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Review

Strategies for development of dengue virus inhibitors

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ABSTRACT

Antiviral drug discovery is becoming increasingly important due to the global threat of viral disease pandemics. Many members of the genus *Flavivirus* are significant human pathogens, among which dengue virus (DENV) alone poses a public health threat to 2.5 billion worldwide, leading to 50–100 million human infections each year. Neither vaccine nor effective therapeutics is currently available for DENV. Development of a DENV vaccine has been challenging, because of the need to simultaneously immunize and induce a long-lasting protection against all four serotypes of DENV; an incompletely immunized individual may be sensitized to life-threatening dengue hemorrhagic fever or dengue shock syndrome. The challenges associated with vaccine development have underscored the importance of development of antiviral therapies for DENV and other flaviviruses. Here we review the strategies to identify inhibitors for DENV therapy. Both viral and host proteins essential for viral replication cycle are potential targets for antiviral development. Inhibitors could be identified by multiple approaches, including enzyme-based screening, viral replication-based screening, structure-based rational design, virtual screening, and fragment-based screening. The strategies discussed in this report should be applicable to antiviral development of other viruses.

Contents

Abbreviations: DENV, dengue virus; WNV, West Nile virus; YFV, yellow fever virus; HCV, hepatitis C virus; HTS, high-throughput screening; RdRp, RNA-dependent RNA polymerase; HIV, human immunodeficiency virus; HSV, herpes simplex virus; UTR, untranslated region; SAR, structure–activity relationship; ER, endoplasmic reticulum; DHF/DSS, dengue hemorrhagic fever/dengue shock syndrome; SPA, scintillation proximity assay; NOAEL, no observed adverse effect level.

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1. Introduction

Family *Flaviviridae* consist of three genera, *Flavivirus*, *Pestivirus*, and *Hepacivirus*. The genus *Flavivirus* consists of more than 70 viruses, many of which are arthropod-borne and cause human diseases including fevers, encephalitis, and hemorrhagic fevers (Gubler et al., 2007). Pathogenic flaviviruses include the four serotypes of dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV). No clinically approved antiviral therapy is currently available for treatment of flavivirus infections. Human vaccines are available only for YFV, JEV, and TBEV. It is therefore a priority of public health to develop therapeutics for flavivirus infections.

Flaviviruses are small enveloped viruses about 50 nm in diameter, containing a single positive sense RNA that is approximately 11 kb in length. The viral genome encodes three structural proteins (capsid [C], premembrane [PrM], and envelope [E] proteins) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5; Fig. 1). The structural proteins form the viral particle. The non-structural proteins participate in the replication of the RNA genome, virion assembly (Kummerer and Rice, 2002; Liu et al., 2003), and invasion of innate immune response (Guo et al., 2005; Liu et al., 2005; Munoz-Jordan et al., 2003, 2005). Of these, only NS3 and NS5 have known enzymatic activities, making them ideal antiviral targets, since the enzymatic activity can be used to develop an assay for HTS campaigns. The N-terminal domain of NS3, together with NS2B, contains a serine protease activity; the C-terminal domain functions as an RNA helicase, an RNA triphosphatase, and an NTPase (Falgout et al., 1993; Wengler and Wengler, 1991, 1993). The N-terminal domain of NS5 contains a methyltransferase activity; the C-terminal domain serves as an RNA-dependent RNA polymerase (RdRp) (Ackermann and Padmanabhan, 2001; Egloff et al., 2002; Ray et al., 2006; Tan et al., 1996). Other non-structural proteins are required for RNA replication, among which NS2A, NS2B, NS4A, and NS4B are transmembrane proteins that form the scaffold for the viral replication complex (Lindenbach and Rice, 1997; Miller et al., 2006, 2007). Although the exact topology of the replication complex remains to be determined, the non-structural proteins without known enzymatic activity are valid antiviral targets.

Many aspects of the flavivirus replication and pathogenesis have been recently reviewed (Dong et al., 2008; Kroschewski et al., 2008; Malet et al., 2008; Perera et al., 2008; Xu et al., 2005). Here we concentrate on antiviral approaches and methodologies, using DENV as an example.

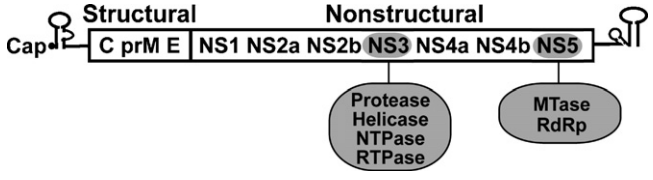

Fig. 1. Schematic of the flavivirus genome. The flavivirus genome encodes three structural proteins that form the virion and seven non-structural proteins, many of which are required for viral replication. Some other known properties of the proteins are indicated.

2. Viral target-based approach

2.1. Protease NS2B/NS3

Viral proteases are a proven antiviral targets. There are currently nine HIV-1 protease inhibitors in clinical use (Menéndez-Arias, 2010) and a few HCV protease inhibitors in various stages of clinical trials (Soriano et al., 2008). DENV protease domain consists of the first 170 amino acids of NS3, with an amino acid homology of >50% among various members of the genus *Flavivirus* (Valle and Falgout, 1998). Like other flaviviruses, DENV protease is a serine protease with a catalytic triad (His51, Asp75, and Ser135) and belongs to the trypsin superfamily (Bazan and Fletterick, 1989). The protease activity is dependent on association with a hydrophilic region of 40 amino acids of the NS2B protein, which acts as a cofactor and actively participates in the formation of the S2 and S3 sub-pockets in the protease active site (Erbel et al., 2006). Both viral and host proteases are required for viral polyprotein processing, which is essential for viral replication and virion assembly. NS2B/NS3pro prefers a substrate with basic residues (Arg or Lys) at the P1 and P2 sites and a short side-chain amino acid (Gly, Ser, or Ala) at the P1′ site (Chambers et al., 1990; Preugschat et al., 1990); the protease cleaves at the P2P1↓P1′ site.

Two main approaches have been taken to develop protease inhibitors. One is through HTS of small molecule libraries, and the other is by designing peptidomimetics, which mimic the natural catalytic substrate. The active site of the NS2B–NS3 protease is relatively flat (Erbel et al., 2006), making it hard to design potent inhibitors by structure-based design (Fig. 2). Because the preferred peptide substrate contains several positively charged amino acids in the non-prime side of the active site, the active site of the protease is negatively charged. Since charged molecules are usually not orally bioavailable, this makes it difficult to design peptidomimetics against dengue protease that are potent inhibitors *in vivo*. The fact that the active site is flat and charged also makes it difficult to find small-molecule inhibitors of the NS2B/NS3 protease. Nevertheless, both HTS and peptidomimetic approaches have been used with varying degrees of success (Leung et al., 2001; Mueller et al., 2008). For HCV, the most advanced clinical candidates are peptidomimetics (Soriano et al., 2008). The major problem of this approach is that the chemical synthesis of peptidomimetics is challenging and time-consuming, greatly increasing the development cost.

For *in vitro* HTS, recombinant NS2B/NS3 protease is readily produced in an *Escherichia coli* expression system. The initial NS2B/NS3 protease construct comprised the minimal NS2B core sequence (residues 53–92) fused in-frame to NS3 protease (residues 1–185) via the C-terminal 11 amino acids of NS2B. Expression of such a construct yielded insoluble protein in inclusion bodies that could be refolded into active NS2B/NS3 protease (Yusof et al., 2000). The solubility problem during NS2B/NS3 protease expression could be overcome by lowering the culture temperature to 30 °C (Leung et

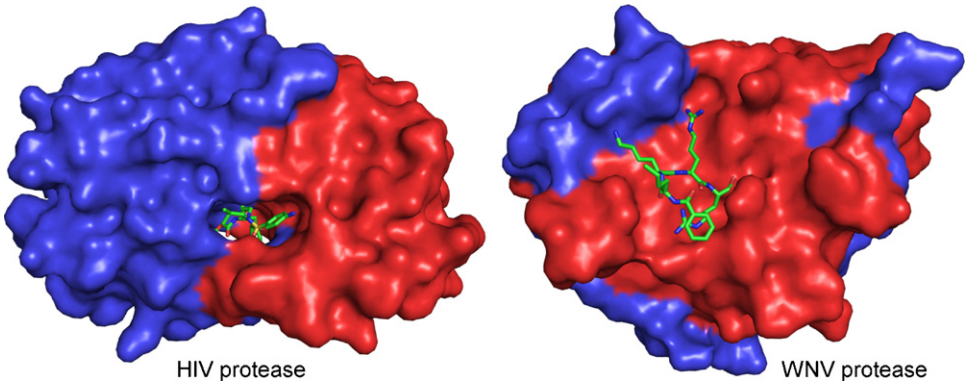

Fig. 2. Comparison of the HIV and WNV protease active sites. The structures of the HIV protease dimer (3EKV), bound to Amprenavir® and of the WNV bound to a peptide inhibitor (2FP7; Erbel et al., 2006) are shown. The flavivirus protease active site is very flat, making it difficult to inhibit with small molecules.

al., 2001). Another problem of the initial NS2B/NS3 protease construct is the auto-cleavage at the NS2B/NS3 junction; this problem was solved by replacing the C-terminal 11 amino acids of NS2B with a $Gly_4SerGly4$ linker (Leung et al., 2001). The availability of these active recombinant constructs enabled a detailed examination of the interaction between the peptide substrate with NS2B/NS3 and the interaction between NS3 and NS2B (Iempridee et al., 2008; Marcon et al., 2008; Prusis et al., 2008; Zuo et al., 2009). They also formed the basis for development of HTS-compatible assays for DENV and other flavivirus proteases for large-scale screening of compound libraries. These assays typically used a peptide substrate that contained dibasic residues at the P1 and P2 positions fused with a fluorophore such as 7-amino-methyl-coumarin (AMC) or p-nitrophenyl (pNA) at the C-terminus. Cleavage by the protease results in an increase in fluorescence due to release of the fluorophore, which can be read on a plate reader or, in the case of pNA, results in a yellow color which can be read by colorimetry (Mueller et al., 2007, 2008).

The optimal activity of DENV NS2B/NS3 protease requires a high pH of 9.0 and up to 20% glycerol (Leung et al., 2001). These two properties make the protease assay less suitable for large-scale compound screening. A high pH can lead to protonation of some classes of compounds, resulting in false-positive or false-negative hits. Furthermore, robotic liquid-handling platforms are often not tolerant of high glycerol content because the higher viscosity increases the error rate from pipetting small volumes into 1536-well plates. Hence, for large-scale compound testing, the protease assay must be run at suboptimal catalytic efficiency which could affect the screen outcome. Besides the suboptimal condition, another important issue is the NS2B/NS3 construct used for the HTS. To date, the soluble NS2B/NS3 construct (described above) has been used for screening, since the presence or absence of the C-terminal domain of NS3 has no effect on the protease activity *in vitro* (Lim et al., unpublished data). However, the activity of this enzyme may not be representative of the NS2B/NS3 protease activity *in vivo* as the rest of NS2B, comprising the three transmembrane helices that anchor active NS2B/NS3 protease to the ER, is absent. This means that the natural environment for this enzyme is different from the artificial assay created *in vitro* and may explain why additives (such as glycerol or gelatin) enhance its catalytic activity *in vitro*. An alternative assay format would be to use full-length NS2B–NS3 in an assay with artificial membranes, but this is not amenable to HTS.

Classical inhibitors of serine proteases are ineffective or have low potency against the dengue NS3 protease. One exception is aprotinin, which envelops the enzyme and prevents the substrate from accessing the protease active site (Aleshin et al., 2007). Despite efforts to find inhibitors for different flavivirus proteases (WNV, DENV and YFV) using different strategies such as diverse library screens, peptidomimetics, and virtual screens, no compounds to date have been shown to have appropriate properties for further drug development. This is either because the scaffold is too labile (Erbel et al., 2006; Shiryaev et al., 2006) or because the inhibitors bind to the enzyme too weakly (Ekonomiuk et al., 2009; Mueller et al., 2007, 2008).

Initial synthetic peptide substrates for *in vitro* assays displayed relatively low catalytic efficiency. The tri-peptide substrates, Boc-Gly-Arg-Arg and Boc-Gln-Arg-Arg, showed slow turnover rates of 172 and 107 $M^{-1}\,s^{-1}$, respectively, with DENV-2 NS2B/3 protease (Yusof et al., 2000). Similarly, hexapeptides comprising the P1–P6 sites of endogenous dengue protease cleavage sites (NS2A/B, 2B/3, 3/4A, 4B/5) were poorly catalyzed (k_{cat}/K_m values ranged from 72 to 275 $M^{-1}\,s^{-1}$) (Leung et al., 2001). By using positional scanning tetrapeptide libraries, we identified peptide substrates with optimal amino acid residues in the P1–P4 and P1′–P4′ sites for DENV1–4 (Li et al., 2005). A high-affinity non-prime substrate, Bz-Nle-Lys-Arg-Arg (K_m = 5.88 μM) was obtained; the substrate was efficiently turned over by DEN1–4 NS2B/NS3 proteases (k_{cat}/K_m ranged from 52,000 to 380,000 $M^{-1}\,s^{-1}$). Furthermore, optimal P1′–P4′ sites were shown to have the preference Ser > Gly-Asp/Glu/Gly-Ser-Gly/X. This high-affinity substrate led to the development of a robust *in vitro* assay to measure dengue protease activity (Li et al., 2005).

More recently, various groups have used internally quenched fluorogenic octapeptides spanning the P1–P4 and P1′–P4′ sites as substrates for dengue protease. These substrates are likely to be better mimics of the natural polyprotein substrate. Lower K_m and higher k_{cat}/K_m values were obtained with peptide substrates encompassing the natural cleavage junctions of the non-structural proteins compared to peptides containing only the non-prime positions (Gouvea et al., 2007). The most active substrate spanned the NS4B/NS5 cleavage junction (TTSTRR↓GTGNIGQ) and gave a k_{cat}/K_m value of 1500 $M^{-1}\,s^{-1}$ for the octapeptide substrate; in contrast, the P1–P6 hexapeptide substrate yielded only 275 $M^{-1}\,s^{-1}$ (Gouvea et al., 2007; Leung et al., 2001). In addition, by examining the hydrolysis of different lengths of peptide substrates, it was shown that the optimal peptide spanned residues P4–P3′ (Niyomrattanakit et al., 2006). The peptide Abz-RRRR↓SAGnY-amide, based on the processing site in the DENV capsid protein was an efficient substrate (k_{cat}/K_m = 11,087 $M^{-1}\,s^{-1}$). Interestingly, the presence of basic residues in the prime sites (P2′ and P5′) also increased the turnover rate of the substrate, with the peptide SAAQRR↓GRIGRLQ having a k_{cat}/K_m of 35,900 $M^{-1}\,s^{-1}$, but addition of Arg in the P1′ site prevented cleavage (Gouvea et al., 2007). It will be interesting to see whether screening with these newly identified octapeptide substrates will produce more promising chemical scaffolds for lead discovery.

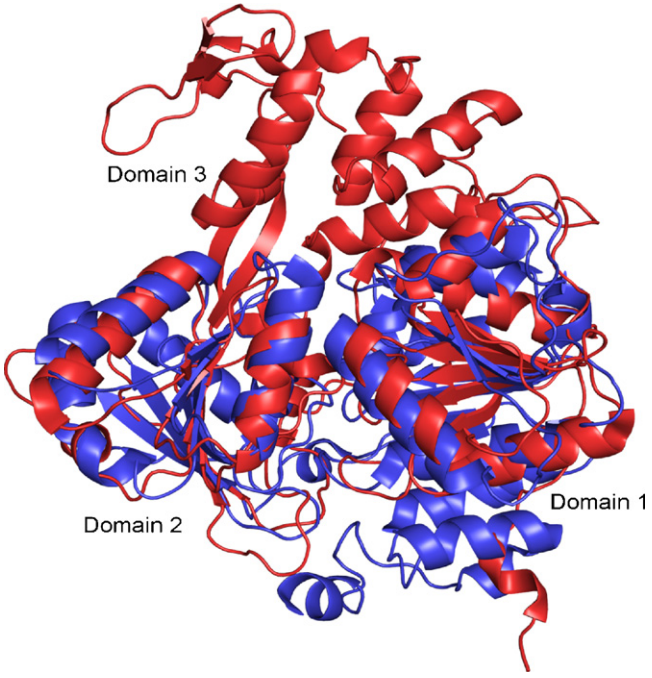

Fig. 3. Alignment of the structures of the dengue helicase (in blue; Xu et al., 2005) and human DDX19B (red; von Moeller et al., 2009), showing that both helicases contain the RecA-like folds (domains 1 and 2), but that only the dengue helicase contains domain 3, indicating that compounds that inhibit the viral helicase are likely to be specific to the this enzyme. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

2.2. Helicase

RNA helicases are abundant in nature, and are essential for all processes of RNA metabolism (Markland et al., 2000; Pyle, 2008). Although these enzymes are called helicases, many of them are not thought to perform RNA-duplex unwinding *in vivo*, but may have different functions, such as single-strand RNA translocation, strand annealing, or protein remodeling (Cordin et al., 2006). Since helicases are essential for all RNA metabolism and often perform very specific functions, many human viruses encode their own helicases, including flaviviruses. All helicases share conserved motifs; however, the helicases from HCV and flavivirus contain an additional domain (domain 3) compared to human helicases such as DDX19B (von Moeller et al., 2009), raising the possibility of developing inhibitors that can specifically inhibit viral helicases (Fig. 3).

Structural and functional studies suggest that targeting DENV helicase for drug discovery is challenging. Crystal structures of DENV NS3 show that the helicase domain does not have any pockets that are likely to bind a small-molecular inhibitor (Luo et al., 2008; Mancini et al., 2007; Wu et al., 2005a; Xu et al., 2005; Yamashita et al., 2008). The ATP-binding pocket in the helicase structure is shallow (with no specificity for ATP over other nucleotides) and polar, making it unlikely to bind a small drug-like molecule. However, it has recently been shown in WNV that NS4A inhibits the ATPase activity of NS3, without affecting its helicase activity (Shiryaev et al., 2009). This raises the possibility of NS4A forming part of the ATP-binding site and therefore making this site more likely to bind a drug-like molecule.

Functional analysis showed poor unwinding activity of recombinant NS3 of DENV *in vitro*. The helicase assays require artificially high enzyme concentrations to detect sufficient activity for HTS (Boguszewska-Chachulska et al., 2004; Kyono et al., 1998). Two types of helicase assays have been reported. One assay uses a fluorescence resonance energy transfer (FRET)-based format with a fluorophore on one RNA strand and a quencher on the other strand (Boguszewska-Chachulska et al., 2004; Earnshaw et al., 1999). When the duplex is unwound, the fluorescence from the fluorophore increases, leading to a signal increase. One problem of this assay is that compounds that naturally quench the fluorophore may also interfere with the signal, resulting in a false positive. Another helicase assay uses a scintillation proximity assay (SPA)-based method (Kyono et al., 1998). One RNA strand is tritiated, whilst the complementary strand is biotinylated. The RNA duplex is attached to the streptavidin SPA beads. If the helicase unwinds the duplex, the tritiated strand is no longer in close proximity to the scintillation beads, leading to a decrease in the scintillation signal. For both assay types, the weak unwinding activity of flavivirus helicase makes it difficult to have a successful HTS.

Despite a significant effort on the HCV helicase, there are still no marketed antivirals that target this enzyme. The majority of leads identified from the HCV helicase HTS were low potency inhibitors of RNA binding (Maga et al., 2005). Similar to the DENV helicase structure, the HCV helicase has little specificity for binding to the base in the ATP-binding pocket, explaining why no high-affinity inhibitors of ATP hydrolysis have been identified. More recently, structure-based design approaches have identified inhibitors with *in vitro* activities against HCV helicase (Kandil et al., 2009; Maga et al., 2005). In contrast, inhibitors of HSV DNA helicase have been identified through HTS (Sivaraja et al., 1998), and have shown promise in pre-clinical experiments in animal models (Betz et al., 2002; Biswas et al., 2007). It should be noted that the helicase of HSV is a large trimeric complex of UL5, UL8, and UL52 with both helicase and primase activities, making it a more suitable drug target. In HIV, the virus does not encode its own helicase, but requires human helicases DDX1 and DDX3 for viral replication (Yedavalli et al., 2004). Inhibitors of the human helicase DDX3 were shown to have anti-HIV efficacy in both cell culture and animal models (Yedavalli et al., 2008). However, with a chronic disease such as HIV, the long-term effect of inhibiting a host enzyme on patients needs to be carefully monitored.

2.3. RNA-dependent RNA polymerases

Viral polymerase inhibitors can be classified into two main categories, nucleoside analogs and non-nucleosides, with the majority of marketed drugs being nucleoside analogs.

2.3.1. Nucleoside inhibitors

Most antiviral nucleoside/nucleotide inhibitors are prodrugs which have to be converted into their corresponding triphosphates to serve as substrates for the viral polymerases (Carroll and Olsen, 2006). A prodrug is any pharmacologically inactive compound that becomes active by spontaneous or enzymatic transformation. For this reason, nucleoside inhibitors are evaluated using cellular assays (see below) rather than *in vitro* enzyme assays. For anti-DENV nucleoside analogs, all phosphorylation events must be carried out by host kinases to form the corresponding triphosphate analogs. The nucleoside analog triphosphate then acts as a substrate mimic and chain terminator upon incorporation into the viral genome. Thus, a potent and selective nucleoside analog must first be recognized by the host nucleoside/nucleotide kinases, but it must also specifically inhibit the viral polymerase over any of the human polymerases.

There are several challenges in development of nucleoside inhibitors. First, interpretation of structure–activity relationship (SAR) of nucleoside analogs is difficult, as the SAR is dependent on the RdRp as well as on the combination of host kinases for each phosphorylation to form the nucleoside triphosphate. A slight change in either the base or ribose moiety could dramatically affect the kinase-mediated phosphorylation efficiency,

resulting in different potency independent from the intrinsic potency of the nucleotide triphosphate analog against RdRp. For this reason, nucleoside analogs are usually discovered serendipitously. Different cell lines may show wide range of discrepancy in antiviral activity due to differences in expression levels of nucleoside/nucleotide kinases or nucleoside transporters (Leary et al., 2002). Therefore, it is useful to evaluate nucleoside inhibitors in more than one clinically relevant cell line.

Nucleosides enter cells by either passive diffusion or via the nucleoside transporter, depending on the presence and abundance of specific nucleoside transporters in a particular cell line and the concentration of the nucleoside analogs (Pastor-Anglada et al., 2005). Generally, nucleoside inhibitors show poor passive diffusion due to their polar nature. This often results in low or variable bioavailibility in animal studies. To circumvent this problem, conjugation of an easily cleavable moiety is often employed. This is normally done by attaching aliphatic acids or amino acids to the alcohol groups on the ribose or acyclic ribose (Kroschewski et al., 2008). The aliphatic attachment improves lipophilicity of the compound, facilitating passive diffusion into cells. The amino-acid conjugation not only improves lipophilicity, but also potentially enables the compound to be recognized by the peptide transporter PepT1, which transports the compound across the plasma membrane (Han et al., 1998). Once absorbed, the compound is rapidly converted into the parent drug by cellular esterases.

Another challenge faced by nucleoside analogs is the conversion of the nucleotide to its monophosphate form by host kinases, which is often the rate-limiting step in tri-phosphorylation. An obvious solution is to synthesize the nucleoside monophosphate or phosphonate prodrugs. An example of a phosphoramidate approach was illustrated through converting previously inactive nucleoside analogs for HCV into potent inhibitors (Perrone et al., 2007a,b). Other examples of approved nucleotide phosphonates include Adefovir Disproxil and Tenofovir Disproxil for the treatment of Hepatitis B virus and HIV, respectively.

A common side effect of antiviral nucleoside analogs is mitochondrial toxicity. This phenomenon was first discovered in nucleoside analogs for the treatment of HIV, which usually involved inhibition of the mitochondrial polymerase gamma, leading to a gradual decline in the levels of mitochondrial DNA (Cote et al., 2002). However, not all nucleoside analog toxicity is associated with mitochondria, indicating that not all toxicity is due to inhibition of DNA polymerase gamma (Moyle, 2000). It should be noted that most nucleoside analogs that cause mitochondrial toxicity are deoxy-nucleoside analogs and involved prolonged exposure of the drug. Nucleoside analogs targeting RNA viruses will typically be ribose nucleosides, thus the effect and extent of mitochondrial toxicity for ribose-based nucleoside inhibitor for acute viral diseases such as DENV remains unclear.

Despite all these disadvantages, nucleoside analogs remain the largest class of antiviral drugs. One advantage in the case of dengue is that nucleotides targeting the RdRp should inhibit all four serotypes of DENV. In fact, the 2′C methyl deaza-adenosine, a nucleoside inhibitor developed for the treatment of HCV, has been reported to be a broad-spectrum anti-flaviviral agent, including DENV (Migliaccio et al., 2003); the compound was recently reported to have robust antiviral efficacy in HCV-infected chimpanzees (Carroll et al., 2009). In addition, nucleoside inhibitors seem to have a higher barrier for development of resistant virus; it takes longer for HCV to develop resistance against nucleoside analogs compared with non-nucleoside polymerase inhibitors or protease inhibitors (McCown et al., 2003).

We recently reported a novel adenosine analog, NITD008, that potently inhibits DENV both *in vitro* and *in vivo* with an EC_{50} of 0.64 μM and no observed cytotoxicity at up to 50 μM compound (Yin et al., 2009). Compared with adenosine, NITD008 contains a carbon substitution for N-7 of the purine and acetylene at the 2′ position of ribose. Besides DENV, the compound inhibits other flaviviruses, including HCV, but it does not inhibit non-flaviviruses. NITD008 functions as a chain terminator during viral RNA synthesis. Treatment of DENV-infected mice with NITD008 suppressed peak viremia and completely prevented the infected mice from death. No observed adverse effect level (NOAEL) was achieved when rats were orally dosed with NITD008 at 50 mg/kg/day for 1 week. However, NOAEL could not be accomplished when rats and dogs were daily dosed for 2 weeks. The results have proved the concept that a nucleotide inhibitor could potentially be developed for treatment of flavivirus infections.

2.3.2. Non-nucleoside inhibitors

The non-nucleoside approach has led to the successful development of reverse transcriptase inhibitors (NNRTI) which act by binding in an induced-fit allosteric pocket (Martins et al., 2008). Like all known polymerases, flavivirus RdRp assumes a right-handed configuration consisting of thumb, fingers and palm subdomains. The crystal structures of DENV-3 and WNV RdRp display a closed conformation, characteristic of a primer-independent RdRp (Yap et al., 2007; Yin et al., 2009). Two and five cavities have been identified in DENV-3 and WNV RdRp, respectively, among which two cavities (located in the thumb subdomain) are shared by both RdRps (Malet et al., 2008). The size and shape of the identified cavities are potential allosteric sites for small-molecular inhibitors. In support of this approach, inhibitors targeting multiple allosteric sites of HCV RdRp have been identified (Wu et al., 2005b).

Non-nucleoside inhibitors are typically identified through HTS of compound libraries. The most common HTS for viral polymerase is a SPA assay of primer extension using homopolymeric RNA annealed to its complementary primer (Pellerin et al., 2002; Yap et al., 2007). The primer is biotinylated so it binds to streptavidin-coated SPA beads. Tritiated nucleotides are usually used in the assay; upon incorporation, the scintillation signal increases due to the proximity of the labeled nucleotides to the SPA beads. This method allows homogeneous detection of radioactive incorporation without washing or removing any solution, thus minimizing pipetting errors. In addition to HTS, other methods can also be used to identify non-nucleoside inhibitors, such as high-throughput docking or fragment-based screening (see below). Once a potent inhibitor has been found and the SAR *in vitro* has been characterized, it is important to test the cellular activity of the compound class. It is common that inhibitors with nanomolar dissociation constants are completely inactive inside cells (Beaulieu et al., 2006). The lack of cellular activity could result from poor cellular penetration of the compounds or inaccessibility of the allosteric site in the context of viral replication complex.

One major challenge facing the non-nucleoside inhibitors is the heterogeneity of viruses, since there is often less selective pressure to conserve the amino acids that form an allosteric binding site. In HCV, allosteric inhibitors that are developed against one genotype are often not potent against another genotype (Pauwels et al., 2007). In addition, it is likely that resistant viruses may already exist in the clinical population of viral quasispecies (Le Pogam et al., 2008), which can quickly emerge upon monotherapy of non-nucleoside inhibitors.

2.4. Methyltransferases

The 5′ end of the flavivirus plus-strand RNA genome contains a type 1 cap structure (m^7GpppAmG) (Cleaves and Dubin, 1979; Wengler, 1981). Flavivirus RNA capping requires four enzymatic reactions: (i) NS3 hydrolyzes the 5′-triphosphate end of the nascent RNA transcript to a 5′-diphosphate by an RNA triphosphatase (Wengler and Wengler, 1993); (ii) an unidentified RNA guanylyl-

transferase transfers the GMP moiety of GTP to the 5′-diphosphate of RNA; (iii) NS5 methylates the N-7 position of guanine (N-7 MTase; GpppA-RNA → m^7GpppA-RNA) (Ray et al., 2006); and (iv) NS5 methylates the 2′-OH position of the first nucleotide ribose (2′-O MTase; m^7GpppA-RNA → m^7GpppAm-RNA) (Egloff et al., 2002). *S*-Adenosyl-L-methionine (SAM) is the methyl donor for both the N-7 and 2′-O methylations, generating *S*-adenosyl-L-homocysteine (SAH) as a by-product.

The flavivirus MTase domain, located at the N-terminus of NS5, catalyzes N-7 and 2′-O methylations of the viral RNA cap in a sequential manner, GpppA-RNA → m^7GpppA-RNA → m^7GpppAm-RNA (Zhou et al., 2007). Compared with host MTases, the flavivirus MTase has two unique features. (i) The flavivirus MTase specifically methylates viral RNA substrates; the N-7 and 2′-O methylation reactions require distinct viral RNA elements (Dong et al., 2007). In contrast, host RNA cap MTases nonspecifically methylate any RNA cap (Furuichi and Shatkin, 2000). (ii) Despite exhibiting two distinct methylation activities, the flavivirus MTase has a single binding site for SAM in its crystal structure. It was hypothesized that substrate GpppA-RNA must be re-positioned to accept the N-7 and 2′-O methyl groups from SAM during the two methylation reactions. In support of this model, structure-guided mutagenesis studies revealed two distinct sets of amino acids on the enzyme surface that are specifically required for the N-7 and 2′-O methylations (Dong et al., 2008).

The biological function of the flavivirus MTase has been validated in the context of full-length virus. WNVs defective in N-7 methylation are non-replicative; however, WNVs defective in 2′-O methylation are attenuated and can protect mice from subsequent wild-type WNV challenge (Zhou et al., 2007). Similarly, DENV-2 defective in cap methylation is attenuated in viral replication (Kroschewski et al., 2008). These results demonstrate that the flavivirus MTase is a valid antiviral target. Crystal structures of MTase from various flaviviruses have been solved, in complexes with SAH and cap analogs (Egloff et al., 2002, 2007; Zhou et al., 2007). The structural information has provided a solid foundation for structure-based antiviral approaches (see below). One obvious challenge for development of viral MTase inhibitors is selectivity because host MTase-mediated methylations of mRNA cap and protein are essential for cellular functions. It remains to be seen whether the flavivirus MTase is a good drug target since some human MTases have proved difficult targets. However, since this enzyme contains two well-defined ligand-binding pockets (Fig. 4) and its activity is essential for viral replication it should certainly be investigated further.

A SPA assay was recently developed for HTS of DENV 2′-O MTase (Kroschewski et al., 2008). The assay used a biotinylated capped RNA substrate and [methyl-^{3}H]-SAM. Upon transfer of the ^{3}H-labeled methyl group to the RNA cap, the ^{3}H-labeled RNA is immobilized onto the streptavidin-coated SPA beads, leading to an increase in the scintillation signal.

One recent study showed that a mutant WNV defective in both N-7 and 2′-O MTase activities can be rescued by a single adaptive mutation (W751R) in the RdRp domain (Dong et al., 2008). Remarkably, the W751R mutation (located at the entrance of the RNA template tunnel of polymerase) improved the polymerase activity of the recombinant full-length NS5 by >5-fold. The result implies that resistance to MTase inhibitors may arise from mutations outside the MTase domain.

2.5. Envelope protein

The flavivirus E protein consists of three domains, the central domain I, the extended finger-like domain II containing the fusion loop, and the immunoglobin-like domain III (Modis et al., 2003; Rey et al., 1995). The flavivirus E proteins are members of

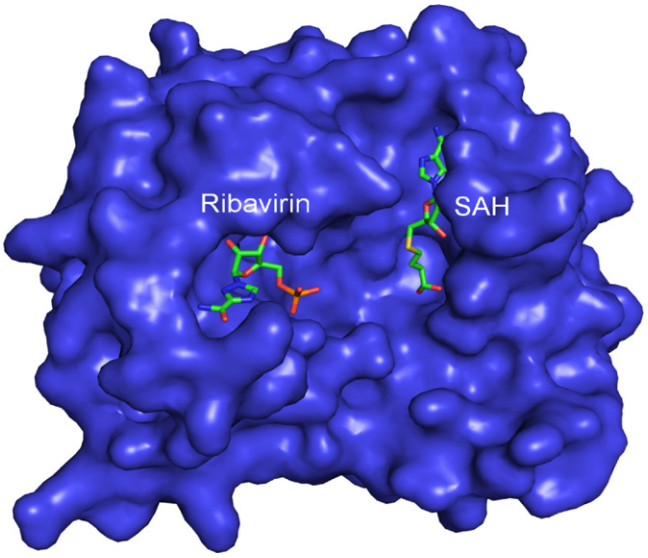

Fig. 4. Structure of the dengue methyltransferase. A surface representation of the dengue methyltransferase shows the positions of the GTP-cap binding site, here bound to Ribavirin, and of the *S*-adenosyl homocysteine/*S*-adenosyl methionine binding site. The two existing ligand-binding pockets suggest that it is possible to inhibit the enzyme with a small molecule.

class II viral fusion proteins, distinct from class I fusion proteins such as gp120/40 of HIV and hemagglutinin of influenza virus (Harrison, 2005). The E protein mediates binding of the virus to cell surface receptors, most notably the ICAM3-grabbing non-integrin receptors DC-SIGN and L-SIGN (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003), but other receptors have also been implicated, such as the high-affinity laminin receptor (Thepparit and Smith, 2004), the mannose receptor (Miller et al., 2008), and Glucose Regulated Protein 78 (GRP78) (Jindadamrongwech et al., 2004). Disruption of the interaction between the E protein and cell receptors would be a potential antiviral approach, similar to the recent development of antagonists for the HIV-1 co-receptor CCR5 that have resulted in the anti-HIV drug Maraviroc (Kuritzkes, 2009). It was recently shown that carbohydrate-binding agents (CBAs), such as plant lectins, could block DENV infection of cells that express DC-SIGN, but did not affect infection of cells that do not express DC-SIGN (Alen et al., 2009). The involvement of multiple receptors on different cell types, and a lack of knowledge of their involvement in the infection process in patients, makes it challenging to specifically target receptor-mediated viral entry.

After attachment to the cell surface, the DENV is internalized by clathrin-mediated endocytosis followed by membrane fusion between the virion and the endosome, resulting in release of the viral RNA into the cytoplasm of the host cell (van der Schaar et al., 2008). The E protein mediates this membrane fusion process by low pH-induced conformational changes, and a rearrangement from a dimeric to a trimeric organization on the surface of the virus particle as depicted in Modis et al. (2004). Disruption of the membrane fusion process is an obvious target for antiviral development, as evidenced by the peptidic HIV entry inhibitor drug Enfuvirtide (Barbas et al., 1997). For DENV, it has been shown that domain III of the E protein can block entry by specifically inhibiting virus fusion (Liao and Kielian, 2005), and that peptides from the E-protein fusion domain can interfere with flavivirus infectivity (Hrobowski et al., 2005). Peptidic drug antivirals, however, suffer from poor absorption in the gastrointestinal tract, often requiring intravenous delivery. They are also likely to be expensive to manufacture and so would be unaffordable for the majority of dengue patients.

The structure of the dengue E protein revealed a hydrophobic pocket occupied by a small detergent molecule (β-N-

Table 1
Potential anti-flaviviral host targets and their likely drawbacks.

Potential host targets		Advantages	Disadvantages
Function	Enzyme		
Proteases	Furin	Cleaves prM protein on the virus surface, which is essential for maturation.	Small-molecule inhibitors of furin have proved difficult to develop.
	Signal peptidase	Cleavage of the C-prM, prM-E, E-NS1, and NS4A–NS4B junctions.	Processes many secretory proteins so inhibition likely to have side effects.
Glucosidases	α-Glucosidase	Responsible for glycosylation of prM, E, and NS1. Inhibitors castanospermin, deoxynojirimycin and iminocyclitol compounds inhibit DENV replication.	Existing inhibitors do not reduce DENV replication sufficiently suggesting an α-glucosidase inhibitor alone is not sufficient.
Kinases	c-Src c-Yes	The c-Src inhibitor, Dasatinib, inhibits virus assembly. Required for WNV maturation.	Role of c-Src not fully understood. Efficacy of inhibitors *in vivo* remains to be shown.
Cholesterol biosynthesis	Mevalonate diphosphate decarboxylase (MVD)	Cholesterol has been shown to be important for WNV, JEV, and DENV entry and replication. MVD required for DENV replication.	An effective inhibitor *in vivo* would require de novo cholesterol biosynthesis to be essential.
Immune response	TNF-α, IL-8, MMP-9	Cytokines, chemokines, are involved in progression to severe dengue.	Roles in vascular leakage not fully understood so further validation required.
	C-type lectin domain family 5, member A (CLEC5A)	DENV binds to CLEC5A to release pro-inflammatory cytokines.	Proof-of-concept as anti-leakage target remains to be shown. Difficult to develop small-molecule inhibitors of protein–protein interactions.
	Platelet-activating factor receptor (PAFR)	Activation essential for dengue pathogenesis. Induces vascular permeability and cytokines. Oral PAFR antagonist delayed DENV lethality in animal models.	DENV lethality not completely reversed in $PAFR^{-/-}$ mice, suggesting inhibition of additional pathways required.

octylglucoside) in a "hinge" region between domain I and II, suggesting the potential for small-molecule inhibitors of fusion (Modis et al., 2003). Virtual screening has been used by several groups to look for compounds that can potentially bind into this pocket and affect viral entry. In a recent study, three classes of small molecules from virtual screening showed 50% inhibition of YFV infection (50% effective concentration; EC_{50}) in the 10–50 μM range (Kroschewski et al., 2008). Focusing on a structure class with a central thiazole ring, compounds were synthesized with improved EC_{50}s down to about 1 μM against YFV. In a study from our lab, virtual screening of the dengue E protein pocket was also used to identify a compound class with a central thiophene ring that showed EC_{50}s against DENV in the 100–300 nM range (Wang et al., 2009). Time of addition experiments showed that the compounds inhibit viral entry, and immunofluorescent labeling of cells showed that the compounds cause accumulation of virus in endosomes, consistent with inhibition of membrane fusion. In the most recent study from our lab, we identified a compound that inhibits E protein mediated membrane fusion in two functional fusion assays, showing antiviral activity when present during the entry phase of dengue infection (Poh et al., 2009). It should be noted that none of these studies demonstrated directly that the compounds bind inside the pocket of the E protein.

An alternative approach to targeting the DENV E protein is the development of neutralizing monoclonal antibodies for therapeutic use. Although the only antiviral monoclonal antibody currently on the market is Palivizumab for prophylactic use against respiratory syncytial virus infections, there have been promising developments for other viruses. Comprehensive studies on neutralizing antibodies and epitopes on DENV and WNV have generated important insights into the mechanism of antibody neutralization of flaviviruses (Gromowski et al., 2008; Lok et al., 2008; Oliphant et al., 2005). The humanized E16 WNV neutralizing antibody is currently being studied for its potential as a therapeutic agent (Zhang et al., 2009). However, the existence of four antigenically distinct serotypes and the phenomenon of antibody-dependent enhancement (ADE) complicates the generation of dengue antibody therapeutics.

ADE is mediated through Fcγ-receptor binding and uptake of non-neutralizing antibody–virus complexes enhancing the level of infection (Halstead, 2003). The risk of non-neutralizing levels of anti-dengue antibodies causing ADE has been a worry for both vaccine and therapeutic-antibody development. However, recent mutation studies on the constant region of the immunoglobulin molecule have identified mutations that abolish Fcγ-receptor binding without affecting other properties (Goncalvez et al., 2007; Hezareh et al., 2001), thus offering a way to avoid the risk of ADE for recombinantly produced antibody therapeutics.

For an anti-dengue drug, a single neutralizing antibody that is cross-reactive against all four DENV serotypes would be the most obvious and cost-effective option. Several cross-reactive epitopes and antibodies have been identified (Chu et al., 2005; Crill and Roehrig, 2001; Lisova et al., 2007), but none is potent enough for therapeutic development. The use of cocktails of antibodies that are specific and highly neutralizing for one or several serotypes could be an alternative, but this approach would have the drawback of increasing the cost of production and the complexity of regulatory issues involving efficacy and safety.

3. Host target-based approach

Flaviviruses utilize host proteins for their entry, translation, polyprotein cleavage, replication, and assembly. The host proteins are potential antiviral targets, provided that they are druggable, and that their inhibition is not toxic for the host (Table 1).

3.1. Host proteases

Besides the viral protease, host proteases are required for processing the DENV polyprotein. The best characterized host proteases are furin and the signal peptidase. Furin cleaves the tick-borne encephalitis virus prM protein on the virus surface into the mature M protein, which is essential for maturation of the virion into an infectious virus (Elshuber et al., 2003; Stadler et al., 1997). The pr–M junction contains the furin consensus cleavage site, R–X–(K/R)–R (where X is any amino acid). Residues outside of the furin consensus sequence may also influence the efficiency of

cleavage in the context of the virus (Sriburi et al., 2001). The signal peptidase is located on the luminal side of the endoplasmic reticulum (ER) membrane and is responsible for the co-translational cleavage of the C-prM, prM-E, E-NS1, and NS4A–NS4B junctions (Chambers et al., 1991). Since the signal peptidase has broad functions (including processing most secretory proteins), its inhibition is likely to have side effects.

3.2. Glucosidase

Glucosidase is a well-known host enzyme responsible for the proper folding and glycosylation of the prM, E, and NS1 dengue proteins (Courageot et al., 2000). After translocation into the ER lumen, a 14-residue oligosaccharide core unit, $Glc_3Man_9GlcNAc_2$ is added co-translationally to glycoproteins. α-Glucosidases I and II trim the terminal glucose residues on the core unit, leaving the protein mono-glucosylated and allowing it to bind to the ER chaperones, calnexin and/or calreticulin for proper folding. Therefore, the α-glucosidases are required for correct folding of many different glycoproteins. Inhibitors such as castanospermin (Whitby et al., 2005), the iminosugar deoxynojirimycin (DNJ) and its derivatives (Wu et al., 2002), as well as iminocyclitol compounds (Gu et al., 2007) are inhibitors of DENV *in vitro* and *in vivo*. α-Glucosidase is also required for the maturation of other viruses such as HIV (Fenouillet and Gluckman, 1991). Specific α-glucosidase inhibitors could be useful as broad-spectrum antivirals.

3.3. Host kinases

Host kinases have recently been shown to be involved in dengue virus assembly and secretion (Chu and Yang, 2007). Dasatinib, an inhibitor of the protein kinase c-Src, was found to inhibit DENV assembly. The role of the kinase c-Src in DENV replication was confirmed by using small interfering RNA (siRNA) to inhibit its expression, but it is not clear how c-Src inhibits viral assembly and secretion. Another member of the Src kinase family, c-Yes, has been shown to influence the maturation of WNV (Hirsch et al., 2005). The *in vivo* efficacy of these kinase inhibitors remains to be determined.

3.4. Cholesterol-biosynthesis pathway

The cholesterol-biosynthesis pathway has been linked to DENV entry and replication as well as to the host immune response (Malet et al., 2007). By chemically removing cholesterol from cellular membranes using β-methyl-cyclodextrin or fillipin, cholesterol has been shown to be important for WNV, JEV, and DENV entry and replication (Hirsch et al., 2005; Lee et al., 2008). Using siRNA against MVD (mevalonate diphosphate decarboxylase, an enzyme essential for cholesterol biosynthesis), we recently showed that MVD was required for DENV replication (Rothwell et al., 2009). These results suggest that the approved drugs for inhibition of cholesterol biosynthesis should be tested for efficacy against DENV.

3.5. Host factors involved in DHF/DSS pathogenesis

Although the pathogenesis of DHF/DSS is not fully understood, host proteins involved in the immune response are important for this process and could be potential targets for vascular leakage and thrombocytopenia. Because of the double role that the immune system plays in both the protection and pathogenesis of severe dengue, it is difficult to identify factors that only cause harmful side effects that are not involved in antiviral protection. Many cytokines, chemokines, and other soluble proteins in the blood are involved in the progression to DHF/DSS, including TNF-α (Shresta et al., 2006), IL-8 (Talavera et al., 2004), MMP-9 (Luplertlop et al., 2006), VEGF/VEGF receptor (Srikiatkhachorn et al., 2007). Although antibodies against TNF-α, IL-8 and MMP-9 all show protection against vascular leakage in mouse models, until we fully understand the role of these factors in human DHF/DSS, it is risky to further develop any of these treatments for humans.

A new lectin receptor, CLEC5A (C-type lectin domain family 5, member A) was identified to trigger the release of pro-inflammatory cytokines without affecting the antiviral response (Chen et al., 2008). DENV binds to CLEC5A but does not use it for entering the cells. Knockdown of CLEC5A by siRNA suppressed the release of TNF-α, IL-6, IL-8, IP-10, but did not affect the production of IFN-α. Anti-CLEC5A antibodies which prevent DENV binding to the receptor significantly reduced vascular leakage and prevented death in $STAT1^{-/-}$ knockout mice infected by the virus. Low-molecular-weight inhibitors preventing the binding of the virus to CLEC5A are difficult to develop, but a proof-of-concept can be tried with humanized antibodies against CLEC5A to further validate the role of this receptor in development of DHF/DSS.

A recent study showed that platelet-activating factor receptor (PAF) is essential for dengue pathogenesis (Souza et al., 2009). Since activation of PAF receptor (PAFR) on endothelial cells and leukocytes induces an increase in vascular permeability and production of cytokines, the authors hypothesized that suppression of the PAFR activation could reduce the systemic manifestations of dengue infection. Indeed, when compared with the wild-type infected mice, the infected $PAFR^{-/-}$ mice showed decreased thrombocytopenia, lower cytokine levels, and delayed lethality. Furthermore, treatment with orally active PAFR antagonist prevented the above-mentioned manifestations, even when started 5 days after virus inoculation. The results suggest that small molecules blocking the host pathways involved in dengue pathogenesis could potentially be developed for treatment.

4. Structure-based approach

4.1. Structure-based rational design

The use of high-resolution protein crystallography for structure-based design is a powerful approach to increase the affinity and potency of lead antiviral compounds. Confirmed hits from screening can be either co-crystallized with the protein target or soaked into pre-formed apo-protein crystals to determine the exact position and manner of binding. This information can then be used in conjunction with docking and molecular modeling to design compounds that would be predicted to have a higher affinity for the protein. However, the above exercise may not be straightforward even with known crystal forms. For example, soaking compounds into crystals is frequently unsuccessful because the compound does not bind or it damages the crystals, greatly decreasing the extent of the crystal diffraction. This may occur if binding of the ligand causes a slight conformational change in the protein. An alternative approach is to co-crystallize compounds with the protein. If the crystallization conditions are known, it is possible to screen around these conditions to obtain crystals containing the ligand–protein complex. However, the ligand itself will frequently influence the crystallization of the protein. If this is the case, high-throughput crystallization screening with a mixture of the protein and compound is required to identify new crystallization conditions for the ligand–protein complex. Screening can be done with as little as 100 nl of the protein–compound mixture per drop and crystals can be harvested directly from the crystallization drop for data collection from a synchrotron radiation source. Molecular replacement can then be used to solve the co-crystal structure. It is important to complement co-crystallography with biophysical binding techniques as well as the IC_{50} to confirm that the compound binds specifically to the protein.

Several physicochemical parameters should be taken into account when trying to increase the affinity of lead compounds. Most synthetic inhibitors bind largely due to hydrophobic interactions, so hydrophobic regions on the protein should be exploited as far as possible. Hydrogen bond donors and acceptors are also important since these can be used to increase the solubility of compounds. Where possible the hydrogen bonds should be optimized in terms of geometry and distance to give the greatest increase in free energy and, thus, increase the affinity for the protein, since in solution the compound is free to make optimal hydrogen bonds with water. Entropic effects are also important since flexible molecules may move rapidly in solution and upon binding to the protein, they may be forced to adopt a particular conformation. Therefore, high-resolution structural information can be used to show the conformation of the compound bound to the target and then changes such as non-rotatable bonds or large bulky groups can be used to 'lock' the molecule in a particular conformation.

4.2. Fragment-based screening

Fragment-based screening is a newer technique that complements HTS techniques. The libraries for fragment-based screening usually contain compounds with molecular weights below 300 Da (around half the molecular weights of compounds from traditional libraries); the libraries typically have fewer compounds (with up to 5000) than traditional libraries (with 1 million or more) (Hesterkamp and Whittaker, 2008). Enzymatic screening can be used to find fragment leads, but it is common to use binding or structural approaches (including protein crystallography or NMR) to identify leads and to determine the manner of the interactions. Because high ligand concentrations are commonly required for enzyme inhibition, ligands with nonspecific inhibition should be filtered out using binding assays. The best fragment-based approach is to use a combination of NMR, X-ray crystallography, and other biophysical techniques to optimize leads. Importantly, the fragments in the library should be highly soluble in water since the initial screening must be performed at high concentrations, even up to the millimolar range. A combination of crystallography, surface plasmon resonance, and enzyme assays has been used on the HCV RdRp to optimize leads from dissociation constants in the low millimolar range to sub-micromolar inhibitors (Fig. 5) (Antonysamy et al., 2008). This is accomplished by combining the interactions of several different fragment leads, as well as by expanding the compounds to pick up new interactions.

The most common use of protein crystallography in fragment-based screening is to soak pre-formed protein crystals into cocktails of compound fragments, typically up to 10 at a time, with compound concentrations around 25–100 mM since the fragments are expected to have low affinity. The fragments are all small and often have similar structures, so it can be difficult identifying the bound ligand. This requires high-resolution data, but has been simplified by the development of automatic ligand-fitting software that scores the possible compounds to rank the likelihood of their binding to a particular site (Mooij et al., 2006).

An alternative approach, often used in parallel with protein crystallography is to use NMR spectroscopy since several different parameters can be used to detect interactions. Chemical shift changes can be used to provide low-resolution structural information about the protein–ligand complex; greater details can be provided by the interpretation of nuclear overhauser effects (NOEs). This requires that the NMR resonances of the protein are first assigned and this in turn requires N15- and C13-labeled protein and can be time consuming. NMR techniques that detect the ligand itself can also be used. This means that there is no limit on the size of the protein that is used or any requirement for isotope labeling of the protein. Relaxation and cross-relaxation of the ligand

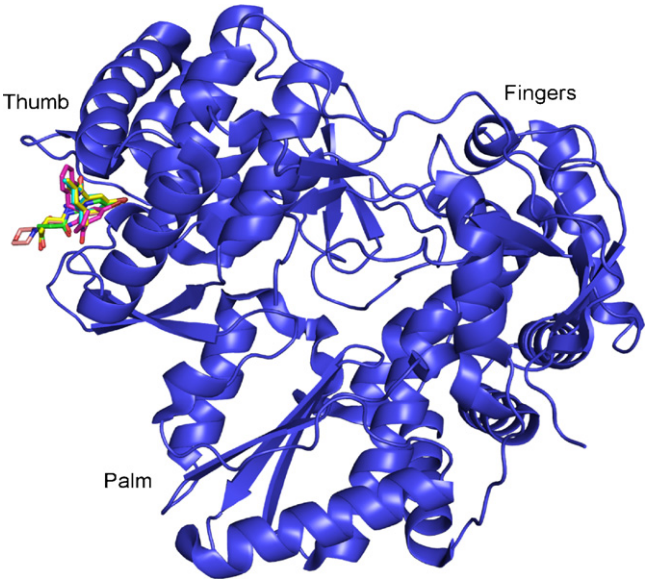

Fig. 5. The structure of the HCV RdRp, showing the position of compounds identified by fragment-based screening bound to an allosteric site in the thumb subdomain of the polymerase. A number of fragments were identified to bind to the same site by fragment-based screening and by including different regions of the different compounds it was possible to increase the affinity 5000 times (Antonysamy et al., 2008).

are most strongly affected by binding to a protein since these are dependent on molecular rotation. Although NMR is much slower than traditional screening it has the advantage that it can measure the purity and aggregation state of the compounds and reduce the identification of false positives. It can also be used to determine the equilibrium dissociation constant (K_d) even for low affinity compounds, as well as the location of the interaction.

Fragment-based screening from these approaches can generate a large number of hits with hit rates as high as 1–5% (Jhoti et al., 2007). Hits can then be developed into a number of separate chemical series. Comparison of different hits can be used to gain information about the requirements for a stable interaction and the addition of different functional groups can be tested. Alternatively, if two or more fragments are identified that bind to separate sites, it may be possible to design a linker between them that would create a higher affinity compound than either fragment alone. Further rounds of structure-based design can then be used to increase the affinity and potency of the compounds.

5. Replication-based HTS approach

A number of cell-based assays have been developed to screen for antiviral compounds. Compared with the target-based approach, viral replication-based assays cover multiple steps and targets involved in a viral infection cycle.

5.1. Live-virus assays

5.1.1. Cytopathic effect inhibition assay

Many viral infections cause cytopathic effect (CPE). Inhibition of CPE is a common method for antiviral screening (Green et al., 2008). The CPE assay for DENV involves infection of susceptible cells with virus in the presence of a test compound. We typically infect Huh-7 cells with DENV-2 New Guinea C strain. After 3–4 days of incubation, the ability of compounds to inhibit virus-induced cell death is determined by measuring the intracellular level of ATP using CellTiter-Glo® luminescent-cell-viability assay (Promega). This CPE-based assay is amenable for HTS because it is

homogeneous. One major advantage of the CPE HTS is that the assay gives a gain-of-signal endpoint; it reduces false-positive hits from compounds that are cytotoxic or interfere with the assay readout. However, the assay has two disadvantages: (1) it uses infectious virus, and the assay should be performed in a biosafety containment; (2) the assay is not a direct measurement of virus replication.

5.1.2. *In situ cellular ELISA*

To directly measure virus replication, we developed a cell-based flavivirus immunodetection (CFI) assay (Yin et al., 2009). The assay is based on quantitative immunodetection of the production of DENV E protein in virus-infected cells. A similar in situ cellular ELISA approach has been reported for testing antiviral agents in many other viruses (Berkowitz and Levin, 1985; Tatarowicz et al., 1991). The CFI assay needs several washing steps, which limit its adaptation to a high-throughput format. Nevertheless, the CFI assay represents a good secondary assay that has many advantages over the plaque reduction assay. It is less labor intensive, with a shorter turn-around time (3 days from the time of virus inoculation versus 6–7 days in the plaque reduction assay); the data collection is performed on a plate reader, avoiding subjective manual plaque counting. The CFI results show a good correlation with those from the plaque reduction assay.

5.2. *Viral replicon assay*

To overcome the safety concerns associated with handling live viruses, an alternative approach is to use a luciferase-based subgenomic replicon system. This system typically encodes a luciferase reporter in place of the viral structural proteins (Ng et al., 2007; Pang et al., 2001; Puig-Basagoiti et al., 2006). The antiviral agents that target any of the steps for viral translation or RNA replication should decrease the luciferase signal. In contrast to the CPE-inhibition assay, the replicon assay has a loss-of-signal endpoint, since inhibition of viral replication decreases luciferase expression. Assays with a loss-of-signal endpoint usually result in a high number of false-positive hits, due to molecules inhibiting the activity of the luciferase reporter or due to compound-mediated cytotoxicity. Therefore, appropriate counter screens, such as a cytotoxicity assay, and a cell-based luciferase screen, have to be run in parallel with the replicon assay. Running multiple assays in parallel significantly increases the cost of screening.

5.3. *Virus-like particle assay*

Another approach to avoid using live viruses for HTS is to use virus-like particles (VLPs). VLPs are generated by co-transfecting cells with a subgenomic replicon and a separate vector expressing the viral structural proteins. Semliki Forest virus or Sindbis virus expression vectors containing the flaviviral structural proteins have been used successfully to package flavivirus replicons (Khromykh et al., 1998; Puig-Basagoiti et al., 2005). Infection of susceptible cells with VLPs leads to expression of the luciferase when the replicon replicates inside cells. Because there is no further supply of structural proteins, the VLP infection is a single round, and significantly reduces the biohazard risk. The VLP assay is similar to the replicon assay, and so suffers from the same problems due to a loss-of-signal end point. However, compared with the replicon assay, the VLP assay has the potential to identify viral-entry inhibitors as well as replication inhibitors.

Compared with enzyme-based assays, the cell-based assays discussed above have a number of disadvantages. An obvious problem is that the targets of hits (derived from the cell-based HTS) are unknown, which makes generating structure–activity relationships difficult. This is even more pronounced when a compound influences more than one target so modifications to the compound then affect the activity of a range of different targets inside the cell. For later pre-clinical drug development, it is important to know the target to be able to predict potential side effects. Another problem is that the library may contain potent inhibitors of viral enzymes, but if the compounds do not penetrate cells, or the compounds are rapidly degraded, they will not be identified in cell-based screens.

6. Concluding remarks

The major goal of anti-DENV therapy is to prevent patients with DF (mild disease form) from development of DHF and DSS (severe disease form). Prospective studies of clinically characterized patients indicated that plasma levels of viremia and viral NS1 were 10- to 100-fold higher in patients with DHF/DSS than in those with DF (Gubler et al., 1981; Libraty et al., 2002). The rationale for anti-DENV therapy is to develop inhibitors that can suppress viremia by $\geq$10-fold *in vivo*. Both host and viral targets have been proven valid for antiviral development. Among the ten viral proteins, the atomic structures of capsid (Ma et al., 2004), envelope (Modis et al., 2003), NS3 (protease domain, helicase domain, and full-length NS3) (Erbel et al., 2006; Luo et al., 2007, 2008), MTase (Egloff et al., 2002), and RdRp (Yap et al., 2007) have been solved. The structural information has provided a solid foundation for the development of inhibitors of viral targets.

Since a host protein usually serves multiple functions, development of inhibitors of host targets for antiviral therapy is expected to be more challenging than development of inhibitors of viral targets. First, the compound should specifically abolish the host activity involved in viral replication without affecting its normal cellular function. Second, the compound should sufficiently knockdown the host factor under physiological conditions *in vivo*. It is possible that in cell culture, a host inhibitor could potently inhibit virus replication, due to the low concentration of the host factor; however, when tested *in vivo*, the compound may not be capable of sufficiently suppressing viral replication, due to a higher concentration of the host factor. However, inhibitors of host factors are expected to have a higher genetic barrier to the emergence of viral escape mutants. Due to the error-prone nature of RdRp, drug resistance to virus-specific inhibitors can quickly emerge. Since viral inhibitors usually bind to defined pockets of viral proteins, mutations in viral genome that disrupt or weaken the binding would reduce the compound efficacy and lead to resistance. In contrast, host inhibitors do not directly bind to viral targets, creating a greater barrier for the emergence of resistant viruses.

One major hurdle for dengue drug discovery is the lack of a good small animal model. A number of mouse models have been established, including the AG129 model (Shresta et al., 2006), wild-type BALB/c model (Atrasheuskaya et al., 2003), and humanized mouse model (Bente et al., 2005; Mota and Rico-Hesse, 2009). The AG129 mouse model was successfully used to examine the *in vivo* efficacy of inhibitors (Lubeck et al., 1980). However, caution should be taken when interpreting the results from the AG129 mouse experiments. Since the AG129 mouse lacks interferon-α/β and interferon-γ receptors, this model may underestimate the real *in vivo* efficacy of compounds.

Since dengue is an acute disease with fever duration of less than a week (Gubler et al., 2007), the length of therapeutic treatment is expected to be less than a week. This is in contrast to the antiviral therapy of chronic diseases (such as HIV and HCV) which require long-term treatment. The difference between the acute and chronic diseases should be considered during pre-clinical development of DENV inhibitors. Given the short period of viremia in DENV-infected patients (about a week after symptom onset), the success of treatment requires early diagnosis of DENV infection (using RT-PCR or measuring viral NS1 protein in the blood), before

the decrease in viremia in patients. Alternatively, during DENV epidemics or outbreaks, fever patients could receive prophylactic treatments after simple clinical and hematological measurements.

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