breakbone fever', is caused by dengue virus (DENV). The four serotypes of DENV constitute the most significant mosquito-borne viral pathogen. The virus is transmitted by infected *Aedes* (*A.*) mosquitoes, primarily *A. aegypti*. Dengue is endemic in over 100 countries across the globe, and on every continent except Antarctica. A recent study estimated that 390 million people are infected with DENV each year, with 96 million infections exhibiting disease symptoms each year (Bhatt et al., 2013). Upon DENV infection, some individuals develop mild disease with flu-like symptoms, whereas a few individuals develop the severe disease – dengue hemorrhagic fever and shock syndrome (DHF/DSS). The acute phase of the illness, characterized by fever and myalgias, lasts 1–2 weeks. Convalescence is accompanied by asthenia, and full recovery often takes several weeks. Dengue is a major cause of morbidity and hospitalization in many tropical and subtropical countries. The greatest documented burden of dengue occurs in Asia and Latin America, but the disease has also emerged in previously unaffected areas. It continues to spread, fueled by changing climates, increasing urbanization, and global travel. The cost of illness to society is considerable, from lost wages and diminished productivity, to costs related to care-giving and direct medical expenses. The cost of dengue in the Western Hemisphere alone is estimated at $2.1 billion per year (Beatty et al., 2011).

Public–private partnership is an excellent model to fight neglected diseases. The Novartis Institute for Tropical Diseases (NITD) was founded in 2002 through funding from Novartis and the Singapore Economic Development Board. One of NITD's missions is to develop novel therapeutics for dengue; such therapeutics will be provided to poor patients in developing countries at a cost price. To seek feedback on potential treatment strategies, a symposium supported by the Novartis Foundation, was held at NITD in 2005, with participation of leading flavivirologists from around the world (Novartis Foundation Symposium 277, 2006). NITD remains the only institution that has a dedicated mission of dengue antiviral development. In the past decade, NITD together with academia and other companies have made significant inroads towards the development of dengue antiviral. The goal of this review is to summarize major progress on this topic.

2. Feasibility of dengue antiviral therapy

One key concern surrounding the utility of antiviral therapy for dengue is the rapid decline in viremia during the natural course of infection. Studies of dengue patients indicate that viremia is reduced up to 10-fold within 24 h and up to 100-fold within 48 h (Libraty et al., 2002a,b; Nguyen et al., 2013). Thus, a DENV antiviral must be fast-acting and will be more effective when used in tandem with rapid diagnostics of early DENV infection. On this note, researchers and clinicians have been searching for prognostic biomarkers for severe dengue diseases. Nevertheless, there is sufficient precedent to suggest that antivirals will work for dengue.

Direct antiviral agents (DAA) have been successfully used to treat chronic viral diseases such as HIV-1, HCV, Hepatitis B virus (HBV), as well as acute viral illness like influenza. Thus, the treatment strategy for DENV would be akin to that for influenza, with a higher likelihood of success when patients are dosed as early as possible, ideally within 40 h of fever onset. In urban settings, this would not be a problem, as clinical assessment, coupled with rapid diagnostic kits (e.g., viral NS1 protein test) can be utilized. In rural areas, however, where laboratory services are not readily available, reliance on experienced medical personnel to identify individuals with traits of DENV infection is crucial. Eventually, the feasibility of dengue antiviral therapy can only be unequivocally addressed in patients using a clinical candidate. When an antiviral becomes available, dengue patients will readily seek treatment; such patient behavior would greatly facilitate early treatment. In addition, an antiviral could potentially be used prophylactically in communities with dengue outbreaks.

These considerations mandate that the optimal and minimally acceptable characteristics of dengue drugs (target product profiles) must encompass a good safety profile, with the eventual intent to treat children, since they make up a significant number of the patient population in developing countries. Other optimal characteristics include rapid resolution of symptoms and reducing risks of severity, comparable inhibition of all four DENV serotypes, oral administration and a long shelf-life to facilitate drug distribution in rural settings. Ideally, an effective dengue drug would be given to patients orally once a day, and for less than a week.

3. Rationale for a direct antiviral agent for dengue

Distinct differences (>10-fold) in plasma viremia between severe (DHF/DSS) and non-severe (DF) patients have been reported in the research literature (Libraty et al., 2002a,b). This suggests that reducing viral levels by antiviral therapy during the early phase of infection could prevent or lessen the chances of patients progressing to DHF/DSS. Both Wang et al. (2003, 2006) and Guilarde et al. (2008) observed the presence of DENV during post-defervescence in patients with severe dengue. These findings imply that the clinical manifestations of severe dengue may, in part, be virus-driven, and support the hypothesis that antivirals given at later stages of illness will still be beneficial.

Based on the clinical results, the current rationale for a dengue antiviral is to rapidly reduce viremia by >10-fold through DAA treatment during the early phase of DENV infection; such reduction of viremia would be expected to translate into clinical benefits and prevent the development of DHF/DSS. However, it remains to be established whether the 10-fold reduction of viremia should be the only criterion to determine the minimal efficacious dose during preclinical development or as the key parameter for PoC (proof-of-concept) in a clinical trial. Ideally, prevention of the infected patients from development into severe diseases (DHF/DSS) should be used as the ultimate PoC criteria. Practically, since <1% of dengue patients develop DHF/DSS (St John et al., 2013), testing the effect of a compound on the prevention of DHF/DSS would require more than several thousand patients in order to draw a statistically sound conclusion. Therefore, it is critical to identify prognostic biomarkers that can predict which patients would develop severe disease. Such prognostic biomarkers would greatly help healthcare workers to stratify dengue patients and to facilitate antiviral trials.

To address this goal, the Early Dengue Infection and Outcome Study (EDEN) was undertaken in 2005 by NITD and researchers from 7 Singapore agencies (Low et al., 2006). This multi-center longitudinal study characterized the epidemiological, clinical, viral and host-specific features of adult dengue disease. Increased expression of IP-10 and I-TAC in the peripheral blood of dengue patients at the early onset of fever was detected and may be useful as early biomarkers (Fink et al., 2007). The study also enabled the generation of decision algorithms using simple clinical and hematological parameters that predicted diagnosis and prognosis of dengue which could be useful in disease management and surveillance (Tanner et al., 2008; Schreiber et al., 2009).

4. Viral genome and replication

DENV belongs to the family *Flaviviridae*, which includes three genera, *Flavivirus*, *Pestivirus*, and *Hepacivirus*. DENV is a member of the genus *Flavivirus*, which includes other disease-causing viruses such as West Nile virus (WNV), yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), and Murray Valley encephalitis virus (MVEV). DENV is spherical in shape, with a diameter of 50 nm. The structures of mature and immature DENV particles have been solved by cryo-electron microscopy (Mukhopadhyay et al., 2005). The virus has a single-stranded, plus-sense RNA genome approximately 11,000 nucleotides in length. The genomic RNA encodes 10 proteins (Fig. 1): the structural proteins capsid (C), envelope (E), and membrane (M) and the nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Structural proteins form the virion: the C protein encapsulates the viral genomic RNA to form the nucleocapsid, and the nucleocapsid is enveloped by a lipid bilayer, in which viral prM and E proteins are embedded (Kuhn et al., 2002). The structures of the E (Modis et al., 2003; Rey et al., 1995), prM (Li et al., 2008), and C (Ma et al., 2004a) proteins have been solved by X-ray crystallography or NMR (reviewed in (Perera and Kuhn, 2008)).

Flavivirus nonstructural proteins are essential for virus replication, virion assembly, and evasion of host immune response (Bollati et al., 2010; Kummerer and Rice, 2002; Lindenbach et al., 2007; Liu et al., 2003; Munoz-Jordan et al., 2003). The nonstructural proteins are primarily localized in the cytoplasm to form replication complexes which are involved in viral RNA synthesis (Lindenbach et al., 2007). The best characterized DENV nonstructural proteins are NS3 and NS5, which are multifunctional proteins containing several enzymatic activities. The N-terminal domain of NS3 is a serine protease (with NS2B as a cofactor) and its C-terminal domain encodes RNA triphosphatase and helicase activities (Lescar et al., 2008; Luo et al., 2008a,b; Noble et al., 2012; Noble and Shi, 2012; Xu et al., 2005). NS5 contains a methyltransferase (MTase) domain at its N terminus and an RNA-dependent RNA polymerase (RdRp) at its C terminus (Ackermann and Padmanabhan, 2001; Dong et al., 2008; Egloff et al., 2002; Ray et al., 2006; Yap et al., 2007). Inhibition of viral enzymes has been proven to be the most successful antiviral approach, as demonstrated by the marketed drugs for HIV, HBV, and HCV. Crystal structures of all DENV enzymes have been solved in the past decade (Noble et al., 2010, 2012); such structural information forms a solid foundation for modern drug discovery.

Other nonstructural proteins (NS2A, NS2B, NS4A, and NS4B) are transmembrane proteins that have no identified enzymatic activities, but are essential for formation of the active viral replication complex (Lindenbach et al., 2007; Miller et al., 2007, 2006). Inhibitors targeting these non-structural proteins could be identified using virus replication-based assays. Indeed, using DENV infection assay or replicon assays, several groups have independently identified compounds that inhibit DENV replication by targeting NS4B (see later sections). Nonstructural proteins with no enzymatic activities could also be targeted for antiviral development, as demonstrated by the success of HCV NS5A inhibitor currently in clinical trial (Gao et al., 2010).

5. Approaches to find dengue virus inhibitors

Over the past ten years, NITD has taken both target-based and cell-based approaches to identify DENV-specific inhibitors. For the target-based approach, we systematically developed *in vitro* biochemical assays for DENV protease (Li et al., 2005), helicase (Krawczyk et al., 2010), methyltransferase (Chung et al., 2010;

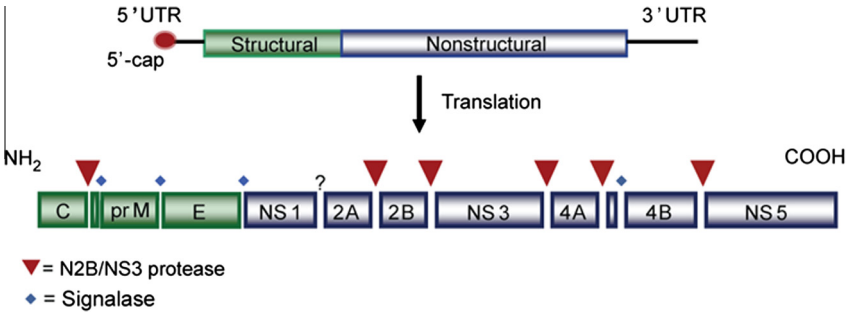


Fig. 1. Schematic diagram of the DENV genome organization and polyprotein processing. At the top is the viral genome, with the 5′-cap, 5′ UTR, the structural and nonstructural protein coding regions, and 3′ UTR. The bottom figure depicts the genome-translated polyprotein that is processed by the viral NS2B/NS3 protease, host signalases or unknown proteases.

Lim et al., 2008), and polymerase (Niyomrattanakit et al., 2010a). Screening campaigns have been performed against each of these targets. Unfortunately, though much effort was made to follow up on hits generated from these screens, none yielded specific inhibitors with good lead-like properties. In addition to the enzyme activity-based HTS, we also applied structure-based *in silico* docking (e.g., E protein inhibitor (Wang et al., 2009)) and rational design (e.g., methyltransferase inhibitor (Lim et al., 2011)). As discussed in the following sections, each target-based project presents its unique set of advantages and challenges.

For the cell-based approach, we performed HTS using three distinct viral replication assays: a DENV infection assay (Noble et al., 2010); a replicon assay (Ng et al., 2007); and a virus-like particle infection assay (Qing et al., 2010). Among the three HTS assays, the virus-like particle infection HTS yielded the least productive hits. Two points are noteworthy for the cell-based approach. First, "hits" identified from these cell-based HTS could potentially inhibit either host or viral targets. The number of host factors required for a productive viral infection cycle is significantly more than 10 viral proteins of DENV. Consequently, the probability of identifying inhibitors of host proteins in cell-based assays is anticipated to be much greater than that for viral proteins. It is therefore critical to biologically profile the "hits" to understand their antiviral mechanisms and targets before launching into chemical derivations of the hit. Secondly, target deconvolution can be challenging. The HTS hits could be tested in the various DENV enzyme assays to screen for viral targets; alternatively, the viral target could be identified through selection for resistant virus (Xie et al., 2011); in addition, a compound-mediated pulldown method could be used to identify host targets (Bonavia et al., 2011; Wang et al., 2011a).

In general, hits optimized in the target-based screening approaches are validated in the DENV cell-based assays for on-target antiviral activity. Depending on its physicochemical property, a compound class from target-based approaches may need to be further improved for cellular permeability. On the other hand, hits from cell-based screening efforts usually do not have this issue. Compounds that show anti-DENV activity in cells are further assessed for cytotoxicity and selectivity against other related viruses (such as WNV, JEV, YFV or HCV) or unrelated viruses (e.g. alphaviruses). In the final stages, lead compounds are selected for *in vitro* and *in vivo* pharmacokinetic profiling including plasma stability (half-life), exposure and oral bioavailability, before being tested in dengue mouse efficacy models (Schul et al., 2007; Yin et al., 2009b) and two-week preclinical toxicity studies in animals (usually in rats and dogs). On this note, selection of starting compounds that have lower molecular weight (MW <300 Da), lower lipophilicity, and fewer hydrogen bonds is more likely to result in optimized leads with acceptable drug-like properties (reviewed in Verheij, 2006). These chemical entities with molecular properties that adhere to Lipinski's "rule of five" (<5 hydrogen bond donors, <10 hydrogen bond acceptors, LogP <5, MW <500 Da) often have lower attrition rates during preclinical and clincal developments (Lipinski, 2004).

The following sections review key dengue drug discovery progress made at the NITD, as well as major discoveries made by academia and other companies in the past ten years.

6. Entry inhibitors

Inhibiting viral entry is a proven antiviral approach, as exemplified by two marketed drugs for HIV-1treatment. Maraviroc interacts with HIV co-receptor CCR5, inhibiting viral attachment to cells and entry (Pugach et al., 2008). Enfuvirtide binds to viral gp41, blocking viral and host cell membrane fusion (Lalezari et al., 2003). In addition, Myrcludex-B, a synthetic lipopeptide derived from the preS1 domain of the hepatitis B virus (HBV) envelope protein, which targets the hepatocytes and blocks *de novo* HBV infection, is currently undergoing phase Ib-IIa trials (Volz et al., 2013). Of the three inhibitors, the latter two are of peptide origin and must be administered by subcutaneous injection.

X-ray crystallography and cryo-electron microscopy studies have revealed that DENV consists of 180 molecules of E and M proteins arranged on a host-derived lipid membrane (Mukhopadhyay et al., 2005). Each monomer of the E ectodomain consists of three domains: I, II, and III. Two features of DENV E protein and DENV membrane fusion could be used for design of entry inhibitors (Fig. 2). First, between domain I and domain II is a hydrophobic pocket that was found to interact with detergent β-N-octyl-glucoside (Modis et al., 2003). Inhibitors that occupy this pocket could sterically block the conformational changes between domains I and II; such conformational change is essential for virus–host membrane fusion. Second, the C-terminal portion of the E protein consists of a stem region, followed by a membrane anchor. The stem region is highly conserved among flaviviruses; during fusion, the stem region needs to fold back onto the trimer of E ectodomain. Similar to HIV Enfuvirtide, a peptide representing the DENV E stem could competitively block membrane fusion (see examples below).

Four main approaches have been taken to search for entry inhibitors of DENV:

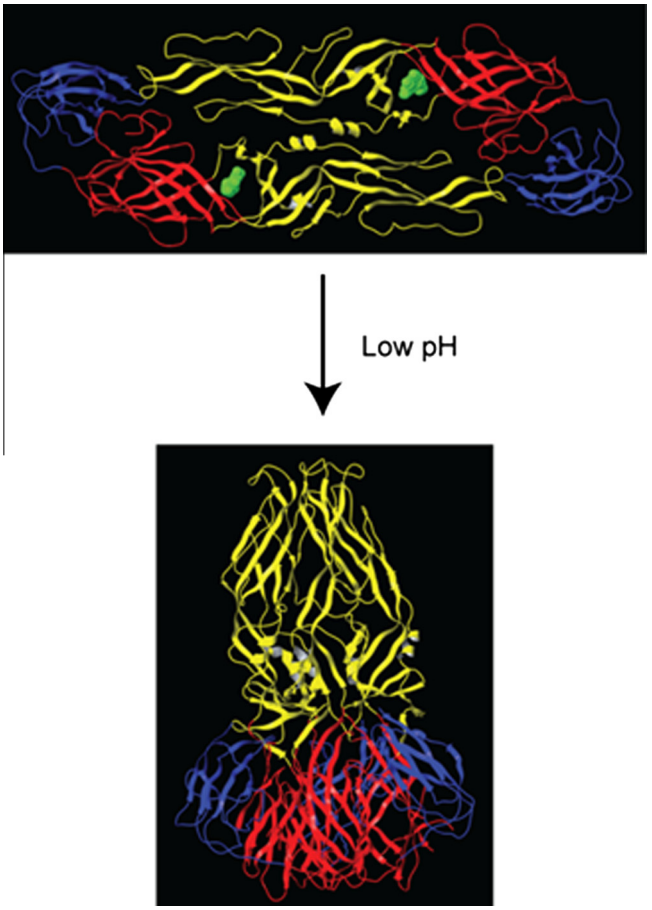


Fig. 2. The DENV E dimer and trimer. The red, yellow and blue parts of each subunit correspond to domains I, II and III of the DENV E protein, respectively. The *n*-octyl-β-D-glucoside (β-OG) is displayed as green spheres. Upon acidification, the prefusion E dimer undergoes a conformational change to the fusion-active E trimer (Modis et al., 2003, 2004).

(i) *In silico* docking to identify compounds that bind to the hydrophobic pocket between domains I and II;
(ii) compound library screening, using an assay that captures a fusion intermediate between a stem-less E trimer and an E stem peptide;
(iii) design of peptides that block the fold–back interaction between the E stem and E trimer during fusion;
(iv) diverse library screens, using a DENV infection assay.

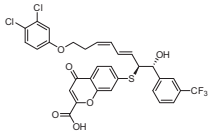
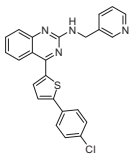
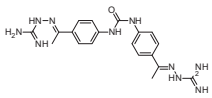
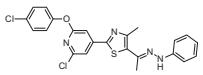
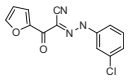
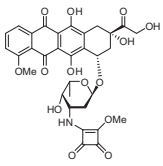
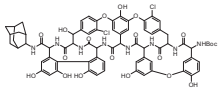
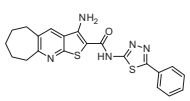
These approaches have produced some promising results.

6.1. Targeting a hydrophobic pocket of the E protein

A virtual screen has been performed to identify small molecules that bind to the hydrophobic pocket for β-N-octyl-glucoside in the DENV E protein (Fig. 2; (Alen and Schols, 2012)). Two classes of compounds were identified from the Novartis compound library. The first class of compounds, represented by NITD-448 (Table 1), inhibited E protein-mediated membrane fusion (IC_{50} 6.8 μM) as well as DENV-2 infection (EC_{50} 9.8 μM) in cell culture (Poh et al., 2009). Due to the large molecular weight of this compound, poor selectivity, and pharmacokinetic properties, this compound series was not further pursued. The second class of compounds (e.g., compound 6; Table 1) contained a quinazoline core, and inhibited various laboratory and clinical isolates of DENV with micromolar to nanomolar EC_{50} (Wang et al., 2009). After significant chemistry efforts to improve the pharmacokinetic properties of this scaffold, its low potency and high plasma protein-binding activity due to lipophilicity prevented further development of the compound.

In silico docking with the NCI library also identified the highly basic compound P02 (Table 1), which inhibited YFV production at micromolar concentrations (Zhou et al., 2008). This compound was confirmed by NMR to bind DENV virions; in addition, it competed with *N*-octyl-β-D-glucoside for binding to the E protein. Kampmann et al. (2009) also performed a virtual screen using the Maybridge database. One compound, A5 (Table 1), was found to have low micromolar activity against DENV, WNV, and YFV. The compound prevented syncytia formation in cells expressing DENV envelope. However, no follow-up studies have been reported for these compounds.

Table 1
Inhibitors of DENV structural proteins (see details in text).

Compound	Structure	Mode of action	Cellular activity (EC_{50}/CC_{50}) μM	AG129 dengue mouse activity	References
Entry inhibitors					
NITD-448		E protein βOG pocket	9.8/48.7	ND	Poh et al. (2009)
Compound 6		E protein βOG pocket	0.119/>20	ND	Wang et al. (2009)
P02		E protein βOG pocket	13[a]/371	ND	Zhou et al. (2008)
A5		E protein βOG pocket	1.2/>100	ND	Kampmann et al. (2009)
1662G07		E stem and E trimer interaction	16.9[b]/>100	ND	Schmidt et al. (2012)
Peptide DN59(stem peptide)	MAILGDTAWDFGSLGGVFTSIGKALHQVFGAIY	Bind to E trimer to block stem binding	10/>30	ND	Hrobowski et al. (2005)
Peptide 1OAN1 (β sheet connecting E domains I and II)	FWFTLIKTQAKQPARYRRFC	Interact with E to block virus attachment	7/>50	ND	Costin et al. (2010), Nicholson et al. (2011)
Antibiotic SA-17		Entry, possibly through E βOG pocket	0.52/43	ND	Kaptein et al. (2010)
Antibiotic LCTA-949		Entry	6.9/>25	ND	De Burghgraeve et al. (2012)
Capsid inhibitor					
ST-148		C protein	0.016/>100	Efficacious	Byrd et al. (2013a)

[a] Activity against YFV is presented.
[b] EC_{90} is presented. ND, not determined.

6.2. Targeting the interaction between E stem and E trimer

Since the stem region of E needs to interact with E trimer during DENV fusion, Schmidt et al. (2012) developed a fluorescence polarization (FP) assay that detects the binding of an E stem-derived peptide to a stem-less E trimer. Using this assay, they uncovered a series of compounds (e.g., 1662G07; Table 1) that inhibit DENV-2. Structure–activity relationship (SAR) studies led to analogs with sub-micromolar IC_{90}s against DENV-2, some activity against DENV-4, but weak activity against DENV-1 and -3. The antiviral spectrum of the compounds needs to be broadened to inhibit all four serotypes.

Besides the small-molecule approach described above, peptides could also be designed to inhibit E-mediated viral entry. Several groups have demonstrated that short peptides derived from the DENV E protein can inhibit DENV in cell culture (Costin et al., 2010; Hrobowski et al., 2005; Liao and Kielian, 2005; Lok et al., 2012). These peptides could also inhibit antibody-enhanced DENV infection (Nicholson et al., 2011). Peptide DN59 (Table 1), representing the pre-membrane stem region (amino acids 412–444) of DENV-2 E, bound to E trimer to inhibit fusion. Such stem peptides inhibited all four DENV serotypes (Schmidt et al., 2010b). Remarkably, DN59 induced holes on virion to eject nucleocapsid, giving rise to empty virions; the mechanism of peptide-induced nucleocapsid ejection remains to be determined (Lok et al., 2012).

Peptide 1OAN1 (Table 1) is a computationally optimized mimic of the first beta sheet strand that connects domains I and II of E protein; the peptide interferes with virus binding to cell through direct interaction with E protein (Nicholson et al., 2011). Both DN59 and 1OAN1 interact with E protein with dissociation constants of about 10^{-7} M (Costin et al., 2010; Schmidt et al., 2010a). Further optimization is needed to improve the potency of these peptide inhibitors. The drawbacks of peptidic inhibitors are the need for intravenous administration and its low-shelf life which could limit their use in clinics. Given that dengue is prevalent mostly in developing countries, it will be challenging to make this class of inhibitors accessible to poor patients living in rural areas.

6.3. Entry inhibitors with undefined mechanisms

Using a DENV infection assay, Neyts and colleagues identified two classes of antibiotics that inhibit DENV with micromolar activities in cell culture. The two classes of antibiotics inhibitors are SA-17 (a derivative of doxorubicin; Table 1) and LCTA-949 (a glycon analog of the antibiotic teicoplanin; Table 1) (De Burghgraeve et al., 2012; Kaptein et al., 2010). Time-of-addition experiments suggest that both compounds inhibit an early step of the viral infection cycle; neither compound inhibited a DENV replicon, which lacks viral entry. These results indicate that these compounds act as entry inhibitors. In addition, LCTA-949 was shown to directly bind to DENV particles and to prevent infection of DENV opsonized with antibodies. *In silico* docking suggests that SA-17 fits into the β-N-octyl-glucoside pocket of the E protein.

Besides the small-molecule approach, monoclonal antibodies have been actively pursued for therapeutic purposes. The main challenge of this approach is to design an antibody that can potently neutralize all four serotypes of DENV. Since this review focuses on small-molecule inhibitors, we will not discuss therapeutic antibodies. Readers are encouraged to refer to an excellent review on this topic (Diamond et al., 2012).

7. Capsid inhibitors

From a DENV infection-based HTS of approximate 200,000 compounds, Byrd et al. (2013a) identified an inhibitor, ST-148 (Table 1), which targets the DENV C protein. This compound inhibited DENV-2 in a viral titer reduction assay with an EC_{50} of 0.016 μM and an EC_{90} of 0.125 μM. It displayed weaker activities against other serotypes, with EC_{50}s ranging from 2.832, 0.512, and 1.150 μM for DENV-1, -3, and -4, respectively. ST-148 resistant DENV-2 harbored a single amino acid change of S34L in C protein. Infectious virus with the engineered mutation showed a 550-fold reduction of susceptibility to ST-148. Binding of ST-148 to DENV C protein was also performed; surprisingly, the compound bound equally well to the wild-type and mutant C proteins, raising the question of the mechanism-of-inhibition of this compound. Ser34 is located in the $\alpha1$–$\alpha1'$ helices of the dimeric C structure. The authors proposed that ST-148 might prevent C interaction with membranes.

Oral and intraperitoneal administrations of ST-148 showed good exposure when dosed at 50 mg/kg/day in the AG129 mice with peak plasma concentrations at 468-, and 7750-fold above the *in vitro* EC_{50}. Similarly, after an intravenous dose of 20 mg/kg/day, mice plasma levels of ST-148 were 6093-fold above the *in vitro* EC_{50}. On average, BID treatment with ST-148 reduced peak plasma viremia by 52-fold and reduced viral load in the spleen and liver by 3- and 20-fold, respectively. The pharmacokinetic data indicate that ST-148 has limited oral bioavailability and fairly rapid clearance but good systemic availability following i.p. administration. Overall, the results warrant further preclinical development of ST-148.

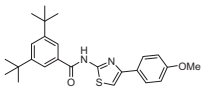
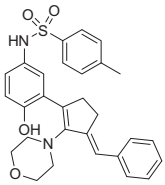
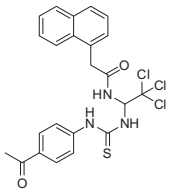
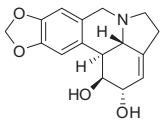
8. NS4B inhibitors

Using an HTS with a cell line harboring a luciferase replicon of DENV-2, we identified a DENV NS4B inhibitor, NITD-618 (Table 2; (Xie et al., 2011)), from a compound library of 1.8 million small molecules. NITD-618 was active against all four serotypes, with EC_{50}s ranging from 1.0 to 4.1 μM, but inactive against other RNA viruses (EC_{50} >40 μM), including the two closely related flaviviruses, WNV and YFV, two plus-strand RNA alphaviruses (chikungunya virus and Western equine encephalitis virus [WEEV]), and a negative-strand RNA rhabdovirus (vesicular stomatitis virus [VSV]). These results demonstrate that NITD-618 selectively inhibits DENV. Mapping of the NITD-618-resistant replicon revealed amino acid changes within the NS4B protein (P104L and A119T); when engineered into the wild-type replicon or DENV, these mutations conferred resistance to NITD-618. This compound could interrupt the NS3–NS4B complex formation, as residue P104L was previously shown to be important for the NS3–NS4B interaction (Umareddy et al., 2006). Unfortunately, the high lipophilicity of NITD-618 resulted in poor pharmacokinetic properties which hindered testing its activity against DENV in AG129 mice. Medicinal chemistry attempts to reduce its lipophilicity resulted in a loss of activity or a reduction in antiviral selectivity against DENV.

Other groups working on related flaviviruses have also found inhibitors that target the NS4B protein. An HTS using pseudo-infectious YFV particles, which express *Renilla* luciferase in a replication-dependent manner, identified two classes of inhibitors targeting the NS4B protein (Patkar et al., 2009). Virus resistant to compounds CCG-3394 and CCG-4088 (Table 2) harbored a K128R mutation in the cytoplasmic loop of NS4B.

Lycorine (Table 2) was reported to reduce viral titers of WNV, DENV, and YFV mainly through suppression of viral RNA replication (Zou et al., 2008). An amino acid change V9M in viral 2 K peptide conferred WNV resistance to lycorine through enhancing viral RNA replication. Initial chemistry synthesis demonstrated that modifications of the two hydroxyl groups of lycorine can increase the compound's potency, while reducing its cytotoxicity. In HCV, an NS4B inhibitor, Clemizole, was also identified from an HTS

Table 2
Inhibitors of DENV NS4B (see details in text).

Compound	Structure	Mode of action	Cellular activity (EC_{50}/CC_{50}) μM	AG129 dengue mouse activity	Reference
NITD-618		NS4B	1.0/>40	ND	Xie et al. (2011)
CCG-3394		NS4B	1.48/31	ND	Patkar et al. (2009)
CCG-4088		NS4B	0.4/13	ND	Patkar et al. (2009)
Lycorine		2 K peptide	0.23/24	ND	Zou et al. (2008)

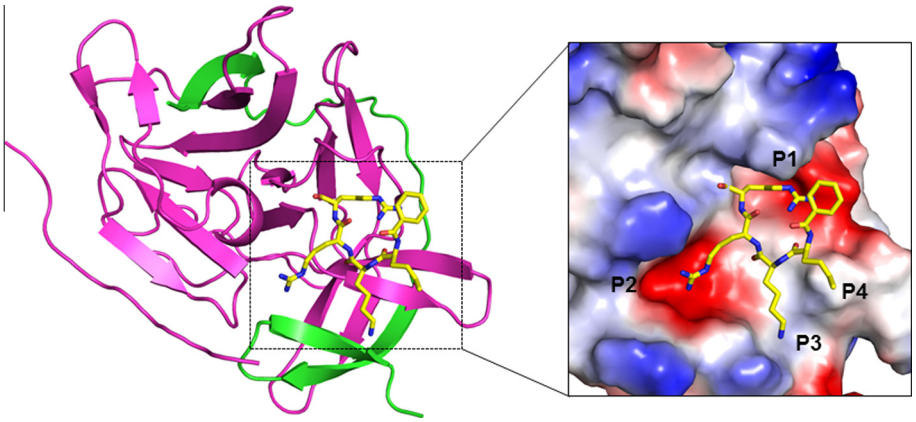


Fig. 3. Structure of the DENV NS2B/3 protease, highlighting the S2, S3 pockets, with the bound nKRR-H inhibitor (Noble et al., 2012). The structure is shown in cartoon representation, with NS3 in pink and NS2B in green. The wrapped NS2B beta-hairpin is indicated. Bz-nKRR-H is shown in stick representation.

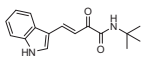
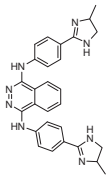
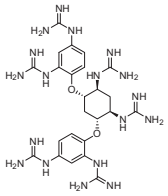
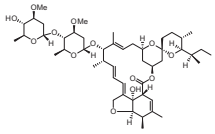
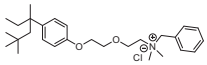
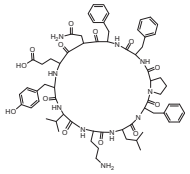
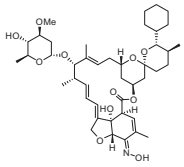
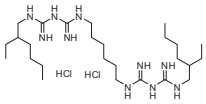
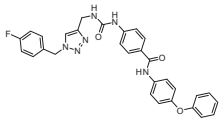
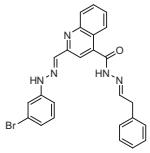
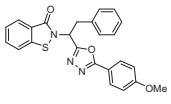
campaign; Clemizole is currently in Phase I clinical trial (Einav et al., 2010).

9. Protease inhibitors

Viral proteases are proven antiviral targets, as evidenced by the clinical availability of ten HIV-1 protease inhibitors (PIs) (De Clercq, 2009) and the two HCV PIs (Wyles, 2013). Thus, it is plausible that a protease inhibitor for DENV would be efficacious in the clinic. However, experience with both HIV-1 and HCV indicates that there are certain drawbacks associated with PIs, and these are important considerations in developing a DENV PI. For the treatment of both HIV-1 and HCV infection, the rapid emergence of PI drug-resistant viral strains was observed. This limits their utility to combination therapy with other DAAs (or additionally, in the case of HCV, with PegIFN/ribavirin), as well their prospects for long-term therapy (Wensing et al., 2010). Furthermore, while the majority of HIV-1 PIs are active across different viral strains, HCV PIs (telaprevir and boceprevir) are active only against genotype 1 (as drug design was based on the protease structure of HCV genotype 1), and are less effective in genotype 1a than 1b (Pockros, 2011). Sequence dissimilarity in the NS3 PI binding sites, occurring as natural polymorphisms across the different HCV genotypes, explains why non-genotype-1 HCV strains are resistant to both PIs (Halfon and Locarnini, 2011). More worrisome, naturally occurring mutations in NS3 have been detected in a significant number of HCV genotype 1 strains in treatment-naïve patients; these mutations result in resistance to the two PIs (Halfon and Locarnini, 2011).

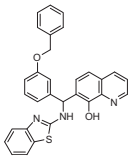
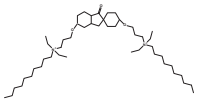
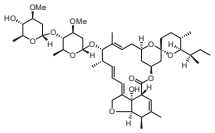
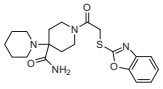
The DENV protease bears strong functional similarity to its HCV counterpart. Like the latter, DENV NS3 protease activity is dependent on association with a hydrophilic region of 40 amino acids of NS2B. Unlike HCV, NS2B functions as a cofactor that actively participates in the formation of the S2 and S3 sub-pockets in the protease active site (Fig. 3; (Erbel et al., 2006; Noble et al., 2012)). Proteases from the four serotypes of DENV share very similar substrate specificity (Li et al., 2005), as well as higher sequence

Table 3
Inhibitors of DENV NS3 protease and helicase (see details in text).

Compound	Structure	Mode of action	Cellular activity (EC_{50}/CC_{50}) μM	AG129 dengue mouse activity	References
Protease inhibitors					
Retro tripeptides	R-Arg-Lys-Nle-NH_2	Mixed inhibition	ND	ND	Nitsche et al. (2012)
Cyclic peptide 9	Cys-Ala-Gly-Lys-Arg-Lys-Ser-Gly	Protease	ND	ND	Xu et al. (2012)
Tetrapeptide	Bz-Nle-Lys-Arg-Arg-$B(OH)_2$	Competitive inhibition	ND	ND	Yin et al. (2006)
Compound 32		Protease	<10/>100	ND	Steuer et al. (2011)
Compound 1		Competitive inhibition	ND	ND	Bodenreider et al. (2009)
166347		Competitive inhibition	ND	ND	Cregar-Hernandez et al. (2011)
ARDP0006		Competitive inhibition	4.2/69	ND	Tomlinson et al. 2009a,b
Ivermectin		Mixed inhibition	ND	ND	Tomlinson and Watowich (2012)
Benzethonium chloride		Mixed inhibition	ND	ND	Tomlinson and Watowich (2012)
Tyrothricin		Competitive inhibition	ND	ND	Tomlinson and Watowich (2012)
Selamectin		Mixed inhibition	ND	ND	Tomlinson and Watowich (2012)
Alexidine hydrochloride		Mixed inhibition	ND	ND	Tomlinson and Watowich (2012)
Aminobenzamide		Protease	ND	ND	Aravapalli et al. (2012)
Compound 23i		Protease	24.7/>100	ND	Deng et al. (2012)
Compound 7n		Protease	ND	ND	Lai et al. (2013a)

(*continued on next page*)

Table 3 (*continued*)

Compound	Structure	Mode of action	Cellular activity (EC_{50}/CC_{50}) μM	AG129 dengue mouse activity	References
Compound 1		Protease	ND	ND	Lai et al. (2013b)
BP2109		Protease	0.17/29.28	ND	Yang et al. (2011)
Helicase inhibitors					
Ivermectin		Helicase	0.7/3.8	ND	Mastrangelo et al. (2012)
ST-610		Helicase	0.272/>100		Byrd et al. (2013b)

similarity, compared to HCV proteases. In 1,500 HCV NS3-protease sequences analyzed from treatment-naive patient samples, only 47% of amino acids were found to be conserved (Cento et al., 2012). Along the same line, sequence similarity across DENV serotypes for NS3 is between 63% to 74%, suggesting that the genetic barrier to develop resistance could also be low for DENV PI. Therefore, a dengue PI would have to be administered in combination with other DAAs.

With the exception of tipranavir, most HIV-1 PIs and the two HCV PIs are substrate mimetics. This approach has been similarly taken to develop DENV PI, but to date, no compounds have advanced to the preclinical stage. The challenges with the peptidiomimetic strategy for DENV protease are the shallow active site (like in HCV) and the need to replace the two basic (mostly Arg) P1 and P2 residues (Fig. 3). Most of the reported DENV peptidic inhibitors possessed a reactive warhead and/or at least one basic residue. As such, they exhibit inhibitory activities in biochemical protease assays (retro tripeptide, cyclic peptide 9, tetrapeptide, and compound 32; Table 3), but not in cell-based viral infection assays (except for compound 32 (Steuer et al., 2011); Table 3) due to their poor permeability and stability (Nitsche et al., 2012; Xu et al., 2012; Yin et al., 2006). Cyclizing the peptides improves their stability and cell permeability, and could be a way forward for DENV PI (Xu et al., 2012).

The most potent DENV peptidic inhibitor is a tetra-peptide with a boronic acid, which is highly unsuitable for oral dosing and is non-DENV specific (Yin et al., 2006). This is because DENV protease shares substrate specificity with the host serine protease, furin (bearing the cleavage site RXRR), and to a lesser extent, other serine proteases like trypsin, thrombin, or elastase, which recognize Arg in the P1 position. We observed that the peptidic inhibitor, Bz-Nle-Lys-Arg-Arg-H was highly potent against these human enzymes when tested *in vitro* (unpublished). Cross inhibition against human enzymes is a safety concern in the design of DENV PIs. Therefore, a larger selectivity panel of host serine proteases would be needed to ascertain DENV specificity.

The second approach to identify DENV PIs relies on diverse library screening. Several campaigns have been carried out with the single-chain linked NS2B/3 protein construct (Leung et al., 2001). A number of inhibitors have been reported. For one particular class of compound (compound 1; Table 3) identified at NITD, the ensuing hit-to-lead activities generated both specific and nonspecific analogs that could be differentiated by biophysical assays (Bodenreider et al., 2009). NMR showed that the closed conformation of the protease was stabilized upon binding of compound 1 and its specific analogs but not of nonspecific compounds. This experience illustrates the pitfalls in relying solely on IC_{50} values to drive the SAR in hit-to-lead optimization. However, these compounds did not show activity in cell-based assays, probably due to poor peameability caused by the presence of positively charged substituents in this scaffold.

Several other compound series have been identified elsewhere. However, many of the lead compounds are weakly active (single-digit micromolar inhibition), have not been shown to directly bind to the DENV protease *in vitro*, and do not show inhibition in cellular assays, prompting speculation that some of these hits may be nonspecific. If co-crystal structures of these compounds bound to the DENV protease can be generated, this will certainly guide lead optimization. A guanidinylated and therefore cationic compound (166347; Table 3) was identified as a competitive inhibitor, suggesting that it bound to the active site of the DENV protease (Cregar-Hernandez et al., 2011). Separately, Tomlinson et al. 2009a,b and Tomlinson and Watowich (2011, 2012) identified several classes of compounds (ARDP0006, Ivermectin, benzethonium chloride, tyrothricin, selamectin, and alexidine hydrochloride; Table 3) that inhibited DENV-2 protease, some of which also inhibited the WNV protease, but were not active against trypsin. Lai and colleagues showed that the protease hits (Aminobenzamide, 23i, 7n, and 1; Table 3) identified from a WNV protease screen could also inhibit the DENV protease; furthermore, these hits could be docked onto the substrate S1–S4 binding site on the protease structure (Aravapalli et al., 2012; Lai et al., 2013a,b).

A rational approach using *in silico* docking identified inhibitors of the DENV protease (compound 23i; Table 3; (Deng et al., 2012)). Although the compounds showed activity in a biochemical protease assay, none of them had TI (therapeutic index = CC_{50}/EC_{50}) of $\geqslant 10$. Yang and colleagues identified a hit (BP2109; Table 3) through a DENV protease HTS (Yang et al., 2011). BP2109 exhibited low micromolar inhibition *in vitro*, but submicromolar activity in a replicon assay. Importantly, a BP2109-resistant DENV replicon revealed two amino acid mutations (R55K and E80K) in NS2B, which when engineered into DENV resulted in the loss of sensitivity to the compound. The R55K and E80K mutations were mapped to the soluble region of NS2B that form part of the active protease.

BP2109 (Table 3) contains two positive charges, but is likely cell permeable due to the formation of micelle and cellular internalization through endocytosis. Although this compound is not a good starting point for lead optimization, it demonstrates that the DENV protease is a druggable antiviral target.

10. Helicase inhibitors

The C-terminal domain of the NS3 protein is a non-processive NTPase/helicase (comprising amino acids 180–618). The flavivirus helicase belongs to the superfamily of "SF2" helicases (Lescar et al., 2008). The helicase/NTPase activities and crystal structures have been reported for DENV (Fig. 4), YFV, and JEV (Lescar et al., 2008; Wu et al., 2005; Xu et al., 2005; Yamashita et al., 2008). The ATPase active site is housed between subdomains 1 and 2 with the substrate, ATP, primarily held through its triphosphate moiety via contacts through a divalent metal ion and residues from motifs I, II and VI (Fig. 4; (Xu et al., 2005)). The lack of specific amino acid interactions with the ATP nucleobase or ribose ring makes it challenging to design inhibitors to interact with this pocket. The crystal structure of DENV helicase–RNA–AMPPNP ternary complex (Luo et al., 2008a,b) showed that the RNA ligand binds to a shallow pocket on the helicase (Fig. 4), making it challenging to design compounds to inhibit RNA–protein interaction.

Despite the availability of a high-resolution structure of the DENV helicase, limited progress has been made towards the rational design of helicase inhibitors. Recently, an *in silico* docking of compounds into the single-strand RNA access site of WNV helicase identified ivermectin (Table 3) which inhibited WNV, YFV, and DENV helicase dsRNA unwinding activity at submicromolar levels. However, ivermectin did not inhibit the NS3 ATPase activity (Mastrangelo et al., 2012). When tested in cell culture, ivermectin inhibited YFV with EC_{50} of 0.5 nM, but was less potent against DENV (EC_{50} 0.7 μM), JEV (EC_{50} 0.3 μM), WNV (EC_{50} 4 μM), and TBEV (EC_{50} 0.2 μM) (Mastrangelo et al., 2012). As mentioned above, ivermectin also inhibits DENV protease activity (Tomlinson and Watowich, 2012); therefore, the observed anti-DENV activity in cell culture could be the inhibition of both protease and helicase activities. Ivermectin is a broadly used anti-helminthic drug. It remains to be determined whether *in vivo* efficacy could be achieved in DENV.

NITD performed one HTS using an *in vitro* unwinding assay. We failed to identify specific helicase inhibitors. After counter-screens, the HTS hits were mostly found to be assay artifacts or nonspecific binding to the RNA substrate. These challenges are shared by other researchers working on other viral or host helicases. Readers are encouraged to refer to an excellent review on targeting helicases for drug development (Shadrick et al., 2013). For the DENV helicase, the need to design inhibitors that do not cross-react with host helicases is another hurdle, given the large number of host helicases in the SF2 superfamily (Lescar et al., 2008).

Byrd et al. (2013b) recently reported a novel small molecule inhibitor (ST-610; Table 3) of the DENV helicase. The compound was identified from an HTS using a DENV infection assay. ST610 potently and selectively inhibited all four serotypes of DENV in cell culture. Sequence analysis of drug-resistant virus found a single amino acid change (A263T) in the helicase domain that confers resistance to the compound. ST-610 inhibits DENV helicase RNA unwinding activity, but does not inhibit its ATPase activity. When dosed intraperitoneally (up to 100 mg/kg BID), ST-610 showed marginal efficacy in the DENV AG-129 mouse model, with <10-fold viremia reduction. For further development, the *in vivo* pharmacokinetic properties of this compound need to be improved.

11. Methyltransferase inhibitors

The N-terminal domain of the flavivirus NS5 protein (amino acids 1–272) functions as a dual methyltransferase (MTase), which can methylate the 5′-end of the viral genomic RNA at the N-7 position of the guanine cap (N-7 MTase) as well as the 2′-OH position of

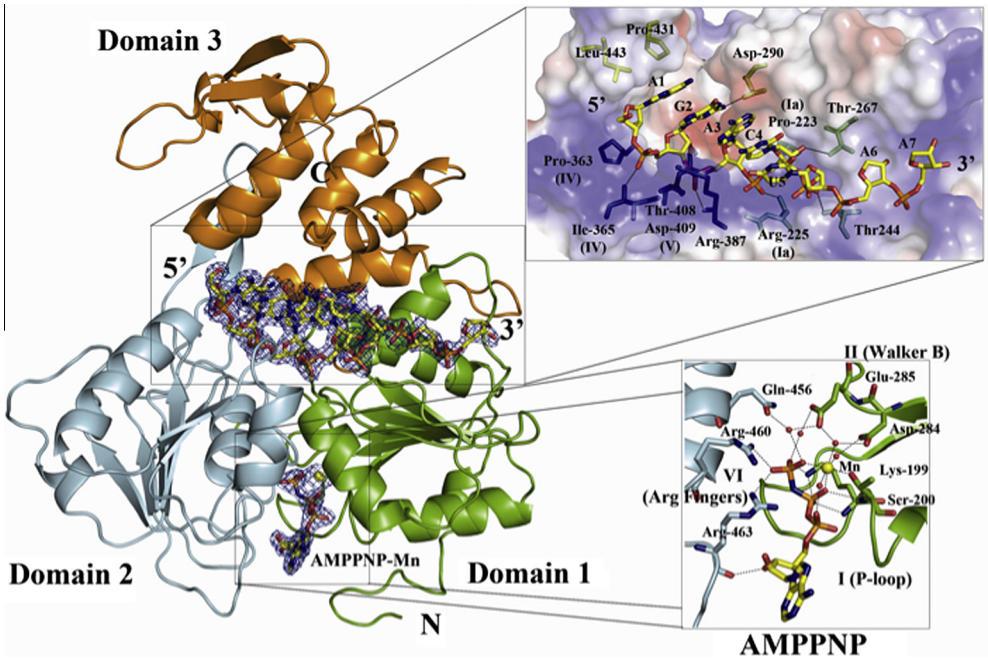


Fig. 4. The NENV helicase–RNA–AMPPNP ternary complex. Cartoon representation of NS3 helicase bound to ssRNA and AMPPNP, a non-hydrolysable ATP analog (AMPPNP) shown as sticks (Luo et al., 2008a,b). Domain 3 of the helicase is colored in orange. The Rec-A-like subdomains 1 and 2 are colored in green and blue, respectively. The manganese ion is represented as a yellow sphere. Top inset: magnified view of interactions between the protein and its RNA ligand (5′-AGACUAA-3′), using a color-coded representation of the helicase electrostatic surface potential. The color scale is 10 kT (red) to þ10 kT (blue). Residues that contact the RNA are displayed as sticks and labeled. Bottom insert: magnified view of the AMPPNP-Mn^{2+} ternary complexes (with bound RNA). Water molecules are shown as red spheres. Hydrogen bonds are indicated by dashed lines.

the first nucleotide ribose (2′-*O* MTase) (Egloff et al., 2002; Ray et al., 2006). It can further methylate internal adenosines of the viral RNA genome and potentially host RNA at the ribose 2′-OH position (internal methylation) (Dong et al., 2012). Both the cap and internal methylations use *S*-adenosine-L-methionine (SAM) as the methyl donor and generate *S*-adenosine-L-homocysteine (SAH) as a by-product. Despite exhibiting two distinct methylation activities, flavivirus MTases show a single binding site for SAM in the apo-crystal structure and display high homology to host MTase (Noble and Shi, 2012).

Functional studies showed that N-7 methylation of RNA cap is critical for efficient translation (Ray et al., 2006). Mutations of MTase that abolish N-7 methylation are lethal for flavivirus replication (Zhou et al., 2007), suggesting that the N-7 MTase is a potential antiviral target. 2′-*O* cap methylation functions to subvert innate host antiviral response through escape of IFIT-mediated suppression (Daffis et al., 2010).

The wealth of structural information has provided a solid foundation for structure-guided antiviral approaches. However, since the core domains of various MTases are conserved, designing flavivirus-selective inhibitors which do not inhibit host MTases, e.g., RNA and DNA MTase or protein MTase, or SAM-binding proteins, will be challenging. Using a structure-guided approach, we recently designed DENV-selective inhibitors by taking advantage of a flavivirus MTase-unique pocket located next to the SAM binding site (Fig. 5; (Dong et al., 2010; Lim et al., 2011)). Specifically, SAM analogs (e.g., compound 10; Table 4) with an extra chemical group that interacts with the flavivirus-unique pocket selectively inhibited viral MTases. Although this provided proof of concept *in vitro*, the compound needs to be improved for cell permeability to achieve antiviral activity in cell culture.

Over the past three years, we have conducted two medium-throughput screens to identify inhibitors of the DENV MTase. Our first screen measured DENV N7 and 2′-*O* activities using the scintillation proximity assay (SPA) format as it is amenable to miniaturization (Chung et al., 2010; Lim et al., 2008). About 60,000 compounds were screened. After counter screens, we failed to identify any specific hits. It is possible that the relatively small pool of compounds tested did not contain any specific inhibitors and screening with a larger number of compounds would increase

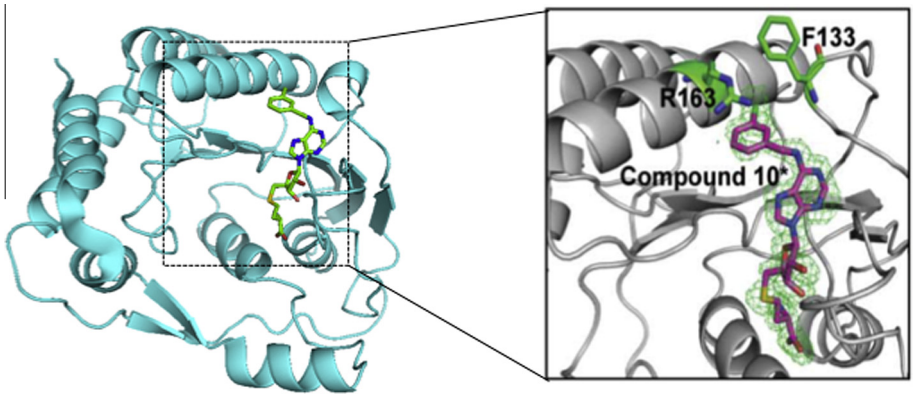
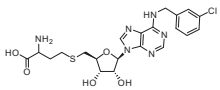
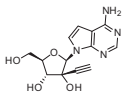
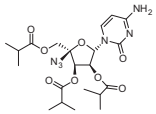
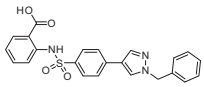
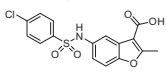


Fig. 5. Crystal structure of the DENV MTase in complex with compound 10. Surface representation of the DENV MTase shows the flavivirus-conserved hydrophobic cavity (yellow) and the bound compound 10 (in stick presentation). Inset magnifies compound 10 bound to the MTase gydrophobic pocket (in cartoon presentation); the confomation of amino acids Phe-133 and Arg-163 was changed upon binding to compound 10 (Lim et al., 2011).

Table 4
Inhibitors of DENV NS5 MTase and RdRp (see details in text).

Compound	Structure	Mode of action	Cellular activity (EC_{50}/CC_{50}) μM	AG129 dengue mouse activity	References
MTase inhibitor					
Compound 10		MTase	ND	ND	Lim et al. (2011)
RdRp nucleoside inhibitors					
NITD-008		Adenosine nucleoside	0.64/>100	Efficacious	Yin et al. 2009a,b
Balapiravir		Cytidine nucleoside	1.9/ND	ND	Nguyen et al. (2013)
RdRp non-nucleoside inhibitors					
NITD-2		RdRp non-nucleoside	ND	ND	Niyomrattanakit et al. 2010a,b
NITD-107		RdRp non-nucleoside	ND	ND	Noble et al. (2013)

the likelihood of success. However, extra screening was not carried out because the costs of the SPA beads, tritiated-labeled SAM, and the capped/biotinylated RNA substrates together were prohibitively high. Our second screen measured DENV 2′-*O* MTase activity using a fluorescent polarization assay (Lim. et al., 2013). We screened 250,000 compounds. After extensive follow-ups, no specific hits were identified. Considering the disappointing outcome of the enzyme activity-based screens, we believe that a structure-guided approach or perhaps a competitive binding assay (e.g. SPR or NMR) may be more suitable for hit identification for flavivirus MTase.

12. Polymerase nucleoside analog inhibitors

The NS5 polymerase is the most conserved flavivirus protein and is an attractive drug target, as it is essential for viral RNA synthesis. Readers are encouraged to refer to several excellent reviews on its structure and function (Bollati et al., 2010; Lescar et al., 2008; Malet et al., 2008; Noble and Shi, 2012).Viral polymerase inhibitors can be classified into two broad categories: nucleoside/nucleotide analog inhibitors (NIs) and non-nucleoside inhibitors (NNIs). NIs, when converted to the corresponding triphosphate, can compete with natural NTP substrates and be incorporated into the growing RNA chain to terminate elongation. Besides RNA chain termination as the main mode of action, some NIs, such as ribavirin, could be incorporated into an RNA chain without termination. When such an RNA chain serves as a template for RNA synthesis, the integrated NIs cause mutations in the daughter RNAs, leading to error catastrophe (De Clercq and Neyts, 2009). NNIs usually bind to allosteric sites in the polymerase; NNIs either "lock" the enzyme into an inactive form or prevent conformational changes required for initiation and/or elongation of RNA synthesis.

NIs represent the largest class of approved antiviral regimens in clinical use. There are clear therapeutic benefits with this class of inhibitors as seen in the treatment of viral diseases such as HIV-1 (Cihlar and Ray, 2010), and HBV infection (De Clercq, 2011). While conceptually easy, there are several key challenges in developing NIs for therapeutics. First, it is often impossible to predict the structure–activity relationship of the compounds, as several enzymatic steps are required to convert them to active triphosphates (Stein and Moore, 2001). This makes it challenging for classical medicinal chemistry approaches to improve potency. Therefore, potent NIs are often discovered serendipitously. Second, the toxicity of nucleoside analogs is unpredictable and is often missed *in vitro*. Although mitochondrial toxicity, such as inhibition of polymerase gamma, has been implicated in most antiviral NIs, especially for those for the treatment of HIV-1 infection, there are other toxicities associated with NIs (Dagan et al., 2002). Thus, animal toxicological studies are often needed to assess the potential side effect of this class of compound, which is often costly in terms of compound and animal requirement. Our experience with NITD-008 (Table 4), an adenosine analog which potently inhibited DENV replication, is a good example (Chen et al., 2010a; Yin et al., 2009b). Treatment of DENV-infected mice with NITD-008 suppressed peak viremia and completely prevented death in a lethal mouse model. No adverse effect was observed when rats were orally dosed with NITD-008 at 50 mg/kg/day for 1 week. However, after 2 weeks of oral dosing, severe side-effects were observed in both rats and dogs. These results led to the termination of NITD-008 for further development for DENV treatment.

There are several advantages which make NIs attractive for drug development. For instance, since NIs target polymerase active sites which are often conserved, it has a high barrier of resistance emergence compared to other classes of inhibitors (Delang et al., 2011). Moreover, NIs usually display equivalent potency against different serotypes/genotypes of a particular virus and even related viruses (Kuntzen et al., 2008). For example, several NIs synthesized by NITD have shown equipotency against all four serotypes of DENV as well as other closely related flaviviruses (Chen et al., 2010a,b; Yin et al., 2009b). Another example is tenofovir, which is effective in both HIV and HBV (Woo et al., 2010). Similarly, we (Yin et al., 2009b) and others (Migliaccio et al., 2003; Nguyen et al., 2013) have observed cross-reactivity of NIs against DENV and HCV.

Balapiravir (Table 4) is the tri-isobutyrate ester prodrug of 4′-azidocytidine (R1479). It was originally developed for HCV treatment by Hoffmann-La Roche. Unfortunately, its clinical development for HCV was terminated due to hematological adverse effects in patients after extended treatment (Nelson et al., 2012). Since R1479 has anti-DENV activity, it was repurposed for a phase II trial for DENV (Nguyen et al., 2013). Dengue patients received balapiravir at doses of 1500 mg ($n = 10$) or 3000 mg ($n = 22$) orally for 5 days. The patients were recruited based on the following criteria: (i) fever with temperature $\geqslant 38\,^{\circ}C$; (ii) a positive NS1 rapid test; (iii) onset of symptoms <48 h prior to initial dosing. Suprisingly, balapiravir did not improve the clinical and virological parameters in patients, including viral levels in the blood, NS1 production, as well as fever duration. The reason for the lack of efficacy remains to be determined. *In vitro* activity of balapiravir against DENV ranged from EC_{50} values of 1.9–11 μM and 1.3–3.2 μM in DENV HuH7 replicon and human PBMC DENV infection assays, respectively. Both doses administered to DENV patients attained plasma drug levels comparable to the EC_{50} values (C_{max} >5.5 and 8.8 μM with 1500 mg and 3000 mg, respectively). It was speculated that perhaps higher plasma levels of the drug are needed to achieve antiviral effects.

Besides the challenges and opportunities described above, the following three categories of parameters make NIs uniquely different from other classes of viral inhibitors.

12.1. Dissolution, absorption, and cellular uptake

NIs are usually quite polar, and thus solubility is usually not an issue for this class of inhibitor, unless large amounts of drug are needed for efficacy. However, a highly polar surface area may not be ideal for cellular uptake; the addition of simple ester (prodrugs) usually improves absorption and exposure (Brandl et al., 2008). Cellular uptake is usually via passive diffusion, which may not be optimal for this class of compounds. In some cases, they pass through the plasma membrane via nucleoside transporters (Cass et al., 1999). The highly polar surface area also makes them unlikely to penetrate the blood–brain barrier. Thus, the use of this class of drugs in viral encephalitis could be limited.

12.2. Metabolism

When dosed as nucleoside analogs, NIs are inactive without transformation into the corresponding triphosphates intracellularly. As a result, their activity can only be evaluated using cellular assays. In addition, due to differences in the expression of enzymes responsible for converting the nucleoside into its active nucleoside triphosphates, they are prone to cell-type variations. It is therefore essential to evaluate these drugs using relevant target cells. For developing DENV NIs, it is also advisable to profile them in physiologically relevant cells, such as hematopoietic cells, where DENV replicates in patients (Noisakran et al., 2010). However, the dependency on cellular activation could be advantageous if the target organ is rich in enzymes which convert the drug into its triphosphate active form. This avenue is indeed employed to target HCV NIs to the liver (McGuigan et al., 2010), or alternatively to use CYP enzymes as the first activation step of a prodrug (Niu et al., 2012).

The conversion of a nucleoside analog into corresponding monophosphate is often the rate-limiting step in nucleoside activation (Stein and Moore, 2001). However, in a few cases, such as zidovudine, the conversion of zidovudine monophosphate into the diphosphate by thymidylate kinase is the rate-limiting step (Lavie et al., 1997). To circumvent the first phosphorylation dependency, newer prodrugs often have pre-attached monophosphates or phosphoramidate/ester which could greatly improve potency (Zhou et al., 2011). The pre-attached monophosphate brings in extra complications, because the monophosphate moiety is very polar, making the final molecule more inefficient to cross plasma membrane. Additional masking groups are needed to protect the monophosphate moiety and to improve plasma membrane penetration; these masking groups have to stay intact throughout the absorption and distribution phase, but should be broken down quickly once the drug reaches the target organ. Since intracellular concentration of modified nucleoside triphosphate is the active inhibitor for viral polymerase, the intracellular level of the triphosphate nucleoside is expected to correlate with the level of viremia reduction. This concept was proved in AZT-treated HIV patients (Fletcher et al., 2000). Along the same line, the pharmacokinetics of the triphosphate nucleoside, rather than plasma exposure of the nucleoside, is a better guide for the dosing regimen (Wang et al., 2004).

12.3. Factors affecting efficacy of the drug

Different cells express a diverse range of enzymes responsible for nucleoside triphosphate conversion; thus, the potency of the nucleoside will not be the same. In addition, factors such as concentration of natural nucleoside triphosphate (Garcia-Lerma et al., 2011) and activation cell state (Gao et al., 1993) can influence the efficacy of NIs as well. A special type of drug-drug interaction occurs when two nucleosides share a common activation pathway (Veal et al., 1997). These factors should be considered in combination when evaluating nucleoside inhibitors.

13. Polymerase non-nucleoside inhibitors

NNIs are typically non-competitive inhibitors. They act by occupying specific pockets in the polymerase, and block conformational changes which are needed for its initiation and/or elongation activities. While there are many good examples of NNIs targeting HIV-1 reverse transcriptase in clinical use (Blas-Garcia et al., 2011), there are as yet no FDA-approved NNIs available for other viral polymerases. Numerous HCV RdRp NNI drug candidates (Sofia et al., 2012) have been tested in clinical settings, but the results, such as for tegobuvir (Gilead; indirect polymerase inhibitor) or filibuvir (Pfizer; thumb II site inhibitor), have been disappointing, as no efficacy was observed as monotherapy (Pockros, 2013). Other compounds, such as setrobuvir (ANA598; palm I inhibitor), BI207127 (thumb I site inhibitor), or VX-222 (thumb II site inhibitor) are currently in phase II trials.

There are several potential drawbacks to the use of NNIs in antiviral therapy. First, the pocket must be well conserved for the compound to be broadly acting across different serotypes or genotypes. Second, the virus may generate amino acid mutations in or near the NNI binding pocket to overcome its inhibitory action. Alternatively, naturally occurring resistant viruses may exist in circulation which are insensitive to a particular NNI, resulting in the expansion of "escape mutants." For these reasons, DENV RdRp NNIs are envisaged to be administered in combination with another drug with a high barrier to resistance (either an NI or another DAA) to prevent the emergence of resistant viruses. On the positive side, highly specific NNIs can translate to lower off-target effects and potentially lower cytotoxicity. This is boosted by the fact that the host has no RdRp protein.

Most first-generation NNIs are often identified through high-throughput screening activities using RdRp biochemical assays. However, during hit-to-lead optimization, obtaining RdRp co-crystal structures with bound inhibitors opens up opportunities to further develop analogs via a structure-guided approach (Melagraki and Afantitis, 2011). Alternatively, strategies such as fragment-based screening may also be considered (Scott et al., 2012). On this note, few allosteric binding pockets are present in the "closed" conformation of the DENV RdRp (Malet et al., 2008; Zou et al., 2011). It remains to be seen if additional pockets would be formed in the "open" conformation upon RNA binding. This information awaits the co-crystal structure of the DENV RdRp–RNA complex.

Since one of the major issues related to NNIs is the likelihood of emergence of resistant viruses, understanding the mode of action and improving the binding mechanism would help to reduce this problem. NNIs that have strong inhibitory effects on enzyme catalysis, as well as high binding affinity with good kinetic profiles (e.g., fast K_{on}, slow K_{off}, and a long residence time) will make better candidates (Winquist et al., 2013).

NITD conducted two HTS campaigns, both screening about 1.8 million compounds, using biochemical assays that measured DENV polymerase elongation activities. The first HTS used an SPR RdRp elongation assay. We identified one compound class (e.g., NITD-2; Table 4) that bound in the RNA tunnel of the RdRp, and likely functions by blocking RNA synthesis (Niyomrattanakit et al., 2010b). Although good SAR was observed from analogs tested in the elongation SPA assay (Yin et al., 2009a), the series could not be advanced due to poor cellular permeability. The second HTS utilized a fluorescent-coupled elongation assay (Niyomrattanakit et al., 2010a). Unfortunately, many of the hits identified from this HTS failed to exhibit binding specificity to DENV RdRp. A few hits that inhibited the enzyme were exemplified by NITD-107 (Table 4; (Noble et al., 2013)). Although NITD-107 binds in the DENV RdRp active site (Fig. 6), it showed only weak inhibitory activity and a flat SAR during chemistry campaign (unpublished). In both HTS, there was a substantial number of false positives which acted by aggregating the protein or interfering with the assay readout. One possible reason for this finding was the poor stability of the recombinant DENV polymerase, making the enzyme sensitive to nonspecific inhibition. Alternatively, the fraction of catalytically active DENV RdRp in the total protein population may be very low, making it susceptible to this problem. Indeed, for HCV, the proportion of active form in the recombinant protein has been reported to be <1% (Carroll et al., 2000). In addition, since the polymerase requires divalent ions to function, the assay is sensitive to inhibition by metal chelators. Improving the stability or specific activity of recombinant DENV RdRp would certainly help to overcome these challenges.

14. Host target inhibitors

Targeting host factors required for the virus life cycle, instead of virus-encoded proteins, opens new perspectives to counteract viral infections and is being applied to different families of viruses (Schwegmann and Brombacher, 2008; Tan et al., 2007). For example, there are currently a few phase I and II HCV clinical trials in which the compounds target host proteins, such as cyclophilin, TLR (agonist), histone deactylase and caspases. This approach offers the advantage of a significantly higher barrier to the emergence of resistance, compared with DAA, since viral mutations are less able to compensate for the loss of an essential host cofactor. Furthermore, different viruses may share a host

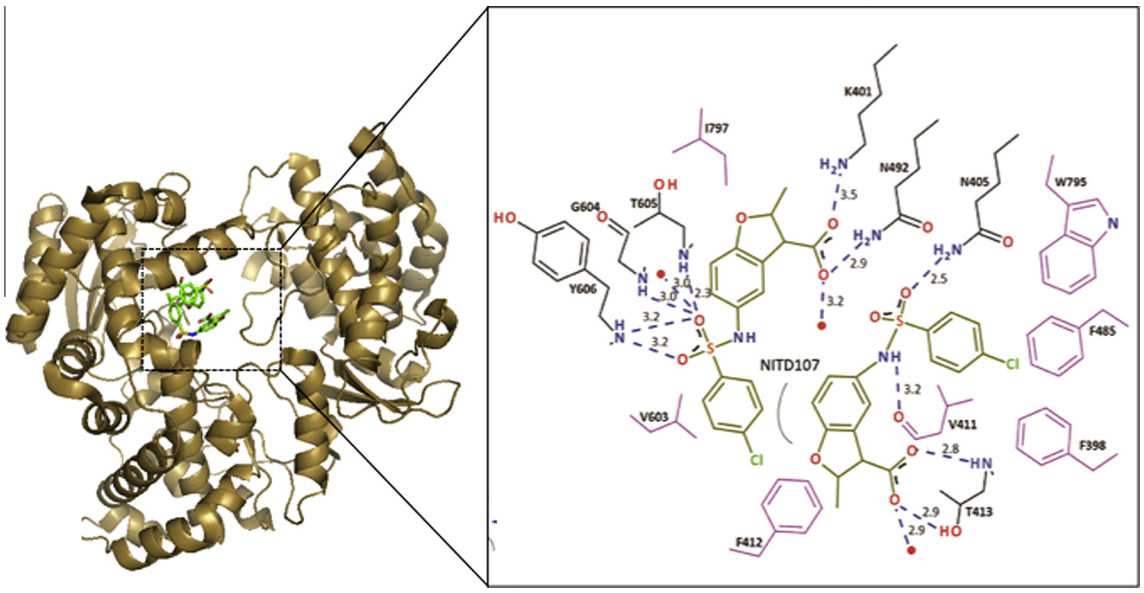
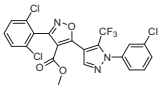
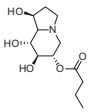


Fig. 6. Crystal structure of DENV RdRp bound to two molecules of NITD-107 (Noble et al., 2013). The protein is shown as a cartoon and the compound molecules as sticks. Boxed section shows the map of amino acid interaction between RdRp and NITD107 dimer. Interactions between RdRp and NITD107 dimer. Interatomic distances between 2.5 to 3.2 Å are depicted.

Table 5
Inhibitors of host targets (see details in text).

Compound	Structure	Mode of action	Cellular activity (EC_{50}/CC_{50}) μM	AG129 dengue mouse activity	References
NITD-451		Translation inhibitor	0.16/57	Efficacious	Wang et al. (2011b)
NITD-982		Inhibit pyrimidine biosynthesis through DHODH	0.0024/>5	Not efficacious	Wang et al. (2011a)
Celgosivir		Alpha-glucosidase	0.22/>100	Efficacious	Rathore et al. (2011), Schul et al. (2007), Whitby et al. (2005)

factor/pathway for replication, so that compounds targeting the shared host factor/pathway could have pan-antiviral activities.

The drawback of targeting host factors is the higher potential for undesirable drug-induced side effects, because these factors are often essential for cell survival or metabolism. However, this approach would encounter fewer hurdles in the treatment of acute diseases, such as dengue, compared with the treatment of chronic diseases like HIV-1 or HCV infection (Schang, 2006). Several host pathways have been shown to be important for DENV replication, including the ubiquitin–proteasome system (Fink et al., 2007; Kanlaya et al., 2010), the unfolded protein response (Umareddy et al., 2007; Sessions et al., 2009) and lipid metabolism (reviewed in Fischl and Bartenschlager, 2011; see also below).

14.1. Viral translation inhibtors

Screening of the Novartis compound library using a DENV-induced cytopathic (CPE) assay led to the identification of a class of compounds with a benzomorphane core as inhibitors of DENV (Wang et al., 2011b). The initial "hit" NITD-2636 exhibited 76% cell protection at 5 μM. NITD-2636 displayed a broad spectrum of antiviral activity. It not only reduced the titer of DENV, but also the titers of other flaviviruses, including YFV and WNV. However, the compound did not efficiently suppress non-flaviviruses such as the alphavirus WEEV or VSV. Synthetic chemistry efforts were carried out using NITD-2636 as a starting point to explore the structure–activity-relationship (SAR), with the aim of improving metabolic stability. Substitution of the metabolic-labile phenolic-OH group with an electron-withdrawing cyano group (NITD-451; Table 5) retained antiviral activity while improving stability.

The mechanism of action of NITD-451 (Table 5), was elucidated using a DENV luciferase-reporting replicon system. NITD-451 suppressed DENV driven luciferase activity as early as 2 h post-transfection, indicating its inhibitory role in RNA translation. Its mode of action was further validated with a cell-free translation system, in which *in vitro* translation of a reporter RNA was directly suppressed by the compound. As DENV uses host machinery for translating its genome, NITD-451 targets a host factor involved in translation. The identity of this factor remains to be determined. The interaction between the compound and its target is specific, as only the S,R,S enantiomer NITD-451 was active in inhibiting DENV replication and RNA translation, whereas the R,S,R

enantiomer NITD-452 was inactive in both assays. *In vivo* efficacy experiments showed that treatment of DENV-infected mice with 25 mg/kg of NITD-451 reduced peak viremia by about 40-fold, without any obvious adverse effects. However, mice treated with 75 mg/kg of compound per day exhibited adverse effects, thus terminating the progression of this class of compound.

Similar to the DENV translation inhibitor, Noueiry et al. (2007) identified compound AP30451that selectively inhibits flavivirus RNA translation. The compound was identified through library screening, using a WNV replicon assay (Noueiry et al., 2007).

14.2. Host pyrimidine biosynthesis inhibitors

In the CPE-based HTS campaign mentioned above, another interesting class of compounds with an isoxazole-pyrazole core was identified (Wang et al., 2011a). One such compound, NITD-982 (Table 5), displayed nanomolar potency against four different RNA virus families, including the *Flaviviridae*, *Paramyxoviridae*, *Orthomyxoviridae*, and *Retroviridae*. The broad-spectrum activity of this compound strongly indicated that a cellular target was involved in its mechanism of action.

To identify the cellular target(s) of NITD-982 (Table 5), a three-channel iTRAQ quantitative chemical proteomics technology was implemented (Bonavia et al., 2011). NITD-102, a bioactive analog of NITD-982, was synthesized and immobilized to a column to capture target protein from virus-infected cell lysates. The protein identified was dihydroorotate dehydrogenase (DHODH), which is a mitochondrial protein that catalyzes the oxidation of dihydroorotate to orotate, the fourth enzymatic step of the *de novo* pyrimidine biosynthesis. An integral approach of biochemical, biophysical, and pharmacological techniques was used for target validation. The results showed that

(i) The compound inhibits the enzymatic activity of the recombinant DHODH;
(ii) an analog of NITD-982 directly binds to the recombinant DHODH;
(iii) the compound-mediated inhibition of viral replication could be reversed by supplementing culture medium with uridine; and
(iv) DENV-2 variants resistant to Brequinar (a known DHODH inhibitor) are cross-resistant to NITD-982.

Collectively, the results indicate that NITD-982 directly inhibits DHODH activity to deplete intracellular pyrimidine pools, leading to the suppression of viral RNA synthesis. However, its *in vitro* efficacy did not translate into *in vivo* efficacy. This lack of antiviral efficacy *in vivo* could be attributed to the uridine uptake from diets that replenish or maintain a high concentration of pyrimidine in the plasma, therefore counteracting compound-mediated inhibition of viral replication.

14.3. Host cholesterol synthesis pathway inhibitors

Cholesterol biosysnthesis and transport have been implicated for the replication of several flaviviruses, including DENV. Decreased levels of plasma cholesterol, lipoproteins, and triglycerides are associated with the severity of dengue disease (Villar-Centeno et al., 2008; van Gorp et al., 2002). Lee et al. (2008) found that disruption of lipid raft formation by cholesterol depletion with methyl-beta-cyclodextrin or cholesterol chelation with filipin III reduced JEV and DEN-2 infection, mainly at intracellular replication steps and, to a lesser extent, at viral entry. DENV infection was also impaired when cholesterol intake in infected cells was disrupted using a cholesterol transport inhibitor, U18666A (Poh et al., 2009). The compound negatively affected viral trafficking in the cholesterol-loaded late endosomes/lysosomes, as well as *de novo* sterol biosynthesis. An additive antiviral effect of U18666A with C75, a fatty acid synthase inhibitor, was observed, suggesting that DENV relies on both cholesterol and fatty acid biosynthesis for its successful replication. In addition, hymeglusin and zaragozic acid A (inhibitors of HMG-CoA synthase and squalene synthase, respectively) inhibited DENV2 NGC replication in K562 cells, while lovastatin (HMG-CoA reductase inhibitor) reduced its replication in human peripheral blood mononuclear cells (Rothwell et al., 2009).

14.4. Cellular glucosidase inhibitor: Celgosivir

Celgosivir (butyl-castanospermine; Table 5) is an oral prodrug of the natural product castanospermine that inhibits alpha-glucosidase I, an enzyme that plays a critical role in viral maturation by initiating the processing of N-linked oligosaccharides of viral envelope glycoproteins. It was tested in a phase II clinical trials in combination with peglated IFN and ribavirin for treatment of HCV infection. It was not further pursued, as it was not better than the standard drug regimen (Durantel, 2009).

Several studies have indicated that castanospermine and Celgosivir also inhibit DENV replication, disrupting the folding of the DENV structural proteins prM and E (Whitby et al., 2005) as well as NS1 (Rathore et al., 2011). Intraperitoneal injection of castanospermine prevented mortality in an intracranial lethal challenge mouse model (Whitby et al., 2005) and significantly reduced plasma virus levels in a viremia mouse model, when dosed orally at 75 mg/kg twice a day (Schul et al., 2007). Subsequent testing of Celgosivir also indicated good efficacy in a lethal ADE dengue infection mouse model. Twice-a-day dosing at 50 mg/kg via intraperitoneal injection for 5 days completely protected all mice from death (Rathore et al., 2011). Treatment with 25 mg/kg BID of Celgosivir was comparable to 50 mg/kg BID of castanospermine, suggesting that the prodrug was twice as efficacious, very likely due to its better cellular permeability (Watanabe et al., 2012).

Based on these encouraging findings, a phase Ib clinical trial is currently ongoing to evaluate the activity, pharmacokinetics, safety, and tolerability of Celgosivir in dengue patients. Using PK simulations, it was anticipated that 200 mg BID in humans would give rise to a similar steady-state C_{min} as 50 mg/kg BID in mice (Watanabe et al., 2012). The efficacy results of Celgosivir in dengue patients will impact on the development of other alpha-glucosidase inhibitors. Besides Celgosivir, other iminosugar analogs targeting the host alpha-glucosidase have been shown to be efficacious against DENV, both in cell culture and the AG129 mouse model (Miller et al., 2012; Yu et al., 2012).

A previous trial in HIV-1 patients identified grade III toxicity in transaminase, creatine kinase (with normal CkMB isoenzyme) and LDH after 14 days of oral dosing at 300 mg BID; similar toxicity was observed after 14 days of oral dosing at 450 mg QD (Roth et al., 1996). Consequently, the dosing regime for dengue patients comprised a loading dose of 400 mg, followed by 200 mg BID for a total of 5 days. The inclusion criteria are similar to the balapriravir trial conducted on DENV patients (Nguyen et al., 2013).

14.5. Severe dengue disease inhibitors

Patients with dengue exhibit a variety of serious complications, including plasma leakage, hypovolemic shock, thrombocytopenia and bleeding. These occur as plasma viremia is resolving, and are consequently thought to be virus-driven immunological responses (Libraty et al., 2002a,b). Inhibitors of immunopathological pathways could broaden the window of treatment time, especially for treatment of patients in whom the viremia duration is short. Unfortunately, the molecular details of the host pathways that lead

to severe dengue (DHF/DSS) are not well defined; the feasibility of such an approach therefore remains low at this time. However, this approach was recently explored by two studies.

Tam and co-workers (2012) conducted corticosteroid therapy in early infected dengue patients. Corticosteriods are potent inhibitors of inflammatory processes and suppress the production of cytokines, chemokines, arachidonic acid metabolites and adhesion molecules. No improvement of clinical and virological parameters was observed in the corticosteroid-treated patients, compared to the placebo control group. St John et al. (2013) reported that treatment of DENV-infected mice with mast cell-stabilizing drugs or a leukotriene receptor antagonist restored vascular integrity, suggesting that these compounds could be evaluated for their effectiveness in improving disease outcomes in viral hemorrhagic fevers. However, the drug-treated animals exhibited a slight increase of viremia; this phenomenon should be carefully monitored during further development.

15. Conclusions and perspectives

Public–private partnership is an attractive model to raise resources for combating neglected diseases. Building on this model, NITD has made significant contributions to dengue biology and drug discovery, through fruitful collaborations with research institutes in Singapore and leveraging expertise available in Novartis research entities. Studies in the collaborative EDEN program generated insights into dengue diagnosis, epidemiology and pathology, through detailed clinical analyses of dengue patients (Christenbury et al., 2010; Schreiber et al., 2009; Tanner et al., 2008; Fink et al., 2007). In collaboration Julien Lescar's group, the NITD contributed to the first crystal structures of the DENV helicase (Xu et al., 2005), full-length NS3 (Luo et al., 2008a,b), and RdRp (Yap et al., 2007). The first catalytically active flavivirus protease structure (represented by WNV protease) was solved together with Novartis colleagues in Basel (Erbel et al., 2006). These structures, together with the structures of other DENV proteins or the same proteins of other flaviviruses solved by various academic groups (Egloff et al., 2002; Li et al., 2008; Ma et al., 2004b; Malet et al., 2007; Modis et al., 2003, 2004; Rey et al., 1995; Wu et al., 2005) have tremendously enabled DENV drug discovery.

As the only institute with a dedicated mission to develop dengue antivirals, the NITD has taken a concerted approach for dengue drug discovery. Although the effort has not yet generated a clinical candidate, the experience accumulated during the past decade has provided a better rationale for the on-going anti-DENV effort. Compared with the resources that have been invested in HCV antiviral development during the past 25 years, those spent on dengue have been much smaller.

Two general strategies can be pursued for any antiviral therapy. The first is to inhibit viral targets; such an approach has generated most of the DAAs currently in clinical use. The second is to inhibit host targets. Two types of host targets could be pursued: (i) host factors that are essential for viral replication. As exemplified by the pyrimidine biosynthesis inhibitor, targeting host factors has extra unknown hurdles, compared with targeting viral factors. (ii) Host targets that participate in the development of disease symptoms. As mentioned before, inhibitors of pathological pathways could broaden the window of treatment time; this is especially critical for the successful treatment of dengue patients, in whom the duration of viremia is short. Unfortunately, the molecular details of host pathways that lead to DHF/DSS are not well defined; therefore, the feasibility of such an approach remains low at this time. However, targeting host factors has the advantage of a high resistance barrier, as exemplified by HCV compounds that inhibit cellular cyclophilin (Puyang et al., 2010).

One bottleneck for dengue drug discovery is knowledge about sites of viral replication in patients, which is critical for designing compounds that can selectively deposit to replication sites. It is generally accepted that the major replication sites during DENV viremia are monocytes, macrophages, and dendritic cells (Durbin et al., 2008; Rothman, 2011). However, autopsies of a few dengue patients detected viral antigen in hepatocytes (Rosen et al., 1999) and Kupffer cells (Jessie et al., 2004). It remains to be determined whether the postmortem autopsy results are relevant to the current antiviral rationale of suppressing viremia during early infection to achieve clinical benefits. Therefore, for DAA to be effective, they should be able to target sites where early virus replication occurs.

Since the amino acids homology among the four serotypes of DENV is about 70% (Lindenbach et al., 2007), it could be challenging to design a compound that potently inhibits all of them. This goal can be achieved by nucleoside inhibitors, because they interact with the flavivirus-conserved active site of the RdRp during RNA synthesis. For the same reason, a nucleoside analog that is active against DENV is likely to be active against other flaviviruses. For other types of DAA, the antiviral spectrum against all DENV serotypes should be closely monitored during hit characterization. In analogy to HCV antiviral therapy, developing a compound efficacious against all four serotypes of DENV is as challenging as the development of a compound that is active against all six genotypes of HCV (with amino acid variation of about 30%).

In summary, great progress has been made in DENV biology and antiviral drug discovery in the past decade. Many lessons have been learned from various approaches. Though challenging, we are optimistic that the continuing effort will lead to an effective anti-DENV therapy in the near future.

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