Antiviral Research 163 (2019) 156–178

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Dengue drug discovery: Progress, challenges and outlook

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ARTICLE INFO

Keywords:
Drug discovery
Antiviral
Dengue virus
Flavivirus

ABSTRACT

In the context of the only available vaccine (DENGVAXIA) that was marketed in several countries, but poses higher risks to unexposed individuals, the development of antivirals for dengue virus (DENV), whilst challenging, would bring significant benefits to public health. Here recent progress in the field of DENV drug discovery made in academic laboratories and industry is reviewed. Characteristics of an ideal DENV antiviral molecule, given the specific immunopathology provoked by this acute viral infection, are described. New chemical classes identified from biochemical, biophysical and phenotypic screens that target viral (especially NS4B) and host proteins, offer promising opportunities for further development. In particular, new methodologies ("omics") can accelerate the discovery of much awaited flavivirus specific inhibitors. Challenges and opportunities in lead identification activities as well as the path to clinical development of dengue drugs are discussed. To galvanize DENV drug discovery, collaborative public-public partnerships and open-access resources will greatly benefit both the DENV research community and DENV patients.

1. Dengue: a growing global health problem and unmet medical need

DENV belongs to the family of Flaviviruses, which includes other disease-causing viruses such as Zika virus (ZIKV), West Nile virus (WNV), Japanese encephalitis virus (JEV) and yellow fever virus (YFV). The virus has a single stranded, plus-sense viral RNA genome of approximately 11,000 nucleotides in length that encodes three structural (C, Env, M) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5; Lindenbach et al., 2007; Chambers et al., 1990). DENV infects host cells such as monocytes by first attaching to cell surface receptors, followed by cell entry via a clathrin-dependent entry pathway (Fig. 1). After fusion of the virus envelope with endosomal membrane, the viral RNA is released into the cytosol and translated on the rough endoplasmic reticulum (ER) membrane. Translated DENV non-structural (NS) proteins re-organize the ER membrane to form replicative complexes within double-membrane vesicles, where viral RNA replication and virus assembly is initiated (Chatel-Chaix and Bartenschlager, 2014).

Dengue virus-associated diseases are major causes of illness and death in the tropics and subtropics, with as many as 400 million people infected yearly (Bhatt et al., 2013; Brady et al., 2012; Shepard et al., 2016). Four serotypes of dengue viruses (DENV1-4), co-circulate in more than 140 countries (Shepard et al., 2016). It is the tenth highest cause of both mortality and morbidity in developing countries and the leading cause of death in children below 15 years old in some South-East Asian countries (Global Health Data Exchange Results Tool, IHME, 2017). Dengue is a worldwide problem due to increased territorial expansion of both dengue viruses and its vector, the *Aedes* (*Ae.*) mosquitoes. With the current trends in human behaviour (population growth, people movement, urbanization, ineffective vector control) and climate changes (due to global warming), continual geographical spread of dengue diseases is anticipated (Halstead, 2008; WHO Fact sheet on dengue).

Co-circulation and frequent large outbreaks of dengue, chikungunya (CHIKV) as well as other flaviviruses such as ZIKV, WNV, YFV in South-

Abbreviations: ADME, Absorption, distribution, metabolism, and excretion; C, Capsid; CC_{50}, Half maximal cytotoxic concentration; DAA, Direct antiviral agent; DENV, Dengue virus; DF, dengue fever; DHF/DSS, Dengue hemorrhagic fever/dengue shock syndrome; dnI, de novo initiation; DSF, Differential scanning fluorimetry; EC_{50}, Half maximal effective concentration; HBV, Hepatitis B virus; HCV, Hepatitis C virus; HIV, Human immunodeficiency virus; HTS, High-throughput screening; IC_{50}, Half maximal inhibitory concentration; IFA, immunofluorescence assay; ITC, Isothermal calorimetry; JEV, Japanese encephalitis virus; KO, knock-out; M, Membrane; MTS, Medium throughput screening; MTase, Methytransferase; NAT, nucleic acid technology; NI, Nucleoside analogue inhibitors; NNI, Non-nucleoside inhibitor; NRTIs, nucleoside analogue reverse-transcriptase inhibitors; NS, Non-structural; PAHO, Pan American Health Organization; PK, Pharmacokinetics; PPIs, protein-protein interactions; RdRp, RNA-Dependent RNA polymerase; SAR, Structure-activity relationship; S/B, Signal-to-background noise; SBBD, structure-based drug design; SPA, Scintillation proximity assay; TDR, Special Programme for Research and Training in Tropical Diseases; TI, Therapeutic index; WHO, World Health Organization; WNV, West Nile virus; YFV, Yellow fever virus; ZIKV, Zika virus

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https://doi.org/10.1016/j.antiviral.2018.12.016
Received 17 September 2018; Received in revised form 22 December 2018; Accepted 25 December 2018
Available online 29 December 2018
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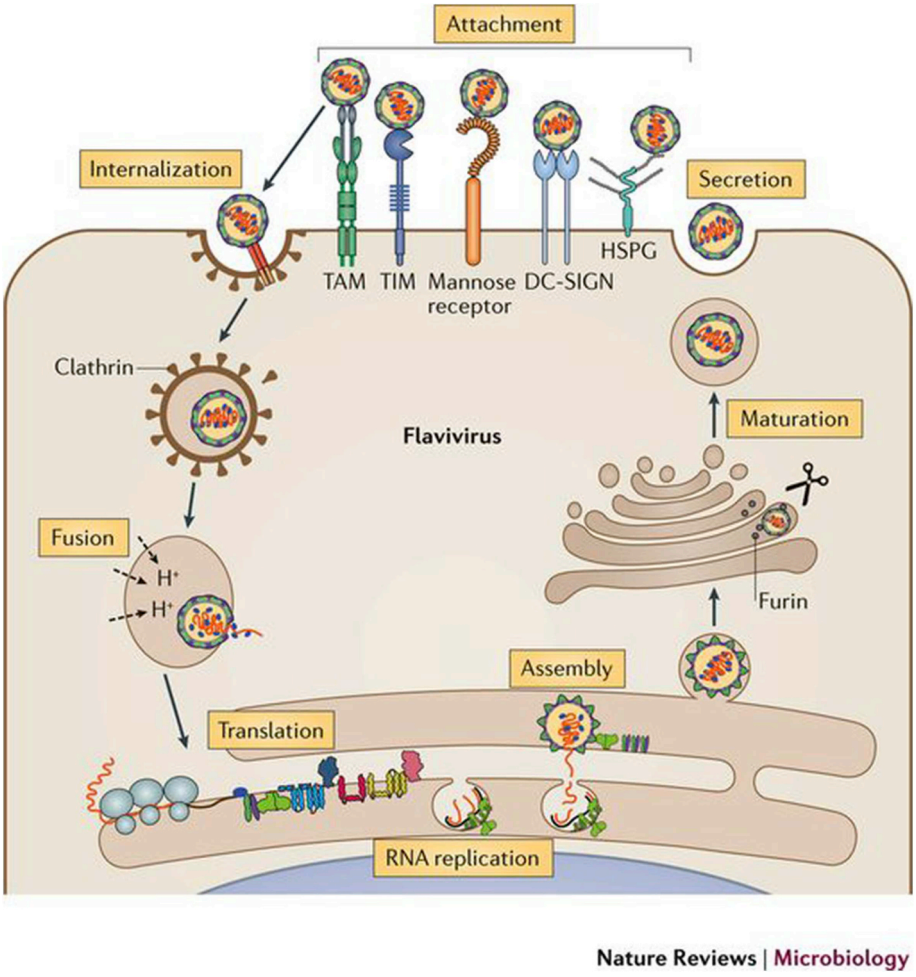

Fig. 1. Dengue virus life cycle. DENV infects host cells such as monocytes by first attaching to cell surface receptors, followed by cell entry via a clathrin-dependent entry pathway. After fusion of the virus envelope with endosomal membrane, the viral RNA is released into the cytosol and translated on the rough endoplasmic reticulum (ER) membrane. Translated DENV non-structural proteins re-organize the ER membrane to form replicative complexes within double-membrane vesicles, where viral RNA replication and virus assembly is initiated. Reproduced with permission from Neufeldt et al. (2018).

east Asia and/or the Americas, pose further public health challenges (Paixao et al., 2018; WHO, 2016–2018, Disease Outbreak news). Patients with these diseases present very similar initial clinical symptoms (flu-like symptoms, high fever, headache, nausea, rash, body pain) with slow and long convalescence phases. Dengue diseases result in approximately 500,000 annual hospitalisations, with increased risks of dengue hemorrhagic/shock syndrome (DHF/DSS) during secondary infections (WHO Fact sheet on dengue). The Pan American Health Organization (PAHO) and WHO recently published guidelines for clinical diagnosis to differentiate between these febrile viral diseases and for the clinical management of infected patients (Tool for the Diagnosis and Care of Patients with Suspected Arboviral Diseases. March-2017, PAHO). In the absence of effective prophylactic and therapeutic measures against DENV, patient management is focused on supportive therapy and the control of onward transmission (WHO Global Strategy for dengue prevention and control, 2012–2020). The economic burden of dengue diseases is estimated at 1.9 million DALYs in developing countries (Global Health Data Exchange Results Tool, IHME, 2017).

Extensive research efforts from a large number of public and private institutions have provided insights into the epidemiology, evolution (Sim and Hibbaerd, 2016; Holmes and Twiddy, 2003) and molecular biology (Lescar et al., 2018; Barrows et al., 2018; Apte-Sengupta et al., 2014) of dengue virus. Similarly, investigations into virus-host interactions (Perera et al., 2017; Acosta et al., 2014) and the immune response to dengue has enabled better understanding of virus pathology (Mathews, 2018; Rivino, 2018; Diamond and Pierson, 2015; Simmons et al., 2015). These knowledge have cumulated in significant undertakings in dengue vaccine development (Silva et al., 2018) and drug discovery (reviewed in Hernandez-Morales and Van Loock, 2018; Whitehorn et al., 2014; Lim et al., 2013a; Noble et al., 2010). Nevertheless, there is still much that we do not know and need to work towards, if we are to deliver safe and effective dengue vaccines (Halstead, 2018; Silva et al., 2018) and therapeutics (Lim et al., 2013a).

Previously, we reviewed the progress in dengue drug discovery made at the Novartis Institute of Tropical Diseases (NITD) as well as the major discoveries made by academia and other companies (Lim et al., 2013a). This review examines the advancements made in this field since 2013, the opportunities and continued challenges that exist and provides perspectives on future directions. Preferred pharmacokinetics and pharmacodynamics profiles of a DENV drug and recommendations for compound progression in the different phases of DENV drug discovery, from hit identification to preclinical testing, as well as challenges in the path to clinical development of dengue drugs, are also discussed. Where applicable, excellent reviews on specific topics will be highlighted and readers are strongly encouraged to refer to these for in-depth study.

2. Feasibility and target product profile of dengue antiviral therapy

Key challenges to the success of dengue therapeutics are the rapid decline in patient viremia (< 1 week duration) during the febrile phase (Libraty et al., 2002a, b; Nguyen et al., 2013) and reluctance (difficulty?) in patients seeking early medical attention. The latter may become less of an issue when an effective antiviral becomes available and necessary infrastructure for drug distribution are put in place. To identify and treat patients early, low-cost, rapid and sensitive diagnostics are critical, especially to discriminate between dengue, and other endemic febrile-causing infectious agents such as malaria, CHIKV and ZIKV. Current commercial immuno-detection assays (rapid lateral flow assays and ELISA for NS1 and anti-dengue envelope antibody detection) and nucleic acid detection technologies (NAT) such as reverse transcription (RT)-qPCR do not meet these requirements for field diagnosis, arguing for the development of more innovative rapid and sensitive diagnostics and identification of predictive biomarkers, especially for assessing progression into DHF/DSS (Nhi et al., 2016; John et al., 2015; reviewed in Low et al., 2018).

Whilst serological IgG/IgM immuno-detection assays are fast and relatively affordable, they have lower sensitivities and are cross-reactive amongst Flaviviruses, making them unsuitable for evaluating individuals with prior flaviviral infections and/or vaccination. Additionally, anti-DENV antibodies are more reliably detected during the later stage of fever/symptom onset as the adaptive immune system takes time to generate humoral response. Although NS1 antigen immuno-detection assays can be used at early infection stages, they suffer from lower sensitivity and variability, depending on the serotype and patient immune status (Chung et al., 2015). Current DENV NAT assays are mainly laboratory-based and are expensive and not rapid (reviewed in Goncalves et al., 2018). That severe dengue diseases is linked to higher viremia levels, supports the hypothesis that reducing viremia early will lower the risks of developing DHF/DSS (Saroch et al., 2017; Lim et al., 2013a). This is also supported by studies that show reduction of virus levels and suppressed inflammatory responses after treatment of DENV infected mice with small molecules against viral (Schul et al., 2007) or host factors (Morison et al., 2017; Pinto et al., 2016). Thus quantitative or semi-quantitative point-of-care NAT could be of value in predicting progression to DHS/DSS. Nevertheless, the validation of this proposition in a clinical setting will be challenging as < 1% of dengue patients develop into DHF/DSS (as discussed in Lim et al., 2013a).

Requisite key characteristics of a dengue drug (target product profile) have been described previously (Hernandez-Morales and Van Loock, 2018; Whitehorn et al., 2014; Lim et al., 2013a; Keller et al., 2006). An ideal DENV drug should be fast-acting (due to rapid viremia decline), equally active against the four DENV serotypes, lessen disease symptoms, shorten days of illness and reduce the risks of disease severity. In terms of drug development, fewer synthesis steps would be advantageous (to reduce the cost of goods for developing countries), as well as oral administration and a long shelf-life, with stability at high temperature and relative humidity (up to 40 °C and 75%, respectively) to facilitate distribution and storage (WHO Technical 62 Report Series). The drug may act via inhibition of either a DENV or host target, but must possess a good safety window (ideally suitable for dosing in young children, pregnant women and elderly) with a low propensity for drug-drug interaction (suitable for combination therapy and for individuals with other health complications). Since DENV is an acute infection, it is envisaged that dosing will not exceed one week. However, this may change, if the drug is shown clinically to be capable of reducing severe DENV diseases or have prophylactic utility.

Emergence of viral resistance to direct acting anti-DENV drugs (DAAs) should be low, when short-term treatment is given. Nevertheless, poor patient compliance or management may still contribute to drug resistance or selection of pre-existing variant strains with drug resistant or compensatory mutant epitopes (Alexander and Bonhoeffer, 2012; Guedj and Neumann, 2010). On this note, the use of new technologies such as next-generation sequencing to study the genetic diversity of virus populations or virus dynamics under selective drug pressure, should shed some light on this area, as is being evaluated for HIV, HCV, Ebola and influenza (Brumme and Poon, 2017; Leung et al., 2017). Likewise, careful consideration of a DENV drug for prophylactic use (i.e. pre-exposure prophylaxis) is important, as a sub-optimal dosing regime may also lead to resistant virus development.

Due to the lack of proof-reading function in dengue virus NS5 polymerase, its nucleotide incorporation error rate is estimated to be around 10^{-5} mutations per nucleotide per replication cycle (Jin et al., 2011; Castro et al., 2005). The resultant virus quasispecies present within the host and also at the population level, can induce the emergence of drug-resistant virus strains following DAA therapy. The actual frequency of inhibitor-resistant mutants is dependent on (i) the number of amino acid substitutions required for resistance, (ii) the genetic barrier (number and type of mutations needed for the amino acid substitutions), and (iii) the fitness cost that the mutations entail. Resistance to inhibitors often requires one or a few mutations, and there are potentially multiple, alternative mutations that can confer resistance to a drug, residing directly in the viral drug target or its interacting viral protein partner(s) (Domingo et al., 2012). To safeguard against escape virus mutants, combination drug therapy that either target different viral proteins (e.g. NS2/3 protease and NS5 polymerase) or host (e.g. alpha-glucosidase) and viral proteins are necessary. This strategy has been shown to be effective, as exemplified by the current treatment of HCV infections. The most recently approved HCV DAA regimens are pan-genotype (GT), once-daily, all-oral DAA combinations of Glecaprevir/pibrentasvir (anti-NS5A/NS4A/3 protease) and sofosbuvir/velpatasvir/voxilaprevir (anti-NS5B (NI)/NS5A/NS4A/3 protease) (reviewed in Crouchet et al., 2018; Vermehren et al., 2018).

Finally, whilst the ideal DENV DAA should be equally effective across all four DENV serotypes, this may be somewhat challenging due to serotype amino acid sequence dissimilarity in the viral structural and non-structural proteins (e.g. NS4B sequence is 78–85% identical in DENV1-4; Lindenbach et al., 2007). This scenario has been observed for HCV DAAs. In the case of the anti-NS5A drug, Daclatasvir (formerly BMS-790052), EC_{50} values in HCV replicon assays for GT-1a and -1b, were 50 pM and 9 pM, respectively, but ranged from pM to low nM for replicons with NS5A derived from GT-2a, -3a, -4a, and -5a (Gao et al., 2010). Similarly, EC_{50} values of HCV polymerase NI, SOVALDI® (Sofosbuvir; formerly PSI-7977) in HCV genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a or 6a replicon assays, ranged from 0.014 to 0.11 μM (SOVALDI® prescription data sheet, Gilead Sciences, Inc). Such divergence in genotypic or serotypic inhibition in DAA treatment argues for virus typing during clinical diagnosis and the application of combination therapy to ensure better treatment outcomes.

3. Pharmacokinetics and pharmacodynamics profiles of a dengue drug

In terms of its pharmacokinetics (PK) profile, a good DENV drug candidate should possess sufficient solubility and stability in the gastrointestinal tract and liver, and be well-absorbed (good permeability), to permit systematically distribution (since DENV has been shown to have wide cell tropism). The physicochemical properties of the drug greatly influence these factors and a good oral drug is based on a balance of these features (Lipinski rule of 5; Lipinski et al., 1997). Limited drug absorption via the gut, early biotransformation and elimination in the liver and kidney can significantly reduce the amount of orally administered drug that enters the systemic circulation. Other preferred PK parameters include good bioavailability and volume of distribution, medium to long half-life, medium to low clearance, to (ideally) enable once a day oral dosing (to facilitate easy patient compliance). Once absorbed systemically, the drug in the blood may be protein or lipid bound or exist in a free soluble form. Only the free soluble form of the

drug is taken up by target organs and cells, as well by the kidney for elimination. Similarly, within a cell, it is the unbound fraction of a drug that exerts therapeutic effect on its target.

An ideal DENV drug should possess a wide therapeutic window (or good safety margin) between the efficacious the dose range and the toxic dose. Drug efficacy is initially determined from *in vitro* assays (e.g. EC_{99}, EC_{90}, EC_{50} and IC_{50} values) and next, in animal models and human patients. However, *in vitro* biochemical and cell-based assays are typically "closed" or isolated systems where the provided drug and target concentrations are in equilibrium during the course of the experiment. *In vivo* systems, on the other hand, are "open" systems, where drug and target concentrations fluctuate with time. Both *in vitro* and *in vivo* efficacy outcomes are strongly influenced by drug-target interactions such as the binding affinity and specificity of the drug to its target (K_a, K_d). For *in vivo* efficacy, drugs with longer residence time (t_R) have been shown to have better biological efficacy (Lu and Tonge, 2010). Improving drug-target residence time (measured as $1/k_{off}$, the reciprocal of the dissociation rate constant, k_{off}) is a key driver during drug discovery to achieve *in vivo* success (reviewed in Copeland, 2016). Other important factors that influence drug efficacy include the highest (C_{max}), and lowest (C_{min}, C_{trough}) drug plasma concentrations achieved after dosing as well as the time (T_{max}) to attain these parameters. To retain pharmacological effects, the free drug plasma concentration should be well-above the EC_{50} value, and ideally above the EC_{90} or EC_{99} values during the entire course of each dosage (trough free drug concentration). This reduces the likelihood that remaining virus attains drug resistance mutations during subsequent rounds of replication (Drusano et al., 2002).

In the absence of an orally bioavailable drug, intravenous drug administration in hospitals or settings that allow patient monitoring should not be ruled out. PK profiles of drug candidates are typically assessed during pre-clinical studies on animal species and in phase I clinical trials on healthy human volunteers. In some developing countries, DENV patient populations may be under-nourished or have other health issues. This may result in changes in drug absorption, distribution, metabolism or elimination (ADME). Thus, DENV drug dosing regimes in phase II/III clinical trials to determine efficacy ought to consider these possible variance. DENV is not reported to cause in utero infection nor is considered a non-neurotropic virus (Li et al., 2017). Dengue drugs, thus need not be specifically designed to pass the blood-brain barrier nor cross the placenta, which may present additional development hurdles.

4. Approaches to find dengue virus inhibitors

Hit finding approaches to obtain new chemical starting points for DENV targets include the use of biochemical, biophysical and cell-based assays to screen compound libraries, either with diverse chemical scaffolds or target-focused compounds (focused library). Aided by available X-ray crystal structures of a number of DENV proteins, researchers utilized computational screening approaches, such as *in silico* compound docking (virtual screening) and rational, structure-based drug design (SBBD) to identify potential inhibitors. These latter methodologies have yielded successful drug candidates, in other infectious diseases, such as HCV and HIV. SBBD may also be applied to the design of new drugs to overcome viral resistance, through mapping resistant epitopes to the X-ray structure of the target proteins, like HIV-1 and HCV protease (Yilmaz et al., 2016) and influenza neuraminidase (Prachanronarong et al., 2016).

The following sections highlight recent discoveries made by researchers (since 2013) using these different methodologies in the quest to identify novel DENV inhibitors (Sections 4.1-4.9) and the extent the hits are validated (Tables 1–6). Criteria and methodologies for compound progression from hit-to-lead, lead optimisation, and candidate drug selection that are relevant to DENV drug discovery, are described in the Supplementary Materials.

4.1. Biochemical assays

Over the years, DENV enzymes, NS2B/3 protease (Erbel et al., 2006; Li et al., 2005; Leung et al., 2001; Yusof et al., 2000), NS3 helicase/NTPase/RTPase (Basavannacharya and Vasudevan, 2014; Wang et al., 2009; Benarroch et al., 2004), NS5 methyl-transferase (Barral et al., 2013; Lim et al., 2013b, 2011; 2008; Chung et al., 2010) and NS5 polymerase (Niyomrattanakit et al., 2015, 2010; Selisko et al., 2006; Nomaguchi et al., 2003) were systematically studied. Biochemical assays for these proteins, based on fluorescence or radioactive nucleotide incorporation were developed and used for inhibitor identification. DENV enzyme functional and structural characterisation, inhibitor identification and profiling in biochemical, binding or cell based assays, have been discussed in-depth for NS2B/3 protease (Leonel et al., 2018; Nitsche, 2018; Luo et al., 2015; Lim et al., 2013a; Noble et al., 2010), NS3 helicase/NTPase/RTPase (Luo et al., 2015; Lim et al., 2013a), NS5 methyl-transferase (MTase) and NS5 polymerase (Lim et al., 2015, 2013a; Bollati et al., 2010).

Different in-house libraries and commercial compound libraries have been interrogated for new chemical starting points with DENV biochemical assays. The compound library sizes ranged from a few hundred to a million compounds (Table 1). More recent screening campaigns have yielded potent inhibitors for NS2B/3 protease (Beesetti et al., 2018; Weng et al., 2016; Balasubramanian et al., 2016; Wu et al., 2015) and NS3 helicase (Sweeney et al., 2015) with activity in DENV cell-based assays. More detailed descriptions of NS2B/3 protease inhibitors have been reviewed by Leonel et al. (2018) and Nitsche (2018). Finding good starting points for NS5 MTase and polymerase from compound screening with *in vitro* enzyme assays have thus far been less fruitful. This may be due to the absence of suitable starting points in the compound libraries and also the highly intractable nature of these protein targets. For example, majority of marketed drugs that against viral DNA (from HSV, HCMV, VZV) and RNA polymerases (from HCV, RSV) and RT (from HIV, HBV) are nucleoside inhibitors (De Clercq and Li, 2016). Many of the hits identified from the Novartis compound library bound in or near the RNA tunnel in the apo-NS5 polymerase (Smith et al., 2015; Noble et al., 2013; Niyomrattanakit et al., 2011; Yin et al., 2009). Subsequent medicinal chemistry follow-ups to generate SAR and improve potency were often challenging due to weak binding affinities, as well as high MWs and lipophilicity of the hits. Using the NS5 de novo initiation assay, Pelliccia et al. (2017) and Benmansour et al. (2016) identified new promising scaffolds, with low micromolar activities in the enzyme and cell-based assays. One frequent trend that occurs from biochemical studies is the lack of hit validation with biophysical assays and confirmation of on-target inhibition in cell-based assays (refer to Table 1). With further compound characterisation and medicinal investigation, recently identified hits can hopefully be ascertained to interact specifically with their respective viral enzyme targets and translate into amenable leads.

4.2. Biophysical assays

A number of biophysical assays such as fluorescence quenching, SPR (surface plasmon resonance), ITC (isothermal calorimetry), DSF (differential scanning fluorimetry or thermal shift), X-ray crystallography, NMR (nuclear magnetic resonance) have been established for DENV NS3 (reviewed in Luo et al., 2015; Noble et al., 2010; Bodenreider et al., 2009) and NS5 (reviewed in Lim et al., 2015). These binding assays are useful tools to validate hits from high throughput screening and for hit-to-lead activities. They have also been used successfully for compound screening (Table 2). Both in-house libraries and commercial compound libraries have been employed for identification of new chemical starting points, with library sizes ranging from a few hundred to several hundred thousand compounds (Table 2). Binding assays to identify inhibitors that prevent protein-protein interaction have also been developed for DENV proteins. Using alphascreen technology, inhibitors have

Table 1
Compound screening with *in vitro* DENV enzyme assays.

Inhibitors Type/Source	Library size	Screening Assay/Method	Most potent Inhibitor	DENV/Host Target	Reference
In-house library	2000	*In vitro* DENV2 NS2B/3 protease assay; 384 well format	BT24 (quinoline derivative), IC_{50} = 5 μM; inhibits DENV1-4 in cell-based assays	DENV NS2B/3 protease; binding to an allosteric site near active site of DENV-2 protease analyzed by *in silico* docking; no biophysical or genetic validation to confirm specificity.	Beesetti et al. (2018)
In-house library of HCV NS5B inhibitors	18	*In vitro* screening with Dengue 3 NS5 RdRp de novo initiation assay with ssRNA polyC template	Compounds **8** and **10** (2,2-dioxido-2,1-benzothiazine benzoates derivatives) with IC_{50} of 0.6 and 0.9 μM; Compound **8** (non-competitive mode of inhibition), EC_{50} > 20 μM; proposed to bind to RdRp N-pocket by molecular modeling.	DENV3 NS5 RdRp; inhibitory specificity confirmed by DLS and Triton-X-100 addition in RdRp assay. No biophysical or genetic validation to confirm specificity.	Cannalire et al. (2018)
Pyrazole analogs	Not stated	DENV NS5 de novo initiation FAPA assay with 3′UTR viral RNA	Compounds **1–3** (pyrazole derivatives): NS5 Pol de novo initiation IC_{50} = 6–8 μM, DENV2 cell-based RdRp and infection assays, EC_{50} = 6–12 μM; compound **3** (10 mg/kg) protected mice from lethal dose of intracerebrally injected DENV.	DENV NS5 RdRp; no biophysical or genetic validation to confirm specificity.	Pelliccia et al. (2017)
In-house library	Not stated	*In vitro* DENV2 NS2B/3 protease assay; fluorescent peptide substrate, Bz-Nle-Lys–Arg–Arg–MCA	Fused bicyclic derivatives of pyrrolidine and imidazolidinone, SAR conducted; compound **2** (DENV2 IC_{50}, K_i and EC_{50} = 1.2, 4.9 and 39.4 μM)	DENV NS2B/3 protease; binding in protease active site analyzed by *in silico* docking; no biophysical or genetic validation to confirm specificity.	Weng et al. (2016)
		In vitro DENV2 NS2B/3 protease assay; fluorescent peptide substrate, Bz-Nle-Lys–Arg–Arg–MCA	Dipeptides of methionine–proline anilides, SAR conducted; compound **1** has DENV2 IC_{50}, K_i and EC_{50} = 1.2, 4.9 and 38.7 μM (competitive inhibitor)	DENV NS2B/3 protease; binding in protease active site analyzed by *in silico* docking; no biophysical or genetic validation to confirm specificity.	Weng et al. (2016); Zhou et al. (2013)
Asinex, TimTec, Biomol, Enamine, Chembridge, ChemDiv, Life Chemicals, Maybridge, MicroSource, NIH, NINDS, Prestwick, Sigma LOPAC, ICBG Fungal Extracts	≤120,000	*In vitro* DENV2 NS2B/3 protease assay; 384 well format; fluorescent peptide substrate, Bz-Nle-Lys-Arg-Arg-AMC	29 hits chosen for reconfirmation; DENV2 K_i, EC_{50} and CC_{50} values of 8 selected compounds (A-H) ranged from 0.22 to 6.9 μM, 0.08 to > 20 μM (plaque assays and replicon) and 29- > 100 μM.	DENV NS2B/3 protease; binding in protease active site analyzed by *in silico* docking; binding validated by competitive fluorescence quench assay with BPTI. No genetic validation.	Balasubramanian et al. (2016)
French National Chemical Library	Not stated	DENV2 NS5 RdRp de novo initiation assay with homopolymeric(U) RNA (picoGreen incorporation)	SAR conducted from hit, compound **1** (IC_{50} = 1.3 μM). Analogues **18**, **27**, **33** and **34** (3-phenyl-5-[(E)-2-(thiophen2-yl)ethenyl]-1,2,4-oxadiazole and 5-phenyl-2-[2-(2-thienyl)ethenyl]-1,3,4-oxadiazole derivatives), IC_{50} = 2–9 μM; EC_{50} from 2 to 12 μM; CC_{50} from 30 to > 100 μM.	DENV2 NS5 RdRp; no biophysical or genetic validation to confirm specificity.	Benmansour et al. (2016)
National Institutes of Health Molecular Libraries Small Molecule Repository	65,423	*In vitro* WNV NS2B/3 protease; fluorescent peptide substrate, Pyr-RTKR-AMC; 384 well format	Compounds **1** and **2** (Pyrazole ester derivative), DENV IC_{50} = 8.5 and 0.5 μM	DENV NS2B/3 protease; binding validated by ESI-TOF MS and NMR with WT and S135A protease mutant, possibly via covalent interaction to active site; binding to DENV2 protease active site analyzed by in silico docking. No validation in DENV cell-based assays.	Koh-Stenta et al. (2015); Johnston et al. (2007)
Focused helicase inhibitor library (ML283 analogues and other compounds)	253	DENV NS3 ATPase colorimetric assay with helicase domain in presence of poly(U)	HTS hit, ML283 (benzothiazole derivative); SAR conducted. Analogue **24** has DENV ATPase and helicase unwinding IC_{50} = 500 nM and 1.5 μM; EC_{50} = 7.1 μM; SI = 17; active against HCV helicase. Second HTS hit class, pyrrolones; compounds **25**-**30** have weak DENV ATPase and cell-based activities, not active against HCV helicase.	DENV NS3 helicase/ATPase; no biophysical or genetic validation to confirm specificity.	Sweeney et al. (2015); Ndjomou et al. (2012)
In-house library	250				Wu et al. (2015)

(*continued on next page*)

Table 1 (*continued*)

Inhibitors Type/Source	Library size	Screening Assay/Method	Most potent Inhibitor	DENV/Host Target	Reference
		In vitro DENV2 NS2B/3 protease assay; fluorescent peptide substrate, Boc–Gly–Arg–Arg–AMC; binding assay based on microscale thermophoresis (MST) technology	Compound **6** (benzothiazole derivatives); DENV2, 3 NS2B/3 protease assay, IC_{50} = 4 and 1 μM respectively (noncompetitive inhibition); DENV2 EC_{50} = 0.8 μM; DENV2 protease cell-based assay EC_{50} = 3.2 μM.	DENV NS2B/3 protease; binding in allosteric site in protease analyzed by *in silico* docking; compound binding validated by MST; no genetic validation to confirm specificity.	
Experimental Therapeutics Centre, Singapore	1600	*In vitro* fluorescent molecular beacon assay (DENV4 helicase unwinding activity); 384 well format	Suramin has K_i of 0.75 ± 0.03 μM (non-competitive inhibitor).	DENV NS3 helicase; no biophysical or genetic validation to confirm specificity.	Basavannacharya and Vasudevan (2014)
Commercial (source not stated)	7000	*In vitro* DENV2 NS2B/3 protease assay; fluorescent peptide substrate, Bz-Nle-Lys-Arg-Arg-AMC	Hit, compound **1** (thiadiazolopyrimidine) has DENV2 IC_{50} = 6.1 μM. SAR conducted; compound **8b** (thiadiazoloacrylamide derivative) has DENV2 IC_{50} = 2.24 μM	DENV NS2B/3 protease; binding in allosteric site in protease analyzed by *in silico* docking; compound binding validated by MST; no genetic validation.	Liu et al. (2014)
Novartis	≤257,000	DENV4 NS5 RdRp (aa266-900) fluorescent de novo initiation FAPA and LCMS detection assays	Various chemical scaffolds, NS5 RdRp IC_{50} < 20 μM, DENV2 replicon EC_{50} < 30 μM	DENV4 NS5 RdRp; biophysical validation with DSF. No genetic validation to confirm specificity.	Smith et al. (2015)
MicroSource Spectrum Collection (MicroSource Discovery Systems Inc., Gaylordsville, CT)	2000	*In vitro* DENV2 NS2B/3 protease assay; fluorescent peptide substrate, Boc–Gly–Arg–Arg–AMC	Tyrothricin (DENV2 K_i = 12 μM; competitive inhibition); Ivermectin (DENV2 K_i = 79 μM; mixed non-competitive inhibition); Selamectin (DENV2 K_i = 15 μM; mixed non-competitive inhibition)	DENV NS2B/3 protease; counter-screens to remove artifacts; specificity checked with trypsin assay; no biophysical or genetic validation to confirm specificity.	Tomlinson and Watowich (2012)
Crude venom extractions of cone snails (comprise disulfide-rich short peptides of 10–40 residues) followed by fractionation	Not stated	*In vitro* DENV2 NS2B/3 protease assay; fluorescent peptide substrate, Bz-Nle-Lys-Arg-Arg-AMC	Initial hit, MrIA (conotoxin from marmoreus, 13-aa peptide), followed by SAR. Cyclic octapeptide, **9** has DENV2 NS2B/3 K_i = 9 and 2.2 μM, respectively	DENV NS2B/3 protease; binding in active site in protease analyzed by *in silico* docking; no biophysical or genetic validation to confirm specificity	Xu et al. (2017)
Chemical Diversity Laboratory (San Diego, CA)	41,600	*In vitro* DENV2 NS2B/3 protease colorimetric assay; peptide substrate acetyl-TTSTRR-para-nitroaniline	BP2109 DENV2 IC_{50} of 15.43 μM, DENV-2 replicon EC_{50} = 0.17 μM.	DENV NS2B/3 protease; compound resistant DENV2 shows R55K and E80K mutations in NS2B; no biophysical validation.	Yang et al. (2011)
Novartis	1 million	DENV4 NS5 Pol elongation SPA assay (homopolyC/oligoG)	Primary hit, NITD-1 (IC_{50} = 7.2 μM, N-sulfonylanthranilic acid derivative); SAR conducted. Most active analogue, NITD-28 (IC_{50} = 260 nM); inactive in DENV cell-based assays	DENV2 NS5 FL; binding confirmed by uv photo-crosslinking studies and MS analysis.	Niyomrattanakit et al. (2010); Yin et al. (2009)

Table 2
Compound screening with DENV protein binding assays.

Inhibitors Type/Source	Library size	Screening Assay/Method	Inhibitor	DENV/Host Target	Reference
Selleck bioactive compound library and the Chemdiv 7 library	21,271	Alpha screen with DENV prefusion E dimer or domain I (DI)-DII protein and GNF-2 inhibitor (4,6-disubstituted pyrimidine derivative)	7 compounds, IC_{50} < 10 μM; DENV2 EC_{50} range from 0.8 to 9 μM	DENV Envelope; no validation of on-target inhibition in DENV-infected cells.	Lian et al. (2018)
Quinazolinone derivatives	Not stated	SPR with DENV2 RdRp (aa272-900)	Q63, K_d = 0.9 μM; DENV-1, -2, -4 EC_{50} = 1.7–2.1 μM.	DENV NS5 RdRp; binding confirmed by ITC, binding site in RdRp analyzed by *in silico* docking. No validation of on-target inhibition in DENV-infected cells.	Yao et al. (2018)
Maybridge Chemical Company; RO3 fragment library 2009 (Cambridge, U.K.)	500	Thermo-denaturation assay with DENV4 NS3 helicase and DENV3 NS5 MTase	No hits for helicase, 7 hits for MTase (IC_{50} range from 0.18 to 9 mM); fragment linking strategy improved 2′-*O* MTase inhibitory activity.	DENV NS3 helicase and NS5 MTase; binding site in MTase analyzed by *in silico* docking. No biophysical or genetic validation.	Benmansour et al. (2017); Coutard et al. (2014)
Novartis in-house fragment library	1408	DENV3 RdRp (aa 273–900) X-ray crystallography	Most potent compounds, **27**, **29**, **29i**; DENV1-4 NS5 de novo initiation (dnI) IC_{50} = 13–172 nM; DENV4 NS5 elongation IC_{50} = 0.43–5 μM; DENV1-4 EC_{50} = 2–14 μM.	DENV NS5 RdRp; binding confirmed by ITC, SPR, DSF, cellular thermo-shift. Compound resistant DENV2 replicons have L512V and E802D mutations in RdRp.	Noble et al. (2016); Yokokawa et al. (2016); Lim et al. (2016)
LOPAC (Library of Pharmacologically Active Compounds; Sigma, St. Louis, MO)	480	AlphaScreen® to inhibit interaction between HIV-1 integrase and nuclear transport receptor importin α/β1 (IMPα/β1)	Ivermectin; inhibition of DENV1/2 NS5- IMPα/β1 interaction, IC_{50} = 1.5–2.3 μM; EC_{50} = 1.6–2.3 μM.	DENV NS5-IMPα/β1 interaction; no validation of on-target inhibition in DENV-infected cells.	Tay et al. (2013); Wagstaff et al. (2011)
Cysteine reactive probes	5	Binding of cysteine reactive probes to DENV NS2B/3 protease cysteine mutant A125C; fluorescent peptide substrate, N-acetyl-Gly-Arg-Arg-AMC	5,5′-dithiobis-(2-nitrobenzoic acid (DTNB) and biaryl chloromethylketone (BAClMK)	DENV NS2B/3 protease; binding confirmed by X-ray crystallography.	Yildiz et al. (2013)
NRSB library, Institute of Chemistry and Cell Biology Longwood Screening Facility, Harvard Medical School	235,456	DENV2 MTase GTP-bodipy displacement assay	Hit class with thioxothiazolidin core. 24 analogues tested. BG-323 has DENV2 guanylation K_i and IC_{50} = 7.5 and 7.3 μM; EC_{50} = 30 μM	DENV MTase; binding site in MTase analyzed by *in silico* docking; no validation of on-target inhibition in DENV-infected cells.	Stahla-Beek et al. (2012)
Compound libraries at NSRB at Harvard Medical School.	≤30,000	DENV2 Env stem peptide (aa419–447) conjugated with FITC- soluble Env trimer FP assay; 384 well format	1662G07 (Maybridge; IC_{50} = 15 μM; EC_{50}/CC_{50} = 16.9/ > 100 μM); and different analogues, IC_{50} = 8–40 μM; DENV2 EC_{50} = 1.5–10 μM	DENV Envelope; binding confirmed by SPR; no validation of on-target inhibition in DENV-infected cells.	Schmidt et al. (2012, 2010)

Table 3
DENV rational drug design.

Inhibitors Type/Source	Library size	Screening Assay/Method	Inhibitor	DENV/Host Target	Reference
Cyclic peptides designed from aprotinin (target DENV protease prime sites)	19	Designs are based on aprotinin-bound DENV3 NS2B/3 protease X-ray structure (PDB code 3U1J). Fluorescence resonance energy transfer (FRET)-based NS2B/3 protease assay; ITC	19 cyclic peptides targeting S3 to S4′ positions made and tested. CP7 (PC*RARIYGGC*A; cyclized through a disulfide bond between two cysteine residues*), DENV3 K_i = 2.9 μM	DENV NS2B/3 protease; molecular simulation to analyze binding in protease active site; no biophysical or genetic validation to confirm specificity.	Lin et al. (2017, 2016)
Peptide inhibitors with CONH2 and boronic acid warheads	8 each	Fluorescence resonance energy transfer (FRET)-based NS2B/3 protease assay	Best compound, **7** (Bz-(4-guanidino)Phe-Arg-B(OH)2; DENV2 K_i = 27 nM; IC_{50} = 36 nM; EC_{50}/CC_{50} = 18/ > 100 μM	DENV and WNV NS2B/3 protease; confirmed by X-ray crystallography; no genetic validation.	Nitsche et al. (2017)
High throughout synthesized cyclic octapeptides	33	*In vitro* DENV2 NS2B/3 protease assay; fluorescent peptide substrate, Boc–Gly–Arg–Arg–MCA	Best peptide, **22** has IC_{50} = 0.95 μM; most cellular active peptide, **33,** has IC_{50} = 2.1 μM and EC_{50}/CC_{50} = 11.4/129 μM.	DENV NS2B/3 protease; binding in active site analyzed by *in silico* docking; no biophysical or genetic validation.	Takagi et al. (2017)
Fused bicyclic compounds of pyrrolidino and imidazolidinone derivatives	Not stated	*In vitro* DENV2 NS2B/3 protease assay; fluorescent peptide substrate, Bz-Nle-Lys–Arg–Arg–MCA	Fused pyrrolidino [1,2-c]imidazolidinone compound, **2** has IC_{50} = 1.2 μM (competitive to nucleotide incorporation) and DENV2 EC_{50} = 39 μM.	DENV NS2B/3 protease; binding site in active analyzed by *in silico* docking; no biophysical or genetic validation.	Weng et al. (2016); Zhou et al. (2013)
Library of active-site metal ion chelator	n.a	*In vitro* filter-binding DENV RdRp assay	DMB220 (pyridoxine-derivative); DENV1-4 RdRp IC_{50} and EC_{50}/CC_{50} = 5–6.7 μM and 2.2–2.8/ > 50 μM; inactive against HIV RT and weakly active against HIV integrase.	DENV RdRp; S600T mutation in RdRp conferred 3-fold increase in IC_{50}. *In silico* docking of compound in RdRp; no biophysical or genetic validation.	Xu et al. (2016); Stranix et al. (2009).
Peptidomimetics (N-capped-Capped (Bz-Arg-Lys- 4-hydroxyphenylglycine-NH_2 tripeptides)	< 100*	Fluorescence resonance energy transfer (FRET) with substrate Abz-Nle-Lys-Arg-Arg-Ser-3-(NO_2)Tyr and HPLC-based NS2B/3 protease assays	**104**, DENV2 K_i = 18 nM; EC_{50}/CC_{50} = 3.4/ > 100 μM. Inactive against thrombin and trypsin.	DENV NS2B/3 protease; binding in active site analyzed by *in silico* docking; competitive fluorescence quench assay with aprotinin confirmed binding to protease active site; no genetic validation.	Behnam et al. (2015*, 2014); Nitsche et al. (2012,2013)
Peptide inhibitors with phenylalanine and phenylglycine analogues as arginine mimetics for S2 pocket.	24	Fluorescence resonance energy transfer (FRET) assay with substrate Abz-Nle-Lys-Arg-Arg-Ser-3-(NO_2)Tyr	Most potent compound, **42a**, DENV IC_{50} = 210 nM, K_i = 139 nM; not active against WNV protease, trypsin.	DENV NS2B/3 protease; binding in active site analyzed by *in silico* docking; competitive fluorescence quench assay with aprotinin confirmed binding to protease active site; no genetic validation.	Weigel et al. (2015)
Capsid peptides	2	Atomic force microscopy-based force spectroscopy, dynamic light scattering, NMR and computational analysis	Peptide comprising aa14-23 of DENV Capsid protein, IC_{50} and EC_{50} not determined	Inhibits capsid binding to perilipin 3 in lipid droplets (LD), ApoE in very low-density lipoproteins (VLDLs). No genetic validation.	Faustino et al. (2015a, b, 2014); Martins et al. (2012)
S-adenosyl-homocysteine analogues	12	*In vitro* DENV3 MTase N7 and 2′-*O* MTase assay	Compound 10, N7 and 2′-*O* IC_{50} = 0.82 and 0.17 μM, respectively.	DENV MTase; compound binding confirmed by X-ray crystallography, No genetic validation.	Lim et al. (2011)

Table 4
Compound screening with *in silico* docking and modeling in X-ray structures of DENV proteins.

Inhibitors Type/Source	Library size	Screening Assay/Method	Inhibitor	DENV/Host Target	Reference
NCI diversity set II library	24,428	*In silico* docking of apo-DENV NS2B/3 protease X-ray crystal structure (2FOM) using AutoDock Vina program; fluorescence-quench NS2B/3 protease assay with heptapeptide; split luciferase complementation (SLC)-based conformational switch assay to monitor NS2B conformational changes upon binding to NS3 protease	Tested top 29 hits; NSC135618, DENV2 IC_{50} = 1.8 μM (hill coefficient = 0.7; non-competitive mode of inhibition); EC_{50} = 0.81 μM; A549 CC_{50} = 48.8 μM; inhibits ZIKV, YFV and WNV cell-based assays at low micromolar activities.	Likely NS2B/3 protease; binding confirmed with biophysical (thermal shift; Tm increased by 2.6–4.8 °C) using WT and mutant proteins and biochemical ZIKV protease cleavage assay. No genetic validation to confirm specificity.	Brecher et al. (2017)
Maybridge database (using ICM software)	110,000	*In silico* docking of DENV Env β-OG binding site; DENV firefly luciferase reporter infectious virus assay	23 hits; compounds **2** and **5** with DENV2 EC_{50} = 3.1 and 5 μM, respectively; BVDV EC_{50} > 50 μM; CC_{50} > 100 μM; compound **2** docked into DENV Env β-OG binding site.	Maybe DENV Envelope. No biophysical or genetic validation to confirm specificity.	Leal et al. (2017)
Pyrazole analogs	Not stated	*In silico* docking of allosteric pocket of apo-DENV NS2B/3 protease X-ray crystal structure (2FOM) using PLANTS; DENV NS2B/3 protease assay with Boc-Gly-Arg-Arg-AMC	Compounds **4**, **5**: NS2B/3 protease IC_{50} = 5–7 μM; DENV2 cell-based protease and infection assays, EC_{50} = 5–8 μM; compound **4** (1 mg/kg) protected mice from lethal dose of intracerebrally injected DENV.	Maybe DENV NS2B/3 protease; no biophysical or genetic validation to confirm specificity.	Pelliccia et al. (2017)
PubChem	210,903	*In silico* docking of apo-DENV NS2B/3 protease X-ray crystal structure (2FOM) using AutoDock Vina program; DENV NS2B/3 protease assay with Boc-Gly-Arg-Arg-AMC.	5 hits tested; CID54681617, CID54692801 and CID54715399; DENV2 IC_{50} values (μM) and viral titer reduction assay = 19.9 & 79.9%; 17.5 & 69.8% and 9.1 & 73.9%, respectively.	Maybe DENV NS2B/3 protease; no biophysical or genetic validation to confirm specificity.	Cabarcas-Montalvo et al. (2016)
PubChem and SuperNatural II database	372792 (47,473 small molecules and 325,319 natural products)	Homology models of DENV1-4 RdRp; *in silico* docking into RdRp RNA tunnel using AutoDock/Vina programs	39 compounds predicted to bind; IC_{50} not reported.	Maybe DENV3 RdRp; no biochemical, biophysical or genetic validation to confirm specificity.	Galiano et al. (2016)
Plant-derived secondary metabolites	2194	*In silico* docking of DENV envelope, helicase, protease, MTase and RdRp X-ray crystal structures and NMR structures using Molegro Virtual Docker (version 6.0, Molegro ApS, Aarhus, Denmark)	25 hits for NS2B/3 protease; 21 hits for NS3 helicase; 15 hits for MTase; 1 hit for RdRp; 31 hits for Envelope. Most frequent binders: Polyphenolic compounds, flavonoids, chalcones, and other phenolics; IC_{50} not reported	DENV envelope, helicase, protease, MTase and RdRp; no biochemical, biophysical or genetic validation to confirm specificity.	Powers and Setzer (2016)
In-house library of HCV NS5B inhibitors	203	*In silico* docking into DENV3 RdRp X-ray crystal structure (2J7U) using AutoDock 4.2 software package	HeE1-2Tyr (pyridobenzothiazole derivative; non-competitive mode of inhibition), DENV3 IC_{50} = 1.5 μM; DENV1-4 EC_{50} = 6.8–15 μM.	DENV3 NS5 RdRp (binding site determined by X-ray crystallography is in Site 1 between fingers domain and the priming loop); binds in the same region as NITD-107.	Tarantino et al. (2016)
ChemBridge Corporation (San Diego, CA), Enamine (Kyiv, Ukraine), Life Chemicals (Niagara-on-the-Lake, ON), Maybridge Chemicals, Thermo Fisher Scientific Inc., Janssen Pharmaceutical (Belgium).	5 million	*In silico* docking of DENV NS2B/3 protease X-ray crystal structure with nKRR-H inhibitor (3U1I) using Molecular Operating Environment Molecular Operating Environment (MOE) software, Chemical Computing Group Inc.) and AutoDock (The Scripps Research Institute); DENV NS2B/3 protease assay with Bz-nKRR-AMC.	14 hits; compound **14** shows 85.3% at 300 μM inhibition in NS2B/3 protease assay; EC_{50} = 5 μM	Maybe DENV NS2B/3 protease; no biophysical or genetic validation to confirm specificity.	Li et al. (2015)
Src tyrosine protein kinase active scaffolds (databases of bioactive molecules from ChEMBL, BindingDB) and internal collection of kinase inhibitors	≤3000 Src inhibitors and ≤10000 virtual analogs	*In silico* docking of DENV3 NS5 RdRp allosteric site (cavity B) using Glide Standard Precision docking protocol and Autodock Vina; NS3−NS5 AlphaScreen assay	22 compound tested; compound **16i** (purine derivative) with DENV EC_{50} = 5.3 μM; CC_{50} = 168 μM	May inhibit DENV NS3−NS5 interaction; no biophysical or genetic validation to confirm specificity.	Vincetti et al. (2015)
(A) Subset of the ZINC database with "drug-like" properties (selected from ChemBridge Corporation, ChemDiv Inc. (San Diego, CA), Ryan Scientific Inc. (Mount Pleasant, SC), Maybridge Chemical Company, Sigma-	(A) 642,769 (B) 45,458	Drug Discovery@TACC portal; *in silico* docking of DENV NS2B/3 protease X-ray crystal structures with (3U1I and 3U1J) and without (2FOM) bound inhibitors using autodock Vina program on supercomputer	ZINC04321905; DENV NS2B/3 protease K_i = 7 μM with mixed noncompetitive inhibition.	Maybe DENV NS2B/3 protease; no biophysical or genetic validation to confirm specificity.	Viswanathan et al. (2014)

(*continued on next page*)

Table 4 (*continued*)

Inhibitors Type/Source	Library size	Screening Assay/Method	Inhibitor	DENV/Host Target	Reference
Aldrich); (B) Focused library from ChemBridge and Maybridge Chemical with dogP filters.		resources at the Texas Advanced Computing Centre; DENV NS2B/3 protease assay with BocGRR-AMC.			
MOE lead-like database	661,417	*In silico* docking of DENV NS2B/3 protease X-ray crystal structure (2FOM) at the NS3-NS2B interaction region using MOE software	39 hits tested; SK-12 (noncompetitive inhibitor); DENV1-4 EC_{50} = 0.7–2.4 μM; JEV EC_{50} = 29.8 μM; in silico modeling in NS2B binding site of NS3 protease.	Maybe DENV NS2B/3 protease; no biophysical or genetic validation to confirm specificity.	Pambudi et al. (2013)
Library of Pharmacologically Active Compounds (LOPAC), Sigma-Aldrich	1280	*In silico* docking (AutoDock 4 software package) of a model of WNV NS3 helicase X-ray crystal structure (2QEQ) with ssRNA.	3 hits tested; Ivermectin (DENV IC_{50} = 500 ± 70 nM; EC_{50} = 700 nM (virus reduction assay) and < 1 μM (CPE assay); K_i = 354 nM; uncompetitive inhibition. Mutations T408A and D409A in helicase protein abolish inhibitory activity. Also inhibits WNV, YFV, TBEV, JEV but not BVDV nor HCV.	Maybe DENV helicase; no biophysical or genetic validation to confirm specificity.	Mastrangelo et al. (2012)

been found for envelope protein (Lian et al., 2018) and NS5 (Tay et al., 2013) which have good potency in DENV cell-based assays. A report by Yao et al. (2018) described the identification of a quinazolinone derivative, Q63, that inhibits NS5 RdRp, following compound screening by SPR. Q63 shows good DENV-1, -2, -4 inhibition in cells with EC_{50} values ranging from 1.7 to 2.1 μM. In addition, researchers have used competitive ligand binding assays to screen for inhibitors. Stahla-Beek et al. (2012) used a fluorescent-labeled GTP (GTP-bodipy) to screen for inhibitors to NS5 MTase and identified BG-323. By generating a A125C NS2B/3 protease mutant, Yildiz et al. (2013) used cysteine reactive probes to develop a screen which can be used to identify protease inhibitors.

Fragment-based drug discovery (FBDD) by X-ray crystallography has recently been successfully used to develop potent cellular active inhibitors against NS5 polymerase (reviewed in Lim et al., 2018). Starting from a hit, JF-31-MG46, that binds weakly to a novel pocket in DENV3 RdRp and is inactive in DENV cell-based assays (DENV1-4 RdRp K_d and *dn*I IC_{50} value = 610 and 734 μM, respectively; DENV1-4 EC_{50} > 50 μM), the most active derivatives (compound **27**, **29**, **29i**) inhibited DENV1-4 infection at low-to-high micromolar concentrations (Table 2). Fragment based screening by DSF with NS5 MTase and NS3 helicase have also been performed. Whilst no hits were obtained for helicase, seven hits were found for NS5 MTase (Coutard et al., 2014). By linking two fragments, the group was able to further improve inhibitor potency Benmansour et al. (2017). One advantage of using fragments as starting points is that they often have higher ligand efficiencies and binding is driven by hydrogen bond interactions. This increases the likelihood that the final optimized ligand will not be too hydrophobic (log P < 5). To date, eighteen drug candidates discovered by FBDD have advanced to clinical trials (Erlanson et al., 2016; Velvadapu et al., 2015). There are two FDA-approved drugs derived from fragment-based approaches, Zelboraf® (vemurafenib, PLX4032; targets B-Raf-V600E mutant enzyme, for treatment of late-stage melanoma; Bollag et al., 2012) and Venclexta (Venetoclax; targets B-cell lymphoma-2 (BCL-2) for treatment of chronic lymphocytic leukemia (CLL) and small lymphocytic lymphoma (SLL); Scheffold et al., 2018). Nevertheless, due to the need for strong computational and medicinal support in FBDD, some groups may be hesitant to embark on this approach.

4.3. Structure based drug discovery

In general, structure based drug discovery (SBDD) for DENV has mainly been used to pursue peptidomimetics against NS2B/3 protease (Table 3). These have resulted in peptidic inhibitors with submicromolar activities in *in vitro* protease assays and high micromolar activities in DENV cell-based assays (Lin et al., 2017, 2016; Nitsche et al., 2017, Takagi et al., 2017). Whilst the focus is on targeting the P1–P4 sites in the NS2B/3 protease (Nitsche et al., 2017, Takagi et al., 2017; Behnam et al., 2015), Lin et al. (2017, 2016) reported success in targeting the P′ sites with cyclic peptides, to inhibit this enzyme. To date, the most potent peptide inhibitor, **103**, was described by Behnam et al. (2015), with DENV2 K_i = 18 nM; EC_{50} = 3.4 μM. The compound library size described by researchers using the SBDD approach, are typically smaller (< 100) as each peptidic inhibitor is custom synthesized. Rational design approaches have also been attempted for DENV capsid (Faustino et al., 2015a; b, 2014), NS5 RdRp (Xu et al., 2016) and MTase (Lim et al., 2011). DMB220, an RdRp inhibitor, showed encouraging pan-DENV1-4 inhibition in both biochemical and cell-based assays (Xu et al., 2016, Table 3). Comprehensive reviews of the DENV envelope peptide inhibitors that block virus entry (Chew et al., 2017) and NS2B/3 (Nitsche, 2018; Chew et al., 2017; Lim et al., 2013a) peptide inhibitors have previously been undertaken and readers are encouraged to refer to them for more details.

Table 5
Compound Screening with DENV cell-based assays.

Inhibitors Type/Source	Library size	Screening Assay/Method	Inhibitor	DENV/Host Target	Reference
Mammalian Ser/Thr, Tyr and lipid kinase inhibitors from Calbiochem (San Diego, CA), Sigma-Aldrich (St. Louis, MO), Pierce (Rockford, IL), re-synthesized clinical-stage inhibitors)	> 120	DENV infection of Vero cells (IFA, fluorescence detection); 384 well format	Imatinib, dasatinib, GNF-2 (4,6 disubstituted pyrimidine), AZD0530, 2,4-diamino pyrimidines, DENV2 EC_{50} from 5 to 20 μM	c-Src, Abl and Fyn kinases and block DENV particle formation by binding to DENV Envelope in ER; validated by siRNA knockdown	de Wispelaere et al. (2018), 2013; Clark et al. (2016); Chu and Yang (2007)
In-house library	Not stated	*In vitro* screen with DENV-2 luciferase reporter replicon assay; inhibitions tested with DENV2 whole virus infection assay	SAR exploration from hit compound, 10e; compound **14l** (phthalazinone derivative); DENV2 EC_{50} value of 0.13 μM	Under investigation (proposed as NS2B/3 protease based on docking studies); no biochemical, biophysical or genetic validation to confirm specificity.	Lu et al. (2018)
Shionogi antiviral compounds library	7000	DENV-induced cytopathic (CPE) assay	Compound-B (benzimidazole derivative); DENV1-4 EC_{50}s = 1.32–4.12 μM	NS4A; compound resistant virus, has C87S mutation in NS4A.	Nobori et al. (2018)
Chemically diverse compounds National Screening Laboratory for the Regional Centers of Excellence in Biodefense and Emerging Infectious Diseases (Harvard Medical School, Boston, MA)	51,000	Human foreskin fibroblasts, stably expressing human telomerase reverse transcriptase and IRF3/IFN-responsive pGreenFire-ISRE lentivector.	AV-C ((2-fluorophenyl)-2-(5-isopropyl-1,3,4-thiadiazol-2-yl)-1,2-dihydrochromeno[2,3-c]pyrrole-3,9-dione); DENV2 EC_{50} from 9.9 μM	Agonist of TRIF signaling pathway, induces IRF3 expression and type I interferon secretion; IPS-1/MAVS involved in Flavivirus replication. Validated with CRISPR/Cas9-mediated genome editing in cells.	Pryke et al. (2017)
MicroSource (2000 known drugs, experimental bioactives, and pure natural products), Prestwick Chemical Library (1119 off- patent drugs), Tocris, CRL, BioFocus, SPECS	5632	DENV2 infection of HEK293 cells (high content imaging, fluorescence detection); 384 well format	SAR exploration of 39 analogs; VGTI-A3, VGTI-A3-03 (DENV2 IC_{90} = 112 and 40 nM, respectively)	Binds to DENV capsid pocket involved in dimerization and associates with secreted virus particles; compound resistant virus, has T25L, L35P, L38M mutations in capsid.	Smith et al. (2018), Shum et al. (2010)
			SKI-417616 (dihydrodibenzothiepines derivatives); DENV2 EC_{50} = 1.2 μM; CC_{50} = 43.2 μM; also inhibits DENV-1, -3, -4, WNV, SINV at similar potency.	Inhibits Dopamine Receptor D4 and Downstream Mitogen-Activated Protein Kinase Signaling (through blocking ERK phosphorylation); chemical validation with known inhibitors.	Smith et al. (2014)
			Methotrexate (MTX) and floxuridine (DENV2 IC_{50} = 90 and 60 nM, respectively); also inhibits DENV-1, -3, -4, WNV at similar potency. No efficacy in lethal DENV mouse model.	MTX = dihydrofolate reductase; floxuridine = uracil analogue, inhibits thymidylate synthase; chemical validation with thymidine precursor.	Fischer et al. (2013)
Chemical Diversity Lab (San Diego, CA)	60,000	DENV-2 luciferase reporter replicon in BHK21 cells	BP13944, DENV2 EC_{50} = 1.03 ± 0.09 μM; active on DENV-1, -3 and -4.	DENV NS2B/3 protease; compound resistant DENV2 replicon has E66G mutation in NS3 protease.	Yang et al. (2014)
Janssen Pharmaceutical in-house library	Not stated	DENV-2 luciferase reporter replicon	JNJ-1A, DENV1-4 EC50 = 0.7 μM; equally potent on DENV-1, -3, -4.	DENV NS4B; compound resistant DENV2 replicon has T108I mutation in NS4B; P104L/A119T mutation in NS4B also abolished inhibition; same as NITD-618.	Hernandez-Morales et al. (2017)
			SDM25N	γ opioid receptor antagonist, DENV EC_{50} = 1.9 μM; compound resistant DENV2 replicon has F164L mutation in NS4B; P104L mutation in NS4B also abolished inhibition; same as NITD-618 and JNJ-1A.	van Cleef et al. (2013)
Chemical library from the Centre for Drug Design and Discovery (CD3); KU Leuven	Not stated	DENV2-induced CPE in Vero-B cells (7 day assay)	Compound **1** (acyl-indole derivative), DENV2 EC_{50}/CC_{50} = 0.078/29 μM; extensive SAR exploration resulted in compound **12a** (dimethoxyaniline analogue, (+)-enantiomer) DENV2 EC_{50}/CC_{50} = 0.007/16 μM	NS4B; compound resistant DENV2 has mutation in NS4B (amino acid not stated).	Bardiot et al. (2018)
			Compound **14** (purine pyrazolyl derivative); DENV2 EC50/CC50 = 1.9/ > 109 μM	Proposed NS5 RdRp; *in silico* docking in cavity B; no biophysical or genetic validation to confirm specificity.	Venkatesham et al. (2017)
			Compounds **6b**, **6d** and **7a** (pyrazine dicarboxamide derivatives), DENV2 EC50/CC50 = 0.5/ > 116 μM	Not determined	Saudi et al. (2016)
			Compounds **15b** (imidazole dicarboxamide derivative), DENV2 EC_{50}/CC_{50} = 2.5/ > 120 μM; compounds **20a** and **20b** (pyrazine dicarboxamide derivatives), DENV2 EC_{50}/CC_{50} = 0.94/ > 117.5 μM.	Not determined	Saudi et al. (2014a)

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Table 5 (*continued*)

Inhibitors Type/Source	Library size	Screening Assay/Method	Inhibitor	DENV/Host Target	Reference
Selleckchem bioactive compound library (FDA-approved drugs and known bioactives)	≤2000	ZIKV infection of U2OS cells (high content imaging, fluorescence detection); 384 well format	Nanchangmycin (IC_{50} not determined); active against ZIKV, WNV, CHIKV.	Receptor Tyrosine Kinase, AXL; proposed as attachment receptor for Flaviviruses; no chemical or genetic validation to confirm specificity.	Rausch et al. (2017)
Novartis in-house library	1.8 million	DENV-2 luciferase reporter replicon; counter-screen with HCV replicon	Hit, compound **1** (spiropyrazolopyridon derivative), DENV2 EC_{50} = 14 nM (HCV replicon EC_{50} > 5 μM); extensive SAR exploration; compounds **14a**; DENV-2 and -3 EC_{50} = 42/76 nM; DENV-1 and -4 EC_{50} > 1 μM; treatment with 5, 25, and 50 mg/kg of compound14a (BID) reduced mouse viremia by 1.7-, 10-, and 39-folds.	DENV NS4B; compound resistant DENV2 has V63 A/L/M/S/T mutations in NS4B.	Wang et al. (2015); Zou et al. (2015)
			NITD-618 (aminothiazole derivative); DENV1-4 EC_{50}/CC_{50} = 1.0–4.1 μM/ > 40 μM	DENV NS4B; compound resistant DENV2 replicon has P104L and A119T mutation in NS4B.	Xie et al. (2011)
		DENV-induced cytopathic (CPE) assay	NITD-982 (isoxazole-pyrazole derivative); DENV2 EC_{50}/CC_{50} = 2.4 nM/ > 5 μM	Inhibits host dihydroorotate dehydrogenase involved in de novo pyrimidine biosynthesis; validated by binding assay, brequinar and uridine addition in DENV cell-based assay	Wang et al. (2011a)
			Hit, NITD2636 has DENV-2 EC_{50} = 0.55 μM; extensive SAR exploration; NITD-451 (benzomorphan derivative); DENV2 EC_{50}/CC_{50} = 160 nM/ > 50 μM; treatment with 25 mg/kg of compound14a (QD) reduced mouse viremia by about 40-folds.	Viral RNA translation; validated with biochemical and cell-based assays.	Wang et al. (2011b)
Not stated	≤200,000	DENV2 infection of Vero cells (CPE measurement); 96 well format	ST-148 (Maybridge); DENV1-4 EC_{50}/CC_{50} = 16–2800 nM/ > 100 μM; treatment with 50 mg/kg of compound (QD) reduced mouse viremia by about 52-folds.	DENV capsid, ST-148 enhanced capsid protein self-interaction; compound resistant DENV2 has S34L mutation in capsid; validated by intracellular BRET assay.	Byrd et al. (2013a); Scaturro et al. (2014)
			ST-610 (benzoxazole derivative; ChemBridge); DENV1-4 EC_{50} = 45–377 nM; treatment with 100 mg/kg of compound (QD or BID) reduced mouse viremia by about 5-folds.	Inhibits DENV helicase unwinding activity but not ATPase activity; compound resistant DENV2 has A263T mutation in helicase.	Byrd et al. (2013b)
Bioactive lipid library Biomol (Enzo) supplemented with additional commercial compounds.	212	DENV infection of Vero cells (IFA, fluorescence detection); 384 well format	4-hydroxyphenyl retinamide (4-HPR); DENV2 EC_{90} = 2 μM; U18666A; also active on WNV, HCV, Modoc virus; treatment with 180 mg/kg of compound (QD) reduced mouse viremia by about 1.7-folds.	Inhibit DENV RNA synthesis likely via host pathway; mechanism of action not determined.	Carocci et al. (2015); Chu and Yang (2007)

Table 6
DENV NIs.

Base	Modification (Name)	Assays	anti-DENV activity: EC_{50}/CC_{50} [IC50] μM	POLRMT SNIR[a] IC_{50} μM [% inhibition]	Reference
Adenosine (A)	2′-C-methyl-A	DENV2 infection in Vero cells [DENV2 in A549 cells]	4/18 [1.1/ > 50]*	62[b]; [> 30[c]]	Migliaccio et al. (2003); Chen et al. (2010a)*
	7-deaza-2′-C-methyl-A (7DMA, MK-0608)	DENV2 infection in Vero cells	15/ > 320	n.d.	Schul et al. (2007); Olsen et al. (2004)
	2′-C-acetylene-A	DENV2 infection in A549 cells	1.4/ > 50	n.d.	Chen et al. (2010a)
	7-deaza-2′-ethynyl-A (NITD008)	DENV2 infection in A549 cells & human PBMCs*	0.7/ > 100; 0.18–28/ > 25*	91[b]	Yin et al. (2009); Chen et al. (2010b)*
	7-deaza-7-F-2′-acetylene-A	DENV2 infection in A549 cells	0.42/44	n.d.	Chen et al. (2010a)
	7-deaza-7-cyano-2′-acetylene-A	DENV2 infection in A549 cells	3.1/ > 100	n.d.	
	7-deaza-7-ethanamide-2′-acetylene-A	DENV2 infection in A549 cells	2/62	n.d.	
	2′-C-acetylene-7-deaza-7-carbamoyl-A (NITD449)	DENV1-4 infection in Vero and A549 cells and human PBMCs	1.62–6.99/ > 50	n.d.	Chen et al. (2010b)
	3′,5′-*O*-diisobutyryl-prodrug of NITD449 (NITD203)	DENV1-4 infection in Vero and A549 cells and human PBMCs	0.1–0.71/ > 50	n.d.	
Guanosine (G)	2′-C-Methyl-G	DENV2 infection in Vero cells	13.6/ > 60	[~70[c]]	Migliaccio et al. (2003)
	aryl-phosphoramidate prodrug of 6-*O*-methyl-2′-C-methyl-G (INX-08189)	DENV2 replicon in Huh7 cells	0.014/ > 1	32[b]	Yeo et al. (2015)
Cytosine (C)	2′-C-methyl-C	DENV2 subgenomic replicon I & whole virus infection in Huh7 cells	11.2 [19.5]	129[b]; [> 60[c]]	Lee et al. (2015)
	2′-deoxy-2′-F-2′-C-methyl-C (PSI-6130)	DENV2 subgenomic replicon & whole virus infection in Huh7 cells	> 50	n.d.	
	4′-azido-C (R1479)	DENV2 infection in human PBMCs	0.1–0.25/ > 25;	2.6[b]; [100[c]]	Chen et al. (2014)
	Tri-isobutyl ester prodrug of R1479 (Balapiravir, R1626)	DENV2 infection in Huh7 cells, human primary macrophages and dendritic cells	1.9–11/ > 2000	n.d.	Nguyen et al. (2013)
Uridine (U)	Phosphoramidate prodrug of 2′-C-methyl-U (24)	DENV2 infection in human PBMCs	0.19/ > 50 [5]	> 300[b] [29.4]	Wang et al. (2018)
	Phosphoramidate prodrug of 2′-C-ethynyl-U (27)	DENV2 infection in human PBMCs	0.45/ > 50 [1.6]	[16.8]	
	Phosphoramidate prodrug of 2′-C-propynyl-U (29)	DENV2 infection in human PBMCs	1.9/ > 50 [2]	[6.7]	
	Phosphoramidate prodrug of 2′-C-methyl-4′F-U (35)	DENV2 infection in human PBMCs	1.1/ > 50 [6.6]	[5.7]	
	Phosphoramidate prodrug of 2′-C-ethynyl-4′-F-U (37)	DENV2 infection in human PBMCs	0.19/ > 50 [0.65]	[3]	
	Phosphoramidate prodrug of 2′-deoxy-2′-F-2′-C-methyl-U (Sofosbuvir; PSI-7977)	DENV2 infection in human PBMCs	1.2/ > 20 [18]	[1.8; < 3[c]]	
		DENV2 CPE and PRNT* assays in Huh7 cells	4.9 μM/ > 100 and 1.4* [14.7]	n.d.	Xu et al. (2017)
		DENV2 subgenomic replicon & whole virus infection in Huh7 cells	> 50	n.d.	Lee et al. (2015)
	3′, 5' -di-*O*-trityl-5-fluoro-2' -dU	DENV2-induced CPE in Vero-B cells (7 day assay)	1.2/ > 50	n.d.	Saudi et al. (2014b)
	3′, 5' -di-*O*-trityl-2′-dU (Compound 2a)	DENV2-induced CPE in Vero-B cells (7 day assay)	2.7/ > 65	n.d.	Chatelain et al. (2013)
	3',5′-di-*O*-trityl-U	DENV2-induced CPE in Vero-B cells (7 day assay)	2/ > 100	n.d.	De Burghgraeve et al. (2013)
	2',5′ di-*O*-trityl-U	DENV2-induced CPE in Vero-B cells (7 day assay)	30/ > 100	n.d.	

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Table 6 (*continued*)

Base	Modification (Name)	Assays	anti-DENV activity: EC_{50}/CC_{50} [IC50] μM	POLRMT SNIR[a] IC_{50} μM [% inhibition]	Reference
Modified nucleobase and nucleoside	Ribavirin nucleobase	DENV2 replicon in Huh7 cells	4.9/ > 1000	n.d.	Lin et al. (2018)
	Ribavirin	DENV2 replicon in Huh7 cells	1.3/20	[< 10[c]]	
	Mizoribine nucleobase	DENV2 replicon in Huh7 cells	2.4/23	n.d.	
	Mizoribine	DENV2 replicon in Huh7 cells	15/33	n.d.	
	T-1105	DENV2 replicon in Huh7 cells	21/ > 665	n.d.	
	T-1105 riboside (T-1106)	DENV2 replicon in Huh7 cells	113/ > 1000	n.d.	
	Diamino-purine	DENV2 replicon in Huh7 cells	3.6/13	n.d.	
	Diamino-purine riboside	DENV2 replicon in Huh7 cells	27/31	n.d.	
	T-705 (favipiravir)	DENV2 replicon in Huh7 cells	110/ > 1000	221[b]; [> 90[c]]	

[a] Mitochondria RNA Polymerase single nucleotide incorporation assay.

[b] Jin, Z., Kinkade, A., Behera, I., Chaudhuri, S., Tucker, K., Dyatkina, N., Rajwanshi, V.K., Wang, G., Jekle, A, Smith, D.B., Beigelman, L., Symons, J.A., Deval, J. 2017. Structure-activity relationship analysis of mitochondrial toxicity caused by antiviral ribonucleoside analogs. Antiviral Res. 143, 1–43.

[c] Arnold, J.J., Sharma, S.D., Feng, J.Y., Ray, A.S., Smidansky, E.D., Kireeva, M.L., Cho, A., Perry, J., Vela, J.E., Park, Y., Xu, Y., Tian, Y., Babusis, D., Barauskus, O., Peterson, B.R., Gnatt, A., Kashlev, M., Zhong, W., Cameron, C.E., 2012a. Sensitivity of mitochondrial transcription and resistance of RNA polymerase II dependent nuclear transcription to antiviral ribonucleosides. PLoS Pathog. 8, e1003030.

4.4. Virtual screening

Many groups have conducted *in silico* compound docking to screen for molecules that bind to DENV envelope, NS2B/3 protease, NS3 helicase, NS5 MTase and NS5 RdRp. However, these virtual hits were not always validated further by biochemical or biophysical assays. Table 4 lists the recent *in silico* docking hits that were further assessed by DENV biochemical and cell-based assays for inhibitory activities, although the confirmation of on-target effect in cells was not reported. If compound resistant DENV replicons or viruses could be raised, it will certainly help to support the lead optimisation phase. Majority of the compounds used for virtual screening originated from commercial or public sources, with library sizes ranging from thousands to millions of compounds. Brecher et al. (2017) reported a highly potent allosteric NS2B/3 protease inhibitor, NSC135618, that has good activity in the biochemical and cell-based assays (DENV2 IC_{50} = 1.8 μM; EC_{50} = 0.81 μM). Similarly, active NS2B/3 protease inhibitors were also identified by docking into the open form of DENV2 NS2B/3 protease (Pelliccia et al., 2017; Cabarcas-Montalvo et al., 2016; Li et al., 2015, Table 4). By docking compounds into the DENV envelope octyl β-glucoside binding pocket, Leal et al. (2017) identified compounds with low micromolar inhibitory activities in DENV cell-based assay. Vincetti et al. (2015) performed virtual docking into the allosteric site (cavity B) of DENV3 NS5 RdRp and identified a compound, **16i**, that may inhibit NS3-NS5 interaction (Table 4). Using an in-house library of HCV NS5B inhibitors, Tarantino et al. (2016) discovered different classes of DENV RdRp inhibitors that showed low to submicro-molar IC_{50} values in the RdRp dnI assay. Whilst compound **8** was inactive in DENV cell-based assays, HeE1-2Tyr inhibited DENV1-4 replication. X-ray crystallography data showed that HeE1-2Tyr bounds in the same region as NITD-107 (Noble et al., 2013) and formed self-interactions.

4.5. Cell-based assays

Phenotypic screens with diverse compound libraries against three different types of DENV cell-based assays (subgenomic luciferase reporter replicon, whole virus infection followed by IFA or cytopathic readouts) have together, yielded a variety of inhibitor classes that act on both viral and host targets (Table 5). Library sizes ranged from a few hundred to more than a million compounds from both commercial and proprietary sources. Earlier hit finding outcomes (till 2013) were extensively discussed in Lim et al. (2013a) and will not be revisited here. Some newly identified host targets that impact DENV replication in cells, include protein tyrosine kinase (Abl, fyn, AXL; de Wispelaere et al., 2018), mitogen-activated protein kinase (Smith et al., 2014) and TRIF-dependent signaling cascade (Pryke et al., 2017, Table 5).

More recent explorations discovered inhibitors to viral targets such as capsid (Smith et al., 2018; Scaturro et al., 2014), envelope (Chu and Yang, 2007), NS4A (Nobori et al., 2018), as well as protease (Lu et al., 2018; Yang et al., 2014). No inhibitors to DENV NS5MTase or polymerase have been identified from phenotypic screens. On the other hand, new chemical entities that act on DENV NS4B continue to be found (Bardiot et al., 2018; Hernandez-Morales et al., 2017, Wang et al., 2015; Zou et al., 2015, Table 5), making it the most frequent hit in screening campaigns (reviewed in Xie et al., 2015). Notably, NS4B residue T108I and the double P104L/A119T mutation conferred resistance to inhibitor, JNJ-1A (DENV1-4 EC_{50} = 0.7 μM, Hernandez-Morales et al., 2017), indicating that like 2DM25N (van Cleef et al., 2013), it targets the same region as NITD-618 (Xie et al., 2011).

Nevertheless, further development of JNJ-1A is challenging as it exhibits suboptimal physicochemical properties, inhibits cytochrome enzyme activities and has *in vitro* mitochondrial toxicity (Hernandez-Morales et al., 2017). Unfavourable physicochemical properties (low solubility, high lipophilicity, instability and short half-lives) were also observed in other NS4B compounds (**12a**, **14a,** NITD-618) including compound **29**, derived by scaffold morphing (Kounde et al., 2017). This

is not surprising, given that NS4B is a membrane protein, and forms extensive interactions with itself and other viral proteins such as NS4A and NS2B-NS3 (Xie et al., 2015). Nevertheless, through chemical derivatization of compound **12a**, Bardiot et al. (2018) obtained a more stable analogue, **100a,** with better stability and solubility and oral bioavailability of 64%. This report indicates that given time and perseverance, physicochemical and PK challenges in NS4B inhibitors can be overcome, as has been seen for the development of HCV NS5A inhibitors (Kohler et al., 2014).

4.6. NS5 polymerase nucleoside analogs

Nucleos(t)ide analogs or inhibitors (NIs) are effective anti-virals, as evinced in the standard treatment of HSV, HCV, HBV and HIV-1. Whilst NIs offer the advantage of broad spectrum activity against different virus genotypes and strains, development of this class of inhibitors presents many challenges. NIs must be delivered to the right host compartments, and be adequately converted by host enzymes (cellular kinases) in the target cell types, to their active triphosphate forms, to exert inhibitory effects. *In vivo* toxicity of NIs, is often a big hurdle, and is not readily predictable. Tissue- and cell-specific toxicity has been strongly correlated with the inhibition of host cell enzymes such as host polymerases, particularly the mitochondrial enzymes, DNA polymerase (Pol) γ (Johnson et al., 2001) and RNA Pol (Fenaux et al., 2016; Feng et al., 2016; Arnold et al., 2012a). Moreover, Pol beta (Brown et al., 2011) and PrimPol (Mislak and Anderson, 2015) have been shown to localise to the mitochondria and to incorporate nucleoside analogue reverse-transcriptase inhibitors (NRTIs). Toxicity caused by NIs may also be due to induction of mitochondrial electron transport chain dysfunction, increase in oxidative stress (NRTIs, Smith et al., 2017; Lund and Wallace, 2004), and reduction in levels of natural NTPs. These effects can be due to interaction with nucleos(t)ide-binding enzymes such as thymidine kinase (AZT, Lynx and McKee, 2006), inosine MP dehydrogenase (ribavirin; Graci and Cameron, 2006) and nucleoside transporters (HSV and HIV-1 NIs; Koczor et al., 2012). Nevertheless, *in vitro* assays for DNA and RNA Pols, cytotoxicity assays have been developed to mitigate NI toxicity (Jin et al., 2017; Young, 2017; Chen et al., 2015; reviewed in Feng, 2018). Structural data of NIs bound to polymerase γ (Szymanski et al., 2015) or RNA Pol (Arnold et al., 2012b), can provide additional guidance.

NIs that have anti-DENV effects are summarised in Table 6. Early NIs were previously described in Chen et al. (2015) and *in vivo* toxicity manifested by many of these NIs, may be partly attributed to inhibition of mitochondrial RNA Pol. A recent paper by Wang et al. (2018) described the anti-DENV activities of a series of 2′-substituted uridine phosphoramidate prodrugs (Table 6) and their *in vitro* toxicity profile. Both 2′-fluoro-2′-C-methyl uridine-triphosphate (the HCV NI, sofosbuvir-triphosphate) and the most active anti-DENV compound, 2′-C-ethynyl-4′-F-uridine (**37;** IC_{50} = 0.65 μM), are poor substrates for mitochondria RNA Pol, did not affect mitochondria protein synthesis and did not show cytotoxicity in three different cell lines tested (CC_{50} > 50 μM in HepG2, K562, MT-4). However, sofosbuvir-triphosphate is a poor inhibitor of DENV RdRp (IC_{50} = 15–18 μM; Table 6). As previously noted in Potisopon et al. (2017), DENV (and ZIKV) RdRp, unlike HCV, are inhibited by NI substitutions in the order: 2′-C-Me > 2′-C-Me-2′-F. Lin et al. (2018) studied the effects of nucleobases as alternative starting points for DENV inhibition. Instead of utilising host kinases to generate monophosphates of the NIs, nucleobases are converted by 5-phosphoribosyl-1-pyrophosphate to give the corresponding nucleoside-5′-monophosphate. Unfortunately, most of the nucleobases tested were either weakly active or rather cytotoxic in Huh7 cells. Trityl-containing uridine analogs were also tested for anti-DENV activity, with several compounds showing low micromolar inhibitory activities biological activity (Table 6). Whilst trityl moieties are generally added as protective groups during chemical synthesis, there have been a few reports indicating that they possess biological activity (Pałasz and Cież, 2015).

4.7. Viral RNA binding proteins

A number of host pathogen recognition receptors (PRRs) such as RIG-I, MDA-5, TLR-3 have been reported to restrict DENV replication through activation of antiviral signaling pathways (Liang et al., 2011; Nasirudeen et al., 2011). DENV RNA synthesis occurs in the viral replicative complex within ER-associated double-membrane vesicles in the cytoplasm (reviewed in Lescar et al., 2018). A number of host proteins have been shown to regulate viral RNA cap formation (e.g. EIF4E), replication (stress granule proteins, P-body proteins), translation (e.g. PABP, EIF4F, ribosomes), and viral RNA encapsidation (e.g. DDX6, NONO and hnRNPM). Some of these host factors positively regulate DENV replication, whilst others are repressive (reviewed in Bidet and Garcia-Blanco, 2018). Identification of small molecules that influence activities of some of these host factors could be one way of modulating DENV replication.

4.8. Host proteins involved in DENV replication

Several host pathways related to lipid metabolism (reviewed in Martín-Acebes et al., 2016; Krishnan and Garcia-Blanco, 2014), ER-golgi trafficking (Miller et al., 2018; Caputo et al., 2018), autophagy and unfolded protein response (Choi et al., 2018) have been shown to be important for DENV life cycle (during entry, replication and maturation) as well as for viral evasion from host immune response. Thus, besides direct anti-virals (NS5 NI, Balapiravir), DENV clinical trials encompassed inhibitors to host proteins (e.g. alpha-glycosidase inhibitors, Celgosivir and UV-4B) to effect DENV inhibition (reviewed in Low et al., 2018; Whitehorn et al., 2014). In the dearth of DAAs, researchers have focused on repurposing approved drugs that affect the above-mentioned host pathways for anti-DENV therapy (reviewed in Botta et al., 2018; Low et al., 2018; Lim et al., 2013a).

Increasingly, kinase inhibitors have been shown to impact DENV replication (Table 5, de Wispelaere et al., 2018, 2013; Smith et al., 2014; Clark et al., 2016; Chu and Yang, 2007). A recent report by Bekerman et al. (2017) revealed that DENV, HCV and Ebola virus exploit two kinases, AAK1 and GAK, that regulate host adaptor proteins AP1 and AP2, for entry and virus production. Treatment with two anti-cancer drugs, sunitinib and erlotinib, protected DENV-infected mice against morbidity and mortality (Pu et al., 2018a; Bekerman et al., 2017). The researchers subsequently synthesized highly specific GAK inhibitors that inhibited DENV replication, after optimising an isothiazolo[4,3-*b*]pyridine derivative from a small library screen against GAK (150 novel drug like compounds were tested in the KINOMEscan screening platform; Kovackova et al., 2015). The analogue with the best SI, **12r**, showed EC_{50}/CC_{50} values of 0.82/ > 25 μM and 3.54/ > 20 μM in Huh7 and human monocyte-derived dendritic cells, respectively (Pu et al., 2018b).

4.9. New host targets from omics approaches

With the advent of new technologies, additional mechanisms by which DENV engage host machinery continue to be elucidated. Proteomics, transcriptomics and genome-scale knock-out (KO) screens are powerful approaches to uncover host factors essential for DENV replication and pathogenesis, providing new candidate targets for antiviral drug development as well as potential biomarkers for prediction of disease severity.

Researchers have utilised different proteomic approaches such as yeast-two-hybrid assays, co-IP or tandem affinity purification followed by MS, stable isotope labeling by/with amino acids in cell culture (SILAC) to map interactions of DENV proteins (envelope, NS3, NS4B, NS5) within the viral RC and with host factors (reviewed in Gerold et al., 2017; Lum and Cristea, 2016). Protein-protein interactions (PPIs)

often have specific interfaces which may be targeted for therapeutic intervention. Of note, Karyala et al. (2016) mined all publicly available literature on dengue–human interactions into a searchable database called DenHunt ((http://proline.biochem.iisc.ernet.in/DenHunt/). A total of 682 direct interactions of human proteins with dengue viral components, 382 indirect interactions and 4120 differentially expressed human genes in dengue infected cell lines and patients were found. This collective information provides a rich resource for new anti-DENV strategies targeting host proteins. The authors highlighted 20 host proteins that are critical for DENV replication and have been commercially validated as drug targets.

Another comprehensive endeavour was conducted by Dey and Mukhopadhyay (2017) who curated published papers as well as virus databases such as VirHostNet and VirusMentha, to compile the free DenvInt database (https://denvint. 000webhostapp.com). The database lists both dengue–human and dengue–mosquito protein interactions and will be updated monthly to incorporate new reports. As of 2017, the total Dengue–human PPI comprised 784 unique interactions, including 535 non-redundant interactions between 335 different human proteins and 10 dengue proteins as well as 249 non-redundant interactions between 140 different mosquito proteins and 10 dengue proteins. Amongst these, 535 dengue–human and 249 dengue–mosquito interaction were studies conducted with DENV2 whilst only about 10% of dengue-human PPI were serotype independent.

Whilst target protein-protein interactions (PPI) may be challenging, drugs that target PPIs are beginning to enter the market and a number of others are undergoing clinical trials (Scott et al., 2016). Some examples of such marketed drugs are Tirofiban (an antiplatelet drug which inhibits interaction between fibrinogen and the platelet integrin receptor GP IIb/IIIa; Hartman et al., 1992) and Lifitegrast (for dry eye treatment by inhibiting interaction between lymphocyte function-associated antigen 1(LFA-1) and intercellular adhesion molecule 1 (ICAM-1); Tauber, 2015). Compounds in clinical trials include Idasanutlin (phase III trial; for anti-cancer application by inhibit MDM2-p53 interaction; Ding et al., 2013) and AZD5991 (in phase I trials for hematologic cancers by inhibiting interaction between Mcl-1 and Bak; Tron et al., 2018).

Transcriptomic approaches such as DNA microarrays have also enabled the dissection of host genes that are altered by DENV infection as well as uncovered potential new biomarkers for profiling disease severity (Banerjee et al., 2017; Becker et al., 2015; Sun et al., 2013). More recently, two independent genome-scale genetic KO screens determined that genes involved in ER-translocation, protein degradation (ERAD), N-linked glycosylation were necessary for the proper cleavage of the flavivirus structural proteins (prM and E) and secretion of viral particles (reviewed in Puschnik et al., 2017). In particular, signal peptidase complex genes (SPCS-1, -2 and -3; Zhang et al., 2016), the oligosaccharyltransferase (OST) complex, and translocon-associated protein (TRAP) complex (Marceau et al., 2016), were found to be important for infectious virus production. These host genes could be investigated as targets for anti-DENV therapy. As a proof-of-concept, the researchers used the oligosaccharyl-transferase inhibitor NGI-1, to inhibit to DENV2 replication (EC_{50}/CC_{50} = 0.85/34.9 μM) and the other three DENV serotypes (Puschnik et al., 2017).

5. Challenges in the path to clinical development of dengue drugs

The goal of the drug discovery process is to perform clinical studies to determine the safety and efficacy of a drug, administered at a particular dose to a specific human target population with acceptable risk/benefit ratios, as specified in the target product profile. Ultimately, the aim is to achieve proof of safety (PoS), mechanism (PoM) and concept (PoC) for the drug candidate whereby defined endpoints (measurables) are met and can be used to obtain regulatory approval.

In the case of clinical development of dengue drugs, the trials conducted to-date use the following parameters as primary measures of drug efficacy: (i) time to fever resolution, resolution of or reduction in (ii) viremia, and (iii) NS1 antigenemia. However, none of these trials have observed a significant difference in these measurables between drug-treated and control groups (reviewed in Low et al., 2018). There are several considerations and challenges in determining the efficacy of a dengue drug. Firstly, due to the rapid decline in viremia seen in DENV patients, it is important to recruit DENV patients for clinical trials at very early stages of infection in order to initiate dosing as early as possible. Yet, there is an absence of rapid and sensitive POC diagnostics which can discriminate between dengue, and other endemic febrile-causing illnesses (discussed in Section 2). Moreover, current DENV IgM/IgG immuno-assays cannot differentiate between an individual with a secondary DENV infection, and a vaccinee who has primary infection. This may pose problems when stratifying patients during late-stage clinical trials to determine drug efficacy and to measure additional secondary endpoints (such as progression to severe disease, or cytokine responses).

Secondly, DENV patients exhibit a wide variance in plasma virus levels during the course of their illness. Reports indicate that amongst both DF and DHF patients, virus levels were about 6-log different within the first 5 days of illness (4–10 $\log_{10}$ RNA copies/ml, Nguyen et al., 2013; 2–9 $\log_{10}$ RNA copies/mL, Guilarde et al., 2008). On the other hand, at > 5 days post-illness, the difference was less pronounced (about 3-logs different) but levels were also substantially lower (1–5 $\log_{10}$ RNA copies/ml; Guilarde et al., 2008). This large divergence in virus titres can make it difficult to confirm if a drug candidate is effective when comparing the treated and control arms. Thus careful segregation of the cohorts are needed, as has been done in some dengue trials conducted (Nguyen et al., 2013).

Thirdly, whilst DENV is known to infect immune cells, it is not clear what additional cell types or tissues are infected in the host, and how they contribute to viral pathogenesis and the spectrum of dengue diseases. Failure to adequately deliver the drug candidate to all the relevant human tissues or compartments in a timely fashion will compromise the efficacy of a drug candidate. This uncertainty can also pose challenges for PK/PD modeling, dose regime design, and determining the therapeutic index. Although current mouse models have been reported to show some signatures of DENV disease (cytokine induction, thrombocytopenia, and systemic infection), they do not fully recapitulate the full spectrum of dengue diseases and may not be suitable for addressing this issue nor for PK/PD evaluation (Chan et al., 2015). Thus, establishment of better predictive pre-clinical models, such as with non-human primates (Omatsu et al., 2012, 2011; Onlamoon et al., 2010) will be advantageous. Ultimately, the use of human challenge studies with DENV vaccine strains may be a way forward to profile clinical drug candidates, post-phase I trials. *In vitro*, compound testing in a wide panel of cell types (particularly in human primary cells) with different clinical DENV1-4 strains should be conducted to build better dose-inhibition relationships.

Besides the lack of efficacy, lack of safety is another common cause of attrition during drug development (Kola and Landis, 2004) and in clinical trials (Harrison, 2016). This latter property is of particular concern in dengue drug development for several reasons: firstly, majority of the target patient population in many South-east Asian countries are young children (< 15 years old) and secondly, adult patients with severe DENV diseases often present with comorbidities. Thirdly, differing physiology and immune status of patients with severe DENV disease compared to those with mild, or intermediate DENV disease (See WHO Dengue classification and levels of severity, 2009), may influence the ADME properties of the drug candidate and hence, alter its safety and efficacy profiles. Hence, careful monitoring of drug concentrations in DENV patients and patient stratification during clinical trials will most likely be required to better understand efficacy outcomes (or lack thereof) and safety issues.

In general, drug-induced toxicities in liver, heart, kidney, and brain currently account for more than 70% of drug attrition and withdrawal

from the market (Wilke et al., 2007). Whilst these may be mitigated by available *in vitro* safety profile panels (Supplementary Tables 3–5), new *in vitro* techniques including cellular thermal shift assays (Molina and Nordlund, 2015; Molina et al., 2013), and 3D organoids of liver, cardiac patch microtissues (Park et al., 2018) are increasingly being utilized in the pharma industry to address safety concerns.

6. Conclusions and perspectives

Intensive efforts from researchers in academia and industry have cumulated in significant knowledge in the life cycle of DENV, its impact on the host and the consequent pathogenesis. A large number of host targets are implicated in regulating the virus, some of which are ubiquitous, whilst others are host (human, mosquito)- and cell-type specific. New technologies that utilise "omics" approaches (proteomics, transcriptomics and genomics-scale KO screens) can provide holistic views of the interactomes between DENV and its host, and have revealed new potential targets (e.g. OST complex) for anti-DENV drug development. This expands the opportunity to repurpose other known drugs, such as metformin (Htun et al., 2018, Soto-Acosta et al., 2017) besides ivermectin, Celgosivir, lovastatin, or HCV inhibitors, for DENV therapy.

Targeting host factors offer the advantages of a significantly higher barrier to emergence of resistance (compared with DAA), due to absence of genetic pathways to resistance as well as pan-antiviral activities. It may also permit the inhibition of related members of the viral genus or family due to conserved pathways in the virus life cycles. Its drawback is the potential of undesirable drug-induced side effects, which may be off- and/or on-target (arising from its physiological function) in nature. As severe dengue diseases significantly impact host physiology, targeting particular classes of host proteins may promote additional unpredicted side-effects and will require close patient monitoring. This also calls for in-depth biological understanding of the mechanism of action of the target gene in cells, in healthy and disease settings, such as using iPSCs, human tissue biobanks and conducting genome editing studies. Nevertheless, since dengue viruses cause acute diseases, these challenges should be surmountable (previously mentioned in Lim et al., 2013a).

Increasingly, targeting PPIs is gaining success in different therapeutic areas, including in infectious diseases, due to better knowledge about the druggability of the interfaces and better screening methodologies (reviewed in Voter and Keck, 2018). New commercial focused chemical libraries designed specifically for PPI screening have also been generated with the aim to overcome limitations of traditional compound libraries (e.g. from Asinex, Chemdiv, Enamine). Thus, screening for inhibitors that block PPIs between DENV proteins or between DENV and host proteins could be a new endeavour for the identification of novel DENV inhibitors.

For phenotypic screens, DENV whole virus infection assays and DENV subgenomic replicon cells have yielded interesting hits to both viral and host proteins. NS4B inhibitors are the most frequent DAAs identified from screening efforts with different diverse compound libraries. Despite dissimilarity in compound structures, some inhibitor classes (JNJ-1A, 2DM25N, NITD-618) share virus resistance phenotypes, suggesting that they target the same region in DENV2 NS4B. Due to differences in DENV1-4 NS4B amino acid sequences, not all NS4B inhibitors exhibit pan-serotype inhibition. As researchers have mainly used DENV2 (in particular, with NGC strain) for phenotypic screens, conducting screens with other DENV serotypes may generate additional new chemical scaffolds for NS4B, or other DENV protein targets.

Development of NS4B inhibitors have been hampered by unfavourable PK properties (due to low solubility and stability). Whilst medicinal chemistry is focused on improving physicochemical characteristics (i.e. adherence to Lipinski's Rule of 5), increasing evidence point to success in developing drugs that fall beyond the rule of 5 (bRo5). These include HCV NS5A and NS3/4A protease inhibitors, and HIV-1 protease inhibitor, ritonavir. Understanding drug development in the bRo5 space has provided some guidance to optimize cell permeability and solubility, to achieve oral bioavailability for compounds with MW $>$ 500 (reviewed in Poongavanam et al., 2018). This may be the way forward for DENV NS4B inhibitors. With continued efforts, it is highly plausible that orally available DENV NS4B drugs can be achieved, as shown by promising efforts in the optimisation of acylindole derivatives (Bardiot et al., 2018).

Amongst DENV enzymatic activities, NS5 RdRp is the most attractive drug target and also plausibly, the most feasible, based on experiences in anti-HCV and -HIV-1 drug approaches. A significant effort was undertaken at NITD to find NS5 RdRp inhibitors. Whilst the previous NI, NITD008 suffered from toxicity issues (Yin et al., 2009), incorporating counter assays in the NI progression flowchart (Wang et al., 2018; Jin et al., 2017; Feng et al., 2016) can help to mitigate this problem. Few amenable NS5 RdRp and MTase hits have been identified from biochemical and phenotypic screens (reviewed in Lim et al., 2015). This may be due to the absence of relevant chemical scaffolds in the libraries, and suggests that new chemical libraries may be required to uncover starting points to these two enzyme activities. On the other hand, developing DENV NS5 NNIs may, like NS4B inhibitors, fall into the category of bRo5 compounds, which necessitate strong medicinal chemistry and PK support. To date, one of the most potent DENV NS5 NNIs belong to the class of N-pocket inhibitors which were derived from FBS by X-ray crystallography (reviewed in Lim et al., 2018). This holds promise in the application of FBS as a suitable hit-finding strategy for DENV protein targets.

Researchers typically employ DENV infection assays with immortalised cell lines. The drawback is that these cells are genetically and metabolically altered. DENV is a complex disease, whereby disease outcome is strongly influenced by the host response. Thus, the use of physiologically relevant cells, such as PBMCs from acutely infected patients or convalescent DENV patients, for compound screening, may provide new chemical entities and insights on DENV-host cell interaction. Applying alternate methods for assay readout (e.g. cytokine production, markers of immune cell activation as surrogate markers for anti-DENV response) may also reveal novel findings. Notably, DENV patients frequently exhibit leukopenia, making it difficult to obtain sufficient quantities of PBMCs for large scale compound or genetic screens. To circumvent this issue, induced pluripotent stem cells (iPSCs) may be generated from somatic cells from symptomatic DENV patients (stratified according to disease severity), and subsequently expanded and re-differentiated to immune cells for drug screening purposes or to study virus pathogenesis. The availability of such cells may also enable explorations into the use of organoid differentiation, organ-on-chips, to model and study DENV disease.

The use of disease-relevant cell-types from patient-derived iPSCs have been successfully applied in the fields of genetic and neurodegenerative diseases (Elitt et al., 2018; Hung et al., 2017). Recently, human pluripotent stem cell (hPSC)-derived hepatocyte-like cells (HLCs) have been demonstrated to support the complete ZIKV replication cycle and the virus is inhibited by 7-deaza-2′-C-methyladenosine (Tricot et al., 2018). Significant differences in the innate immune response against ZIKV and antiviral drug sensitivity were observed when comparing hPSC-HLCs and hepatoma cells.

DENV is the most important arthropod-borne viral disease in the world. In the last 20 years, anti-DENV drug discovery activities have yielded diverse hits against both viral and host factors. Nevertheless, a number of hits, especially those derived from *in silico* docking exercises from academic researchers, were not verified in DENV biochemical or cell-based assays. As well, some hits identified from compound library screens are not fully characterized, perhaps due to insufficient expertise in specific techniques or medicinal chemistry support. In some instances, hits identified from biochemical screens with DENV enzymes were not confirmed by biophysical or binding assays whilst DENV cell-active inhibitors are not checked for on-target inhibition within cells.

Thus potential inhibitors languish in the hit-finding phase, and are not pursued further for medicinal chemistry interrogation. The creation of an open-source database for all reported anti-DENV hits would enable researchers to review and re-visit interesting hits.

In this regard, the proposal to have a DENV drug/vaccine consortium made up of private-public partnerships (PPP; Hernandez-Morales and Van Loock, 2018), would enable researchers to collaborate, share resources and expertise, and to consolidate activities required for hit and lead characterisation. This can help to advance global DENV drug discovery efforts in a concerted fashion. An effective PPP will hopefully, draw greater medicinal chemistry expertise to appraise promising hits and conduct lead optimisation exercises. On this note, the collection of a set of validated (such as NS3 protease and NS4B inhibitors, NITD008, NS5 RdRp N-pocket inhibitors) and unvalidated compounds from anti-DENV screens, curated by a central repository, could be a useful tool-box to researchers who are conducting new screening exercises for DENV or other related Flaviviruses. Such compound collections have been generated for malaria and successfully applied to drug discovery in other neglected tropical diseases (Duffy et al., 2017; Van Voorhis et al., 2016).

The formation of a virtual research organization dedicated to DENV drug discovery, which operates in an open-access format, can allow researchers to tap into an international network of expertise from both academia and industry. Besides benefiting the DENV drug discovery community, it can likely boost drug discovery efforts on other emerging flavi- and non-flavi-viruses and enable rapid response in emergency outbreaks. Importantly, DENV patients will also be better served by such an initiative. Key stakeholders for a successful PPP are (i) public-sponsored organizations: governments, government sponsored organizations (e.g. CDC, NIH, Pasteur Institute, INFECT-ERA), international health organizations (e.g. WHO, TDR) and academia (e.g. Centre for Drug Design and Discovery (CD3) at the University of Leuven, Belgium) (ii) for-profit: pharmaceutical industry, biotechnology companies (iii) not-for-profit and philanthropic organizations (e.g. Gates foundation, Welcome Trust, DNDi, GHIT).

Given that DENV affects more than 140 countries worldwide, there is a strong vested interest in the governments of these countries to join forces to combat this disease. For this to come to fruition, strong leadership and commitment from the afore mentioned stakeholders are needed. To note, WHO, through the Special Programme for Research and Training in Tropical Diseases (TDR), is exploring the possibility to host a pooled fund, raised by the WHO, to support R&D for diseases primarily affecting low-to-middle-income countries (LMICs; type I-III diseases). This fund is intended to support a Scientific Working Group (SWG) to establish disease target product profiles and manage R&D project portfolios to accelerate the development of diagnostics, vaccines and treatments for these diseases, through transparent, efficient, non-political governance (WHO, TDR, 2016). Under WHO classification, DENV is a type II disease, and therapeutic explorations for combating DENV could be hosted under this framework.

Acknowledgements

I thank ex-colleagues who worked on dengue drug discovery at the Novartis Institute for Tropical Diseases (Singapore), Novartis Institute for Biomedical Research (Basel, CH; Cambridge, USA; Emeryville, USA), and the Genomics Institute of the Novartis Research Foundation (San Diego, USA), as well as ex-collaborators (Julien Lescar, Nanyang Technological University, Singapore; Gottfried Otting, Australian National University, Canberra, Australia) for invaluable discussions and insights.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.antiviral.2018.12.016.

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