Investigation of Approaches to Inhibit Dengue Virus

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CERTIFICATE

This is to certify that the thesis entitled **“Investigation of Approaches to Inhibit Dengue Virus”** submitted by **Hemalatha Beesetti,** ID No **2013PHXF001H,** for award of Ph.D. degree of the Institute embodies original work done by her under our supervision.

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Abstract

Dengue is a fast spreading vector-borne viral disease posing a public health threat to half the global population. It is caused by four distinct serotypes of dengue viruses, DENV-1, -2, -3 and -4. All four DENVs co-circulate in many tropical and sub-tropical countries of the world. It has been estimated that there may be about 390 million DENV infections annually. These infections, while being mild in majority of cases, can cause potentially fatal disease associated with endothelial damage leading to plasma leakage, fluid accumulation and bleeding in <1% of the infected population. Effective tools to combat dengue are unmet needs. As each of the four DENVs can cause dengue illness, a vaccine or a drug needs to effectively target all four DENVs. This thesis is focused on exploring the feasibility of identifying pan-DENV inhibitors and antiviral strategies.

The work entailed (i) the expression, purification and characterization of the DENV-2 protease, (ii) establishing and validating a high throughput antiviral drug screening assay, (iii) developing recombinant adenoviral vectors to deliver DENV-specific siRNA, and (iv) establishing cell-based assays to determine multiple parameters which included DENV antigen synthesis, genomic RNA replication and infectious virus production, in the presence and absence of different putative antiviral agents (protease inhibitors as well as DENV genome-specific siRNAs).

This work has: (i) identified two small molecule inhibitors, MB21 (a benzimidazole derivative), and BT24 (a quinoline derivative), of DENV-2 NS3 protease from an in-house library of ~3000 compounds; (ii) identified a conserved siRNA target site (sh5c) in the DENV genome to silence DENV viral gene expression; (iii) developed a replication-defective adenovirus vector (rAd-sh5c) to deliver an siRNA targeting this site into DENV-infected cells. The results demonstrate that that the compounds MB21 and BT24, as well as the sh5c siRNA delivered using rAd-sh5c vector, are independently effective at inhibiting all the four DENV serotypes.

This thesis presents the results obtained and discusses some of their implications, limitations and future scope of the work.

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Abbreviations

µl	Microliter
µm	Micromolar
3’	3 prime end
5’	5 prime end
6x-His tag	6x Histidine tag
aa	Amino acid
Ab	Antibody
Ad5	Adenovirus type 5
ADE	Antibody Dependent Enhancement
Ag	Antigen
AMC	7-amino 4-methylcoumarin
ANOVA	Analysis of variance
ATCC	American Tissue Culture Cell type
bp	Base pair(s)
C	Capsid
Cast	Castanospermine
CC_{50}	Concentration (of drug) causing 50% cell toxicity
cDNA	Complementary DNA
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
CPE	Cytopathic effect
CQ	Chloroquine
CS	Complementary sequence
C_t	Cycle threshold
CYD	Chimeric Yellow fever Dengue vaccine
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DENV	Dengue virus
DENV-1	Dengue virus serotype-1
DENV-2	Dengue virus serotype-2
DENV-3	Dengue virus serotype-3
DENV-4	Dengue virus serotype-4
DF	Dengue Fever
DHF	Dengue Haemorrhagic Fever
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNJ	Deoxynojirimycin
DSS	Dengue Shock Syndrome
E	Envelope
EC	Expression Cassette
EC_{50}	Half maximal effective concentration (concentration which results in half maximal response)
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
F/T	Freeze/Thaw
FACS	Fluorescence Activated Cell-Sorting

FBS	Fetal Bovine Serum
GuHCl	Guanidine hydrochloride
HCV	Hepatitis C Virus
HEPES	4-(2-hydroxyethyl)-1 piperazine ethanesulfonic acid
HRP	Horse Radish Peroxidase
HTS	High Throughput Screening
I	Induced
IC_{50}	Concentration causing 50% inhibition
ICAM-3	InterCellular Adhesion Molecule 3
iDC	Immature monocyte derived Dendritic Cell
IFN	Interferon
IgG H&L	Immunoglobulin heavy and light chain
IL	Interleukin
IPTG	IsoPropyl Thio Galactoside
ITR	Inverted Terminal Repeat
Kb	Kilobase(s)
KDa	Kilo Dalton
LA	Left Arm
LD	Lipid Droplets
M	Marker
m.o.i	Multiplicity of Infection
mAb	Monoclonal Antibody
mM	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N/NC	Negative control
NA	Nucleoside Analog
NaCl	Sodium chloride
NCT	National Clinical Trial registry number
NEC	No Enzyme Control
NIH	National Institutes of Health
Ni-NTA	Nickel-NitriloTriacetic Acid
nM	Nanomolar
NMR	Nuclear Magnetic Resonance
NS proteins	Non-Structural proteins
NS3Hel	NS3 Helicase
NS3Pro	NS3 protease
nt	nucleotide
NT_{50}	Serum dilution/antibody concentration causing 50% neutralization of virus infectivity
NTC	No Template Control
NTR	Non Translated Region(s)
NVBDCP	National Vector Borne Disease Control Program
OD	Optical Density
ONC	Overnight Culture
Ori	Origin of replication
P	Pellet
P value	Probability value
PBS	Phosphate Buffer Saline

PCI	Phenol/Chloroform/Isoamyl alcohol
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
pmoles	picomoles
prM	Pre-Membrane protein
PRNT	Plaque Reduction Neutralization Test
qPCR	Quantitative PCR
RA	Right arm
rAd virus	Recombinant Adenovirus
RdRp	RNA-dependent RNA polymerase
RFU	Relative Fluorescence Units
RISC	RNA Induced Silencing Complex
RNAi	RNA interference
RPLC	Reverse Phase Liquid Chromatography
RPM	Revolutions per minute
RT	Room temperature
RT-PCR	Real time PCR
S	Supernatant
SD	Standard Deviation
SDS-PAGE	Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
sh	Short hairpin
shRNA	Short hairpin RNA
shscr	Short hairpin scrambled
SI	Selectivity Index
siRNA	Small interfering RNA
SMPBS	Skimmed Milk in Phosphate Buffer Saline
ss	Single stranded
$TCID_{50}$	Half maximal Tissue Culture Infective Dose
TD buffer	Trypsin-EDTA buffer
TLC	Thin Layer Chromatography
TMB	3,3’,5,5’-Tetramethylbenzidine
TMD	Trans-Membrane Domain
TVD	Tetravalent Dengue Vaccine
UI	Un-Induced
VC	Virus control
WHO	World Health organization
WNV	West Nile Virus
wt	Wild type
λem	Emission wavelength
λex	Excitation wavelength

INTRODUCTION

Dengue viruses (DENVs) are mosquito-borne single-stranded RNA viruses of the genus *Flavivirus*, family *Flaviviridae.* There are four distinct serotypes of DENVs (DENV-1, DENV-2, DENV-3 and DENV-4), each of which can cause dengue disease The disease manifestations range from an influenza-like fever known as dengue fever (DF) to a severe, sometimes fatal disease characterized by hemorrhage and shock, known as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), respectively.

The occurrence of dengue worldwide has increased ~30-fold in the past five decades. The increased urbanization, transport of vector through global trade within and across the countries and ineffective means to control the disease spread have jointly led to the present situation of ubiquitous dengue prevalence. About 3.6 billion people around the world are at risk of contracting dengue infections.

Significant efforts all over the world are being employed to develop a preventive dengue vaccine, with several candidates in clinical trials. One of these has recently been approved for human use in countries such as Brazil and Philippines. However, this dengue vaccine is reported to have an efficacy of only ~60% and appears to be associated with antibody-dependent enhancement (ADE) of disease severity, especially in children <9 years of age. Drug discovery efforts have not yielded any promising candidate drugs that could be taken to clinical trials so far. As a result, drugs for other indications are being re-purposed for dengue to explore the feasibility of fast-tracking dengue drug development. Thus, celgosivir, a drug initially intended for treating hepatitis C virus infection (HCV), was tested for antiviral efficacy against dengue. After promising results in preclinical trials, celgosivir failed in a proof-of-concept human trial concluded in Singapore recently. Thus, there is a need for continued efforts to develop dengue vaccines and drugs.

Dengue drug development efforts are in early stages and essentially focus on one DENV serotype. It is not known if a single drug can inhibit all four DENVs. It is not known if such a drug can effectively counter the emergence of drug resistant escape mutants. A pragmatic solution to address this possibility is to develop a combination of drugs. Thus, the feasibility of developing multiple pan-DENV drugs is an area that needs to be explored. This represents a significant gap in the context of dengue drug discovery efforts. This thesis is focused on antiviral strategies to target dengue virus replication. Specifically, this work has explored the feasibility of targeting the DENV protease, which is essential for successful completion of the viral life cycle, and the DENV RNA genome, which doubles as a template for both translation and replication. The work presented in this thesis shows that it is possible to inhibit all four DENVs, either by targeting the DENV protease, with small molecule inhibitors, or the viral RNA genome, by deploying endogenous RNA interference (RNAi). Inhibition of DENV was assessed by multiple criteria such as viral antigen synthesis, genomic RNA replication and infectious virion production. Thus, this work demonstrates the feasibility of developing more than one drug and more than one approach to achieving potent inhibition of all four DENVs. This thesis describes the results of this work and their implications.

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CHAPTER 1: REVIEW OF LITERATURE

1.1 Dengue as a global public health problem

Dengue is a fast-spreading vector-borne viral disease of the 21st century (*Gubler, 2012; Swaminathan & Khanna, 2009*). Recent decades have witnessed dramatic increase in the prevalence and incidence of dengue around the world (*WHO, 2012*). It is caused by four distinct serotypes of dengue viruses, DENV-1, -2, -3 and -4, spread to humans by the *Aedes* mosquitoes. Dengue is endemic to >100 tropical and sub-tropical countries (Figure 1.1), placing nearly 3.6 billion people (*Gubler, 2012*), accounting for ~50% of the global population, at risk. The emergence of dengue as a significant global public health problem is attributed to multiple factors such as massive unplanned urbanization, overpopulation, increasing global travel and importantly the failure to eradicate mosquitoes. (*Gubler, 1998, 2002, 2012; Swaminathan & Khanna, 2009; Guzman & Harris, 2015*). Currently, it is estimated that the number of dengue infections is ~390 million per year (Figure 1.1). India alone was found to account for 34% of the global dengue burden (*Bhatt et al, 2013*).

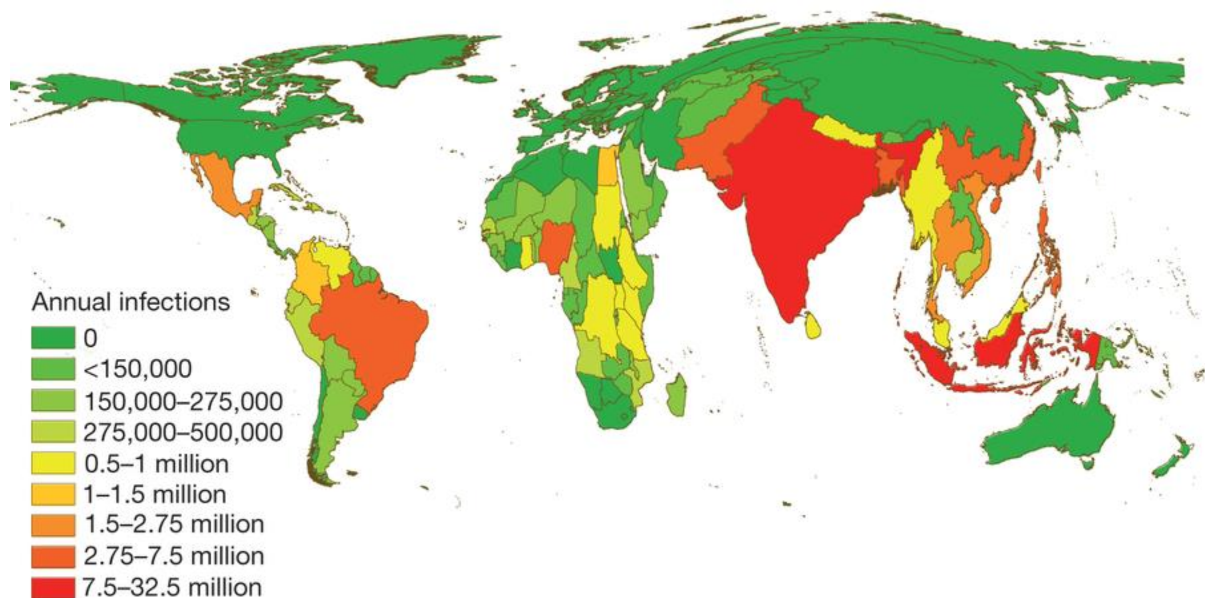

Figure 1.1: The global distribution and burden of dengue. *The severity of the disease is represented by its colour distribution where red represents more occurrences and green represents absence of the disease (from Bhatt et al, 2013)*

This is at variance with data reported by India's national agency for vector-borne disease surveillance (Figure 1.2, page 3). It is widely believed that lack of an effective surveillance system and under-reporting, stemming from the fact that ~80% medical care is in the private sector, have contributed to a gross under-estimation of the magnitude of dengue burden. All four DENVs as well as the mosquito vectors are prevalent in India. Coupled to the high population density, dengue is a serious public health problem in India, as evidenced by the periodic dengue outbreaks. The realization that dengue can spread beyond the borders of the tropical world has created global awareness and the urge to develop ways and means of addressing the public health threat posed by this disease (*Swaminathan & Khanna, 2009; WHO, 2012*).

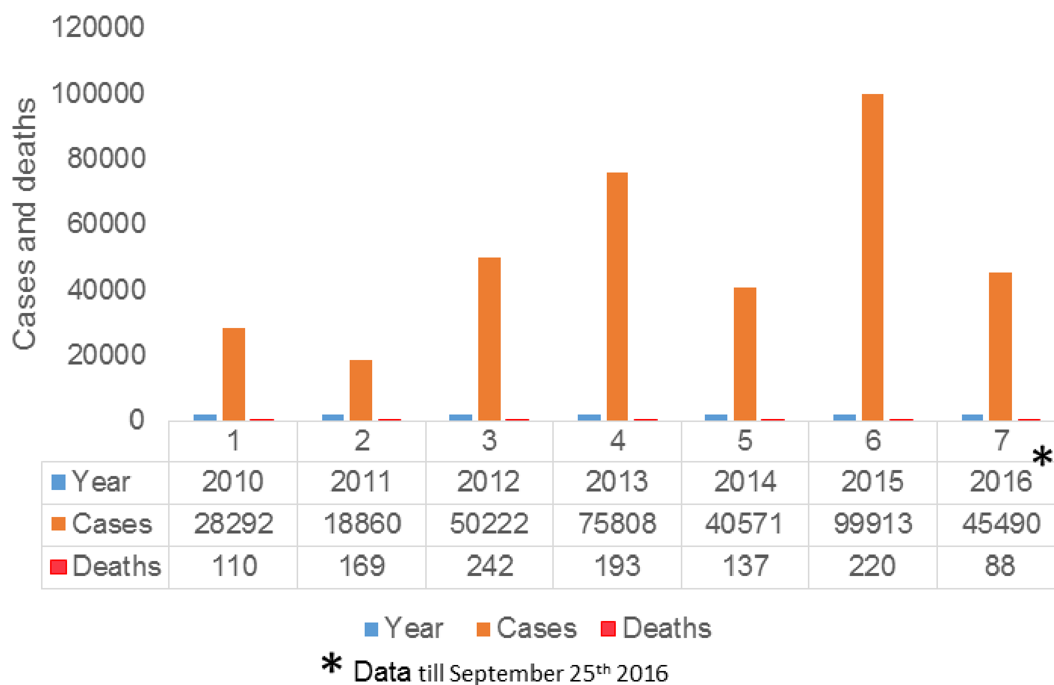

Figure 1.2 Dengue disease surveillance data in India. *Compiled from information provided at the NVBDCP website (http://nvbdcp.gov.in/den-cd.html).*

1.2 Dengue viruses

The DENVs are members of family *Flaviviridae,* belonging to the genus *Flavivirus.* The mature DENV viral particles are ~50nm in diameter. Each DENV particle consists of nucleocapsid core containing a single-stranded plus sense genomic RNA molecule complexed to a basic protein called the capsid protein, C, surrounded by a host-derived lipid bilayer coated with two structural proteins, the membrane protein, M (plus some of its unprocessed precursor, prM) and the envelope protein, E (*Kuhn et al, 2002*).

1.2.1 Genomic organization

DENVs have a single-stranded, 5'-capped, poly A tail-lacking, positive sense RNA genome of about 11,000 bases which contains a single open reading frame (ORF), and is flanked by 5' and 3' non-translated regions (NTRs). These NTRs contain multiple sequence and secondary structural elements that are critical for viral replication and life cycle (*Swaminathan & Khanna, 2009; Filomatori et al, 2006; Alvarz et al, 2005*). The single ORF is translated into a large polyprotein precursor of >3000 amino acid (aa) residues, which is processed by the combined action of host and viral proteases into three mature structural and seven non-structural proteins, as depicted in Figure 1.3 , page 4 . The three structural proteins are capsid (C), membrane (M), envelope (E), and the seven non-structural (NS) proteins are NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (*Lindenbach et al, 2013).* The essential properties and functions of these proteins are summarized in Table 1.1, page 5.

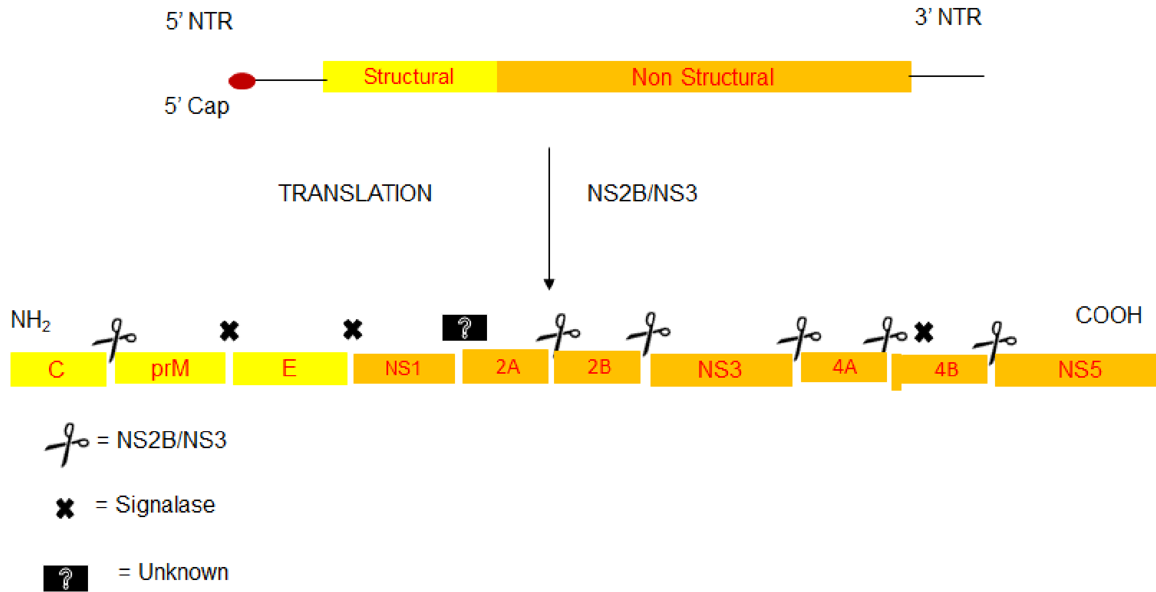

Figure 1.3: The DENV genome and polyprotein. *The top part shows the DENV genome with 5' cap (at the left), the 5' non-translated region (5'NTR), the 3' NTR and the single ORF comprising the structural (yellow) and non-structural (orange) protein-encoding regions. The bottom part depicts the polyprotein translated from the genome with its amino (NH_2) and carboxy (COOH) ends indicated. The symbols above the polyprotein denote the sites of host signalase (**X**), unknown host protease (**?**) and viral protease ( ) cleavage. (from Lim et al, 2013)*

1.2.2 Life cycle

The life cycle of DENV involves two hosts, the mosquito vector and the human host. The bite of an infective female *Aedes* mosquito which transmits DENV into the human host initiates the intrinsic phase of the viral life cycle, which lasts 4-7 days and culminates in viremia, the release of DENV into the circulation. When an uninfected mosquito feeds on such a viremic host, it acquires the virus, which now undergoes an 8-12 day long extrinsic phase of viral life cycle, at the end of which the mosquito becomes infective for the remainder of its lifespan (*Gubler, 1998, WHO 2016).*

The E protein on the surface of the DENV particle mediates host receptor recognition (*Crill & Roehrig, 2001*). Rceptor binding triggers clathrin-mediated endocytosis (*Van der schaar et al, 2008*). The acidic pH within the endosome causes trimerization of the E protein, which leads to fusion of viral and host membranes with concomitant release of the viral genome into the host cell cytoplasm. Recently it has been shown that the release of viral genome into the cytoplasm is linked to DENV capsid degradation, mediated by the host ubiquitin-proteasome system (*Byk et al, 2016*). The viral genomic RNA serves as a template for both translation and replication. It is first translated to give rise to the polyprotein precursor, which matures co- and post-translationally into the three structural and seven non-structural DENV protens mentioned above. Polyprotein maturation depends on proteolytic processing mediated by both host as well as viral proteases (Figure 1.3). Viral genomic RNA replication, which also occurs in the cytoplasm, is chiefly mediated by NS3 and NS5 proteins, which together function as the viral replicase. The synthesis of viral genomic RNA proceeds through a complementary minus sense RNA intermediate. The switch between the (+) RNA and (-) RNA is regulated by the concentration of NS3 and NS5 (*Garcia-Blanco et al, 2016*); Completion of viral protein and RNA synthesis sets the stage for assembly of immature virions, covered with prM-E heterodimers (*Zhang et al, 2003*). The immature

virions mature during transit through the trans-Golgi network. Final maturation is achieved when prM is cleaved by host furin and this is coupled to egress of the mature virions into the extracellular space (*Swaminathan S & Khanna N, 2009; Mukhopadhyay S et al, 2005; Sampath & Padmanabhan, 2009, Screaton et al, 2015*). The key steps of the viral life cycle are represented pictorially in Figure 1.4 (page 6).

Protein	MW (Kda)	Important functions	References
C	11	C proteins make up the nucleocapsid. C interacts with E protein during the translocation of the viral genome into cytoplasm.	Lidenbach and Rice 2003,Lidenbach et al 2013,Freier et al,2015
prM/M	26/8	Premature, acid-induced fusion of E is prevented through association with prM. Furin mediated prM cleavage releases mature virus out of the cell.	Heinz and Allison,2000 Li et al, 2008;Yu et al, 2008
E	50	Major virion surface molecule which mediates binding and membrane fusion. TMD involved in particle assembly and maturation	Lidenbach and Rice 2003,Lidenbach et al 2013 Blazevic et al, 2016
NS1	46	Interaction between NS4A and NS1 is critical for replicase function. Interacts with E, prM and C proteins and plays a critical role in production of infectious virus particles. Interacts with C1q which plays an important role in antibody dependent enhancement NS1 along with hnRNP C1/C2,K and vimentin helps in virus propagation NS1 interacts with various TLRs and is implicated in DENV pathogenesis	Lidenbach and Rice 1999. Scaturro et al, 2015 Silva et al, 2013 Noisakran et al, 2008; Kanlaya et al, 2010 Modhiran et al, 2015
NS2A	22	DENV RNA synthesis, virion assembly and maturation	Xie et al, 2013,2015
NS2B	14	Forms a complex with NS3, and is a necessary cofactor for the serine protease domain of NS3 . TMD involved in viral replication and assembly	Falgout et al, 1991, Yusof et al, 2000 Li et al,2016
NS3	70	Multifunctional protein. Serine Protease, Nucleoside triphosphatase, RNA helicase, RNA triphosphatase.	Preugschat et al,1990; Li et al, 1999;Kadare and Haenni,1997; Wengler 1993
NS4A	16	Involved in virus replicase complex Enhances viral Replication	Miller et al, 2007 ; Roosandal et al, 2006 McLean et al, 2011
NS4B	27	Blocks interferon signaling during virus infection Interacts with NS3 to enhance helicase activity of NS3	Munoz-Jordan et al, 2003,2005 Umareddy et al, 2006
NS5	104	Multifunctional protein. RNA-dependent RNA polymerase , Methyltransferase. RNA capping RNA synthesis initiation by methyltransferase domain and elongation by polymerase domain Modulates splicing	Bartholomeusz et al 1994; Egloff et al, 2002. Zhao Y et al, 2015 Potisopon et al,2014,2016 De Maio et al, 2016

Table 1.1: *Essential properties and functions of flaviviral proteins.*

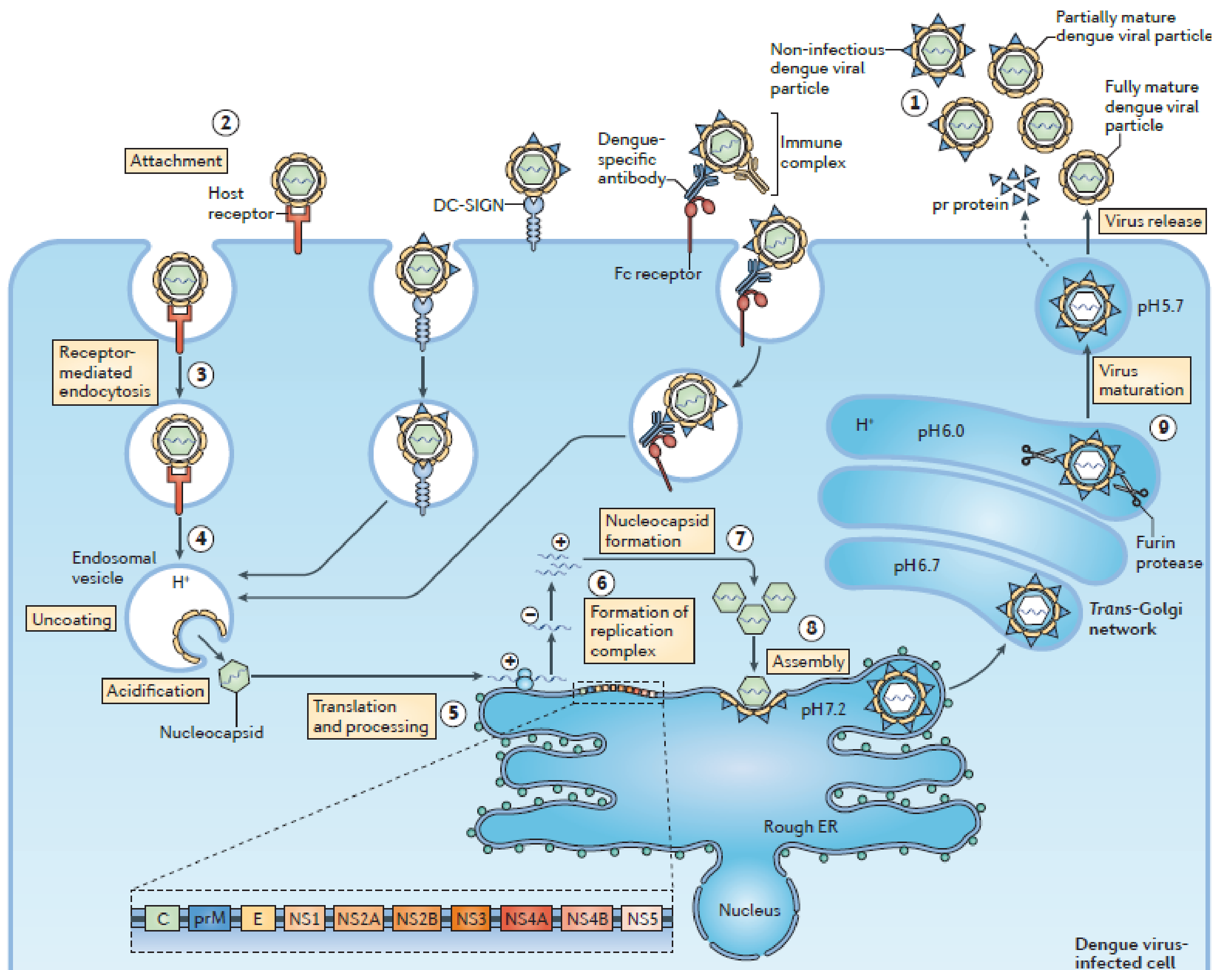

Figure 1.4: Life cycle of DENV.

DENVs can enter susceptible cells using one of multiple pathways. For example, partially mature DENVs may interact with the Fc receptor to effect entry (1), while fully mature virions utilize specific cell surface receptors (2). Subsequent entry involves receptor-mediated endocytosis (3), followed by endosome acidification (4). The acidification triggers conformational changes in the DENV E protein leading to fusion of viral and endosome membranes and release of the viral genome into the cytoplasm of the host cell. The viral RNA is translated into a single polyprotein which is processed (5) by viral and host proteases into the mature viral proteins. Availability of the viral proteins leads to formation of the viral replicase and formation of the replication complex (6). The viral genome switches to replication via a 'minus' RNA intermediate to generate new copies of the 'plus' sense genomic RNA (6). The newly replicated viral genomic RNA complexes with the C protein to form the nucleocapsid (7), which buds into the ER, assembling into immature viral particle (8), surrounded by host-derived lipid bilayer containing prM and E proteins. As the immature DENV particles pass through the Golgi and trans-Golgi network, the accompanying acidification induces conformational changes in the immature virion exposing the furin cleavage sites on prM. Host furin cleaves the prM protein to form M protein and 'pr' peptide. The latter stays associated with the virion until it is released by exocytosis into the extracellular milieu (9). DC-SIGN, dendritic cell-specific ICAM3-grabbing non-integrin; NS proteins, non-structural proteins (from Screaton et al, 2015).

1.3 Dengue disease

Infection with any one of the four DENVs can result in a range of symptoms. Traditional classification of dengue, based on multiple clinical parameters (*WHO, 1997*) has more recently been replaced by a single parameter-based classification(*WHO, 2009*).It has been suggested that the various clinical manifestations may be viewed as representing progressively severe stages of a continuous spectrum of dengue disease (*Swaminathan & Khanna, 2013*).

1.3.1 Clinical manifestations & diagnosis

Infection with any of the DENVs may be asymptomatic or result in a wide spectrum of clinical outcomes ranging from mild fever, hemorrhage, thrombocytopenia and plasma leakage (*WHO, 2016; Pierson & Diamond, 2013*). Classic dengue fever (DF) which manifests itself as sudden onset of high fever lasts about 5-7 days is usually self-limiting. The febrile phase coincides with viremia. A small proportion of DF patients may progress quite rapidly to more severe dengue hemorrhagic fever (DHF). Plasma leakage is the major feature that distinguishes DHF from DF. When the hemorrhage is uncontrolled, it leads to potentially fatal hypovolemic shock knowns as dengue shock syndrome (DSS). Bleeding, thrombocytopenia and ≥20% hemoconcentration, associated with abdominal pain, profuse sweating and hypothermia mark the DHF to DSS progression. It has been observed that viremia levels in DHF/DSS compared to DF is significantly elevated (*Vaughn et al, 2000; Libraty et al, 2002*). Recently, the World Health Organization has re-classified dengue disease into three categories: (i) dengue without warning signs, (ii) dengue with warning signs and (iii) severe dengue (*WHO, 2009*). More recently, the severe forms of dengue disease, DHF/DSS, have been referred to as dengue vascular permeability syndrome, DVPS (*Halstead, 2015, 2016*).

As many signs and symptoms of dengue illness are also seen in many other febrile conditions, clinical diagnosis of dengue infection needs to be confirmed by laboratory tests. This may be done based on the identification of the virus itself, its genomic RNA, its antigens or the antibodies it elicits (*Guzman et al, 2010*). If patient sera are collected during the viremic phase, DENV isolation by mosquito inoculation or cell culture is possible. Alternatively, viremic sera may be used for the detection of viral RNA through coupled reverse-transcription polymerase chain reaction (*Lanciotti et al, 1992; Harris et al, 1998; Johnson et al, 2005*) or isothermal RNA-specific amplification (*Wu et al, 2001*). Commercial diagnostic kits, in different formats, have been developed to detect anti-DENV IgM and IgG antibodies (*Guzman et al, 2010*) as well as DENV NS1 antigen (*Alcon et al, 2002; Young et al, 2000; Dussart et al, 2006, 2008*). A pitfall associated with many of the antibody detection kits is the issue of cross-reactivity stemming from the significant degree of similarity among the flaviviral E proteins. To some extent this has been circumvented by developing designer antigens based on recombinant proteins which contain exclusively DENV-specific epitopes (*AnandaRao et al, 2006; Hapugoda et al, 2007*). It is notable that these designer antigens are being used in the diagnostic kits available in the Indian market (*Dengue Day 1 Test, J. Mitra & Co. Pvt. Ltd*). The NS1 antigen detection kit is being increasingly used for the early detection of DENV infection. In addition, multiple reports have now demonstrated the utility of NS1 detection as a test for ongoing DENV replication in infected cells (*Schule et al, 2007; Ludert et al 2008; Korrapati et al, 2012*).

1.3.2 Pathogenesis

Severe manifestations of dengue disease tend to occur in individuals having prior immunity to a heterotypic DENV. Thus, DHF/DSS occurs during a secondary infection of individuals with a different DENV serotype or during primary infection of infants born to dengue-immune mothers. While several hypotheses have been proposed to explain the pathogenesis of severe dengue disease, the most widely accepted one is based on antibody-dependent enhancement, ADE (*Halstead, 2003, 2015*). According this hypothesis, pre-existing heterotypic antibodies, acquired either actively through secondary infection, or passively through placental transfer, form immune complexes with DENVs and facilitate their FcR-mediated entry into cells of monocytic lineage, leading to an increase in infected cell mass and an increase in virus production. This in turn leads a disproportionate anti-inflammatory response, characterized by a cytokine storm, accompanied by endothelial damage and vascular leakage (*Swaminathan & Khanna, 2009*). AG129 mouse-based *in vivo* ADE models have successfully reproduced many features of severe dengue disease such as high virus levels in blood and tissues, thrombocytopenia, cytokine storm, increased vascular permeability, hemoconcentration and hemorrhage (*Milligan et al, 2015; Zellweger et al, 2010; Balsitis et al, 2010*).

Recent work has implicated a role for DENV NS1 in pathogenesis. DENV NS1 has been shown to interact with TLR4 receptors on monocytes, macrophages and endothelial cells in culture and induce the release of many cytokines characteristic of DHF/DSS accompanied (*Modhiran et al, 2015*). NS1 has been shown to increase endothelial cell permeability *in vitro* and cause lethal vascular permeability *in vivo* in a mouse model (*Beatty et al, 2015*).

1.4 Vaccine development

The fact that dengue illness can be caused by four antigenically distinct DENVs which elicit antibodies that are cross-reactive, but not cross-protective, has made it mandatory that a safe and effective dengue vaccine must be tetravalent. It must confer protection against all the four prevalent DENV serotypes. This requirement imposes one of the greatest challenges in dengue vaccine development. Additionally, the tetravalent immune response must be balanced and durable. If the immune responses are not balanced, it is possible that the vaccine recipient may be sensitized to severe disease through ADE (see Sec.3.2). Yet another challenge is that the basis of protective immunity is not well understood. Finally, one of the biggest hurdles has been the lack of an appropriate animal model system to evaluate experimental dengue vaccines. Clinical testing and long-term evaluation are the only means of assessing safety, immunogenicity and reactogenicity of experimental vaccines.

Despite such hurdles, several vaccines have advanced to clinical trials (*Swaminathan et al, 2010; Swaminathan and Khanna, 2013*). Of these, the most advanced is CYD-TDV, a mixture of four live attenuated vaccine viruses. All four are derived from the live attenuated yellow fever virus (YFV) vaccine YF17D backbone by replacing the prM and E genes of YFV with prM and E genes of DENV-1, -2, -3 and -4. Both the proof-of-concept Phase IIb (*Sabchareon et al, 2012*) as well as multiple Phase III (*Capeding et al, 2014; Villar et al, 2015*) trials revealed the CYD-TDV vaccine to possess overall sub-optimal efficacy, as it manifested very low protective efficacy towards one of the DENV serotypes. In addition, it was found to increase risk of hospitalization in <9 year old children (*Hadinegoro et al, 2015*). These shortcomings notwithstanding, the enormity of the dengue situation has led to the

licensing of this vaccine in some Asian and Latin American countries. Aside from CYD-TDV, several other experimental vaccines are also in clinical development stages as summarized in Table 1.2 (*Schwartz et al, 2015*). These include tetravalent LAVs based on attenuated DENV-2 or DENV-4 backbones, which are essentially similar in design to the CYD-TDV described above, as well as others base on purified inactivated DENVs, DENV E-encoding plasmid DNA vaccines and recombinant envelope protein-based subunit vaccines.

Vaccine Candidate	Vaccine type	Group involved	Phase	Start and end dates	NCT* Number	Status
CYD	Live attenuated	Sanofi Pasteur	III	June 2011-November 2017 June 2011- April 2018	NCT01373281 and NCT01374516	Ongoing Ongoing
DENVax	Live attenuated	Takeda	II	November 2011- April 2016	NCT01511250	Completed; data not available
TV003/TV005	Live attenuated	NIAID⋆and Buatntan Institute	II	October 2013- December 2018 December 2014-February 2019	NCT01696422 and NCT02302066	Ongoing Ongoing
TDENV PIV	Purified inactivated	GSK⋆and WRAIR⋆	I	November 2014–December 2019	NCT02239614	Recruiting
V180	Recombinant subunit	Merck	I	July 2012- December 2014	NCT01477580	Completed
D1ME100	DNA	NMRC ⋆	I	January 2006- April 2009	NCT00290147	Completed

Table 1.2: Dengue vaccines in clinical trials *(from Schwartz et al, 2015).*
**- U.S. NIH clinical trials.*
★- *NIAID: National Institute of Allergy and Infectious Diseases; GSK: GlaxoSmithKline; WRAIR: Walter Reed Army Institute of Research; NMRC: Naval Medical Research Center.*

1.5 Drug development

The expectation that a preventive dengue vaccine could be developed had for long contributed to the neglect of focused efforts at developing drugs for treating dengue infections. The increasing realization of the tough challenges faced in dengue vaccine development, together with the ever-increasing dengue burden, has spurred recent efforts to explore the feasibility of developing drugs for treating dengue infections.

Currently, a therapeutic drug against DENV is not available. The only treatment option available to a DHF/DSS patient is supportive fluid therapy under medical supervision in a hospital setting. An antiviral drug against DENV must be able to rapidly downregulate viral replication and thereby help minimize the viral load to prevent progression of DF to DHF/DSS. Theoretically, such a drug may target one or more of the critical steps in the viral life cycle (Figure 1.4, page 6). Such a drug may inhibit the function of viral proteins or host factors required by DENV for successful completion of its life cycle. An alternate approach, which does not require specific knowledge of the viral or host target, utilizes whole cell-based assays to evaluate inhibition of viral replication. Such endeavours have become possible with the development of high throughput screening (HTS) strategies against known or unknown targets. The expectation from an ideal dengue drug and its potential benefits are summarized in Table 1.3. Available information on dengue drug development, gleaned from a survey of the patent literature, published literature as well as from global clinical trial data are summarized below (Figure 1.5, page 11 and Figure 1.6, page 12).

Attributes of an ideal drug	Potential advantages of an DENV drug
Effective against all 4 DENVs	Prophylactic in endemic regions
Rapid action Capacity to reduce viral titers ≥ 2 logs	Therapeutic in preventing progression of dengue fever to dengue Hemorrhagic fever/dengue shock syndrome.
Low or no significant toxicity	Treating complications from unequal immune protection
High genetic barrier to emergence of resistance	Curbing transmission during outbreaks
Stable to high temperature and humidity	Lowering disease burden, healthcare costs
Affordable in dengue-endemic countries	

***Table 1.3: Attributes and potential benefits of DENV drug*.**

1.5.1 Drugs that block DENV entry

The DENV E glycoprotein mediates viral entry into susceptible host cells. Thus, inhibitors that can serve as entry blockers are designed to target the viral E glycoprotein-host cell receptor interaction. These inhibitors may target the E glycoprotein, the host cell surface receptor or compromise the integrity of the host-derived lipid membrane on the DENV surface, as summarized in Table 1.4.

Therapeutic Candidates	Target	Activity[a]	Mouse efficacy[b]
mAb 14c10.8	E	0.328μg/ml[c]	+ (D1)
mAb 9E-H2L2	E	0.021-0.167μg/ml[d]	+ (D2)
mAb 9F12	EDIII	0.02-1.95ng/μl[d]	+ (D2)
RDW031	Bilayer	-	-
Dendrimer	DC-SIGN	-	-
-	Syndecan	-	-

Table 1.4: Entry Blockers

[a]Entry blocking activity of mAb was assayed in terms of virus neutralization in a cell culture based assay; values shown are mAb concentrations that can reduce virus infectivity by 50%

[b]In vivo efficacy tested using AG129 mouse model; the "+" sign indicates that the drug manifested in vivo efficacy when tested against DENV-1 (D1) or DENV-2 (D2)

[c]Tested against DENV-1

[d]mAb active against all four DENVs

"-" Data not reported/not available.

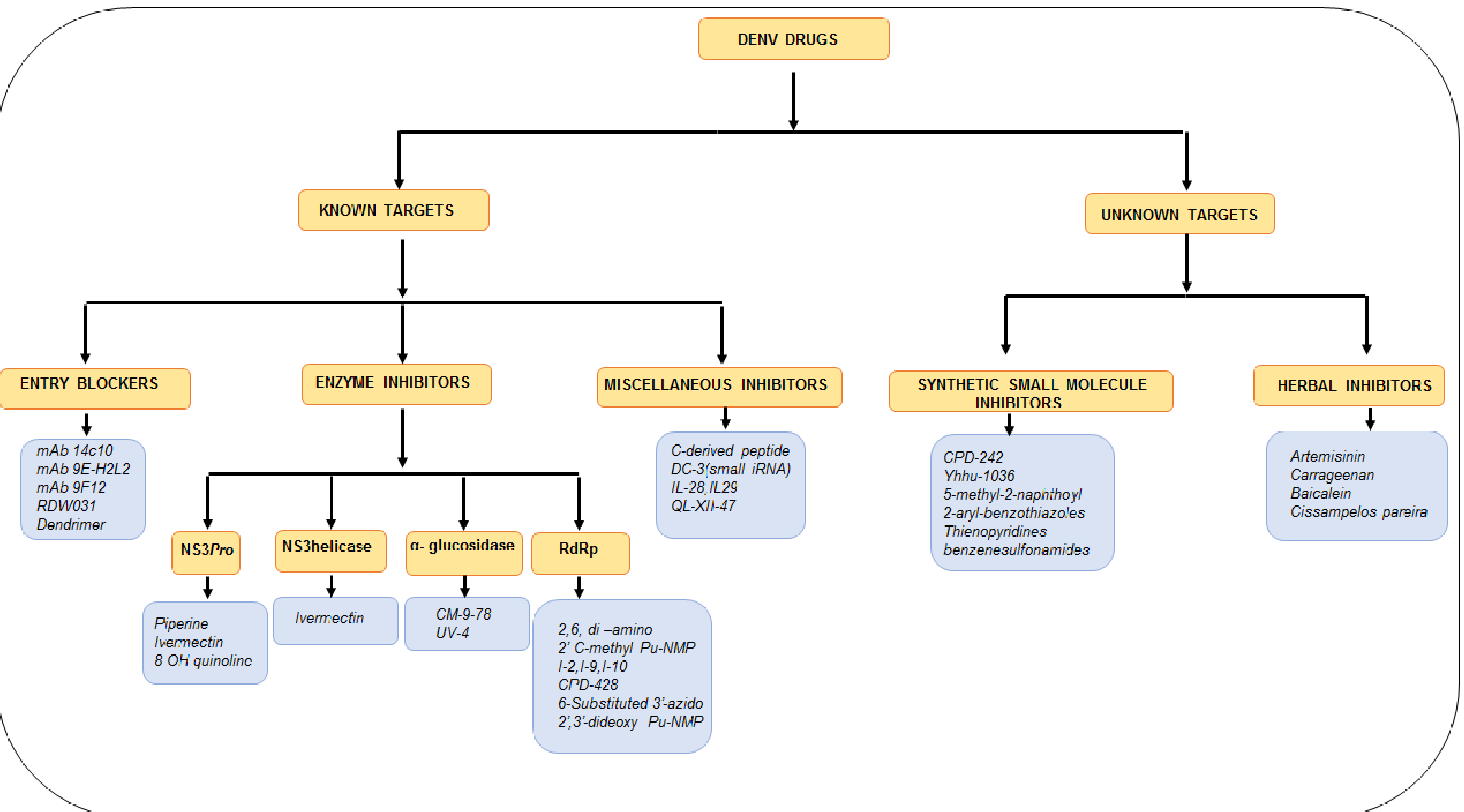

Figure 1.5: Summary of drugs available in literature.

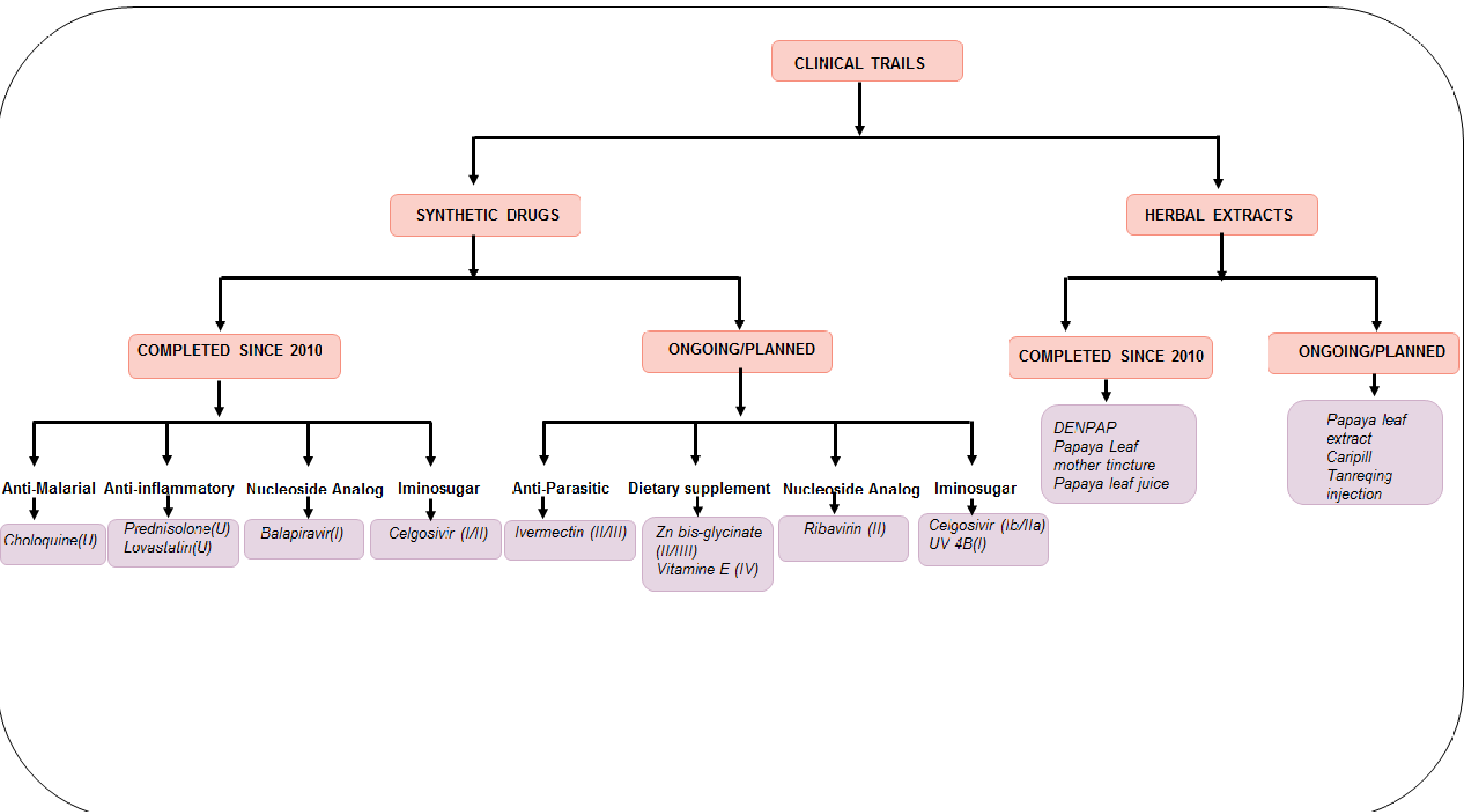

Figure 1.6: Summary of drugs in clinical trials. *The drug phases (I, Ib, II, IIa, III, IV) are given with in parenthesis. U= Unknown status.*

Investigators from Singapore have isolated a DENV-1-specific anti-E monoclonal antibody (mAb), mAb 14c10.8, using Epstein-Barr virus-immortalized memory B cells from a patient who had recovered from a recent DENV-1 infection *(NUS patent, 2013*). Based on cryo-electronmicroscopy of the mAb bound to DENV-1, it was shown that it binds to a quaternary epitope on the E dimer. This mAb which specifically neutralized DENV-1 ($PRNT_{50}$ titer=0.328μg/ml) did not cause heterotypic ADE in Fc receptor (FcR)-bearing K562 cells. Using AG129 mouse, a double knock-out strain lacking interferon (IFN) α/β and γ receptors (*Schul et al, 2007*), it was reported that mAb 14c10.8 was capable of protecting against DENV-1 challenge. Interestingly, this protection was evident regardless of whether the mAb was administered either 24 hours before or 24 hours after DENV-1 challenge.

Using a similar approach as mentioned above, a panel of DENV mAbs was isolated from secondary dengue patients (*ASTRS patent, 2013*). These mAbs manifested 50% neutralization titres (NT_{50}) in the range 0.01-1 μg/ml, using a Fluorescence activated cell sorting (FACS)-based virus neutralization assay. Though all these mAbs targets were specific to DENV E, none was found to be specific to EDIII, the domain of E involved in mediating DENV entry into host cells. This is consistent with the observation reported earlier that the proportion of anti-EDIII antibodies elicited during natural DENV infections is not significant (*Wahala et al, 2009*). Interestingly, 8% of the mAbs in this panel was found to be pan-DENV specific, with one, mAb 9E-H2L2, that displayed NT_{50} titers of 0.04, 0.044, 0.167 and 0.021 μg/ml, against DENV-1, -2, -3 and -4, respectively. However, this mAb, when passively transferred into the AG129 mouse model mentioned above (*Schul et al, 2007*), conferred protection only against DENV-2 challenge. It is noteworthy that the secondary dengue patient from whom mAb 9E-H2L2 was isolated had been exposed to DENV-2 primary infection. This, taken in conjunction with AG129 results, suggests that mAb 9E-H2L2 is a DENV-2 mAb and its ability to neutralize the remaining DENV serotypes is not an indication of protective efficacy, but a manifestation of cross reactivity.

In contrast to mAb 9E-H2L2, mAb 9F12 is reported to be specific to EDIII. This mAb, whose epitope has been mapped to amino acid residues K-305, K-307, K-310 and G-330 located within EDIII, implicated in host receptor recognition and binding (*Crill and Roehrig, 2001*), can potentially block the entry of DENV into host cells. It was found that mAb 9F12 can inhibit all four DENVs effectively, with $PRNT_{50}$ values in the range 0.2-1.95ng/μl (*NTU patent, 2010*). *In vivo* efficacy of this mAb was tested only against DENV-2 using the AG129 challenge model. mAb 9F12 conferred a reasonable degree of protection on the basis of ~4 fold reduction in viremia and ~2 fold reduction in NS1 antigenemia (*NTU patent, 2010*).

Host cell entry of enveloped viruses, such as DENV, can be blocked by disrupting the integrity of the lipid membrane (surrounding the virus particle) with lipophilic molecules such as butylated hydroxyl toluene (BHT) and its derivative, 3,5-di-t-butyl-4-hydroxy benzoic acid (BG4). A novel compound 'RDW031', which is a glucose ester of butylated hydroxyl benzoic acid has been shown to exert potent antiviral activity against Herpes Simplex Virus (HSV-1) and it has been speculated that DENVs, being enveloped viruses themselves, may also be susceptible to RDW031 (*Vachy patent, 2010*).

While the entry blockers discussed above target the virus, it has been suggested that alternate approaches to prevent virus entry may target host cell molecules such as syndecan (*UR patent, 2013*) and DC-SIGN (*IT-ES-FR patent, 2011*), implicated in facilitating virus entry into susceptible host cells. Recent studies have shown that DENV-2 (strain 16681) can

infect several endothelial cells using syndecan. Syndecan is a heparin sulfate-containing proteoglycan known to be involved in cell-to-cell and cell-to-matrix interactions, as well as in migration, proliferation and differentiation (*Alexopoulou et al, 2007; De Rossi et al, 2013*). Studies of mutations in the heparin-binding sites on DENV E as well as syndecan knockdown using RNA interference have demonstrated that DENV-2 utilizes syndecan-2 and syndecan-4 to infect dermal microvascular endothelial cells and umbilical vein endothelial cells, respectively. Similarly, DC-SIGN, is another cell surface component that can mediate pathogen entry. It is known to bind to highly mannosylated glycans of pathogens such as HIV-1 (*Geijtenbeek et al, 2000*). The role of DC-SIGN in mediating DENV entry is well-documented (*Smit et al, 2011*). In this context, the observation that HIV-1 p24 expression can be inhibited significantly by a synthetic pseudomannotriose-containing dendrimer designed to bind DC-SIGN, suggests that targeting DC-SIGN could offer a means to blocking DENV entry as well.

1.5.2 Enzyme inhibitors

Drugs that can specifically inhibit DENV-encoded enzymes as well as host enzymes are potential antiviral agents (Table 1.5). Viral enzymes that have been targeted for drug development include the NS3 protease (NS3Pro), the NS3 helicase (NS3Hel) and NS5 encoded RNA-dependent RNA polymerase (RdRp). Thus far, only one host enzyme, the endoplasmic reticulum (ER) α-glucosidase, has been targeted for anti-DENV drug development.

Drug	Target	Activity (assay)
Piperine[a]	NS3Pro[b(D2)]	-
Ivermectin[c]	NS3Pro	-
8-OH-quinoline	NS3Pro[b(W)]	2.35-63.12μM[d] (DENV-2 replicon assay)
Ivermectin[c]	NS3Hel[b(Ku/Ko)]	0.7μM[d] (DENV-2 VYRA)
2,6, di-amino 2' C-methyl Pu-NMP	RdRp	1.3μM[d] HCV replicon assay

Table 1.5: Enzyme inhibitors

[a]*Obtained from the plant Piper nigrum.*

[b]*Tested in biochemical assay using recombinant enzyme from* [D2]*DENV-2,* [W]*West Nile virus,* [Ku/Ko]*Kunjin and Kokobera viruses.*

[c]*Hits identified by in silico screening of small molecule libraries.*

[d]*Activity data shown are* EC_{50} *values.*

1.5.2.1 NS3 protease (NS3pro) inhibitors

Potential inhibitors of the DENV NS3Pro have been identified based on screening *in silico* (*TU patent, 2013*) or screening *in vitro* (*GU patent, 2014; MU patent, 2010*) using cloned DENV NS3Pro. An anthracene-based molecule, ARDP0006, has been identified by high throughput computational screening (*Wang et al, 2007*). Using the EUDOC docking program, it was found that ARDP0006 interacts with NS3Pro active site and P1 pocket residues. This drug not only inhibited DENV-2 NS3Pro *in vitro*, but also inhibited DENV-2 replication in cultured cells with half maximal effective concentration (EC_{50}) of 4.2±1.9μM (*Tomlinson et al, 2009*). Following an analysis of structure and activity relationship of several

ARDP0006 analogs, it was concluded that the anthracene ring system may serve as robust scaffold for developing potent NS3Pro inhibitors (*TU patent, 2013*).

Another computational screening campaign that included ~2000 chemically diverse compounds from the MicroSource Spectrum Collection, found several putative DENV-2 NS3Pro inhibitors such as ivermectin, selamectin, tyrothricin, alexidine hydrochloride, hematoxylin pentaacetate and methylbenzethonium chloride (*TU patent, 2013*). Interestingly, these compounds are used for other indications. Ivermectin and selamectin are used in treating parasitic infections in humans (ivermectin) and animals (ivermectin and selamectin). Methylbenzethonium chloride is effective in the treatment of cutaneous leishmaniasis (*Kim et al, 2009*). Tyrothricin (*Mogi et al, 2009*) and alexidine hydrochloride (*McDonnell & Russell, 1999*) possess antibiotic activities, with latter also known to possess anticancer activity (*Yip et al, 2006*).

Using cloned West Nile virus (WNV) NS3Pro in combination with a fluorogenic substrate, to screen a collection of several small molecule libraries, investigators from Georgetown University identified several 8-hydroxyquinoline derivatives to be potent WNV NS3Pro inhibitors (*GU patent, 2014*). Further, some of these molecules also inhibited the replication of WNV replicons in cultured cells. These inhibitors also inhibited DENV-2 replicons, but less effectively than they did the replication of WNV replicons. Interestingly, one of these, compound 36, appeared to be specific to DENV-2 replicons only.

Apart from such synthetic molecules, piperine, a naturally occurring plant alkaloid has been found to be a moderate inhibitor of recombinant DENV-2 NS3Pro *in vitro* (*MU patent, 2010*). It has been suggested that piperine could be the starting molecule to develop potent DENV protease inhibitors.

1.5.2.2 NS3 helicase (NS3hel) inhibitors

Inhibiting NS3hel activity could be an effective antiviral strategy as a DENV-2 helicase mutant fails to replicate (*Matusan et al, 2001*). However, as the mechanism of action of NS3hel is not fully understood, designing potential inhibitors has been a challenging task (*Sampath & Padmanabhan, 2009*). A European consortium performed *in silico* analysis to identify the site of single-stranded (ss) RNA access site on NS3hel molecule. Next, they screened a library of >1200 compounds from a commercially available Library of Pharmacologically Active Compounds (LOPAC library, Sigma-Aldrich) for potential NS3hel binders that target this ss RNA access site. Interestingly, this screen identified ivermectin, the NS3Pro inhibitor mentioned above (*TU patent, 2013*), to also be a NS3hel inhibitor. It not only was found to inhibit cloned WNV NS3hel, but also to curtail the replication of DENV-2 in a virus yield reduction assay with an EC_{50}=0.7 µM (*Roman/French patent, 2012*). Available data suggest that the ivermectin's anti-DENV activity could be due to its inhibitory effect on both NS3Pro and NS3hel activities.

1.5.2.3 RdRp inhibitors

Nucleoside analogs (NA) can inhibit viral polymerases and serve as antiviral drugs. NAs are essentially prodrugs, which interfere with the replication machinery once they are converted to their nucleoside triphosphate form by host kinases. RdRp inhibitors have been developed against the polymerases of Human immunodeficiency virus-1(*Cihlar & Ray, 2010*), Hepatitis B virus (*De Clercq, 2011*) and HCV (*Fried et al, 2002*). Hoffman La Roche

has reported three NAs, I-2, I-9 and I-10, which can inhibit DENV-4 viral RNA replication in immature monocyte-derived dendritic cells (iDC) from human volunteers, with half maximal inhibitory concentrations (IC_{50}) of 1.9µM, 16µM and 15µM, respectively (*Hoffmann-La Roche patent, 2012*).

The NA, 2, 6-diamino 2'-C-methyl purine nucleoside monophosphate pro-drug, effective against recombinant HCV NS5b RNA polymerase *in vitro* and HCV replicon replication in cultured cells (EC_{50}=1.3µM) has been suggested to be a potential inhibitor of DENV RdRp as well (*RFS –EU patent, 2012*). NA derivatives based on 6-substituted 3'-azido-2', 3' dideoxy purine nucleoside monophosphate have been proposed to be superior broad spectrum antiviral agents (*RFS –EU patent, 2010*). It has been suggested by Novartis that 2-branched nucleosides may be useful as anti-flaviviral drugs (*Novartis patent, 2014*).

It has been shown that purine analogs in which the imidazole ring is replaced by 1, 3-thiazole/1, 3-oxazole ring, in conjunction with certain substituent combinations on these scaffolds possess significant antiviral activity. Purine analogs, such as compound 428, have been demonstrated to possess antiviral activity against HCV using a replicon assay (*KU patent, 2013b*). However, none of the NA and purine analogs has been tested for their putative anti-DENV inhibitory potential.

1.5.2.4 α-Glucosidase inhibitors

Final maturation and release of infectious DENV (and other enveloped viruses) depends on host ER α-glucosidases. During virus maturation, these enzymes facilitate the interaction of the viral surface glycoproteins with chaperones in ER by trimming the high-mannose N-linked glycan moieties on them (*Parodi, 2000*). Deoxynojirimycin (DNJ) is an iminosugar which is a substrate analog of α-glucosidase. It can serve as a broad spectrum antiviral agent against enveloped viruses, as it is a competitive inhibitor of α-glucosidases which can prevent virus maturation (*Durantel et al, 2007*). However, the utility of iminosugars is limited by efficacy and toxicity issues. A DNJ derivative, CM-9-78, has been shown to be a superior inhibitor of DENV-2 (EC_{50} =6.8µM) (*IHVR patent, 2011*). Another DNJ derivative, CM-10-18, is reported to act synergistically together with ribavirin, against DENV-2 both in cell culture and in AG129 animal model systems (*IHVR patent, 2011*). Ribavirin, which belongs to the NA class of antiviral drugs mentioned above, is used in treating HCV infections (*Hoffmann-La Roche patent, 2012*). Iminosugars UV-1 through UV-5, which are also DNJ derivatves have been reported to inhibit DENV-2 replication in Vero cells in a dose-dependent manner (*OU-UT patent, 2013*). Of these, the iminosugar UV-4, at low dosage (10mg/kg body weight), was found to significantly ($p<0.05$) improve the survival of AG129 mice against challenge by DENV-2 strain S221 (*Zellweger et al, 2010*).

1.5.3 Anti-DENV drugs against unknown targets

High throughput cell-based assays have been developed that are based on the use of cell lines stably transfected with DENV reporter replicon constructs or DENV-infected cells to screen small molecule libraries and herbal extracts for putative inhibitors. It is to be noted that this approach identifies agents that manifest antiviral activity without identifying the potential targets of their antiviral action.

1.5.3.1 Synthetic small molecules

Whole cell-based high-throughput assays have identified potential DENV inhibitors. CPD-242, a phenylethanone, is reported to inhibit DENV replication in Vero cells with EC_{50} values of 0.04, 0.04, 0.57 and 7.87 µM, respectively, for DENV-1, -2, -3 and -4 (*KU patent, 2013a*). It was also found to be effective based on *in vivo* testing using AG129-based dengue viremia (*Schul et al, 2007*) and dengue mortality (*Tan et al, 2010*) models. Another molecule, 5-tert-butoxy-2, 4-diaminoquinazoline (compound Yhhu-1036), has been reported to be a potential DENV-2 inhibitor (IC_{50}=0.009µM) based on a DENV-2 reporter replicon screening (*SIMM patent, 2013*). Finally, HTS screening of >200,000 compounds has identified 2-aryl-benzothiazoles (*Byrd et al patent, 2012*), thienopyridines (*Siga patent, 2013b*) and benzenesulfonamides (*Siga patent, 2013a)* as potential DENV inhibitors based on their ability to protect Vero cells against DENV-induced cytopathocity.

1.5.3.2 Herbal inhibitors

Several assorted phytochemicals have been reported in literature to possess antiviral activity against DENV. The plant alkaloid piperine has been found to have a modest inhibitory on DENV protease (*MU patent, 2010*). The anti-malarial compound, artemisinin, a sesquiterpene lactone from the *Artemisia annua*, mixed with berberine, an alkaloid from *Berberis* plants, is reported to have anti-DENV activity (*USP patent, 2013*). Similarly, the linear sulfated galactose polysaccharide from red seaweeds, carrageenan, is reported to be a DENV-2 inhibitor (*Talarico et al, 2011*). Another phytocompound, 5, 6, 7-trihydroxy flavone, from *Scutellaria baicalensis*, has been shown to inhibit DENV-2 replication in Vero cells (*MU patent, 2013*). An alcoholic extract, of unknown composition, prepared from the plant *Cissampelos pareira*, has been recently demonstrated to be inhibitory to all DENV serotypes in cultured cells. Interestingly, this extract afforded significant protection against DENV-2 challenge in AG129 mice (*Ranbaxy patent, 2010*).

1.5.4 Miscellaneous inhibitors/antiviral strategies

Aside from the targets discussed above, additional host/viral targets being explored for antiviral drug development include host lipid droplets (LDs), putative cysteine-reactive host factors and DENV genomic RNA (Table 1.6). The interaction of DENV C protein with endoplasmic reticulum-derived LDs is essential for successful completion of the viral life cycle (*Samsa et al, 2009*). Peptides derived from the amino-terminal domain of C protein, which can disrupt C-LD interaction, may serve as DENV inhibitors (*Universidade Federal Do Ro De Janeiro patent, 2012*). A tricyclic quinolone, QL-XII-47, is a potential broad spectrum antiviral agent that is capable of inhibiting all four DENV serotypes as well. Based on the fact that QL-XII-47 is a cysteine-directed reagent, it has been speculated that this drug targets a member of host's reactive 'cysteinome' (*Harvard patent, 2013; Weerapana et al, 2010*). Another strategy to achieve antiviral effect has been to stimulate the Interferon (IFN) pathway using interleukin (IL)-28 and IL-29 (*ZG patent, 2012*). IL-28 and IL-29 treatment have been shown to protect DENV-infected Vero cells against cytopathicity with EC_{50} values in the range of 0.0075-0.032µg/ml.

Therapeutic Candidates	Target	Activity	Mouse efficacy
C-derived Peptide	C-LD interaction	-	-
DC-3 (siRNA)	Genomic RNA	>2 log reduction in virus titers (pan DENV active)	+
IL-28, IL-29	PKR, OAS, Mx1 [a]	0.0075-0.032 µg/ml (DENV CPE[b] assay)	-
QL-XII-47	Cysteinome	>3 log reduction in virus titers (pan-DENV active)	-

Table 1.6: Miscellaneous drugs

a- PKR: RNA activated Protein Kinase; OAS: oligoadenylate synthetase; Mx1: IFN-induced GTPase involved in host antiviral response.

b- CPE: Cytopathic effect

1.5.4.1 RNA interference

RNAi is an evolutionarily conserved phenomenon involved in regulating gene expression in eukaryotes. This utilizes small interfering RNAs (siRNAs) to direct the endonuclease activity of cytoplasmic RNA-induced silencing complex (RISC) to mRNAs that possesses complementary siRNA target sequence. The ensuing mRNA cleavage essentially silences gene expression (*Dykxhoorn & Lieberman, 2006; Castonotto & Rossi 2009*).

The fact that RNAi-mediated gene silencing relies on sequence complementarity makes it possible to deploy it as an antiviral strategy capable of discriminating the virus from the host. The utility of RNAi has been tested as an antiviral tool against different viruses (*Dykxhoorn & Lieberman, 2006; Leonard & Schaffer, 2006; Grimm & Kay, 2006*). A couple of reports in the literature have explored the feasibility of deploying the endogenous RNAi pathway against DENV infections. A synthetic siRNA, DC-3, targeting the highly conserved 5' CS element on the DENV genome, has been reported to decrease viral titers of each of the four DENV serotypes by ~ 2 logs *in vitro* and attenuate disease severity *in vivo* in an AG129-based ADE model (*OHSU patent, 2013*). Another group has used a recombinant adenoviral system to deliver a short hairpin (sh) RNA into infected cells. This shRNA, shown to give rise to the corresponding siRNA within infected cells, effectively targets the genomic RNA of all four DENV serotypes (*Korrapati et al, 2012*).

1.6 Drugs in clinical development

The drug discovery initiatives reviewed above have so far not yielded any potential clinical candidates for efficacy testing in human volunteers. However, to accelerate dengue drug development, a few drugs for other indications have entered clinical testing for assessment of their possible utility as DENV antiviral agents (*Borges et al, 2013; Low et al, 2014;Nguyen et al, 2013a, b; Tam et al, 2012; Tricou et al, 2010;Whitehorn et al, 2012, 2015*). These include drug candidates initially developed for HCV therapy (*Klumpp et al, 2006; Brok et al, 2005*), anti-parasitic drugs against malaria (*Borges et al, 2013; Tricou et al, 2010*) and helminth infections (*Omura, 2008; Mastrangelo et al, 2012*), and anti-inflammatory agents (*Nguyen et al, 2013b; Whitehorn et al, 2012, 2015; Tam et al,*

2012).These are summarized in Table 1.7, page 20. Additionally, some of the developing dengue-endemic countries have commenced clinical trials of traditional herbal medicines for dengue summarized in Table 1.8, page 21.

1.6.1 Anti-parasitic drugs

The lysosomotropic alkalinizing action of the anti-malarial drug chloroquine (CQ) can potentially interfere with endosome acidification required for successful DENV infection. CQ has been shown to reduce the efficiency of DENV infection of mammalian cells (*Navarro-Sanchez et al, 2003*). CQ has been tested against dengue in two different trials. One, conducted in Viet Nam, administered CQ at a dosage normally used to treat malaria to >300 adult dengue patients and found no significant therapeutic benefit compared to untreated dengue patients (*Tricou et al, 2010*). The second CQ trial, involving a smaller number of volunteers, carried out in Brazil, again did not find any significant difference between CQ-treated and placebo arms of the study (*Borges et al, 2013*). The failure of CQ in these trials is unexplained. It is likely that the pH-modulating action of CQ which is demonstrable *in vitro* does not manifest *in vivo*. Alternatively, CQ may not have achieved the necessary *in vivo* concentrations to be inhibitory.

The broad-spectrum anti-helminthic drug, ivermectin, documented to inhibit DENV enzymes NS3Pro, NS3hel and RdRp (*Mastrangelo et al, 2012; Wagstaff et al, 2012*), is being tested in a phase II/III trial in >350 dengue patients in Thailand (*NCT02045069,* Table 1.7, page 20). Results are not available yet.

1.6.2 Anti-inflammatory agents

Diseases such as sepsis and atherosclerosis are associated with acute inflammatory response and endothelial damage (*Penn & Kamath, 2012*). Such a situation also occurs in viral hemorrhage associated with DHF/DSS. The plasma leakage in severe cases of dengue is a result of capillary endothelial damage. This provides the rationale for testing anti-inflammatory drugs normally used for non-viral illnesses for therapeutic efficacy against severe dengue illness as well. Pilot studies have shown that pentoxifylline, an inhibitor of the pro-inflammatory cytokine TNF-α, may help treat DSS (*Salgado et al, 2012*).

A clinical trial of the corticosteroid prednisolone was conducted in Viet Nam recently (*Tam et al, 2012*). Prednisolone was given orally to >200 laboratory confirmed dengue patients during the febrile phase. Results from this trial revealed that there was no statistically significant difference in fever clearance times and cytokine levels between prednisolone-treated and untreated groups. There was no evidence of any immunomodulation based on an analysis of cytokine profiles during the acute and convalescent phases of the corticosteroid-treated patients (*Nguyen et al, 2013b*).

1.6.3 Statins

Statins which can mediate anti-inflammatory effect, besides lowering cholesterol, are implicated in minimizing inflammation-induced capillary endothelial damage (*Li & Losordo, 2007*). Lovastatin has also been implicated in disruption of virion assembly based on *in vitro* studies (*Martinez-Gutierrez et al, 2011; Rothwell et al, 2009*). However, data from recent phase I and II lovastatin trials have not revealed any clinical benefit to dengue patients, based

on a comparison of a clinical and virological endpoints in lovastatin versus placebo groups (*Whitehorn et al, 2012, 2015*).

Drug Class	Drug (developer)	Trial Identifier[a]	Phase (n; age)	Trial Site	Start and end dates	Comments
Iminosugar	Celgosivir (Singapore Gen Hospital, Duke NUS Med School and 60 Deg Pharma LLC, Singapore)	NCT02569827[b]	Phase Ib / IIa (72; 21–65 year)	Singapore	July 2016–August 2018	Yet to initiate
	UV-4B (Unither Virology and Quintiles, Inc.)	NCT02061358	Phase I (64; 18–45 year; healthy subjects)	Kansas, USA	July 2014–September 2015	Ongoing/data awaited
Anti-parasitic	Ivermectin (Mahidol University, Thailand)	NCT02045069	Phase II/III (360; >15 years)	Bangkok, Thailand	February 2014–March 2016	Ongoing
Nucleoside analogs	Ribavirin (Guangzhou 8th People's Hospital, China)	NCT01973855[c]	Phase II (300; 18–65 year)	China	January 2012–December 2015	Ongoing/data awaited
Dietary supplement	Zn bis-glycinate (Srinakharinwirot University, Thailand)	TCTR2015111000	Phase II/III (60; 1–15 year)	Thailand	November 2015–June 2016	Ongoing
	Vitamin E (University of Colombo, Sri Lanka	SLCTR/2015/012	Phase IV (100; 5–12 year)	Sri Lanka	June 2015–September 2015	Ongoing/data awaited

Table 1.7: Ongoing/planned dengue therapeutic trials.

[a] *Trial identifiers retrieved from WHO's International Clinical Trials Registry Platform web portal ; trial identifiers with 'NCT' prefix are from the US NIH Clinical Trials Registry and Results database ; TCTR: Thai Clinical Trials Registry ; SLCTR: Sri Lanka Clinical Trials Registry .*

[b] *In addition to celgosivir, this trial will also assess the effect of modipafant (inhibitor of platelet activating factor, an inflammatory mediator).*

[c] *Ribavirin is being tested in combination with Chinese herbal drug.*

Herbal extract (developer)	Trial identifier[a]	Phase[b] (n; age)	Trial site	Start and end dates[b]	Comments
DENPAP (Goan Pharma)	CTRI/2014/06/004660 (www.ctri.nic.in)	U (15; 18–60 year)	Karnataka, India	May 2014–U	Completed; data not Available
Caripill (Micro Labs)	CTRI/2015/05/005806 (www.ctri.nic.in)	U (300; 18–60 year)	Karnataka and Andhra	June 2015–U	Ongoing
Papaya leaf extract (Bioextract & JIPMER, India)	CTRI/2014/10/005120 (www.ctri.nic.in)	Phase IV (60; 18–60 year)	Puducherry, India	U	Yet to initiate
Papaya leaf mother tincture (Fr Mueller Homeopathic Med College, India)	NCT02016027 (www.clinicaltrials.org/)	Phase I (60; 18–25 year; healthy subjects)	Fr Mueller's College, India	September 2013–December 2013	Completed; data not available
Papaya leaf juice (University of Sri Jayewardenepura, Sri Lanka)	SLCTR/2013/005 (www.trials.slctr.lk/.)	U (82; 18–60 year)	Colombo, Sri Lanka	January 2013–June 2014	Completed; data not available
Tanreqing injection (Guangdong Provincial Hospital of Chinese Medicine and Shanghai Kai Bio Pharma)	ChiCTR-IPR-15006778 (www.chictr.org.cn)	U (316; 18–70 year)	China	September 2015–U	Ongoing

Table 1.8: Dengue therapeutic trials using herbal extracts from traditional medicine

[a] *Trials retrieved from WHO's International Clinical Trials Registry Platform web portal ; trial identifiers with 'NCT' prefix are from the US NIH Clinical Trials Registry and Results database ; SLCTR: Sri Lanka Clinical Trials Registry; CTRI: Clinical Trials Registry-India ; ChiCTR: Chinese Clinical Trials Register.*

[b] *In some instances, the trial phase and/or trial dates have not been specified; this is indicated as 'U' (unknown).*

1.6.4 RdRp inhibitors

Balapiravir and ribavirin are NA inhibitors of the flaviviral RdRp. Originally these drugs which were developed for treating HCV infections, have been re-purposed for evaluation of their utility for dengue therapy. As the flaviviral RdRp is one of the most conserved of viral proteins, it is likely that these molecules may be effective against DENV RdRp as well.

Balapiravir, which is an ester prodrug of the NA, 4'-azidocytidine, is well-tolerated and can decrease HCV viremia in a time- and dose-dependent manner (*Roberts et al, 2008*). As HCV causes chronic infection, any drug therapy needs to be for extended durations. The toxicity of balapiravir associated with extended therapy has rendered it unsuitable for HCV therapy. However, as dengue is an acute illness requiring drug therapy for shorter duration, it is likely that balapiravir may be of utility in treating dengue patients. An exploratory trial of balapiravir in dengue patients was carried out by Hoffman-La Roche in 2010-11 (*Nguyen et al, 2013a*). A 5-day treatment of oral balapiravir therapy, initiated within 48 hours of fever onset, was followed by evaluation of viremia and DENV NS1 antigenemia. Despite the drug achieving adequate *in vivo* levels, the kinetics of both virologic markers were similar in both drug-treated and placebo arms of the study. Recent investigations have linked balapiravir's failure to confer therapeutic benefit to dengue patients to impaired prodrug conversion in DENV-infected cells (*Chen et al, 2014*).

Ribavirin, a broad-spectrum RdRp inhibitor, has been documented to manifest efficacy against HCV infection only in combination with IFN-α (*Feld & Hoofnagle, 2005*), but not when administered alone (*Brok et al, 2005*). A clinical trial of ribavirin in conjunction with traditional Chinese medicine to evaluate its efficacy in dengue patients has been concluded and results are yet to be disclosed (NCT01973855, Table 1.7, page 20).

1.6.5 Iminosugars

Iminosugars such as castanospermine (Cast) and deoxynojirimycin (DNJ) which can inhibit ER α-glucosidases, co-opted by many viruses for the final morphogenesis steps in their life cycles, can serve as broad-spectrum antivirals. Two iminosugars, celgosivir, a derivative of Cast, and UV-4, derived from DNJ, are being evaluated as possible dengue drugs in clinical trials.

Celgosivir which has been tested in human trials against HCV (*Durantel, 2007*) and abandoned for reasons cited above, was tested recently in dengue patients in a double-blind placebo-controlled proof-of-concept trial (*Low et al, 2014*). A total of 220mg celgosivir was administered every 12 hours over a 5-day period. Multiple parameters like viremia, time to NS1 clearance and degree of fever, were not significantly different for the celgosivir and placebo groups. Further, diarrhea, an early clinical feature of dengue, was more frequent in the celgosivir group. A phase Ib/IIa trial of celgosivir is planned. This trial will use reduced celgosivir dosage, administered at shorter intervals. Another putative dengue drug, modipafant (inhibitor of platelet activation factor), will also be tested in this trial (NCT02569827, Table 1.7, page 20).

The hydrochloride salt of N-9-methoxynonyl-DNJ, known as UV-4B, was identified as a drug which could protect AG129 mice from lethal DENV challenge. DENV challenge of UV-4B treated mice resulted in lower viremia, reduced tissue virus load and decreased

inflammatory cytokine production (*Perry et al, 2013*). Nextgen sequencing has revealed that emergence of UV-4B resistant DENV mutations is unlikely (*Plummer et al, 2015*). A clinical trial designed to test the safety and tolerability of UV-4B in healthy volunteers is currently in progress (NCT02061358, Table 1.7, page 20).

1.6.6 Traditional medicine

Many of the dengue-endemic nations in the developing world rely on traditional medicine for treatment. This, coupled to the absence of a robust pipeline of promising drug candidates, either from new drug discovery or re-purposing initiatives, has led some Asian countries to commence exploration of indigenous traditional medicine for dengue therapy (*Tang et al, 2012; Lee et al, 2013; Sarala and Panikar, 2014; Sood et al, 2015*). Recently, an Indian group has found that *Cissampleos pareira* extracts not only possess antiviral inhibitory potency against all four DENV serotypes *in vitro*, but also afford significant protection *in vivo* against DENV-2 challenge in the AG129 mouse model (*Sood et al, 2015*). India, Sri Lanka and China have embarked upon clinical trials of various herbal formulations (Table 1.8, page 21). Neither the active ingredient nor the target of action is known for any of these herbal formulations. Except for the Sri Lankan trial, which in an interim report disclosed some evidence of clinical benefit, no data is yet available from the other trials.

1.7 Summary of the current Dengue situation

Dengue disease which poses a public health risk to ~3.6 billion of the world population (*Gubler, 2012*) in >100 tropical and sub-tropical countries is regarded by the WHO as the fastest spreading arntropod-borne illness of the century (*WHO, 2012*). Multiple factors that include population explosion, unplanned urbanization, increased global travel, climatic changes, poor public health infrastructure and the failure to implement sustained vector control measures, have all contributed to the emergence of dengue as a public health problem of global peoportions (*Guzman & Harris, 2015*).

In recent years there has been an increased awareness of the magnitude of the public health risk posed by dengue. This in turn has spurred significant efforts geared towards developing tools to combat dengue. As each of the four DENVs can cause the full spectrum of the disease, any therapeutic or prophylactic has to be effective against all four DENV serotypes. Herein lies the challenge of tackling dengue illness.

Until recently, there has been an emphasis on developing a preventive dengue vaccine. Vaccine development has turned out to be very complex and challenging due to the fact that antibodies are involved both in protection against, as well as pathogenesis of, dengue disease. Recent phase III trial data have revealed that the CYD-TDV vaccine being developed by Sanofi Pasteur is not effective against DENV-2 (*Capeding et al, 2014; Villar et al, 2015*). As mentioned earlier, the enormity of the dengue problem has compelled some countries to approve the use of CYD-TDV despite its sub-optimal efficacy in the last few months.

The unexpected results of the CYD-TDV trials have led to two outcomes. One, it has accelerated interest in developing alternate vaccine candidates, many of which have entered clinical trials, and two, it has kindled interest in fast-tracking drug discovery and development efforts. However, resources invested in dengue drug discovery so far have been modest. Drug discovery efforts have not as of date identified a potentially promising antiviral drug candidate(s) against dengue that could progress to the clinical development phase. The

last few years have witnessed a shift in strategy that has focused on re-purposing existing drugs for their possible efficacy against dengue. Data from a limited number of clinical trials thus far have not been encouraging. An effective dengue drug is currently not available.

1.7.1 Gaps in existing knowledge in the context of developing dengue drugs

In general, antiviral drugs are designed to target an essential viral factor or an essential host factor required for successful completion of the viral life cycle. In the context of dengue, drug discovery efforts are predominantly focused on targeting DENV E, NS3 and NS5 proteins, based on an understanding of the roles of these viral proteins. Not much is known regarding other viral factors that could serve as targets. It is anticipated that when the roles of the other non-structural proteins become understood in greater detail, more potential viral targets would emerge. Likewise, in regard to host factors, not much is known apart from the role of ER α-glucosidases in late maturation phase of the viral life cycle.

Most dengue discovery efforts described in the literature have analysed the antiviral effect of an experimental drug, regardless of whether it targets a viral or host factor, on one DENV serotype alone. The assumption underlying such studies is that an inhibitor against one DENV would be inhibitory towards other DENVs as well. Thus, the feasibility of developing an antiviral drug that is pan-DENV inhibitory is unknown. As DENV replication relies on an error-prone RdRp enzyme, the possibility that an antiviral drug can lead to the emergence of drug resistance cannot be ignored. The most obvious means of raising the barrier to the emergence of resistance is to use a combination of drugs. Thus, the feasibility of developing multiple pan-DENV drugs is an area that needs to be explored.

Despite the availability of RNAi technology, its utility as an antiviral strategy, though proven for other viruses (*Castonotto & Rossi, 2009*), has not been explored in the case of DENV. As DENV genomic RNA doubles as a template for translation as well as replication, it may serve as a potentially useful viral target for antiviral strategies. Further, in combination other antiviral drugs, RNAi may offer an effective means of overcoming issues of possible emergence of resistance.

This work seeks to address some of these gaps in the context of dengue drug development. Thus, this thesis describes efforts to identify more than one pan-DENV small molecule inhibitor as well as investigate the use of RNAi in curtailing the replication of all four DENV serotypes.

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CHAPTER 2: AIMS & OBJECTIVES

2.1 Scope

Dengue disease has emerged as a significant public health problem in recent decades. Four distinct serotypes of dengue viruses (DENV-1, -2, -3 and -4), spread to humans by mosquitoes, cause dengue disease. The full spectrum of the disease can be caused by any of the four DENVs. Thus, a preventive vaccine or a therapeutic drug must be capable of acting against all four DENV serotypes. Efforts to prevent dengue infection with a vaccine have recently met with very limited and uncertain success despite decades of effort. The realization that an effective preventive vaccine is still an elusive goal has prompted efforts to explore the feasibility of dengue drug development. Developing drugs has become all the more relevant as diagnostic tests for early detection of dengue infection have become available recently. As on date there is no approved drug to treat dengue patients. Though several inhibitors have been reported in the literature, most of them have been tested against just one DENV serotype. The initial overall objective of the work presented in this thesis was to explore the feasibility of identifying inhibitors of the DENV protease, which is critical for the successful completion of the viral life cycles, and assess their potential to serve as pan-DENV drugs capable of inhibiting all four DENV serotypes. To accomplish this, the following specific aims were identified.

2.2 Specific Aims

- Expression and purification of cloned DENV-2 NS2b-NS3protease enzyme.
- Setting up of an enzymatic assay to measure the activity of the purified DENV-2 protease.
- Screening of a small molecule library for potential DENV-2 protease inhibitors using the above assay.
- Analysis of chosen hits in a cell-based assay against: (a) DENV-2; & (b) the remaining DENV serotypes (DENV-1, -3 & -4).

Accomplishment of the above specific aims identified pan-DENV inhibitors but also revealed the persistence of residual viral RNA in the presence of these inhibitors. It was felt that in addition to the protease, the DENV RNA itself could be targeted to achieve more effective inhibition. Thus, the feasibility of deploying RNAi to target DENV RNA was investigated. This entailed addressing additional aims as listed below.

2.3 Additional specific aims

- Identification of putative siRNA target site(s), in the coding region of the DENV genome, conserved among all four serotypes.
- Construction of adenovirus type 5 (Ad5)-based recombinant siRNA-delivery vector(s).
- Characterization of the recombinant adenoviral (rAd) vectors.
- Evaluation of the ability of the rAd-delivered siRNA in inhibiting all four DENVs in cultured cells.

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CHAPTER 3: MATERIALS & METHODS

3.1 Host cells

3.1.1 Bacterial hosts

E. coli strains DH5α for routine cloning work, and BL21 (DE3) for recombinant protein expression were purchased from Invitrogen Life Technologies, Carlsbad, USA. *E. coli* strain BJ5183 for recombinant adenoviral genome assembly by homologous recombination was a kind gift from Dr. Bert Vogelstein, Johns Hopkins University, MD, USA.

3.1.2 Mammalian cell lines

The monkey kidney cell line Vero for DENV propagation and experimentation, and the human embryonic kidney cell line HEK 293, for rAd creation and propagation, were obtained from American Type Culture Collection (ATCC), VA, USA. Both cells lines were propagated in Dulbecco's Modified Eagle Medium (DMEM) + 10% heat-inactivated fetal bovine serum (ΔFBS) containing gentamicin and fungizone (each at 50µg/ml), in a 10% CO_2 incubator maintained at 37°C. Tissue culture media, FBS and antibiotic supplements were procured from Invitrogen Life Technologies.

MTT [3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide] assay kit, to assess cytotoxicity of drugs and viruses on cell viability, was also purchased from Invitrogen Life Technologies.

3.1.3 Viruses

Dengue viruses serotypes DENV-1, DENV-2, DENV-3 and DENV-4 were from the supervisor's former laboratory at the International Centre for Genetic Engineering & Biotechnology (ICGEB), New Delhi, transferred to the Virology laboratory at BITS Pilani, Hyderabad campus through formal Material Transfer Agreement (MTA). These viruses were originally provided by Dr. Andrew Falconar, University of Oxford, UK [DENV-1 NI (U88535.1); DENV-2 NGC (AF038403); DENV-3 H87 (M93130); DENV-4 Dom (M1493.2)]. Wild-type adenovirus and the rAd-sh5b vector were obtained from ICGEB through formal MTA process.

3.2 Plasmid vectors

3.2.1 DENV-2 NS2b-NS3Pro expression plasmid

Plasmid pET-NS2b-NS3Pro containing a synthetic DENV-2 protease encoding gene under the control of an isopropyl thiogalactoside (IPTG)-inducible prokaryotic promoter was obtained from ICGEB, New Delhi. This plasmid has a kanamycin selection marker.

3.2.2 Plasmids for RNAi work

Plasmid pLKO.1 TRC vector (*Moffat et al, 2006*) for human U6 promoter driven shRNA expression was from the non-profit plasmid repository, Addgene, MA, USA. Plasmids for rAd construction, pShuttle (a promoter-less vector which provides essential adenoviral *cis*-acting sequence elements and has a kanamycin resistance marker) and

pAdEasy-1 (a plasmid which provides the major portion of the adenovirus type 5 genome, lacking early region 1 sequences, and the ampicillin selection marker) were from Dr. Bert Vogelstein, Johns Hopkins University, MD, USA. The latter two plasmids contain overlapping sequences to permit homologous recombination (*He et al, 1998*).

3.3 Reagents for molecular biology work

3.3.1 Restriction endonucleases & DNA modifying enzymes

Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase and alkaline phosphatase used in routine cloning work were purchased from MBI Fermentas (Thermo Fisher Scientific, MA, USA).

3.3.2 Kits for nucleic acid isolation, reverse transcription and real-time PCR

Plasmid DNA isolation (QIAprep Spin miniprep kit) and gel extraction kits (MinElute kit) were purchased from Qiagen, Hilden, Germany. QIAmp viral RNA minikit for viral genomic RNA isolation from DENV-infected culture supernatants, was also from Qiagen. Verso cDNA synthesis kit for reverse transcription of DENV genomic RNA was from Thermo Scientific. KAPA SYBR FAST qPCR kit, for real time quantification of reverse-transcribed DENV RNA, was procured from KAPA Biosystems, Inc., MA, USA.

3.4 Reagents for protein work

3.4.1 Recombinant NS2b-NS3Pro purification and characterization

Ni^{2+}-NTA Sepahrose superflow chromatographic resin was obtained from Qiagen, Hilden, Germany. Nitrocellulose membrane for Western blotting was procured from Invitrogen Life Science Technologies, Carlsbad, USA. Protein size markers (both unstained and pre-stained) were from Puregene. Bradford reagent for protein estimation was purchased from Biorad Inc., USA.

3.4.2 NS2b-NS3Pro substrate

The peptide, benzoyl-Nle-Lys-Arg-Arg-4-methyl coumarin-7-amide (Bz-nKRR-MCA) was custom synthesized by Peptides International, Louisville, KY, USA.

3.4.3 Small molecule compound library

The ‘in house’ small molecule library containing ~3,000 compounds belonging to several categories such as benzimidazoles, benzothiazoles, quinolines, acridines, thiazoles, thiazolidines, azetidines and spiropiperidones, was a kind gift from Dr. Sriram, Department of Pharmacy, BITS Pilani-Hyderabad Campus.

3.4.4 Detection of DENV NS1 antigen

NS1 ELISA kit for the determination of DENV NS1 antigen secreted into culture supernatants of DENV-infected cells was provided by J. Mitra & Co. Pvt. Ltd., New Delhi.

3.5 *In silico* resources

Molecular docking was done using Schrodinger software (Lingprep programme, Glide XP algorithm, Desmond programme) available in the Pharmacy Department, BITS Pilani, Hyderabad Campus. Statistical analysis was done using Graphpad Prism v5.

3.6 General miscellaneous consumables

3.6.1 Primers

Oligonucleotide primers for PCR, reverse transcription, and sequencing (Table 3.1) were custom synthesized By Sigma-Aldrich, Bangalore. India.

Primer	Sequence(5'-3')	T_m°C
D1	TCAATATGCTGAAACGCGCGAGAAACCG	78.3
D2	TTGCACCAACAGTCAATGTGTTCAGGTTC	73.7
pLKO-For	ACAAGGCTGTTAGAGAGATAATTGG	62.5
pLKO-Rev	TCTCTGCTGTCCCTGTAATAAACCC	67
Ad5E1-For	CATATTATCTGCCACGGAGG	61.9
Ad5E1-Rev	AATGACAAGACCTGCAACCG	65

Table 3.1: List of primers used in this study

3.6.2 Primary and secondary antibody-enzyme conjugate and substrate

Penta-His monoclonal antibody (mAb) for Western blotting was from Qiagen. Hybridomas HB-112 and HB-114, for the preparation of mAbs 4G2 and 2H2, respectively, were obtained from ATCC, VA, USA. Goat anti-mouse IgG-horse radish peroxidase (HRP) conjugate was obtained from Calbiochem, CA, USA. True Blue HRP substrate was from Kirkegaard & Perry Laboratories Inc., MD, USA.

3.6.3 Others

DNA size markers (1kb, 1Kb plus, 100bp DNA ladders), low molecular weight protein markers and protein pre-stained markers for SDS-PAGE gels were procured from Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA. Methyl cellulose, Isopropyl thiogalactoside (IPTG), membranes and other general lab chemicals are procured from EMD Millipore., Billerica, USA.

3.7 Expression, purification and characterization of DENV-2 NS2b-NS3Pro

3.7.1 Preparation of competent *E. coli* BL21 (DE3)

Competent cells were prepared using $CaCl_2$ method. An isolated well-separated colony of *E. coli* BL21 (DE3) cells from a freshly streaked LB plate was inoculated into 3 ml LB medium and incubated in a shaker overnight (37°C, 200rpm). Using this overnight culture, a shake-flask culture was inoculated (1% inoculum, 50 ml) and allowed to grow in the shaking

incubator for ~1-2 hours. The culture was harvested in log-phase (OD_{600}=~0.6), chilled on ice for 15 minutes and pelleted in a centrifuge (2,000 rpm, 10 minutes, 4ºC). The supernatant was discarded and the cells rinsed twice in ice-cold 100mM $CaCl_2$ solution, re-suspended in 0.5ml $CaCl_2$ and let sit on ice overnight.

3.7.2 Transformation of *E. coli* BL21 (DE3)

To 100µl competent cells prepared above, taken in a sterile Eppendorf tube, purified plasmid pET-NS2b-NS3Pro (3-5ng) was added. After incubating this on ice for 30 minutes, the cells were subjected to a heat-shock (42ºC, 90 seconds) and chilled immediately for 5 minutes on ice. Next 0.9 ml antibiotic-free LB medium was added to the tube and the cells placed in a shaking incubator (37ºC, 200rpm) for ~45 minutes to allow revival. A suitable aliquot of the transformed cells were plated on LB + kanamycin (25µg/ml) and incubated overnight (37ºC, 16-18 hours). A master plate was prepared from the resulting colonies.

3.7.3 Expression-screening of transformants

Several transformant clones from the master plate were grown in 3ml test tube cultures (LB + 25µg/ml kanamycin) and induced with 0.5mM IPTG at log-phase. After ~3-4 hours of induction, equivalent numbers of cells (normalized based on OD_{600} readings) from the cultures were pellet down by low-speed centrifugation and lysed by boiling in SDS-PAGE loading buffer. Appropriate aliquots of the resultant lysates (IPTG-induced) were analyzed by SDS-PAGE along with aliquots of un-induced lysates, prepared in parallel. The gels were stained with Coomassie blue to visualize the induced protein band. Based on visual inspection of induced band intensity, the best-expressing clone was taken for further work. This clone, retrieved from the master plate, was used to optimize the following induction parameters: concentration of IPTG, induction duration and induction temperature. All optimization experiments were done using small-scale test tube cultures. Based on this, the following induction conditions were chosen: 0.25mM IPTG, 18 hours at 18ºC. These conditions were chosen to facilitate recovery of recombinant NS2b-NS3Pro from the soluble fraction of the induced cell lysates under native conditions.

3.7.4 Purification of NS2b-NS3Pro from induced cells

A pre-culture of *E. coli* BL21 (DE3) harboring the pET-NS2b-NS3Pro plasmid grown in a shaker at 18ºC, 200rpm overnight (~12-14 hours) was inoculated into a 1 liter (L) LB medium (containing 25µg kanamycin/ml) in a 4L Haffkine flask and allowed to grow at 18ºC at 120 rpm until it reached log phase (OD_{600}=0.6). At this point, the culture was induced with 0.25mM IPTG and allowed to grow for a further 2 hours. Induced culture was spun down in a Sorvall centrifuge (GS3 rotor, 5000 rpm, 4ºC, 20 minutes). The resulting induced cell pellet was rinsed with 100 ml 0.9% NaCl and re-suspended gently and thoroughly in ice-cold 50 ml lysis buffer [50mM HEPES, pH 7.5/300mM NaCl/5% (v/v) glycerol] and allowed to chill on ice for 30 minutes. The chilled cell suspension placed in an ice-bath was sonicated for 3 minutes (10 second bursts with 10 seconds off-time) in a Vibra-Cell sonicator, and centrifuged in a Sorvall centrifuge at high speed (SS34 rotor, 17000 rpm, 4ºC, 30 minutes). The supernatant was mixed with 5ml of Ni^{2+}-NTA resin pre-equilibrated with 5 volumes of lysis buffer for batch binding which was allowed to proceed with gentle stirring for 30 minutes at 4ºC. Next, the resin+sample slurry was packed into a Sigma chromatographic column (1cm x 20cm, ~16ml) and washed successively with 10 volumes lysis buffer and 5 volumes Buffer A (Lysis buffer containing 20mM imidazole). This was followed by elution

using 5 volumes of Buffer B (Lysis buffer containing 100mM imidazole). Fractions of 5ml volume were collected and analyzed by SDS-PAGE. Peak fractions were pooled and dialyzed [against 50mM HEPES, pH7.5/300mM NaCl/5% (vol/vol) glycerol] to remove imidazole, passed through 0.22µM filter, aliquotted, quick-frozen and stored at -80°C. Typically, this resulted in yields in the range of 12-15mg purified NS2b-NS3Pro protein/L starting culture.

3.7.5 Western blot analysis

The Ni^{2+}-NTA affinity-purified recombinant DENV-2 NS2b-NS3Pro was run on SDS-15% PAGE along with pre-stained protein markers on an adjacent lane and electro-transferred to nitrocellulose membrane. The membrane was blocked overnight with 1% polyvinyl pyrrolidone containing 0.2% horse serum, and then incubated with primary antibody (anti-His mAb at 1:7,500 dilution) for 45 minutes at RT. The membrane was washed three times with 1x PBS containing 0.1% Tween 20 (PBS-T), and then incubated in anti-mouse IgG-alkaline phosphatase conjugate solution at a dilution of 1:5,000 for 45 minutes at room temperature (RT). The membrane was washed as above and the protein band visualized by incubating in substrate (5-bromo-4-chloro-3-indolyl phosphate plus nitroblue tetrazolium) for 15 minutes at RT.

3.8 Design of high throughput enzyme and inhibitor assay

3.8.1 Protease assay

Protease assays were carried out in 100µl volume in microtiter wells of 96-well plates. A typical protease reaction (100µl) contained 5nM purified DENV-2 rNS2b-NS3Pro enzyme (15ng protein) in assay buffer (50mM Tris-HCl, pH 8.5/1mM CHAPS/20% glycerol). The reaction was initiated by the addition of peptide substrate Bz-nKRR-AMC (10mM stock) to a final concentration of 10µM. The reaction was incubated at 37°C for 20 minutes. Protease activity was measured in terms of the increase in fluorescence that accompanied cleavage of the peptide substrate (λ_{ex}: 380nm; λ_{em}: 450nm), using a Perkin-Elmer Victor™ X-Multilabel plate reader. Control reactions in which the protease was omitted were run in parallel to correct for background fluorescence of the substrate.

3.8.2 Protease inhibitor screening assay

To measure protease inhibition, the test compound was incorporated into the protease reaction prior to substrate addition. Enzyme control (EC) reactions set up in parallel contained an equivalent amount of the vehicle (DMSO) without any inhibitor. Half maximal inhibitory concentration (IC_{50}) was defined as the inhibitor (test compound) concentration that decreased protease activity by 50%, with reference to the EC reaction (which was taken as 100%), under the experimental conditions. All assays were run in duplicates or triplicates and each experiment was performed at least twice independently.

3.9 Preparation and titration of DENV stocks

3.9.1 Preparation of DENV stocks

Vero cells were seeded in T-75 flasks supplemented with 10ml DMEM + 10% ΔFBS and incubated at 37°C/10% CO_2. When cells were ~80% confluent (24-36 hours post-seeding), DENV infection was set up as follows. Media was aspirated from each flask. To the monolayer, added 2ml of DENV sample (diluted in DMEM + 2% ΔFBS) and returned to the

incubator for 1.5-2 hours. Infections were set up with each of the four DENV serotypes. Two T-75 flasks were infected per DENV. After 2 hours, each flask was supplemented with 13ml DMEM + 10% ΔFBS and returned to the incubator for 7 days. At the end of this period, the infected culture supernatants were harvested and supplemented with ΔFCS to a final concentration of 20%. This was clarified, aliquoted and stored at -80ºC.

3.9.2 Plaque assay for determination of DENV titres

The DENV titres in the stocks prepared above were determined by plaque assay as follows. Vero cells were seeded in 6-well plates at 5×10^5cells/well in 2ml DMEM+10%ΔFBS and placed in the incubator (37ºC/10% CO_2). When the monolayer was near full confluency, cells were infected with serial dilutions (in DMEM+2%ΔFBS) of the DENV stocks prepared above. For each dilution two wells were infected (250µl/well). The plates were returned to the incubator for 2 hours. Following this, the inoculum was aspirated off and the monolayer overlaid with 1% methylcellulose suspension + 6% ΔFBS (2ml/well). The plates were returned to the incubator for 3 days. At the end of this period the methylcellulose overlay was gently discarded into bleach and the monolayer rinsed twice with 1x PBS (2ml/well, each rinse). The monolayers were next fixed with 80% methanol for 10 minutes at RT. The methanol was removed completely and the wells were blocked by incubating with 5% SMPBS (skimmed milk in 1xPBS, 2ml/well) at RT for 10 minutes. After removal of the blocking solution, the wells were treated with 0.5ml of 4G2 mAb solution (diluted 1:1,000 in 2.5% SMPBS) and incubated for 1 hour at 37ºC. After this incubation, the wells were washed with 2.5% SMPBS and then incubated with 0.5ml of goat anti-mouse IgG-HRP conjugate (diluted 1:3,000 in 2.5% SMPBS). Following a further hour of incubation at 37ºC, the wells were washed with 1x PBS twice, dried partially and treated with 0.5 ml True Blue peroxidase substrate and incubated for 1-5 minutes at RT with gentle shaking. As soon as plaques appeared, the substrate was discarded and plaques counted manually. Titers were calculated as plaque-forming units (PFUs)/ml based on the dilution factor and inoculum size used for infection in the beginning.

Virus	Titre (PFU/ml)
DENV-1	5.4×10^6
DENV-2	2.8×10^6
DENV-3	1.1×10^6
DENV-4	1.9×10^7

Table 3.2: DENV stocks prepared for this study

3.10 Creation of rAds for mediating RNAi

3.10.1 Design of U6 promoter-driven shRNA expression cassettes

Double-stranded 58-mer oligonucleotides encoding shRNAs targeting each of the selected sites (sh5c, sh5a & sh3c) on the DENV genomic RNA were inserted in place of the 1.9 Kb stuffer sequence of pLKO.1 TRC vector (*Moffat et al, 2006*), between the unique *Age* I and *Eco* RI sites, under the transcriptional control of the human U6 promoter as described earlier (*Korrapati et al, 2012*). The shRNA-encoding inserts in all three constructs, pLKO-sh5c, pLKO-sh5a and pLKO-sh3c, were verified by sequence analysis.

3.10.2 Construction of rAd vectors encoding shRNAs

The human U6 promoter-driven shRNA expression cassettes (ECs) of the three plasmids above, were retrieved as *Not* I-*Bam* HI fragments, and inserted into the pShuttle vector between *Not* I and *Bgl* II sites, to create three new plasmids: pShuttle-sh5c, pShuttle-sh5a and pShuttle-sh3c. This maneuver placed the shRNA EC downstream of essential *cis*-acting Adenoviral sequence elements, namely the Left-Inverted Terminal Repeat (L-ITR) plus the encapsidation signal (Ψ), provided by the pShuttle vector. Next, each of the shRNA EC together with upstream Adenoviral *cis*-acting sequence elements, was inserted into the E1 region of the Adenoviral genome of plasmid pAdEasy-1 by *in vivo* recombination in *E. coli* BJ5183 (*He et al, 1998*). This resulted in three recombinant adenoviral plasmids: pAdEasy-sh5c, pAdEasy-sh5a and pAdEasy-sh3c.

The recombinant adenoviral plasmids above were digested separately with *Pac* I to eliminate plasmid sequences and transfected into HEK 293 cells using lipofectamine 2000. The transfected HEK 293 cells manifested characteristic cytopathicity at the end of a week. These were harvested, lysed by three rounds of alternate freeze/thaw cycles and inoculated onto fresh HEK monolayers to rescue the shRNA-encoding rAd viruses. Three rAds, one harboring the sh5c EC (rAd-sh5c), the next one harboring the sh5a EC (rAd-sh5a) and the last one harboring sh3c (rAd-sh3c) were created for this study.

3.10.3 Preparation of rAd viral genomic DNAs

Viral DNA corresponding to the three rAds was isolated using a slight modification of the classic Hirt extraction procedure (*Hirt, 1967*). Briefly, HEK 293 cells were seeded in T-75 flasks supplemented with DMEM + 10% FBS (10ml/flask) at a seeding density to achieve ~80% confluency in 24-36 hours, and placed in a 37ºC incubator fed with 10% CO_2. Prior to infection, the growth medium was aspirated out and replaced with DMEM + 5% FCS (5ml/flask). To this a small volume of rAd inoculum was added and the infected cells returned to the incubator. Cells in T-25 flask were infected separately with each of the rAds described above. Dilution of the rAd was optimized in a previous pilot experiment to result in >80% cytopathic effect (CPE) in 3-4 days. At this stage, infected cells were dislodged by tapping the flasks and harvested by low speed centrifugation (2,500 rpm, 5 minutes) at RT. The cell pellets were rinsed once with 1 ml 1x PBS, re-suspended in 400µl 1x PBS, mixed with 10µl RNAse A (10mg/ml stock) and incubated at 37ºC for 1 hour. This was followed by the addition of an equal volume of 2x Hirt lysis buffer (10mM Tris-HCl, pH 8.0/0.5M EDTA, pH 8.0/0.6% SDS) plus 10µl pronase enzyme (20mg/ml stock prepared in 1M Tris-HCl, pH 7.5/10mM NaCl), gentle mixing by inversion of the tube 3-4x and incubation for a further 1 hour at 37ºC. Next, the entire contents were mixed gently with 200µl 5M NaCl, and incubated overnight at 4ºC. The lysate was then centrifuged (13,000 rpm, 25 min, 4ºC) and the clear supernatant extracted gently with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1) and ethanol-precipitated with 2.5 volumes absolute ethanol. The resulting DNA precipitate was washed once with 1ml 70% ethanol, air-dried and re-suspended in 20µl 1xTE buffer.

3.10.4 Genomic analysis of rAd viral DNAs

The rAd viral DNAs were analyzed on a 0.6% agarose gel to estimate their concentrations. This was followed by digesting the viral genomic DNA using different restriction enzymes using ~2µg rAd genomic DNA/20µl reaction in 1x potassium glutamate

buffer (100mM potassium glutamate, 25mM Tris-acetate, pH 7.6, *McClelland et al, 1988*) and 0.8% agarose gel analysis of the resulting band patterns. The presence of the shRNA-encoding insert and the absence of Adenoviral E1 region in the rAd genomic DNAs were verified by PCR using insert-specific primers (pLKO-For and pLKO-Rev primers) and E1 region-specific primers (AdE1-For and AdE1-Rev), respectively. PCR reactions were performed in 25µl final reaction volume; PCR parameters were: initial denaturation at 95ºC/5 minutes, followed by 30 cycles of denaturation at 95ºC/45 seconds, annealing at 50ºC/45 seconds & extension at 72ºC/1 minute, followed by a final extension at 72ºC/7 minutes. PCR products (10µl) were analyzed on a 2% agarose gel. The identity of the amplified PCR product was confirmed by *Xho* I digestion (based on the introduction of the *Xho* I recognition sequence in the loop part the encoded shRNA).

3.10.5 Preparation of rAd stocks

For each rAd, four T-75 flasks of HEK 293 cells were infected, essentially as described above. Apart from the 3 rAds created for this work, two additional rAds, rAd-sh5b and rAd-shscr, reported earlier (*Korrapati et al, 2012*) were also included. Cells were monitored daily under the microscope and harvested when >80% CPE was evident. Cells plus media from all four T-75 flasks of a given rAd infection were collected into a single sterile 50ml tube. All 5 tubes, were subjected to 3 round of alternate freeze/thaw cycles and clarified by low speed centrifugation. The resulting rAd-containing culture supernatants were aliquoted and stored at -80ºC.

3.10.6 Determination of viral titres of rAd stocks

HEK 293 cells were seeded in a 96 well plate at 10,000 cells/well in 100µl DMEM + 5%FBS. Twenty-four hours later, the cells were infected with serial dilutions (ranging from 10^{-3} to 10^{-13}) of the rAd stocks (100µl/well). Eight replicate wells were infected for each dilution. One set of 8 wells were mock-infected and served as control. The plates were incubated at 37ºC/10%CO_2 incubator for several days. At day ~4/5 post-infection, ~50µl medium was added to each well. Wells were monitored microscopically for CPE on a daily basis starting from day 7/8 and were scored as either CPE-positive or CPE-negative. When two consecutive and consistent observations were made, either CPE^+ or CPE^-, for each well on the plate, these were recorded. Tissue Culture Infective Dose was computed as the virus dilution that resulted in 50% of the replicate wells manifesting CPE ($TCID_{50}$ titre) by cumulative averaging (*Burleson et al, 1992*). These titres were multiplied by 0.7 to convert the rAd titres to PFUs/ml (Table 3.3).

Virus	Titre (PFU/ml)
Ad5 wt	1.4×10^{14}
rAd-shscr	1.4×10^{8}
rAd-sh5b	0.8×10^{8}
rAd-sh5c	3.3×10^{8}
rAd-sh5a	7.8×10^{6}
rAd-sh3c	1.9×10^{8}

Table 3.3: Adenovirus stocks prepared for this study

3.11 Cell-based DENV inhibition assays

3.11.1 Determination of the effect of small molecule drugs on DENV

For testing the inhibitory effect of small drugs, cell-based assays were performed as follows. Vero cells were seeded in 48-well plates ($4x10^4$ cells/well in 0.5ml DME+10%ΔFBS) and incubated for 24 hours (37ºC, 10% CO_2). For testing drugs, monolayers were aspirated and treated with 0.5ml DME+0.5%ΔFBS containing 30µM final concentration of the test compound. After 1 hour incubation medium + test compound was removed and saved. The monolayer was infected with DENV (m.o.i =0.1; 200µl/well in DME + 0.5% ΔFBS). After 2 hours, the virus inoculum was removed and replaced with medium containing test compound. The plate was returned to the incubator. Drug concentration was maintained by the addition of 5µl stock compound (equivalent 30µM final concentration) solution into each well at the appropriate concentration, on days 3 and 5 post-infection. Aliquots of culture supernatant were withdrawn at indicated time points up to 7 days for estimation of NS1 antigen (by ELISA), DENV genomic RNA levels (by qRT-PCR) and infectious virus production (by plaque assay). Appropriate virus controls (VC) for each DENV serotype wherein the drug treatment was omitted were run in parallel. All infection experiments were done twice independently. Inhibition by a test compound was assessed with reference to VC which was taken to represent 100% infectivity.

3.11.2 Determination of the effect of rAd-mediated RNAi on DENV

For testing the effect of rAd-mediated RNAi on DENV replication, the experimental design was similar to that above with the following changes. Vero cells, seeded 24 hours earlier in 96-well plates ($2x10^4$ cells/well in 100µl DME+10%ΔFBS) were infected with different rAds at m.o.i. ranging from 5-20 PFU/cell (100µl/well) and placed in the incubator (37ºC, 10% CO_2). At 24 hours post-rAd infection the cells were infected with each DENV at m.o.i=0.025 (50µl/well) and returned to the incubator. Culture supernatants were collected on day 5 post-DENV infection for analysis of NS1 antigen, DENV genomic RNA and infectious virus titres, as above. These experiments which involved rAd infection first and DENV next have been designated as 'AD' experiments. In contrast, in some later experiments, the order of infections was reversed, with DENV infection first, followed 24 hour later with rAd infection. The rest of the details were exactly the same. These have designated as 'DA' mode experiments.

In all instances, all experiments were performed in triplicate wells and repeated at least twice independently. Aliquots of culture supernatants (20µl/well) for NS1 ELISA were frozen until analysis as such. On the other hand, culture supernatants for viral RNA analysis and plaque assay (~100µl/well) were supplemented with ΔFBS to a final concentration of 20% and stored in the freezer (-80ºC) to preserve the stability of the virus until later analysis.

3.12 Analysis of DENV inhibition in culture supernatants

3.12.1 Determination of DENV NS1 antigen levels

Culture supernatants collected at various time points (from the cell-based assays described above), which were stored frozen at -20ºC, were thawed and diluted appropriately (1:100 to 1:1000 in DME+0.5%ΔFBS). Suitable aliquots (50µl) of this were used to detect DENV NS1 antigen using a commercially available Dengue NS1 ELISA kit (J. Mitra & Co.,

India), as per the manufacturer's protocol. This kit uses N- and C-terminal domain-specific anti-NS1 antibodies to detect the NS1 antigen produced by all four DENV serotypes.

3.12.2 Real-time analysis of DENV genomic RNA

Suitable aliquots (50-100µl) of culture supernatants were made up to 140µl with DMEM+5% ΔFBS). Viral RNA was extracted from this using QIAmp Viral RNA kit as per the manufacturer's protocol. Viral RNA was eluted in two rounds in a final volume of 60µl supplemented with 1U/µl Ribolock RNase inhibitor and stored at -80ºC. For first strand cDNA synthesis 10µl viral RNA was reverse transcribed with consensus primer D2 (*Lanciotti et al, 1992*) in a final volume of 25µl (45ºC, 60 minutes incubation) using Verso cDNA kit. Appropriate dilutions of the cDNA reaction were subjected to a q-PCR reaction with primers D1 and D2 (*Lanciotti et al, 1992*) using 1x KAPA SYBR green master mix. Cycling conditions were: initial denaturation at 95ºC/5 minutes, followed by 30 cycles of denaturation at 95ºC/45 seconds, annealing at 50ºC/45 seconds & extension at 72ºC/1 minute, followed by a final extension at 72ºC/7 minutes. Specific amplification was confirmed by 2% agarose gel electrophoresis of the RT-PCR product at the end of the experiment.

3.12.3 Determination of infectious virus titres

Viral titres in the collected supernatants were determined by plaque assay as described above. Serial dilutions of the culture supernatants were analyzed in duplicate wells of a 24-well plate seeded with Vero cells a day before. The protocol followed was essentially similar to that described above for DENV stock titre determination (See Sec 3.9.2).

3.13 Miscellaneous methods

3.13.1 MTT assay

The effect of different drugs and different viruses (rAds + DENV infections) on Vero cell viability was evaluated using the MTT assay. Vero cells were seeded in a 96-well microtiter plate (5,000 cells in 200µl DMEM+5%Δ FBS). To test cytotoxicity of drugs, the cells were exposed to different test compounds at a range of concentrations (2-100µM) for four days at 37ºC in a 10% CO_2 incubator. Control wells received an equivalent amount of DMSO vehicle without the test compound. Cell viability was assessed based on the reduction of MTT assay read out using a commercial kit as per the manufacturer's instructions. In the case of experiments involving viruses, monolayers at the end of the experiment (day 5 post-DENV infection), after collection of culture supernatants for analysis, were checked for viability using MTT.

3.13.2 *In silico* studies

The 3D co-ordinates of the DENV-2 NS2b-NS3Pro were exported from Protein Data Bank (PDB) (www.rcsb.com) with PDB ID: 2FOM. The hydrogen bonds, bond orders and bond energies were optimized using (OPLS) 2005 force field to prepare the protein using Protein Prep Module using 500 cycles of SD and 5000 cycles of CG methods.

The structures of the ligands MB21 and BT24 were drawn in Chemdraw and exported in mol2 format. The drugs in their Mol2 formats were imported into the work space of Schrodinger and the individual molecules were prepared using the Lig Prep module.

Molecular docking of MB21 and BT24 onto the three-dimensional crystal structure of DENV-2 NS2b-NS3Pro was performed around the catalytic triad with X:-8.5621; Y:-11.3034; Z: 6.3229, given as the grid co-ordinates using GLIDE extra precision module (Glide v5.7, Schrodinger, LLC, New York, NY) as described (*Friesner et al, 2004*).The XP Glide Score was used to rank the best fit of the compounds with the target protein that includes the hydrogen bonds visualized in both 2D and 3D plots.

3.13.3 Statistical analysis

The statistical significance (of differences in viral replication between untreated and test compound-treated samples) was assessed using unpaired *t* test with Graphpad software. Differences were considered statistically significant and very significant when the probability levels (P) were <0.05 and <0.01, respectively.

***

CHAPTER 4: RESULTS

4.1 Overview of work plan

The main objective of the work was to evaluate DENV NS2b-NS3Pro and DENV genomic RNA as antiviral targets and identify ways and means of developing drugs/strategies to inhibit the replication of all four DENV serotypes. Essentially the work strategy comprised two arms, as depicted schematically in Figure 4.1 below: (i) the first arm focused on DENV-2 NS2b-NS3Pro as a putative antiviral target. To this end, the work involved the expression and purification of recombinant NS2b-NS3Pro enzyme of DENV-2, its characterization, and the setting-up and validation of a high throughput assay to screen an in-house library of ~3000 small molecular weight compounds. This resulted in the identification of two compounds: MB21, a benzimidazole derivative, during the first phase of screening, and, BT24, a quinoline derivative, in the second phase of screening; (ii) the second arm of the work focused on deploying the endogenous RNAi pathway against the DENV genomic RNA. This entailed the identification of conserved 21 nucleotide sequences which may serve as putative siRNA target sites. Only one such site could be identified within the single ORF of the DENV genome. This and two putative siRNA target sites previously identified in the DENV NTR were chosen for further work. Recombinant adenoviral vectors (rAd-sh5c, rAd-sh5a and rAd-sh3c), targeting these three putative siRNA target sites were created and tested for their ability to decrease the synthesis of the viral antigen NS1 using an ELISA approach. This resulted in the identification of rAd-sh5c as best choice for further analysis. Both arms converged in the final phase of the work, wherein, the two drugs, MB21 and BT24, and the rAd-sh5c vector, were systematically tested against each one of the four DENVs for their inhibitory potential. For this final phase, cell-based assays were established and standardized to evaluate reduction in three parameters as indicators of inhibitory efficacy: viral antigen synthesis, genomic viral RNA replication and infectious progeny virus production, using appropriate assays. The results of these experiments are presented in the following pages of this chapter.

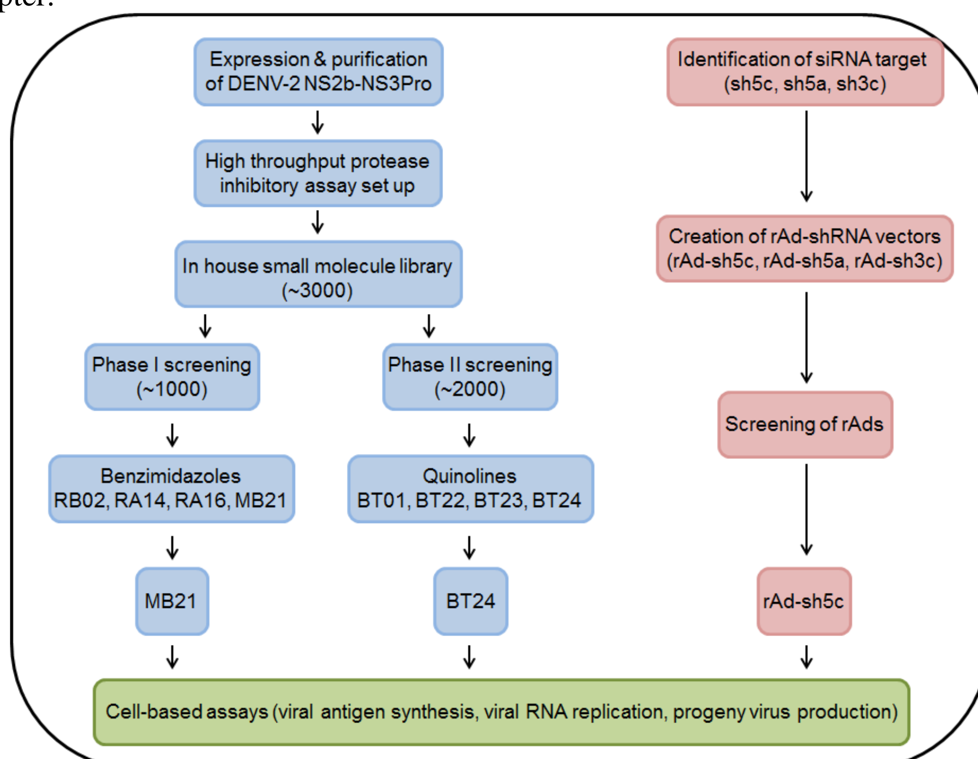

Figure 4.1: Overall strategy of the work carried out. *The two arms of the initial part of the work focusing on the viral protease and the viral genomic RNA, as outlined in the text above, are shown in blue and red, respectively. The final phase of cell-based assays, at which the two arms converge, is shown in green.*

4.2 Expression and purification of DENV-2 NS2b-NS3 protease

The plasmid construct used for bacterial expression of NS2b-NS3Pro of DENV-2 (NGC strain) is shown in Figure 4.2. This plasmid, pET-NS2b-3Pro, contains a synthetic *NS2b-NS3Pro* gene codon-optimized for *E. coli* expression, placed under the control of an IPTG-inducible promoter of the vector pET28b (Figure 4.2A). The synthetic DENV-2 *NS2b-NS3Pro* gene (Figure 4.2B) contains DNA sequences encoding the carboxy-terminal 48 amino acid (aa) residues of NS2b (to provide co-factor function), a flexible octapeptide linker, the amino-terminal 184 aa residues of NS3Pro and a 6x-His tag (to facilitate purification). This recombinant NS2b-NS3Pro enzyme encoded by this synthetic gene is 246 aa long with a predicted molecular weight of ~30kDa. This plasmid was introduced into *E. coli* BL21 (DE3) for NS2b-NS3Pro expression.

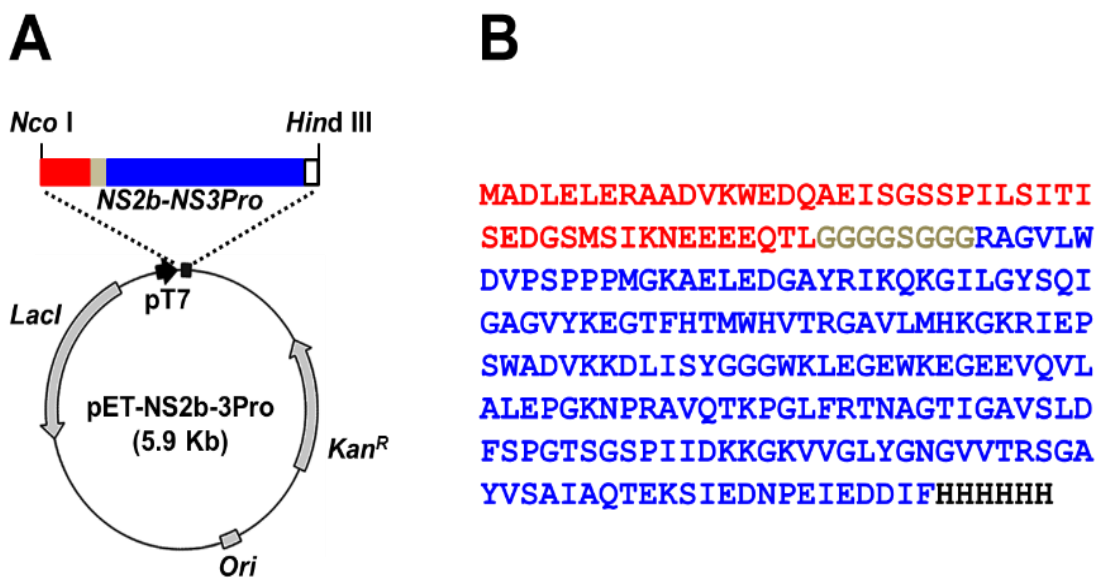

***Figure 4.2**: DENV-2 protease expression plasmid and predicted primary structure of rNS2b-NS3Pro enzyme.* *(A) Map of the rNS2b-NS3Pro E. coli expression plasmid. The salient features of this plasmid include: the phage T7 promoter (pT7), Lac repressor gene (Lac I), Kanamycin marker (Kan^R) and replication origin sequences (Ori). The synthetic NS2b-NS3Pro gene is inserted under the control of pT7. The chimeric gene consists of NS2b-derived sequences encoding the hydrophilic co-factor domain (red box) fused in frame to the protease-encoding domain of NS3 (blue box). These two are fused in frame through a non-cleavable peptide linker-encoding sequence (grey box). The white box at the 3' end denotes sequences encoding 6x His tag. (B) The amino acid sequence of the protein encoded by the rNS2b-NS3 gene is shown. The different sequence components are indicated using the same colours as shown in panel A.*

Expression of recombinant NS2b-NS3Pro was induced by the addition of IPTG at 37ºC. As a preliminary experiment showed that the recombinant protein was associated with the insoluble fraction of the induced cell lysate, induction was attempted at 30ºC. Figure 4.3A (page 39) shows an SDS-PAGE analysis of total native lysates prepared from un-induced (U) and 0.25mM IPTG-induced (I) cells. The data show induction of the recombinant protein, which displayed an electrophoretic mobility consistent with its predicted size of ~30kDa. However, when the induced sample was separated into soluble (supernatant, S) and insoluble (pellet, P) fractions and analysed by SDS-PAGE, the induced protein was once again

associated with the insoluble ‘P’ fraction. As next step, the induction temperature was lowered to 18°C and the induction performed overnight (0.25mM IPTG for 12 hours). A localization experiment performed using the induced cell lysate, shown in Figure 4.3B, revealed that a small proportion of the total induced protein was associated with the soluble supernatant (S) fraction, with the major proportion still associated with the insoluble ‘P’ fraction. All these optimization experiments were performed using test tube cultures.

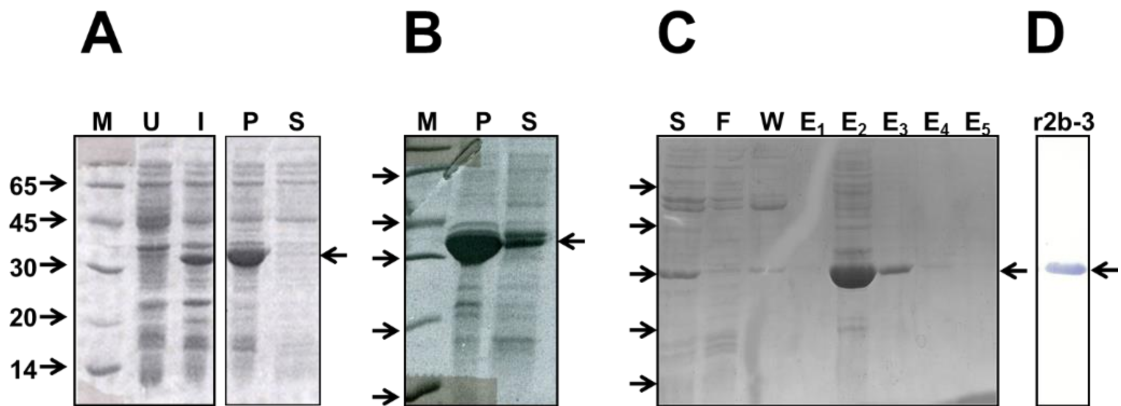

Figure 4.3: E. coli-expression of DENV-2 rNS2b-NS3Pro and its affinity purification. *(A) SDS-PAGE analysis of rNS2b-NS3Pro expression following IPTG induction at 30°C. This panel displays the Coomassie-stained polypeptide profiles of total extracts prepared from un-induced (U) and induced (I) E. coli cells harbouring the plasmid shown in Figure 4.2A (page 38). Lanes ‘P’ and ‘S’ display polypeptide profiles in the pellet and supernatant fractions, respectively, of induced cell extracts. (B) This panel shows polypeptide profiles obtained from P and S fractions of cells induced at 18°C. (C) Native Ni^{2+}-NTA affinity purification of NS2b-NS3 from E. coli induced at 18°C. The panel shows the SDS-PAGE polypeptide profiles at different stages of the purification, which are indicated by the following abbreviations above the lanes: S (supernatant fraction from induced cell lysate), F (flow-through), W (20mM imidazole wash), E_1 to E_5 (fractions eluted using 100mM imidazole). Positions of the protein markers are shown to the left of the panel. (D) Immunoblot analysis of pooled fractions (r2b-3) from panel C using penta-His mAb. Pre-stained protein size markers were run in lanes marked ‘M’ (panels A&B). Their sizes (in kDa) are indicated to the left (panels A-C). The arrow on the right of each panel indicates the position of the rNS2b-NS3 protein.*

Having optimized the conditions to obtain a fraction of the recombinant NS2b-NS3Pro under native conditions in the soluble fraction, a 1-litre shake-flask culture was set up and induced in mid-log phase with 0.25mM IPTG for 12 hours. Induced cells were pelleted down, rinsed once in 1x PBS buffer and re-suspended in native lysis buffer and subjected to sonication. The lysate was clarified by centrifugation and the clear supernatant was mixed with a slurry of Ni^{2+}-NTA-Sepharose beads, which had been pre-equilibrated in binding buffer and incubated for 30 minutes to allow binding of induced protein *via* the 6x-His tag engineered into its C-terminus. After washing away unbound protein with 20mM imidazole, the bound material was eluted using 100mM imidazole. SDS-PAGE analysis performed during Ni^{2+}-NTA affinity purification is shown in Figure 4.3C. As seen from this data, the bulk of the bound induced protein was eluted in fraction E_2. A Western blot of the purified protein using a 6x-His tag-specific monoclonal antibody identified the predicted ~30kDa protein (Figure 4.3D). As this purified protein displays an apparent size of ~30kDa on SDS-PAGE and possesses the engineered 6x-His tag it can be concluded that it is recombinant NS2b-NS3Pro. It was estimated that ~15mg purified recombinant NS2b-NS3Pro of ~90% purity (based on densitometric scanning) was obtained from one litre of induced culture.

4.2.1 Functional characterization of the purified NS2b-NS3Pro enzyme

The protease activity of the purified recombinant NS2b-NS3Pro was evaluated using a synthetic fluorogenic peptide substrate which has previously been shown to be specifically cleaved by proteases encoded by all four DENV serotypes (*Li et al, 2005*). The structure of this peptide substrate, benzoyl-Nle-Lys-Arg-Arg-4-methylcoumarin-7-amide (Bz-nKRR-AMC) is shown in Figure 4.4.

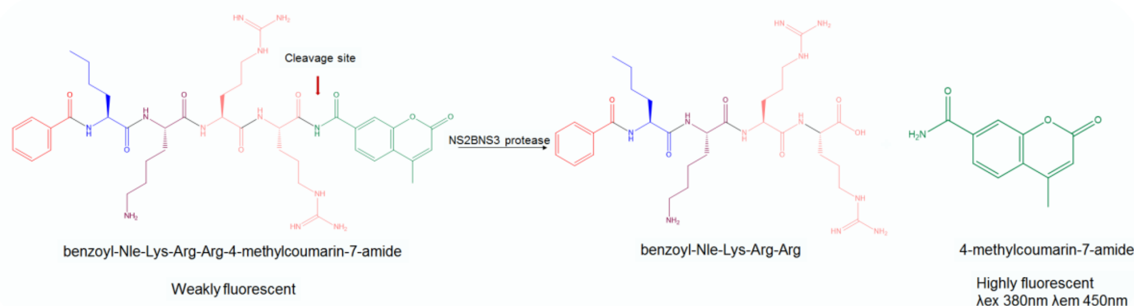

***Figure 4.4**: Enzymatic reaction catalysed by the recombinant protease*

DENV proteases mediate cleavage of the R-MCA amide bond with accompanying release of the fluorophore (4-methylcoumarin-7-amide). Incubation of the purified recombinant NS2b-NS3Pro with Bz-nKRR-AMC peptide was accompanied by an increase in fluorescence as a function of peptide substrate concentration (Figure 4.5A) as well as enzyme concentration (Figure 4.5B). These observations demonstrated that the recombinant DENV-2NS2b-NS3Pro is enzymatically active. Using these data, which identified enzyme and substrate concentration ranges compatible with a linear dose-response, protease assay conditions were optimized.

As a next step, the optimized NS2b-NS3Pro enzyme assay was validated as a prelude to initiating a DENV protease-inhibitor screening campaign. Aprotinin, a serine protease inhibitor has been reported in the literature to bind strongly to cloned DENV-2 protease (*Li et al, 2005*) and inhibit its catalytic activity potently at nanomolar concentrations (*Leung et al, 2001*). Testing a series of aprotinin concentrations on the catalytic activity of the purified recombinant DENV-2 NS2b-NS3Pro revealed that aprotinin could inhibit enzyme activity with an IC_{50} of 20nM, as shown in Figure 4.5C.

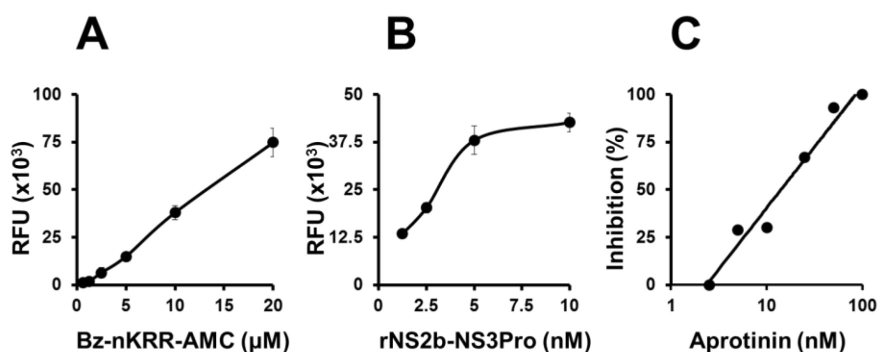

***Figure 4.5: DENV NS2b-NS3 enzyme assay optimization and validation**. (A) Kinetics of NS2b-NS3Pro action as a function of substrate concentration (at 5nM enzyme). (B) Rate of enzyme catalysis as a function of enzyme concentration (at 10 µM substrate). (C) Activity of cloned NS2b-NS3Pro as a function of aprotinin concentration (5nM enzyme, 10µM substrate, 20 min incubation). Activity in the absence of aprotinin is taken as 100% (RFU = Relative Fluorescence Units).*

4.3 Benzimidazoles inhibit DENV-2 NS2b-NS3Pro

As a next step, the functionally validated DENV-2 NS2b-NS3Pro enzyme assay described above was used to screen for putative NS2b-NS3Pro inhibitors from a small molecule library of ~1000 compounds. This 'in-house' small molecule library has been found to contain anti-microbial compounds before (*Jean Kumar et al, 2013; Poyraz et al, 2013*). In initial screening, these compounds were tested against the DENV-2 NS2b-NS3Pro at a single concentration of 25µM each. This screening effort identified 25 compounds with the capacity to inhibit the *in vitro* enzyme activity of DENV-2 NS2b-NS3Pro by >80%, compared to control enzyme reactions, performed in parallel in the absence of any added inhibitor. These 25 compounds were tested again for their inhibitory potency in two independent experiments before progressing further. The top four compounds in terms of their inhibitory efficacy were all benzimidazoles and are shown in Figure 4.6.

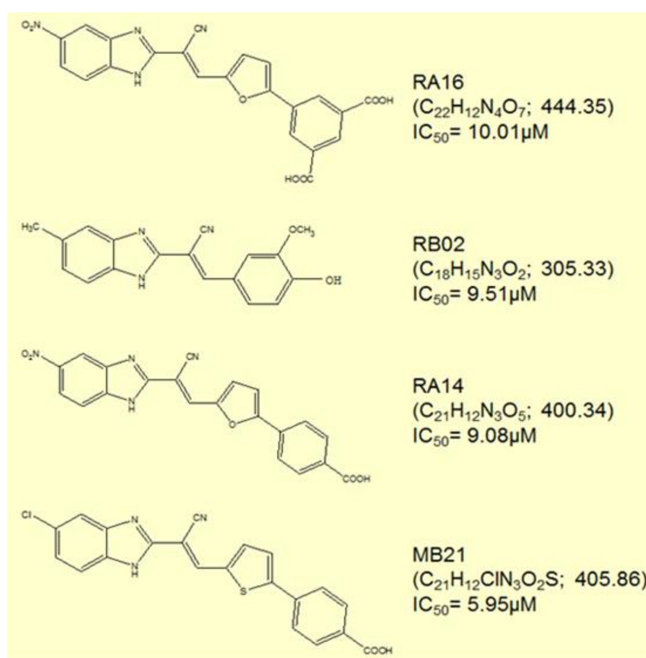

Figure 4.6: Small molecule inhibitors of cloned DENV-2 NS2b-NS3Pro. *Shown to the right of each molecule is the name of the compound, its molecular formula and weight (in parenthesis) and the DENV-2 protease inhibitory potential (IC_{50} value). MTT assay showed that at 30µM concentration RB02 was cytotoxic to Vero cells, while the remaining three were not. When these latter molecules were tested for antiviral activity against DENV-2 in infected Vero cells, RA16 was without inhibitory effect, presumably because it failed to enter the cells. RA14 and MB21 inhibited DENV-2 by ~30% and ~70% respectively.*

Among these, the most potent inhibitor was MB21, with an IC_{50} of 5.9µM (Figure 4.7A, page 42). The remaining benzimidazoles were RB02, RA14 and RA16, all of which manifested IC_{50} values closer to ~10 µM. These data suggested that the NS2b-NS3pro may preferentially bind to benzimidazoles. To investigate this further, *in silico* molecular docking of MB21 on the available crystal structure of NS2b-NS3Pro was performed. This study showed that MB21 embeds well into a hydrophobic cleft of an allosteric site on the protease molecule (*Mukhametov et al, 2014*), in the vicinity of the catalytic triad. The *in silico* docking results are presented in Figures 4.7B and 4.7C (page 42). The available data suggest that MB21 binds to an allosteric site on the protease and thereby manifests inhibition.

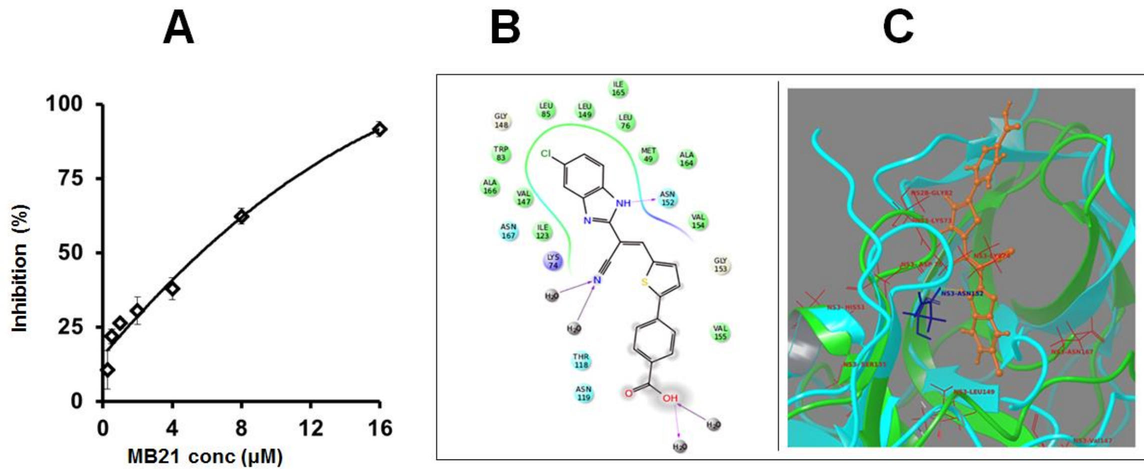

Figure 4.7: Inhibition of DENV-2 NS2b-NS3Pro by MB21 and in silico analysis of the interaction between the two. *(A) Inhibition of protease activity of cloned DENV-2 NS2b-NS3Pro as a function of MB21 concentration. (B) Computer generated 2D ligand interaction picture depicting the interaction between MB21 and DENV-2 NS2b-NS3Pro. Hydrophobic residues are shown in green. (C) Interaction of MB21 at the allosteric pocket in the vicinity of the catalytic triad.*

4.3.1 MB21 is a pan-DENV inhibitor

The question that needed to be addressed next was: will MB21, which inhibits the cloned DENV-2 NS2b-NS3Pro *in vitro*, be able to inhibit the replication of the parent virus in infected cells? Before this could be experimentally addressed, it was necessary to ascertain if MB21 was cytotoxic to Vero cells, the cell line to be used for DENV replication studies. To this end an MTT assay was performed (Figure 4.8A). Vero cells were exposed to different concentrations of MB21 (dissolved in 1% DMSO vehicle). Treatment of the cells with 1% DMSO alone resulted in ~30% loss in cell viability, compared to cells that were not exposed to any DMSO. Interestingly, the viability of cells treated with MB21 up to 100µM final concentration was essentially comparable to that observed with cells treated with the DMSO vehicle alone. These data lead to the conclusion that MB21 is essentially without any discernible cytotoxic effect on Vero cells.

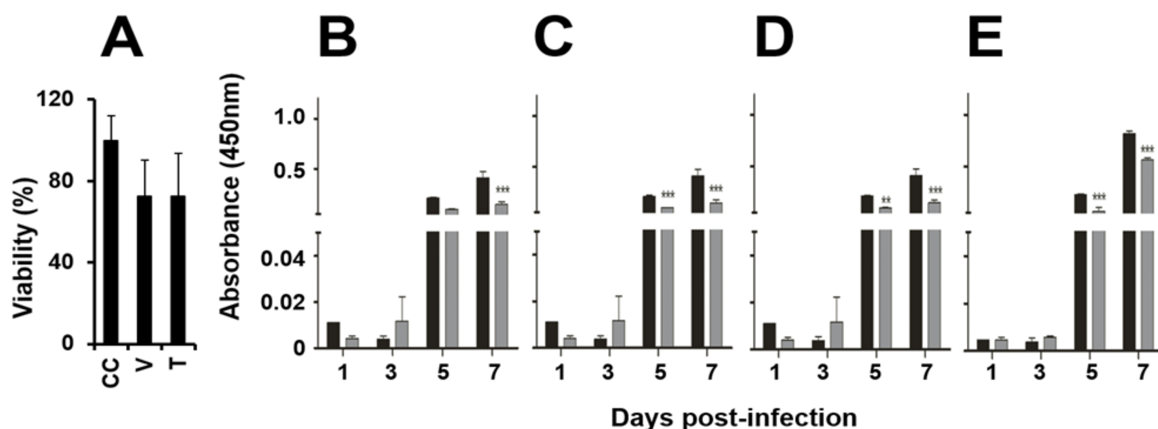

Figure 4.8: Evaluation of antiviral activity of MB21 using cell-based assay. *(**A**) Histogram showing the viability of Vero cells that received no drug ('CC', cell control), 1% DMSO vehicle alone ('V') or 30µM MB21 in 1% DMSO vehicle ('T', test). Panels B-E depict the effect of MB21 on NS1 secretion by DENV-infected cells. Vero cells were infected with DENV-1 (**B**), DENV-2 (**C**), DENV-3 (**D**) or DENV-4 (**E**), either in the absence (black bars) or presence (grey bars) of MB21. Culture supernatants withdrawn at the indicated time points (on the x-axis) during the 1 week experiment, were tested for viral antigen levels using the Dengue NS1 ELISA kit (the NS1 ELISA absorbance scale on the y-axis is the same for panels **B-E**). Data shown are mean values (n =*

3). The vertical bars represent standard deviation, SD. Two-way ANOVA and Bonferroni post-test analysis was done using GraphPad Prism. P values were either significant (**) or very significant (***).

Based on the high degree of functional similarity documented for the NS2b-NS3Pro enzyme of DENV-1, -2, -3 and -4 (*Li et al, 2005*), it may be hypothesized that MB21, demonstrated to inhibit DENV-2 NS2b-NS3Pro *in vitro*, may in fact be able to inhibit the replication of all four DENVs in infected cells. To test this hypothesis, Vero cells were infected with each of the four DENVs separately, in the presence and absence of MB21 at a final concentration of 30µM, followed by assays for assessing residual DENV replication.

The synthesis of the viral antigen NS1 has been shown to be a marker of DENV replication (*Ludert et al, 2008; Korrapati et al, 2012*). NS1 levels were measured over a 1 week period in culture supernatants of cells infected with DENVs in the presence and absence of MB21. The data are presented in Figure 4.8B-E (page 42). These data demonstrate a significant reduction in NS1 levels for all four DENVs in the presence of MB21. Given that NS1 levels mirror viral replication these results predict that viral titres must also be decreased in the presence of MB21. The validity of this prediction was investigated by measuring DENV titres in a virus yield reduction assay. In this experiment, DENV-infected cell culture supernatants (taken from MB21 treated as well as untreated) were collected on day 3 post-infection and subjected to a standard plaque assay. At all dilutions tested, culture supernatants from MB21-treated cells showed significantly lesser number of plaques compared to those from untreated cells (Figure 4.9).

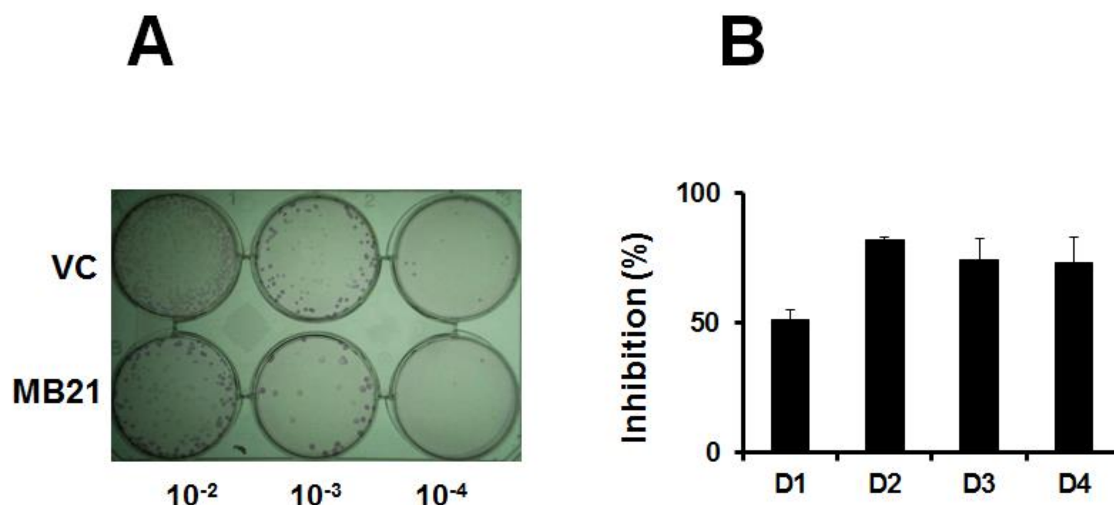

Figure 4.9: The effect of MB21 on infectious progeny DENV generation. *Vero cells were infected separately with each of the four DENVs in the presence or absence of 30µM MB21. DENV titers in culture supernatants drawn on day-3 post-infection were determined by standard plaque assay. (A). This panel shows a typical plaque assay experiment performed with different dilutions (10^{-2} to 10^{-4}) of supernatants obtained from Vero cell cultures infected with DENV-4 in the absence (VC) or presence of MB21 (MB21). (B). Histogram showing the percent inhibition of DENV-1 (D1), DENV-2 (D2), DENV-3 (D3) and DENV-4 (D4) titers in the presence of MB21 with reference to those in its absence (taken as representing 0% inhibition). Data shown are mean values (n=3). The vertical bars represent standard deviation, SD.*

The actual viral titres calculated from plaque counts in this experiment are summarized in Table 4.1 (page 44). From the data in this table, it is evident that MB21 caused inhibition of DENV-1, -2, -3 and -4 by 50, 82, 75 and 73%, respectively. This was found to be statistically significant.

DENV serotype[c]	Without MB21	With MB21[d]	P value[e]
1	1.06 ± 0.03	0.52 ± 0.02	0.0028***
2	1.54 ± 0.12	0.27 ± 0.01	0.0045***
3	0.59 ± 0.01	0.14 ± 0.04	0.0065***
4	0.22 ± 0.02	0.05 ± 0.03	0.031**

Table 4.1: DENV titers[a] (x10^6 pfu[b]/ml) in the absence and presence of MB21

[a]*Titers were determined by plaque assay on Vero cells.*
[b]*Pfu = plaque forming units.*
[c]*The viral strains used were: DENV-1: West Pac 74; DENV-2: S-16803; DENV-3: CH54389; and DENV-4: TVP-360.*
[d]*MB21 used at 30 µM final concentration.*
[e]*P values were calculated using Graphpad software; P values were either significant (**) or very significant (***).*

Taken together, the available data support the conclusion that MB21 is a pan-DENV inhibitor, which appeared to be relatively more efficient at inhibiting DENV-2, -3 and -4 than it was at inhibiting DENV-1. Given that DENV genomic RNA replication is an error-prone process, it is desirable to have additional inhibitors so that one may envisage the future use of a cocktail of inhibitors to suppress the replication of all four DENVs more efficiently. Additionally, this would also raise the barrier against the emergence of drug resistance mutants.

4.4 Acridines and quinolines also possess NS2b-NS3Pro inhibitory potential

A second round of *in vitro* DENV protease inhibitor screening was carried out with an additional ~2,000 compounds, drawn from the 'in-house' small molecule library using the cloned DENV-2 NS2b-NS3Pro-mediated fluorogenic peptide substrate cleavage described in section 4.2.1 (page 40). A preliminary screen of each of these drugs at a single drug concentration (as done in the first screening round) identified 8 molecules with potent DENV-2 NS2b-NS3Pro inhibitory activity (>50% inhibition at 25µM). These 8 molecules, shown in Figure 4.10, represented four quinoline derivatives (BT series) and four acridine derivatives (BU series). These 8 compounds were tested once again at the single concentration to confirm that they could be studied further.

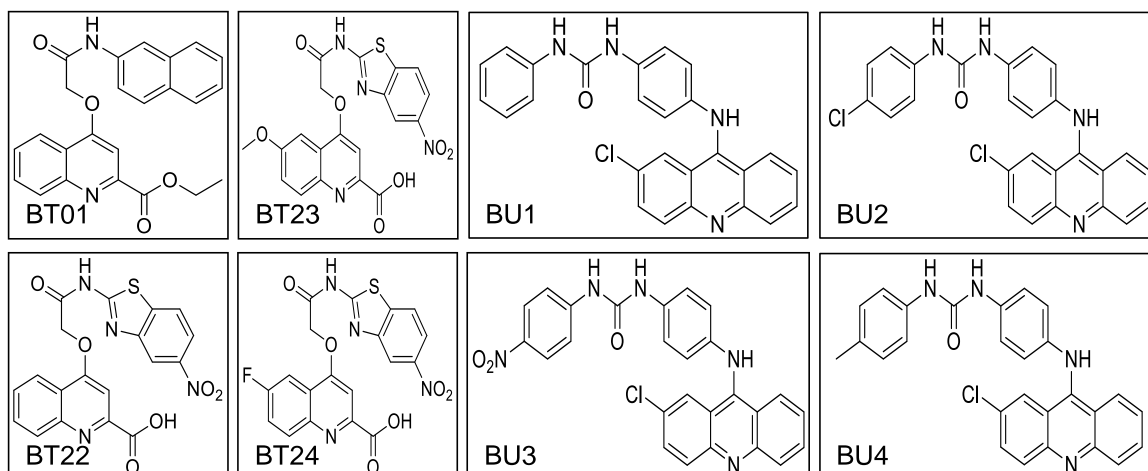

Figure 4.10: BU and BT series compounds identified in the second round of screening.

Having confirmed that these 8 molecules did possess DENV-2 NS2b-NS3Pro enzyme inhibitory activity, the IC_{50} values were determined for each one. For this, a series of dilutions of each of the 8 compounds was tested against the purified cloned DENV-2 NS2b-NS3Pro enzyme in the fluorogenic peptide cleavage assay. This experiment was repeated independently twice and the resultant data are summarized in Figure 4.11.

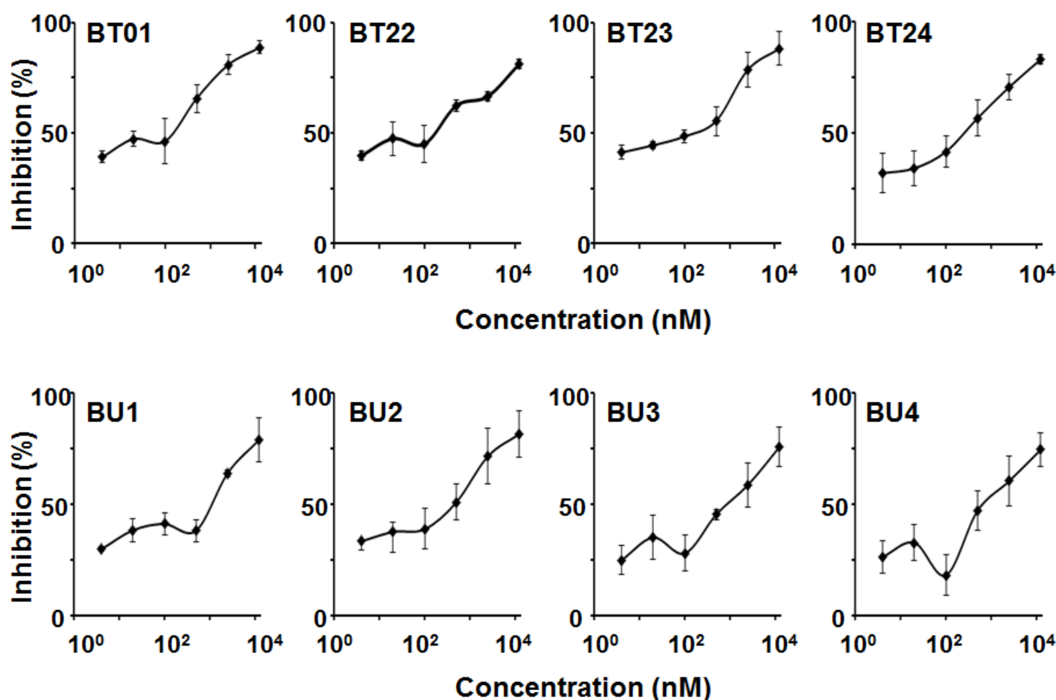

***Figure 4.11: Inhibition of Denv-2 NS2b-NS3Pro by BT and BU series**. Percent inhibition shown on the vertical axis was calculated with respect to NS2b-NS3Pro enzyme activity in the absence of added drug taken to represent 0% inhibition. The data represents mean values (n= 3) ±SD.*

The BT series of compounds were relatively more potent as NS2b-NS3Pro inhibitors, with IC_{50} values ranging from 0.24-0.50µM, than the BU series whose IC_{50} values ranged from 1.22-2.41 µM (Table 4.2).

Code	Compound Name	Mol wt	IC_{50} (µM)
BT01	Ethyl 4-(2-(naphthalen-2-ylamino)-2-oxoethoxy)quinoline-2-carboxylate	400.43	0.27±0.042
BT22	4-(2-((5-nitrobenzo[d]thiazol-2-yl)amino)-2-oxoethoxy)quinoline-2-carboxylic acid	424.39	0.24±0.053
BT23	6-methoxy-4-(2-((5-nitrobenzo[d]thiazol-2-yl)amino)-2-oxoethoxy)quinoline-2-carboxylic acid	454.41	0.33±0.077
BT24	6-fluoro-4-(2-((5-nitrobenzo[d]thiazol-2-yl)amino)-2-oxoethoxy)quinoline-2-carboxylic acid	442.38	0.50±0.007
BU1	1-(4-((2-chloroacridin-9-yl)amino)phenyl)-3-phenylurea	438.91	1.40±0.087
BU2	1-(4-((2-chloroacridin-9-yl)amino)phenyl)-3-(4-chlorophenyl)urea	473.35	1.22±0.183
BU3	1-(4-((2-chloroacridin-9-yl)amino)phenyl)-3-(4-nitrophenyl)urea	483.91	2.41±0.007
BU4	1-(4-((2-chloroacridin-9-yl)amino)phenyl)-3-(p-tolyl)urea	452.93	1.92±0.070

***Table 4.2: IC_{50} values of BT and BU series**. The concentration of drugs that can cause 50% inhibition of the enzyme activity of NS2b-NS3Pro was calculated using data shown in Fig 4.11.*

4.4.1 Quinolines are relatively less cytotoxic

As done before, the cytotoxicity of the BU and BT series of compounds was tested on Vero cells. Each of the 8 compounds was tested at doses ranging from 1-125µM final concentration. Each drug dose level was evaluated in three replicate wells. Cells were exposed to the drug for 3 days and then viability assessed using the MTT assay. It was observed that all BU series of compounds were uniformly cytotoxic at concentrations as low as ~10µM. Therefore, the BU series of compounds were eliminated from further testing in cell-based assays. Cytotoxicity data for BT series are summarized in Table 4.3.

Drug	$IC_{50(\mu M)}$	$CC_{50(\mu M)}$	SI
BT01	0.27	>125*	>462.9
BT22	0.24	>125*	>520.8
BT23	0.33	24	72.7
BT24	0.50	75	150

Table 4.3: Selectivity index (SI) of BT series. *SI is IC_{50}/CC_{50} ratio. *As the cells exposed to these drugs did not show 50% cytotoxicity even at 125µM concentration, the CC_{50} of these compounds were taken as 125µM for SI calculation.*

It was observed that both BT01 and BT22 were quite non-cytotoxic even at the highest concentration tested. On the other hand, BT23 and BT24 manifested discernible cytotoxicity, with the former being relatively more cytotoxic (Figure 4.12). However, based on the selectivity indices, it was evident that BT24 was a more promising candidate for testing in cell-based antiviral efficacy assays.

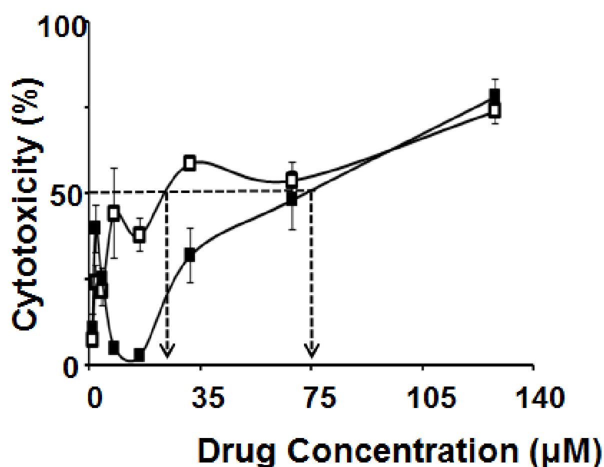

Figure 4.12: Vero cell cytotoxicity of BT23 and BT24. *MTT data from Vero cells cultured in the absence of added drug (control cells) represents 100% viability or 0% cytotoxicity. MTT data obtained from drug treated cells are expressed as percent cytotoxicity with respect to control cells. The dashed lines with arrowheads pointing towards the x-axis indicate drug concentration that results in 50% cytotoxicity. The data represents mean values (n=3).The vertical bar represents standard deviation, SD.*

As done earlier for MB21, an *in silico* molecular docking analysis was performed to examine the interaction of BT24 with DENV-2 NS2b-NS3Pro. The result of this analysis is shown in Figure 4.13 (page 47). Putative interaction of BT24 with Trp 83, Thr 118, Thr 120

and Asn 152 may be critical to its inhibitory efficiency. Based on previous studies (*Mukhametov et al, 2014*) it is likely that BT24-Asn 152 interaction precludes NS2b-NS3 interaction, which is critical to enzymatic function of the protease.

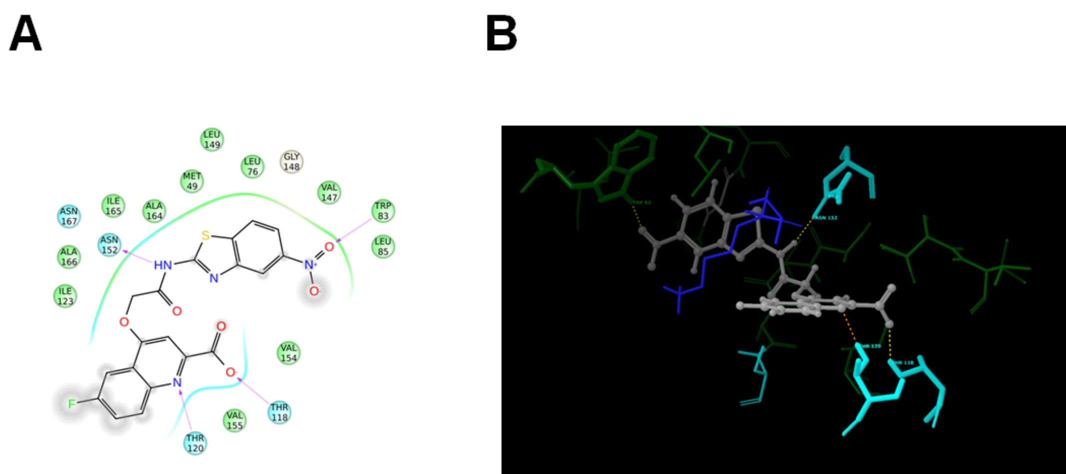

Figure 4.13: In silico analysis of the interaction between BT24 and DENV-2 NS2b-NS3Pro*. (A). Computer generated 2D ligand interaction picture depicting the interaction between BT24 and NS2b-NS3Pro; hydrophobic residues are shown in green. (B). 3D rendition of the interaction in panel 'A' indicating H-bonds (dashed lines) of BT24 (grey colour) with NS2b-NS3Pro.*

4.4.2 BT24 is also a pan-DENV inhibitor

As with MB21, the question to be addressed at this juncture was: would the protease inhibitory activity of BT series of compounds reflect inherent DENV inhibitory potential? To this end, Vero cells were infected with DENV-1, -2, -3 and -4, separately (at m.o.i of 0.025), either in the presence or absence of each of the 4 BT series derivatives, each at a final concentration of 30µM. Culture supernatants were analysed on day 5 post-DENV infection for DENV NS1 antigen levels using the commercial ELISA kit. Taking the NS1 levels observed in the cognate DENV-infected cells in the absence of added drug as 0%, the magnitude of inhibition (expressed as percentage) of each DENV serotype is presented in Figure 4.14 (page 48). This experiment revealed that BT01 and BT22 manifested discernible inhibition of DENV-1 and DENV-2, with the former being inhibited by ~50%, and the latter ~25%. The inhibitory potential of these two drugs on DENV-3 and DENV-4, was either quite low (BT22) or virtually non-existent (BT01).

In contrast, both BT23 and BT24 manifested pan-DENV inhibitory activity in the NS1 ELISA assay. However, it is likely that the BT23 mediated inhibition measured in this assay may be clouded by the cytotoxicity of this compound at 30µM concentration (BT23 CC_{50}=24µM, Table 4.3, page 46). It is to be pointed out that experience from MB21 experiments had indicated that at drug concentrations <30µM, it is difficult to discern antiviral effect in cell-based assays.

This leads to the conclusion that BT24, which is not cytotoxic at 30µM concentration, is a promising pan-DENV inhibitory molecule that merits further study. It is to be noted that while BT24, in the NS1 assay (Figure 4.14, page 48), manifested >75% inhibition against DENVs-1, -2 and -4, was relatively less effective against DENV-3 (~50% inhibition).

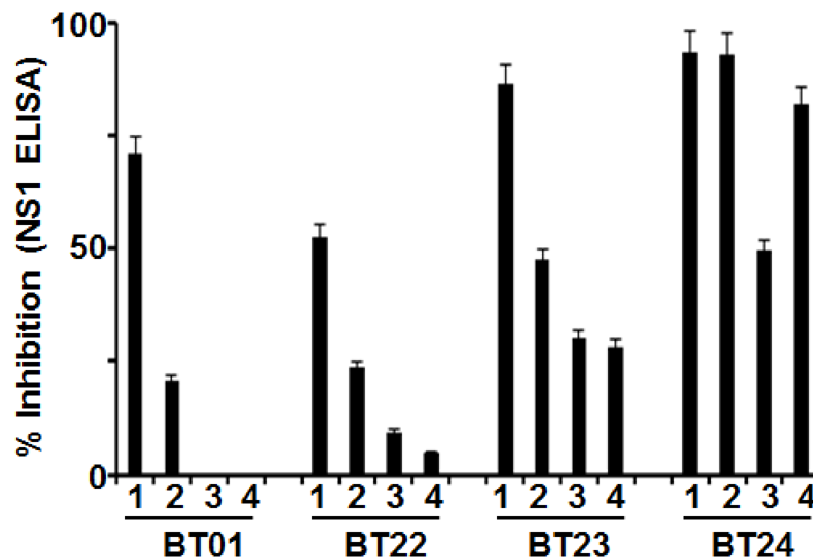

Figure 4.14: Effect of BT series compounds on DENV NS1 antigen synthesis. *The reduction in NS1 levels observed in Vero cells infected with DENV in the presence of added drug, compared to those observed in cells infected with DENV in the absence of any added drug (representing 0% inhibition) is expressed as the % inhibition on the vertical axis. The data show mean values (n=3). The vertical bars represent standard deviation, SD.*

To address if the reduction in NS1 synthesis observed in the presence of BT24 reflects a decrease in viral genomic RNA levels, a quantitative reverse-transcription PCR (qRT-PCR) experiment was performed. First, standardization work was carried out to establish optimal conditions for DENV genomic RNA extraction, cDNA synthesis and q-PCR. Vero cells were infected with DENV-1 at different dosages, ranging from 50 to 400 PFUs/well for 5 days, followed by viral genomic RNA extraction, cDNA synthesis and q-PCR. This experiment was done twice and the real-time amplification profiles from one of these trials, shown in Figure 4.15A clearly demonstrate incremental rightward shift, indicating increasing C_t values,

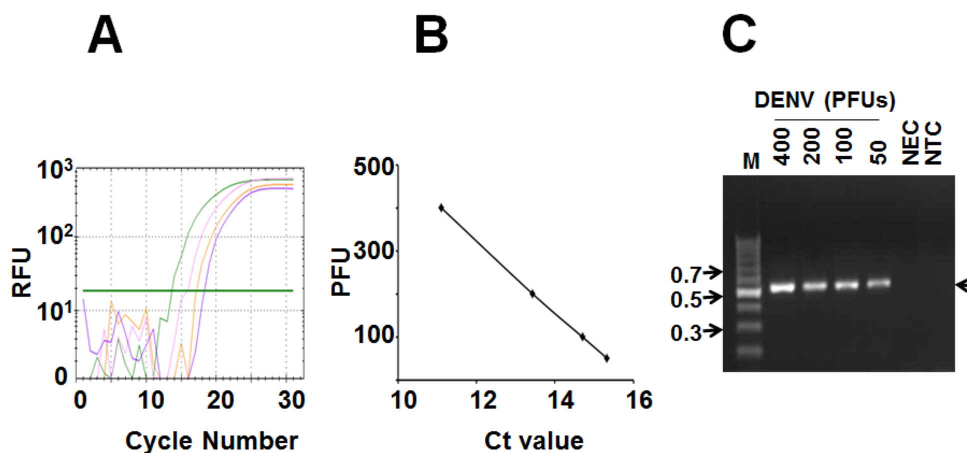

Figure 4.15: Optimization of qRT-PCR experiment. *(A). Real-time amplification profiles of reverse-transcribed viral genomic RNA, 5 days after infecting Vero cells with 50-400 PFUs of DENV-1. (B). A plot of initial virus dose used in infection versus the C_t values obtained in the real-time experiment shown in panel 'A'. (C). Agarose (2%) gel analysis of the specific amplified product at the end of the qRT-PCR experiment. DNA size markers were run in lane 'M'. Their sizes (in Kb) are shown to the left. The arrow to the right indicates the position of the 511bp amplified product. NEC= No enzyme control, NTC= No template control.*

with decreasing DENV-1 PFUs used for infection. A plot of the observed C_t values as a function of DENV-1 PFU dosage at infection, revealed a perfectly linear relationship, shown in Figure 4.15B (page 48). Agarose gel electrophoretic analysis of the reaction products, at the end of the qRT-PCR experiment, revealed specific amplification of the predicted 511bp amplicon (Figure 4.15C, page 48). This experiment established that it should be possible to measure any BT24-mediated reduction in DENV genomic RNA reliably and accurately, under the optimized experimental conditions.

Next, a Vero cell experiment was set up wherein the cells were pre-treated with BT24 (30µM final concentration) overnight, followed by infection with DENV-1, -2, -3 or -4 at an m.o.i. of 0.025 (which is within the linear range of DENV infection optimized above, Figure 4.15). Parallel DENV infections (for all four serotypes) were set up in the absence of any drug pre-treatment. At day-5 post-infection, DENV genomic RNAs were isolated from all samples and subjected to qRT-PCR, using the conditions optimized in the earlier experiment for each of the steps (Figure 4.15, page 48). The real-time amplification profiles of the BT24 treated and untreated samples, for each of the four DENV serotypes are presented in Figure 4.16. The rightward shift in the amplification profiles, of the drug-treated sample (green curves) when compared to those not treated with the drug (pink curves), in each of the four cases clearly demonstrates that there is a decrease in the viral genomic RNA levels.

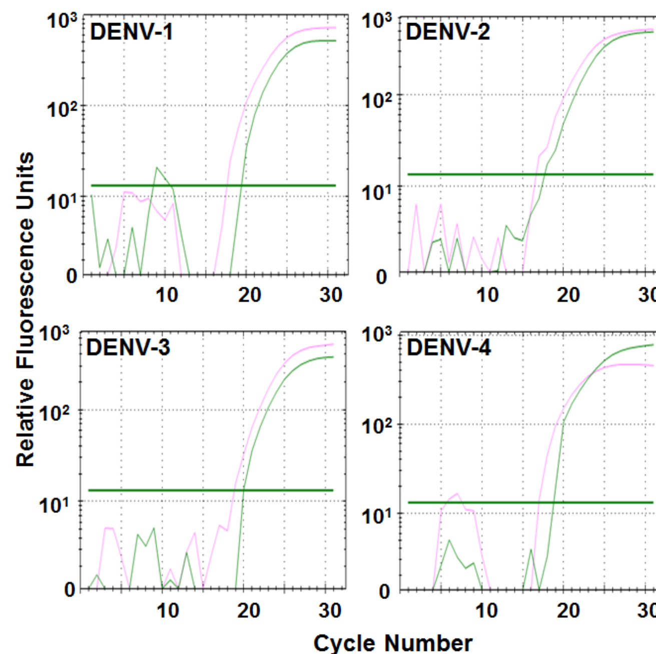

Figure 4.16: Effect of BT24 on DENV genomic RNA replication. *Real-time amplification profiles obtained by subjecting DENV genomic RNA, isolated 5 days post infection to qRT-PCR. DENV genomic RNA was prepared from infected cells that were treated with BT24 (green curve) or untreated (pink curve).*

With respect to DENV genomic RNA levels found in the absence of BT24 treatment taken as 100%, the reduction in BT24-treated samples was in the range of 50-70% for the four DENV serotypes, as shown in Figure 4.17A (page 50). This difference was found to be statistically significant. Once again, the credibility of the qRT-PCR data was corroborated by demonstrating that amplification was specific to DENV genomic template alone, as evidenced by the detection of the expected 511bp product alone at the end of the real-time amplification experiment (Figure 4.17B).

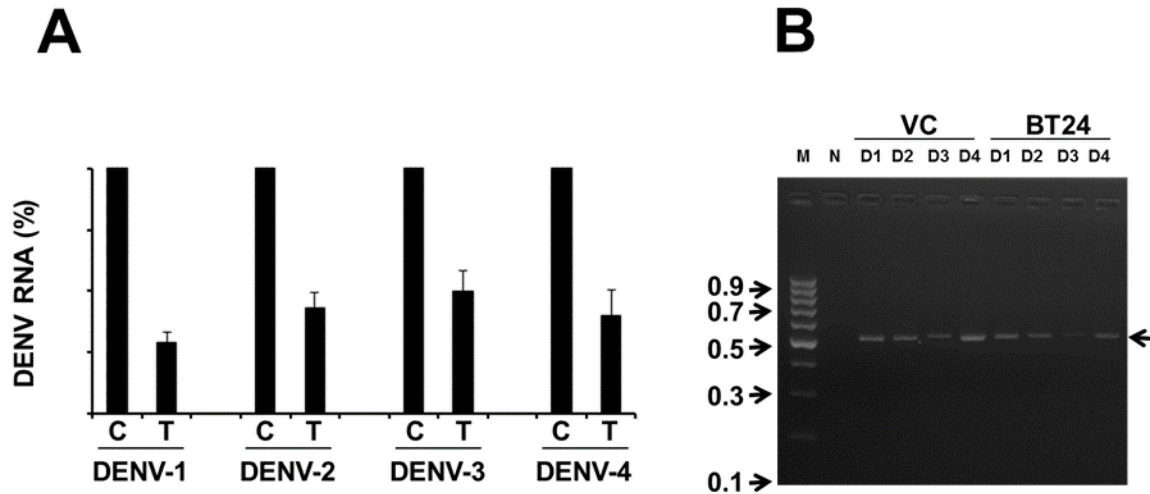

Figure 4.17: Reverse transcriptase q-PCR analysis of the effect of BT24 on DENV genomic RNA levels. *(A). The histogram depicts relative levels of viral genomic RNA determined either in the absence (C) or presence (T) of BT24. Viral RNA levels in the absence of BT24 were taken to represent 100%. (B). Post q-PCR agarose gel analysis showing specific amplification of the 511bp region of the DENV genome from DENV-1 (D1), DENV-2 (D2), DENV-3 (D3) or DENV-4 (D4) infected culture supernatants either in the absence (VC) or presence of BT24 (BT24). 'N' denotes negative control (no template). DNA size markers were analysed in lane 'M'. Their sizes (in Kb) are indicated to the left of the panel.*

The observed reduction in DENV genomic RNA levels should translate to lower viral titers in the presence of BT24. That this indeed is the case is corroborated by the results of a plaque assay performed on the samples used for the qRT-PCR experiment above. The plaque assay data are summarized in Table 4.4. These results showed that DENV-1, -3 and -4 were inhibited quite efficiently (>90%) by BT24, while DENV-2 alone was inhibited much less effectively (~43%). Comparison of the plaque assay-based inhibition data in Table 4.4 with NS1-based inhibition data in Figure 4.14 (page 48), reveals an interesting inconsistency. For DENV-2 and DENV-3, data from these two different approaches yield discordant inhibitory data. While based on NS1 ELISA, BT24 inhibits DENV-2 efficiently (~90%), the inhibition based on plaque assay is modest. Likewise, DENV-3 is apparently inhibited by BT24 much less effectively in the NS1 assay (~50%), but quite effectively in the plaque assay. However, both these methods reveal consistent inhibitory potency of BT24 against DENV-1 and DENV-4. This may reflect subtle serotype-specific differences in the role of NS1 in mediating viral genomic RNA replication and/or virion morphogenesis.

Experiment	Titre (PFU/ml)	Inhibition (%)
DENV-1	1.70×10^6	-
DENV-1+BT24	0.15×10^6	~91
DENV-2	1.02×10^4	
DENV-2+BT24	0.58×10^4	~43
DENV-3	0.02×10^4	
DENV-3+BT24	0.001×10^4	~95
DENV-4	1.30×10^6	-
DENV-4+BT24	0.07×10^6	~95

Table 4.4: Effect of BT24 on infectious DENV production.

Collectively, the data thus far, as exemplified by MB21 and BT24, showed that NS2b-NS3Pro-targeted small molecule drugs can inhibit the replication of DENVs in infected cells. However, the inhibition is not complete as evidenced by qRT-PCR data, which is corroborated by plaque assay data. These observations suggested that it may be possible to suppress DENV replication more effectively if the viral genomic RNA were to be targeted directly.

4.5 RNA interference as an antiviral strategy

A recent comprehensive analysis of the NTR sequences of the DENV genomes of the four serotypes identified a highly conserved siRNA target site located just upstream of the single ORF (*Korrapati et al, 2012*). An siRNA targeting this conserved site could serve as a pan-DENV inhibitor capable of attenuating the replication of all four DENV serotypes. This siRNA was delivered into DENV-infected cells as the corresponding shRNA precursor (designated as sh5b) with the help of a replication-defective recombinant adenoviral (rAd) vector (*Korrapati et al, 2012*). As this study found low levels of DENV genomic RNA in infected cells in the presence of sh5b RNA, it was felt that identification of additional conserved siRNA target(s) in the single ORF of the DENV genome may help raise the genetic barrier to minimize the generation of escape mutants.

4.5.1 Identification of 'sh5c' as a putative site for RNAi-targeting

A multiple sequence alignment of the ORFs of all four DENVs [DENV-1 NI (U88535.1); DENV-2 NGC (AF038403); DENV-3 H87 (M93130); DENV-4 Dom (M1493.2)] was performed using the Clustal W2 tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) available at the Expasy Bioinformatics Resource Portal (www.expasy.org). This analysis revealed that there was not a single continuous stretch of 21 *nts* conserved across all four serotypes within the single ORF. However, one putative site of 21 *nts* encoding the N-terminal *aa*s of the C protein was found to be conserved completely between DENV-2 and DENV-4; further, 20 of the 21 *nts* in this sequence, were conserved in DENV-1 and DENV-3 as well. The single mismatch was at nt 17 for these two latter serotypes (Figure 4.18). This putative site is designated as 'sh5c' in this study. Of note, it was demonstrated in the previous study that the 'sh5b' siRNA could inhibit DENV-4 despite a 3-*nt* mismatch at the 3' end (*Korrapati et al, 2012*). It was therefore felt that a shRNA based on the 'sh5c' target site could perhaps serve as pan-DENV inhibitory agent. Thus, a 'sh5c' shRNA-encoding rAd vector was designed, created and characterized as follows.

```
sh5c      ------------------AATATGCTGAAACGCGAGAGA--------------------- 21
DENV-4    GTGGTTAGACCACCTTTCAATATGCTGAAACGCGAGAGAAACCGCGTATCAACCCCTCAA 179
DENV-2    GCGAGAAATACCCCTTTCAATATGCTGAAACGCGAGAGAAACCGCGTGTCGACTGTACAA 177
DENV-3    ACGGGAAAACCGTCTATCAATATGCTGAAACGCGTGAGAAACCGTGTGTCAACTGGATCA 175
DENV-1    ACGGGTCGACCGTCTTTCAATATGCTGAAACGCGCGAGAAACCGCGTGTCAACTGTTTCA 175
                            **************** ****
```

Figure 4.18: Identification of putative conserved siRNA target site in the ORF of DENVs. *The asterisks indicate the nucleotides conserved across the 4 DENV serotypes.*

4.6 Design and construction of an sh5c-encoding rAd vector

An ‘sh5c’-encoding double-stranded (ds) oligonucleotide, shown in Figure 4.19 below, was designed first. The top-strand oligo (sh5c-F, 58-mer) consisted of 21 *nts* sense sequence (identical to the ‘sh5c’ target sequence in Figure 4.18, page 51) and its corresponding 21-*nt* ‘antisense’ sequence, separated by a short ‘loop’ sequence. The bottom-strand (sh5c-R) is complementary to sh5c-F. Further, both sh5c-F and sh5c-R oligonucleotides had additional 5’ terminal *nts* to provide appropriate cloning overhangs. These two were phosphorylated at their 5’ ends (ATP+T_4 Polynucleotide Kinase) and annealed to result in the double-stranded sh5c oligonucleotide with 5’ *Age* I and 3’ *Eco* RI sticky ends (top part of Figure 4.19).

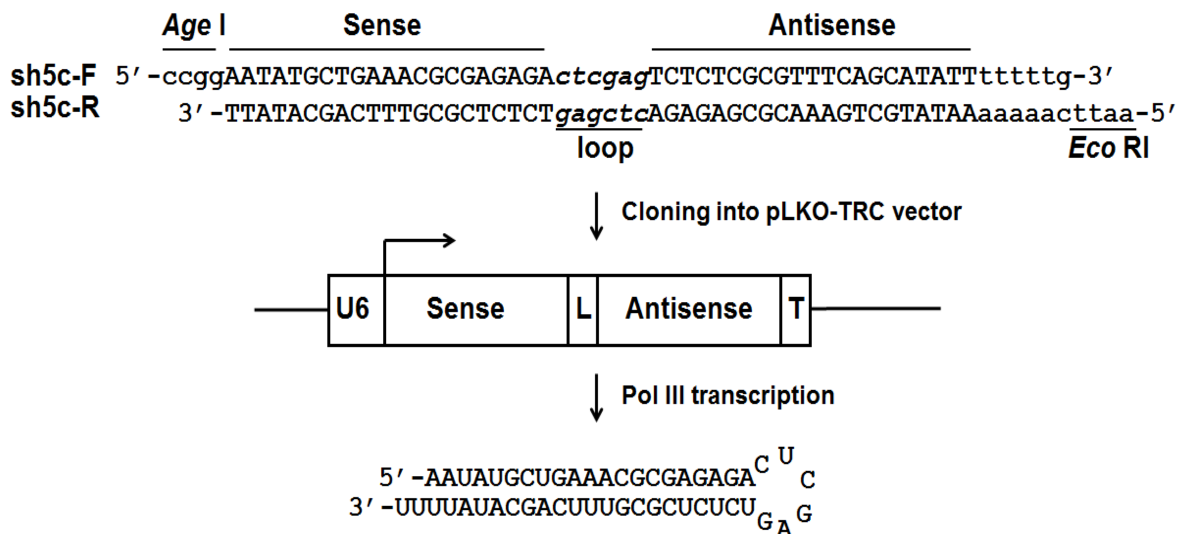

Figure 4.19: Design of sh5c and the creation of a U6 promoter-driven expression cassette.

This ds-oligonucleotide was cloned downstream of the U6 RNA Pol III promoter (in pLKO.1 vector) to create the sh5c expression cassette (sh5c EC) shown in the middle part of Figure 4.19 (L denotes the ‘loop’ sequence and T denotes the transcription terminator). Upon Pol III transcription the sh5c EC is predicted to generate a single-stranded RNA which can fold back upon itself (through base-pairing between the ‘sense’ and ‘antisense’ sequences) to form the sh5c RNA (bottom part of Figure 4.19).

Cloning the sh5c’-encoding double-stranded oligonucleotide into the *Age* I/*Eco* RI sites, downstream of the U6 promoter in the pLKO.1 TRC vector mentioned above, resulted in the creation of the plasmid pLKO-sh5c. The loop sequence in the sh5c-encoding double-stranded oligonucleotide insert was designed to contain an *Xho* I site (Figure 4.19). This was used to screen for insert-containing pLKO-sh5c clones. One of the clones was verified by additional restriction analysis. That this plasmid carried the sh5c insert was confirmed by sequencing both strands using forward and reverse primers (Table 3.1) that annealed to the vector sequences flanking the cloning site. A schematic map of the pLKO-sh5c plasmid is shown in Figure 4.20 (left side) on the next page.The sh5c EC, shown in red, is flanked by a *Not* I site at the 5’ side and a *Bam* HI site at the 3’ side. In order to incorporate this into a rAd vector, it is first necessary to move the U6 promoter-driven sh5c EC into an adenoviral shuttle vector, pShuttle.

This pShuttle plasmid provides essential *cis*-acting elements, namely the Left-Inverted Terminal Repeat (L-ITR) plus the encapsidation signal (Ψ), required for replication and packaging of the rAd vector. This plasmid also provides two adenoviral sequences known as the Right Arm (RA) and Left Arm (LA), to facilitate homologous recombination with the adenoviral backbone DNA carried in plasmid pAdEasy-1 (below). The *Not* I-*Bam* HI fragment, containing the 'sh5c EC', from pLKO-sh5c was transferred into the *Not* I and *Bgl* II sites of pShuttle, to obtain the second construct, pShuttle-sh5c (Figure 4.20, middle).

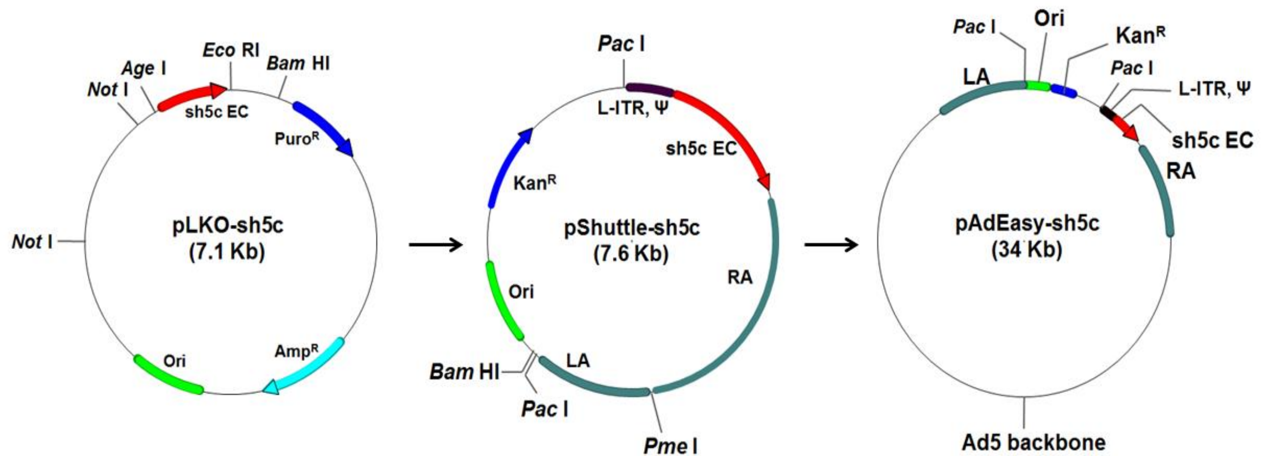

Figure 4.20: Overall strategy adopted to incorporate sh5c EC into adenoviral genome

The pShuttle-sh5c plasmid was thoroughly characterized by restriction analysis to ensure it contains the sh5c EC. A few of restriction sites tested are shown in Figure 4.21. Panel 'A' shows the location of the restriction sites on pShuttle-sh5c and panel 'B' shows the corresponding restriction fragments visualized by agarose gel electrophoresis. This data essentially confirms successful creation of the pShutte-sh5c construct.

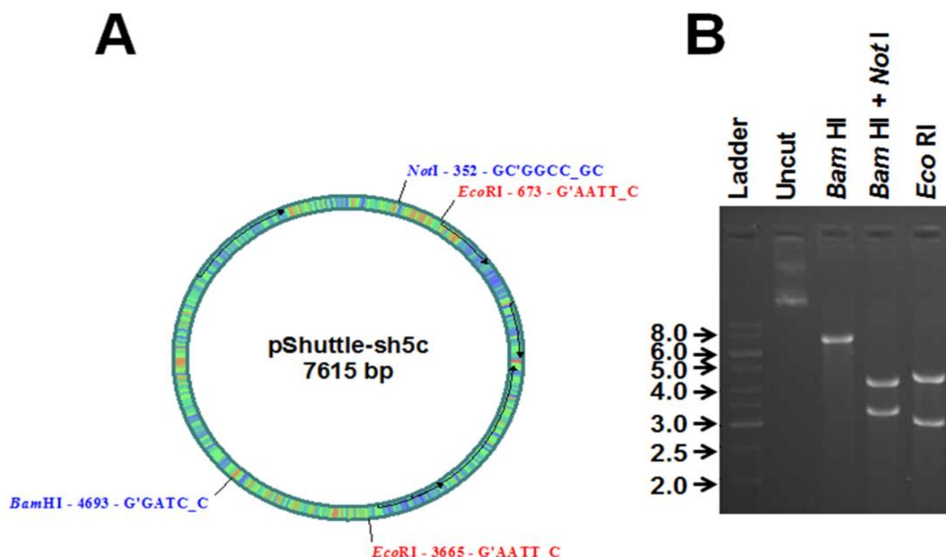

Figure 4.21: Confirmation of pShuttle-sh5c by restriction analysis. *(A).The pShuttle-sh5c plasmid (shown in Figure 4.20, middle) was redrawn to show only the restriction sites used for characterizing the plasmid. The location of the restriction site along with the recognition sequence is shown next to the restriction enzyme's name. (B). Agarose gel analysis of the plasmid shown in panel 'A'. DNA size markers were analysed in the lane marked 'ladder'; their sizes (Kb) are shown to the left. Plasmid DNA before digestion (uncut) and after digestion with Bam HI, Bam HI+Not I and Eco RI (shown on top) were analysed in the remaining lanes.*

As a next step, the sh5c EC from pShuttle-sh5c plasmid was incorporated into the genomic DNA of adenovirus carried in a plasmid called pAdEasy-1. This plasmid contains Ad5 genomic DNA which lacks *nts* 1-3533 encompassing L-ITR and Ψ plus early region 1 (E1). The lack of E1 region is intended to make the resultant rAd vector replication-defective, as a safety feature. The RA and LA sequences of pShuttle are identical to sequences at either end of the adenoviral genomic DNA inserted into pAdEasy-1. To incorporate pShuttle-sh5c portion containing the Ad5 L-ITR, Ψ and the U6-promoter-driven sh5c EC, the plasmid was linearized with *Pme* I (which cuts between RA and LA) and co-transformed into recombination-competent *E. coli* BJ5183. Homologous recombination between pShuttle-sh5c and pAdEasy-1 *via* the RA and LA sequences generated the third construct, pAdEasy-sh5c (Figure 4.20, right side, page 53). The pAdEasy-sh5c plasmid contains the entire rAd genome (with the sh5c EC inserted into the E1 region) flanked by *Pac* I sites. The identity of this plasmid was confirmed by restriction analysis (Figure 4.22). As before, panel 'A' shows the locations of the restriction sites tested on the pAdEasy-sh5c map and panel 'B' shows the band pattern obtained experimentally. As the predicted and actual band patterns were as expected, it was concluded that pAdEasy sh5c plasmid had been created successfully.

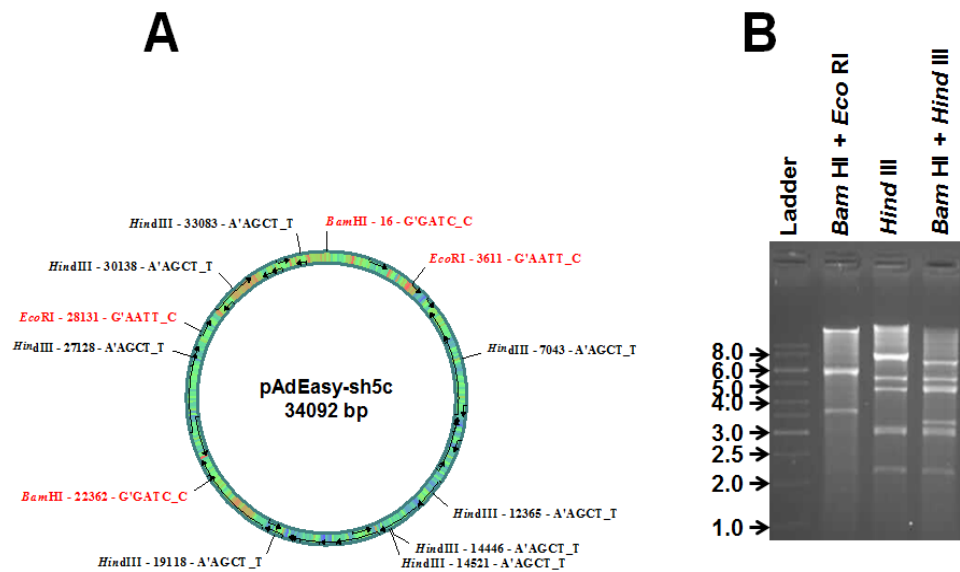

Figure 4.22: Confirmation of pAdEasy-sh5c by restriction analysis. *(A). The pAdEasy-sh5c plasmid (shown in Figure 4.20, extreme right, page 53) was redrawn to show only the restriction sites used for characterizing the plasmid. The location of restriction site along with the recognition sequence is shown next to the restriction enzyme's name. (B). Agarose gel analysis of the plasmid shown in panel 'A'. DNA size markers were analysed in the lane marked 'ladder'; their sizes (Kb) are shown to the left. Plasmid DNA before digestion (uncut) and after digestion with Hind III, Bam HI+Hind III and Bam HI+Eco RI (shown on top) were analysed in the remaining lanes.*

In order to create the rAd vector encoding the sh5c-EC, the rAd genome in the pAdEasy-sh5c construct was rescued as follows. The sh5c-encoding rAd genome was retrieved by *Pac* I digestion of the pAdEasy-sh5c plasmid and introduced into the Ad5 E1-supplementing cell line HEK 293, through lipofection. This resulted in the generation of recombinant sh5c-encoding rAd virus, rAd-sh5c. The initial viral lysate from transfected cells was amplified by serial propagation in HEK 293 cells to obtain stock virus for further characterization and experimentation. A schematic representation of the rAd-sh5c virus is shown in Figure 4.23A (page 55). Shown below is the sh5c RNA encoded by the rAd-sh5c

virus. The locations of some of the restriction enzyme recognition sites used to characterize the rAd-sh5c genome are indicated by line maps below (Figure 4.23B).

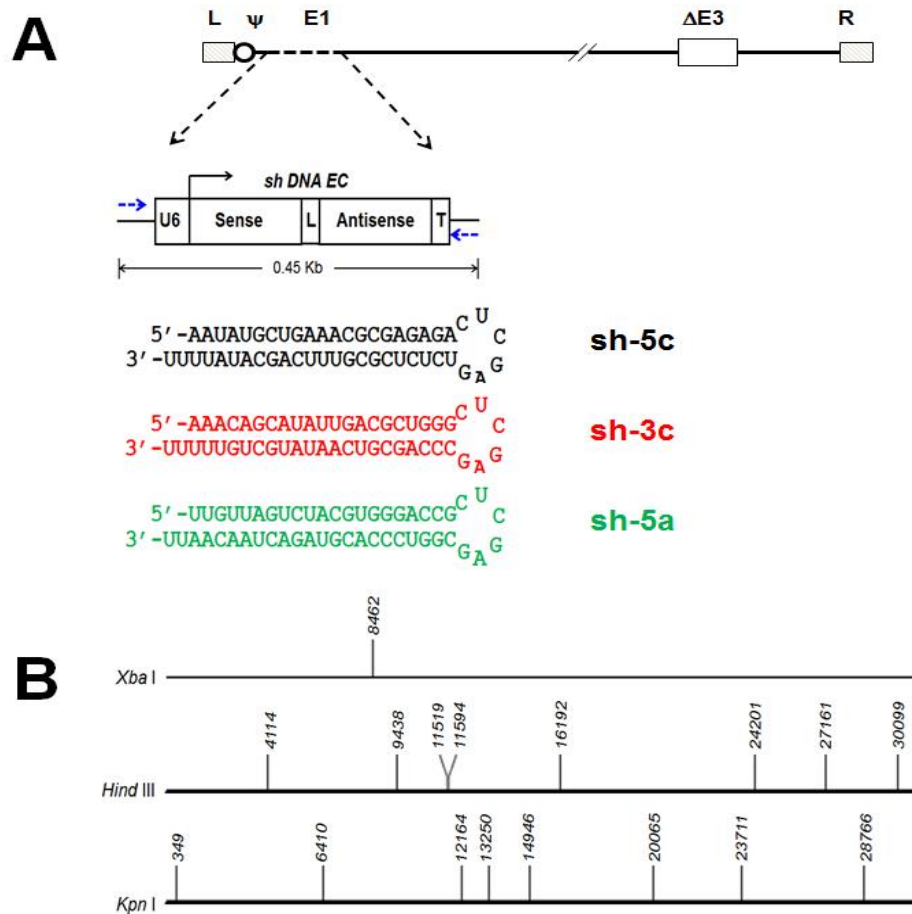

Figure 4.23: Design of the rAd-sh viruses. *(A). The linear genome of the rAd-sh viruses (rAd-sh5c, rAd-sh3c and rAd-sh5a) constructed for this study. In constructing the rAd-sh virus, the E1 region (dashed line) is replaced by the sh DNA expression cassette (sh DNA EC),consisting of the U6 promoter (U6),the shDNA insert with the sense and antisense arms of 21 base pairs each, followed by the U6 terminator (T).'L' denotes the 6-basepair loop sequence connecting the sense and antisense arms. The lines flanking the expression cassette represent plasmid vector sequences. Other elements of the rAd-sh genome include a ~2.7 Kb deletion in the E3 region (Δ E3), the left (L) and right (R) inverted terminal repeats, and the packaging signal (Ψ). Shown below are the nt sequences of the shRNAs: sh-5c, sh-3c and sh-5a. (B). Line maps of the rAd-sh genomes representing the locations of restriction sites (Xba I, Hind III and Kpn I). The numbers indicate the position of the restriction enzyme cleavage site starting from the left end of the genome. Note: The three rAd-sh genomes differ only in the sh DNA EC which does not contain recognition sites for any of the 3 restriction enzymes tested. Therefore, all three rAd-sh viral genomes are predicted to yield identical restriction patterns with the enzymes tested.*

4.6.1 Physical characterization of rAd-sh5c

Viral DNA was extracted from rAd-sh5c-infected HEK 293 cells using the Hirt lysis protocol and analysed by restriction digestion using *Xba* I, *Hin*d III and *Kpn* I. For comparison, the viral genomes corresponding to rAd-shscr (rAd vector encoding a scrambled shRNA EC in the E1 region) as well as wild-type (wt) Ad5 were analysed in parallel (Figure 4.24A, page 56). Both the rAd-sh5c and rAd-shscr viral DNAs are expected to give identical restriction patterns as they differ only in the shRNA encoding sequences which possess none of these three restriction sites. It is to be noted that the wild type (wt) Ad5 genome contains

an intact E1 region which can be identified by the presence of a unique *Xba* I site within it, about 1.4 Kb from the left end of viral genome. That the E1 region is absent in the rAd-sh5c (and the rAd-shscr) genomic DNA is evident from the absence of this ~1.4 Kb band upon *Xba* I digestion. Similarly, restriction with enzymes such as *Hin*d III and *Kpn* I also revealed the predicted differences between the wt Ad5 genome and rAd-sh genomes.

As a next step, The Hirt viral DNAs were used as PCR templates in conjunction with insert-specific and E1 region-specific primer pairs. In this experiment wt Ad5 DNA served as the positive control for the presence of the E1 region-specific PCR product and the previously characterized rAd-sh5b viral DNA as the positive control for the presence of the shRNA encoding insert. As shown in Figure 4.24B below, the E1 region-specific primers amplified a ~0.5 Kb PCR product when wt Ad5 viral genomic DNA was the template (lane 'wt'), but not when viral genomic DNA from rAd-sh5b (lane '5b') or rAd-sh5c (lane '5c') was used as template. Similarly, the insert-specific primer pair amplified the predicted PCR product (also ~0.5 Kb in size) from viral genomic DNAs corresponding to either rAd-sh5b (lane '5b') or rAd-sh5c (lane '5c'), but not to wt Ad5 (lane 'wt'), as can be seen from Figure 4.24C below. According to design, a unique *Xho* I site is located between the sequences encoding the sense and antisense strands of the shRNA (Section 4.6, Page 52). The presence of this site was used to confirm the identity of the insert-specific PCR product. As shown in Figure 4.24C, the shDNA EC-insert-specific PCR products from both rAd-sh5b as well as rAd-sh5c carry this unique *Xho* I site (lanes '5b + X' and '5c + X', Figure 4.24C) as evidenced by the digestion of each of these PCR products into two fragments (of equal size, ~0.25 Kb).

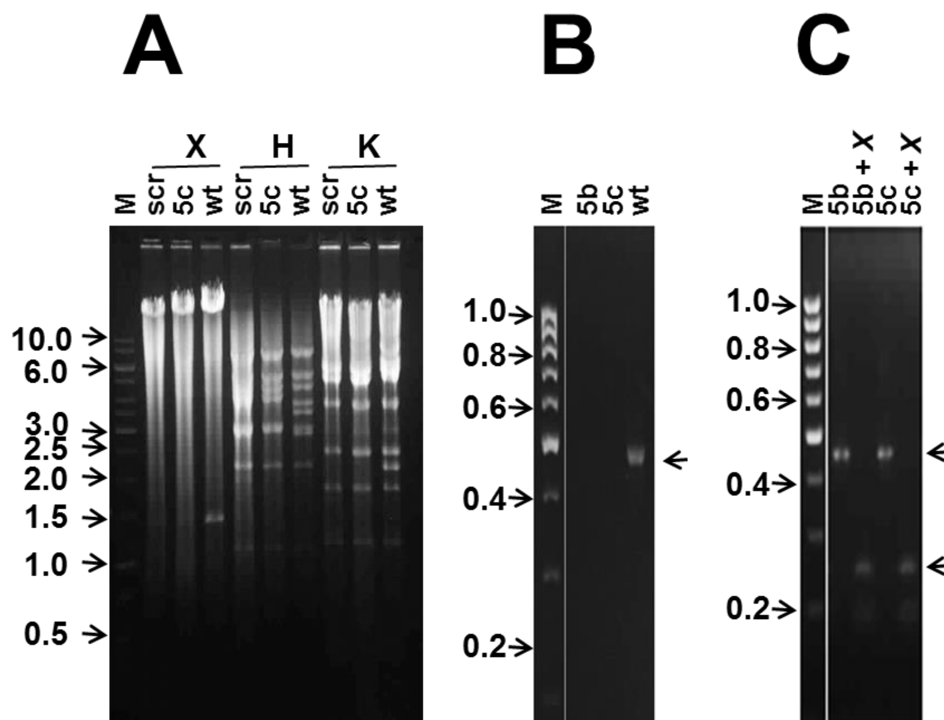

Figure 4.24: Physical characterization of the rAd-sh5c genome. *(A). Agarose gel analysis of rAd-sh5c viral DNA (5c) digested with Xba I (X), Hind III (H) and Kpn I (K).For comparison, viral DNA from rAd-shscr (scr) and the parental Ad5 (wt) were analysed in parallel. It may be noted that rAd-shscr (Korrapati et al, 2012) is similar in design to the rAd-sh viruses as previously described. As a result, rAd-sh5c is predicted to result in the same restriction profile as rAd-shscr. (B). PCR analysis of rAd-sh5c (5c) using Ad5 E1-specific primers. Viral DNAs of rAd-sh5b (5b) reported by Korrapati et al, 2012, and parental Ad5 (wt) were also analysed in parallel. The arrows to the right of the panel denote the positions of the predicted Ad5 E1 region-specific amplicon. (C). PCR analysis of rAd-sh5c (5c) using insert-specific primers. Viral DNAs of rAd-sh5b (Korrapati et al, 2012)*

and parental Ad5 (wt) were also analysed in parallel. The identity of the PCR products was confirmed by the presence of the sh-DNA EC loop-encoded Xho I site in the PCR product. DNA size markers (sizes in Kb shown to the left) were analysed in lane 'M'. The arrows to the right of the panel denote the positions of insert-specific PCR product before (5b and 5c, upper arrow) and after (5b+X and 5c+X, lower arrow) Xho I digestion. As before, DNA size markers (sizes in Kb shown to the left) were analysed in lane 'M'.

Previous studies had identified two additional putative siRNA target sites, sh5a (conserved in DENVs-1, -2 and -3) and sh3c (conserved among all four DENV serotypes) in the 5' and 3' NTRs respectively (*Korrapati et al, 2012*). Following a strategy similar to the one described for the construction of pAdEasy-sh5c, constructs pAdEasy-sh5a and pAdEasy-sh3c, corresponding to these two additional siRNA target sites, were developed. These also carry the U6 promoter-driven shRNA EC integrated into the E1 region of a replication-defective adenoviral genome. Starting from these 2 plasmids, two additional rAds, rAd-sh5a and rAd-sh3c, were generated and characterized as done for rAd-sh5c above (*data not shown*). The siRNAs encoded by these two rAds are shown in Figure 4.23A, page 55, below the sh5c siRNA sequence. Stocks of all three rAds were made and titrated on HEK 293 cells using standard protocols (Table 3.3).

4.7 Effect of rAd-mediated RNAi delivery on DENV replication in Vero cells

It has already been established that rAd vectors do not affect the replication of DENV in infected Vero cells using real time PCR analysis of DENV genomic RNA as shown in Figure 4.25 (*Korrapati et al, 2012*).

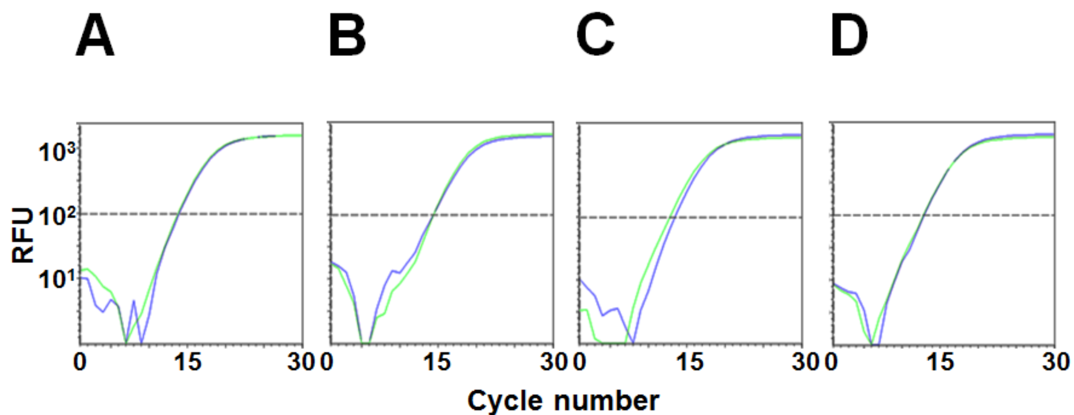

***Figure 4.25: rAd-sh vector does not affect the replication of DENV RNA**. Vero cells were either mock-infected or infected with rAd-shscr for 24 hours followed by infection with DENV-1 (A), DENV-2 (B), DENV-3 (C), DENV-4 (D) (blue curve). Total cellular RNA (green curve) was isolated on day 7 post-DENV infection and analysed for DENV 'plus' sense genomic RNA by real time PCR. The horizontal dashed line indicates the baseline used to determine Ct values (Korrapati et al, 2012).*

Next, preliminary functional characterization of the three rAds constructed above were carried out. For this, Vero cells were infected with each of the three newly constructed rAds (rAd-sh5c, rAd-sh5a and rAd-sh3c), separately. Additionally, separate Vero cell infections were set up with two previously characterized rAds, rAd-shscr and rAd-sh5b (*Korrapati et al, 2012*), as negative and positive controls, respectively. Each rAd infection was performed in 4 replicate wells. At 24 hours post rAd-infection, cells were infected separately with DENV-1, DENV-2, DENV-3 and DENV-4. At 5 days post-DENV infection, culture supernatants were analysed for the presence of the DENV viral antigen NS1, as an

index of viral replication. NS1 antigen levels were estimated using commercial ELISA kit, as before. At the time of culture supernatant collection, varying levels of cytopathicity was observed in cells pre-infected with rAd-sh5a and rAd-sh3c. This was not seen with the remaining three rAds. To compensate for variations arising from well-to-well differences in cytopathicity, MTT assay was performed on all residual cells in the wells. The viable fraction of cells in each well was used to normalize the NS1 levels determined for that well. These data are depicted in Figure 4.26. This experiment revealed that, of the three rAds screened, rAd-sh5c was the best choice as it inhibited all four DENVs by at least ~65% without any appreciable cytopathicity.

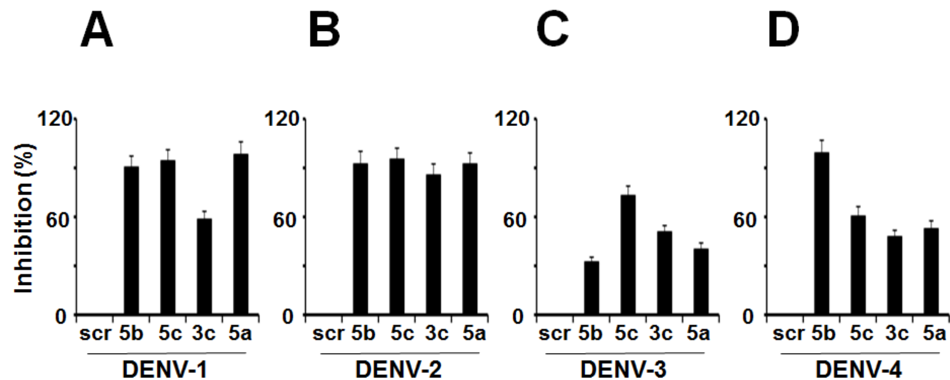

Figure 4.26: Comparative analysis of the effect of different rAd-sh vectors on DENV NS1 antigen secretion. *Vero cells were pre-infected with different rAd-sh vectors (scr: rAd-shscr; 5b: rAd-sh5b; 5c: rAd-sh5c; 3c: rAd-sh3c; 5a: rAd-sh5a) at m.o.i of 10. Twenty-four hours post rAd-sh infection, the cells were re-infected with DENV-1 (A), DENV-2 (B), DENV-3 (C) and DENV-4 (D), all at m.o.i= 0.025. Culture supernatants were analysed for NS1 antigen 5 days post DENV infection using a commercially available ELISA Kit (J. Mitra & Co.). Inhibition of NS1 antigen secretion was computed taking NS1 antigen levels secreted by DENVs in the presence of rAd-shscr as 100%. The data represent the mean of triplicates. To normalize for differences in cell numbers, the inhibition data were normalized to cell viability data obtained in an MTT assay performed on the monolayer after collecting culture supernatant for NS1 ELISA.*

4.7.1 The antiviral effect of rAd-mediated RNAi persists for several days

In the next series of experiments, the duration of the rAd-sh5c delivered RNAi-mediated suppression of DENV infection was examined (Figure 4.27). Essentially, the above experiment was repeated using rAd-shscr, rAd-sh5b and rAd-sh5c for the first infection,

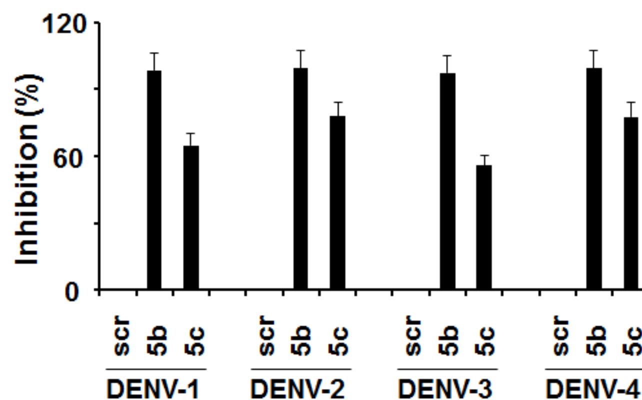

Figure 4.27: rAd-shRNA vector-mediated RNAi effect is long lasting. *Vero cells were separately pre-infected with rAd-shscr (scr), rAd-sh5b (5b) and rAd-sh5c (5c). Twenty four hours later cells were infected with DENV-1, DENV-2, DENV-3 and DENV-4, separately. Culture supernatants were collected on day 9 post-DENV infection and analysed for NS1 antigen levels using ELISA. NS1 levels were normalized to cell numbers based*

on MTT assay. Inhibition of NS1antigen was computed taking the NS1 antigen levels secreted by DENVs in the presence of rAd-shscr as 100% (equivalent to 0% inhibition). The data represents the mean of triplicates.

followed by DENV-1, -2, -3 and -4, separately, for the second infection. However, this time the culture supernatants were periodically collected on days 3, 5, 7 and 9, and analysed for NS1 levels. The data obtained with culture supernatants collected on day 9 post-DENV infection, normalized for viable cell content, are presented in Figure 4.27 (page 58). The data demonstrate that the RNAi-mediated suppression is more or less preserved even as long as day 9. This has implications for potential therapeutic utility of RNAi as an antiviral strategy against DENV infections.

4.7.2 rAd-mediated RNAi decreases NS1 antigen synthesis during ongoing DENV infection

Up until this point, all experiments designed to examine the antiviral effect of rAd-mediated RNAi involved pre-infection with different rAd-encoding shRNAs followed by DENV infection 24 hours later (referred to as 'AD mode' of infection for adenovirus followed by dengue virus). It may be argued that establishing RNAi prior to DENV infection may not reflect the natural situation. That is, will rAd-delivered RNAi be effective enough to curtail an ongoing DENV infection? To answer this, a 'DA mode' experiment was set up, wherein Vero cells were initially infected with DENVs, followed 24 hours later by infection with rAd-shRNA encoding virus. The results of NS1 analysis from a typical DA mode experiment, using culture supernatants collected 5 days later, are shown in Figure 4.28 below. This experiment revealed that the rAdsh-5b-mediated RNAi was quite effective at suppressing NS1 antigen expression by all four DENVs. Under the experimental conditions used, sh5b siRNA inhibited NS1 production of DENV-1, DENV-2, DENV-3 and DENV-4 by 97%, 98%, 95% and 93%, respectively. That the rAd-sh5b-delivered RNAi is effective against ongoing DENV-2 infection was observed in our earlier studies also (*Korrapati et al, 2012*). Importantly, the current data demonstrate that rAd-sh5b is equally potent at curtailing NS1 antigen expression during ongoing infection by the remaining three DENV serotypes as well.

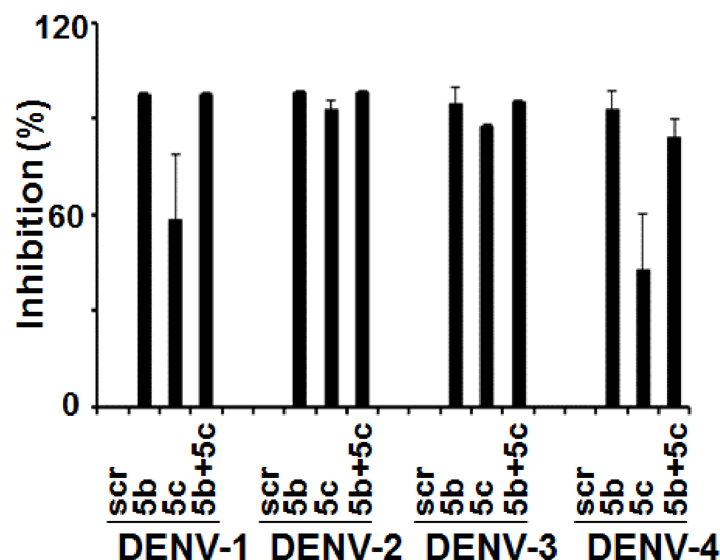

***Figure 4.28: Effect of rAd-mediated RNAi on NS1 antigen secretion after establishing DENV infection**. Vero cells were first infected with the four DENVs (m.o.i = 0.01) separately. Twenty four hours later the cells were infected with rAd-shscr (scr), rAd-sh5b (5b), rAd-sh5c (5c), or a mixture of rAd-sh5b+rAd-sh5c (5b+5c). All rAds were at 10 PFU/cell. Culture supernatants were collected on day 5 post-DENV infection and analysed for NS1 antigen levels using ELISA. NS1 levels were normalized to cell numbers based on MTT assay. Inhibition of*

NS1 antigen secretion was calculated taking the NS1 antigen levels secreted by DENVs in the presence of rAd-shscr as 100% (equivalent to 0% inhibition). The data represent the mean of triplicates (vertical bars represents SD).

When a mixture was rAd-sh5b and rAd-sh5c was tested for NS1 expression inhibitory potency (Figure 4.28, '5b+5c' bars), in the DA mode assay, it is found that the serotype-specific levels of inhibition noticed were essentially comparable to those seen with rAd-sh5b alone (DENV-1:~98%; DENV-2: ~98%; DENV-3: ~95%; & DENV-4: ~84%). However, that this is not a reflection of lack of inhibitory potency of rAd-sh5c is evident from experiments wherein rAd-sh5c was tested singly in the absence of rAd-sh5b. Interestingly, rAd-sh5c, which by itself was highly effective against ongoing DENV-2 (~92% inhibition) and DENV-3 (~87% inhibition) infection, was moderately effective against DENVs-1 (~58% inhibition) and -4 (~43% inhibition), reflecting subtle serotype differences in the accessibility of the sh5c target site. This leads to the conclusion that both sh5b and sh5c siRNA target sites on DENV genomic RNAs are susceptible to RNAi.

4.7.3 A mixture of rAd-sh5b and rAd-sh5c inhibits ongoing viral RNA accumulation potently

Next, the effect of rAd-sh5c-mediated RNAi on the DENV viral genomic RNA replication in cells after establishing DENV infection first (DA mode) was examined. As before, rAd-sh5c was analysed singly and in combination with rAd-sh5b. For comparison, rAd-sh5b was also tested separately by itself in the DA mode experiment. DENV genomic viral RNA was isolated from culture supernatants, reverse-transcribed to cDNA and then quantified by SYBR green-based qRT-PCR. Real time amplification profiles of DENV genomic RNA in culture supernatants obtained in the DA mode experiment are presented in Figure 4.29. Visual inspection of the data reveal that the DENV viral RNA amplification profile is shifted to the right by both rAd-sh5b (curves marked '*b*' in the 4 panels) as well as rAd-sh5c (curves marked '*c*'), with this shift being more pronounced in case of the former.

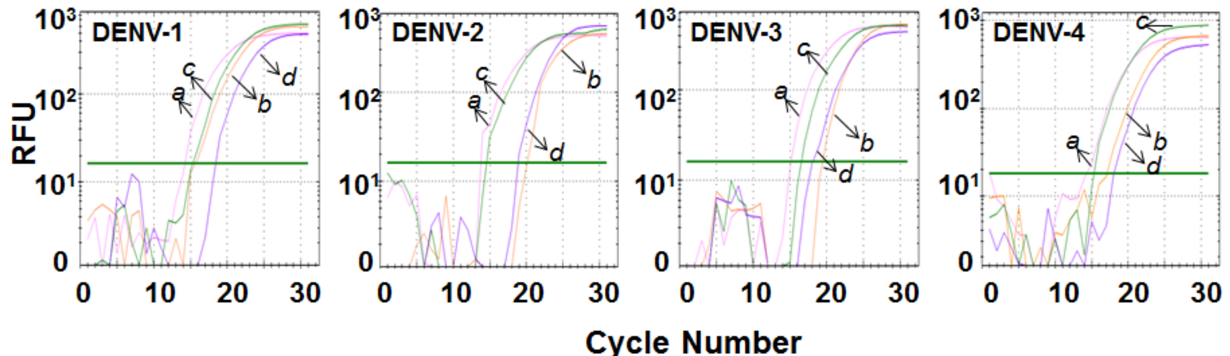

***Figure 4.29: The effect of rAd-mediated shRNA expression on DENV RNA accumulation**. (A) Vero cells were infected separately with DENV-1, DENV-2, DENV-3 and DENV-4. Twenty four hours post DENV-infection, cells were infected with rAdsh-scr (a), rAd-sh5b (b), rAd-sh5c (c), or a mixture of rAdsh5b +rAd-sh5c (d). Real-time analysis was performed with DENV RNA extracted 5 days post DENV-infection.*

When a mixture of rAd-sh5b and rAd-sh5c (curves marked '*d*') was used, this rightward shift was either comparable to or greater than that seen with rAd-sh5b alone. The C_t values are summarized in Table 4.5 (page 61). These data essentially mirrored the NS1 data and corroborate the conclusion that rAd-sh5c does indeed target DENV genomic RNA for RNAi-mediated degradation. One pitfall of the SYBR green method for qPCR is that non-specific amplification can cloud the data obtained. However, the primers and the experimental

	DENV-1	DENV-2	DENV-3	DENV-4
scr	14.15	13.56	15.09	14.27
sh5b	15.61	20.07	19.32	17.07
sh5c	15.24	14.38	16.42	15.20
sh5b+sh5c	18.28	18.75	18.10	18.03

Table 4.5: Real time reverse transcription PCR analysis of DENV genomic RNA in the presence of different rAds*. Data shown are Ct values observed when Vero cells in which DENV infection is established are infected with rAd-shscr (scr),rAd-sh5b (5b), rAd-sh5c (5c) or a mixture of rAd-sh5b + rAd-sh5c (5b+5c).*

conditions used ensured specific amplification of a defined region of the DENV genome based on melt curve analysis as well as agarose gel analysis of the PCR products at the end of the q-RTPCR experiment, as shown in Figure 4.30. Interestingly, the band intensities of the

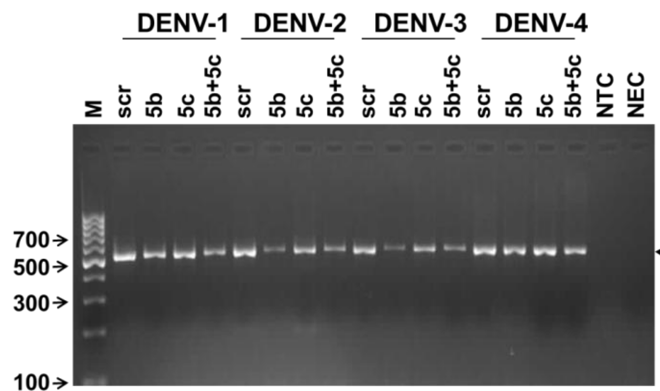

Figure 4.30: Post real-time PCR agarose gel analysis. *Samples generated in experiment shown in Figure 4.29 (page 60) were assessed for specific amplification of the expected 511bp product on 2% agarose. Abbreviations are as follows, scr: rAd-shscr; 5b: rAd-sh5b; 5c: rAd-sh5c; 5b+5c: rAd-sh5b+ rAd-sh5c; NTC: No template control; NEC: No enzyme control. DNA size markers were analysed in lane 'M'. Their sizes (in bp) are shown to the left. The arrow on the right indicates the position of 511bp amplicon.*

amplified products reflect the q-RTPCR data to a certain extent. Quantification of the q-RTPCR data is presented in the bar diagram in Figure 4.31. This figure depicts the percent

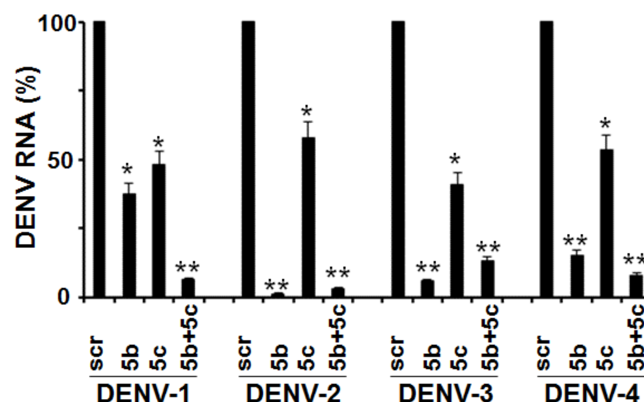

Figure 4.31: Relative quantification of the effect of rAd-mediated shRNA expression on DENV viral genomic RNA accumulation. *Data represent % residual viral genomic RNA in the presence of rAd-sh5b (5b), rAd-sh5c (5c) or a mixture of rAd-sh5b+rAd-sh5c (5b+5c) with respect to DENV RNA levels in presence of rAd-shscr (scr) taken as 100%. The single and double asterisks above the bars represent significant (p<0.05) and very significant (p<0.01) inhibition.*

residual DENV RNA in culture supernatants after treatment with different rAdsh vectors with respect to viral RNA levels in the presence of rAd-shscr taken to represent 100%. These data show that while rAdsh5b and rAdsh5c are both effective at reducing DENV replication during an ongoing infection (DA mode experiment), the former is slightly better than the latter. Importantly, a mixture of both rAdsh5b and rAdsh5c resulted in a more potent reduction in DENV RNA replication across all the four DENV serotypes.

4.7.4 rAd-sh5b plus rAd-sh5c mixture inhibits infectious DENV generation

Finally, the ability of rAd-sh5c to inhibit the release of infectious virus in the DA mode experiment, singly and in combination with rAd-sh5b, was determined. For this, culture supernatants collected on day 5 post rAd infection in a typical DA mode experiment were analysed for DENV titers in a standard plaque assay. The data are summarized in Table 4.6. These data revealed that rAd-sh5b can inhibit secretion of infectious virus ~65% in the case of DENV serotypes 1 and 4, and almost completely in the case of DENV serotypes 2 and 3. The observation of the inhibitory effect of rAd-sh5b on DENV-2 in the DA mode corroborates earlier studies (*Korrapati et al, 2012*). This work has extended this finding to the remaining three DENV serotypes and demonstrated the pan-DENV inhibitory potential of rAd-sh5b. Additionally, this work also shows that rAd-sh5c also behaves in a similar fashion, albeit at slightly lesser efficacy. Importantly, a mixture of rAd-sh5b and rAd-sh5c caused >90% inhibition in the release of infectious DENV of all four serotypes.

	DENV-1	DENV-2	DENV-3	DENV-4
scr	56 (0)	18 (0)	1.2 (0)	60 (0)
sh5b	20 (64.3)	0.2 (98.9)	0 (100)	20 (66.7)
sh5c	28 (50)	2.8 (84.4)	0 (100)	30 (50)
sh5b+sh5c	2 (96.4)	0 (100)	0 (100)	6 (90)

Table 4.6: Effect of rAd-mediated shRNA expression on DENV secretion. *Vero cells were infected separately with DENV-1, DENV-2, DENV-3 or DENV-4. At 24 hours post-DENV infection, cells were infected with rAdsh-scr (scr), rAd-sh5b (sh5b), rAd-sh5c (sh5c) or a mixture of rAd-sh5b and rAd-sh5c (sh5b+sh5c). Culture supernatants collected on day 5 post rAd infection were analysed for the presence of infectious DENV using a standard plaque assay. Data shown are DENV titres ($x10^4$ PFU/ml). Values in parenthesis indicate % inhibition with respect to DENV titer in the presence of rAd-shscr taken as 0% inhibition.*

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CHAPTER 5: DISSCUSSION

Dengue is a viral illness caused by four antigenically distinct viruses, DENV-1, -2, -3 and -4, of the genus *Flavivirus*, family *Flaviviridae* (*Pierson and Diamond, 2013*). It is spread to humans by *Aedes* mosquitoes and at the present time it is documented to be one of the fastest-spreading arboviral diseases, prevalent in >100 tropical/sub-tropical countries, placing ~3.6 billion of the global population at risk (*Gubler et al, 2012*). The increasing prevalence and incidence of dengue is held to be the outcome of multiple contributory factors which include global travel, climatic changes, lack of public health infrastructure in the resource-poor countries and a failure to implement sustained vector control measures (*Guzman & Harris, 2015*). Recent estimates suggest that there may be ~390 million dengue infections annually, of which nearly 75% may be asymptomatic (*Bhatt et al, 2013*). Symptomatic illness is recognizable as distinct clinical entities, DF, DHF and DSS, associated with fever, hemorrhage, plasma leakage and thrombocytopenia (*Swaminathan & Khanna, 2013; WHO, 2016*). The risk factors that determine progression of the relatively mild DF to potentially fatal DHF/DSS are not clearly understood. However, a positive correlation has been observed between very high levels of viremia and DHF/DSS in secondary DENV infections (*Vaughn et al, 2000; Libraty et al, 2002*). It believed that pre-existing antibodies from primary DENV infection facilitate uptake of heterotypic DENV into cells of the monocyte/macrophage lineage *via* FcR (*Halstead, 2003*).

Effective tools to combat dengue are unmet needs. The realization that dengue is not limited by geographic borders has heightened the awareness about it as a significant public health problem in recent years. In turn, this has spurred efforts globally to develop tools aimed at limiting and hopefully preventing the spread of dengue (*Swaminathan & Khanna, 2009*). Many of these efforts are focusing on developing an effective dengue vaccine (*Swaminathan & Khanna, 2009, 2013; Swaminathan et al, 2010; Schwartz et al, 2015*). However, the enigmatic role of antibodies in conferring protective immunity as well as in mediating severe and potentially fatal disease has been posing significant challenges to vaccine development. A key requirement for a safe and efficacious dengue vaccine, mandated from the recognition of the ADE phenomenon referred to above, is that it must provide robust and durable immunity against all four DENV serotypes. The challenges inherent in dengue vaccine development are evident from the recent phase III efficacy trial data of the lead dengue vaccine candidate, CYD-TDV, which shows poor efficacy towards DENV-2 (*Capeding et al, 2014; Villar et al, 2015*). This result, while underlining the need to develop alternate dengue vaccine candidates, has also stimulated interest in exploring the feasibility of developing drugs that may be of utility in treating dengue infections.

Comparison of DF versus DHF/DSS patients reveals that viremia levels in the latter are an order or two higher in magnitude compared to the former (*Vaughn et al, 2000; Libraty et al, 2002*). This observation provides the rationale underlying dengue drug development efforts. Thus, a drug that is able to decrease viral titers ~2 logs may be of potential utility in altering the disease course towards favourable prognosis. In most dengue endemic countries, all four DENV serotypes tend to co-circulate together. This makes it necessary for a dengue drug to be capable of inhibiting all four DENV serotypes equally effectively. In addition, as the progression of a DF patient to DHF/DSS can be very rapid, an effective drug also needs to be rapid-acting, capable of aggressively bringing down viremia quickly. Finally, effective therapeutic intervention is possible only if dengue infection can be diagnosed early. It has been observed that NS1 antigen appears in the circulation as early as one day after DENV infection (*Alcon et al, 2002; Young et al, 2000*). In this context, it is noteworthy that several dengue NS1 antigen detection diagnostic tests have become available in recent years (*Dussart*

et al, 2006; 2008). This makes it possible to initiate dengue antiviral therapy, once available, within 24 hours of infection and provides an additional rationale for developing dengue drugs.

The fact that antiviral drugs have been developed for several other viruses such as human immunodeficiency virus type 1 (HIV-1), hepatitis B virus (HBV) and influenza virus (*Lim et al, 2013*) suggests that it is feasible to develop drugs against DENVs also. This work has explored the feasibility of developing antiviral drugs and strategies against dengue infections. As each of the four DENVs is capable of causing the full spectrum of the disease, and all four serotypes tend to co-circulate in hyper-endemic regions (*Guzman et al, 2010; Swaminathan and Khanna, 2009*), it is essential that a useful antiviral drug should be a pan-DENV inhibitor, effective against all four DENV serotypes. Thus, this work has focused on identifying pan-DENV inhibitors and strategies that may be of potential utility in curtailing the replication of all four DENVs. Two viral targets have been explored as antiviral drug targets: the viral enzyme NS2b-NS3Pro and the viral genomic RNA itself.

5.1 Identification of DENV protease inhibitors

Viral proteases critical to the life cycle of the virus in the infected host cell are regarded as potential drug targets. Indeed, viral protease inhibitors capable of inhibiting several viruses have been developed (*Lim et al, 2013*). In the cases of DENVs, the amino-terminal 180 *aa* region of NS3 protein has been found to possess protease activity which, in concert with host proteases, plays a critical role in the generation of the mature viral proteins (*Lidenbach et al, 2013*). It has also been documented in literature that the specificity and efficiency of the NS3Pro enzyme cleavage is dependent on the C-terminal ~40 *aa* residues of the DENV NS2b protein (*Li et al, 2005*). Thus, it is likely that inhibitors of NS2b-NS3Pro may have the potential to serve as antiviral drugs against DENVs. This led to the decision to develop an *in vitro* inhibitor screening assay using an in-house library of small molecular weight compounds previously reported to contain antimicrobial compounds (*Jeankumar et al, 2013; Poyraz et al, 2013*).

A plasmid encoding DENV-2 NS2b-NS3Pro was already available in the laboratory (*Figure 4.2, page 38*). Using this plasmid, recombinant NS2b-NS3Pro was expressed and purified it to >90% homogeneity, using Ni^{2+}-NTA affinity chromatography and native conditions (*Figure 4.3, page 39*). It was demonstrated to possess catalytic activity using a flurogenic peptide substrate, Bz-nKRR-AMC (*Figure 4.4, page 40*), mimicking the endogenous NS3Pro cleavage site (*Li et al, 2005*). Protease assay conditions were carefully optimized and the utility of the protease assay in inhibitor screening demonstrated (*Figure 4.5, page 40*), using a known protease inhibitor, aprotinin (*Leung et al, 2001*).

5.1.1 MB21

The in-house library was screened initially to shortlist compounds that could inhibit the recombinant NS2b-NS3Pro >80% at 25µM final concentration. This drug concentration was used to allow identification of as many potential inhibitors as possible. From ~1000 small molecular weight compounds, 25 compounds were selected for further analysis. From this subset, 4 molecules, designated as RB02, RA14, RA16 and MB21 were found to inhibit the cloned DENV-2 protease quite efficiently. Interestingly, all four were found to be benzimidazole derivatives (*Figure 4.6, page 41*). Of these 4, MB21 was the most potent inhibitor (IC_{50} ~6µM). *In silico* molecular docking analysis revealed that the benzimidazole

moiety of MB21 fits into a hydrophobic cleft in the vicinity of the catalytic triad (*Figure 4.7B & C, page 42*). This cleft has been reported to be part of an allosteric site on the protease molecule reported in other studies (*Mukhametov et al, 2014; Othman et al, 2008*). Based on these data it is likely that MB21 binding to the allosteric site may adversely affect substrate binding, leading to inhibition of enzymatic function.

Having demonstrated that MB21 can inhibit DENV-2 NS2-NS3Pro *in vitro*, the question to address was: would MB21 inhibit replication of the parent virus, DENV-2? Further, as the NS2b-NS3Pro enzymes among the four DENV serotypes manifest a high degree of functional similarity (*Li et al, 2005*), would MB21 be capable of inhibiting the remaining three DENV serotypes as well? To answer these questions, a cell-based viral infection assay was set up using Vero cells. First, the cytotoxicity of the drug on Vero cells was assessed. This showed that the viability of cells treated with MB21 (stock in 1% DMSO) at concentrations up to 100µM was essentially similar to that seen in cells treated with DMSO alone. This led to the conclusion that MB21 *per se* is not cytotoxic at this concentration (*Figure 4.8A, page 42*). However, as the DMSO treated cells (in the absence of added MB21) manifested some loss in viability compared to cells that were not exposed to DMSO, further studies were carried out using MB21 stock prepared in 0.5% DMSO.

In order to investigate the effect of MB21 on DENV replication, Vero cells were infected separately with each of the four DENVs, both in the absence and presence of MB21, added to a final concentration of 30µM. Culture supernatants were withdrawn at periodic intervals to assess the degree of inhibition. In this context, it is to be noted that replication of DENV in cultured cells (*Korrapati et al, 2012; Ludert et al, 2008*) and in animal models (*Schul et al, 2007*) can be evaluated by measuring the levels of NS1 antigen secreted by DENV-infected cells, into the culture supernatant or the circulatory system, respectively. Accordingly, NS1 antigen levels were determined in the culture supernatants collected above, using a commercial ELISA kit. This analysis revealed that MB21 was indeed capable of inhibiting all four DENVs significantly (*Figure 4.8B to E, page 42*).

If indeed NS1 reduction mirrors the downregulation of viral replication, it follows that MB21 must be capable of reducing the generation of infectious progeny virus in DENV infected cells. To examine if MB21 has an effect on the final viral titres, a standard plaque assay was performed with culture supernatants collected from MB21-treated and untreated DENV-infected cells. Analysis of the resultant data revealed that MB21 did reduce viral titres in the case of each of the four DENV serotypes, to varying extents (*Figure 4.9, page 43*). Furthermore, the observed reduction was statistically significant (*Table 4.1, page 44*).

Collectively the data showed that, MB21 identified initially as an inhibitor of the NS2b-NS3Pro of DENV-2, not only inhibits the parent DENV-2 virus, but is in fact a pan-DENV inhibitor, capable of inhibiting all four DENV serotypes. Since the replication machinery of the DENVs lacks proof-reading function, one may envisage the emergence of drug resistant mutants. The only practical solution to such a scenario would be to identify additional inhibitors to minimize the possibility of resistance emergence. Therefore, a second round of screening was carried out with an additional ~2000 compounds from the same in-house library.

5.1.2 BT24

The second round of screening followed essentially the same strategy discussed above using the NS2b-NS3Pro enzyme inhibition assay in conjunction with the Bz-nKRR-AMC peptide substrate. This effort resulted in the short-listing of eight potential inhibitors, of which 4 were acridines and the rest quinolones (*Figure 4.10, page 44*). These manifested more potent DENV-2 NS2b-NS3Pro inhibitory activities compared to the benzimidazoles discussed above. Their IC_{50} values ranged from 1-2.5µM (acridine derivatives) and 0.25-0.5µM (quinoline derivatives) (*Table 4.2, page 45*). The finding that quinolines are inhibitors of DENV-2 NS3 protease is consistent with recent disclosure that 8-OH-quinoline is an inhibitor of WNV NS3Pro (*GU patent, 2014*). Once again a prior cytotoxicity assay was performed before proceeding to examine their inhibitory potency on DENVs in cultured cells. Based on the results, the acridines were eliminated due to high levels of cytotoxicity associated with them. The quinolines (BT01, BT22, BT23 and BT24) were investigated further using the cell-based assay described earlier for testing the effect of these compounds on DENV replication in infected cells.

NS1 ELISA data in the cell-based experiments did not correlate with the DENV-2 NS2b-NS3Pro inhibitory activities of BT01 and BT22. While BT01 and BT22 inhibited the parent virus, DENV-2, only weakly, they manifested very low to almost no inhibitory efficacy against DENV-3 and DENV-4. Surprisingly, DENV-1 was inhibited by ~50%. While the reason for this inconsistency between enzyme inhibition in the biochemical assay and the cell-based bioassay is not clear, one contributory factor may have been inefficient uptake of BT01 and BT22 into the cells. As both these compounds were not pan-DENV inhibitory these two were abandoned. Of the remaining two, BT23 and BT24, the latter was more effective against all the four DENV serotypes than the former. Interestingly, molecular docking analysis revealed that the BT24 molecule can potentially interact with multiple *aa* residues in the vicinity of the active site of the NS2b-NS3Pro enzyme. Of these, its interaction with Asn-152 is likely to disrupt NS2b interaction with NS3Pro rendering it catalytically inactive (*Figure 4.13, page 47*).One possible reason for the comparatively lower efficacy of BT23 may have been due to it being relatively more cytotoxic than BT24 to Vero cells. Thus, further work focused on BT24.

As a next step, the replication of viral genomic RNA, and its subsequent packaging into infectious virions were investigated using qRT-PCR and plaque assay, respectively. Real-time analysis revealed that viral genomic RNA levels were decreased by at least 50% in the case of all four DENV serotypes by BT24. In contrast, viral titres were reduced ~90% for DENV-1, -3 and -4 and ~43% for DENV-2. The relatively inefficient inhibition of DENV-2 virus production is surprising, considering that BT24 was chosen based on its ability to inhibit the purified protease derived from DENV-2 quite efficiently. Viral RNA replication and infectious virus production, though linked, are distinct processes and could be impacted differently by the added drug. This apparently anomalous observation may perhaps be a reflection of subtle yet-to-be understood serotype differences. Clearly additional studies are necessary for better understanding.

Collectively, the data thus far support the conclusion that two drugs, the benzimidazole MB21, and the quinoline BT24, are both pan-DENV inhibitors. While it is likely that the viral protease may be the target of these drugs in infected cells, other mechanisms may also be contributing to the observed inhibitory effects in cell based assays.

For example, host fatty acid synthase is recruited by the viral protease in infected cells (*Heaton et al, 2010*). The binding of the added drugs to the viral protease as well as to possibly other viral as well host targets may perturb the virus host interactions in unknown ways. The precise mechanism of the action of these inhibitors needs elucidation. However, it is noteworthy that both MB21 and BT24 represent the first NS2b-NS3Pro-targeted inhibitors reported in literature to be effective against all four DENV serotypes.

5.2 RNAi

Though the NS2b-NS3Pro inhibitors identified in this study, MB21 and BT24, were found to be pan-DENV inhibitory, data from different experiments revealed that inhibition was not complete. For example, a direct assessment of residual DENV titres in MB21-treated samples showed that the magnitude of inhibition ranged from 50-82% with DENV-1 and DENV-2 being the least and most susceptible to inhibition, respectively (*Table 4.1, page 44*). A similar experiment using BT24 demonstrated that infectious virus production could be inhibited by >90% in the case of DENVs-1, -3 and -4, with DENV-2 showing only ~43% inhibition (*Table 4.4, page 50*). A qRT-PCR analysis of BT24-treated samples revealed that viral genomic RNA levels were decreased by 50-70% for the four DENV serotypes (*Figure 4.17A, page 50*). Given the error-prone nature of the DENV genomic RNA replication machinery, it may be hypothesized that replication of this residual viral RNA could potentially give rise to escape mutants that are no longer sensitive to the drug(s).

The above consideration led to the conclusion that an effective antiviral strategy must perhaps directly target the viral genomic RNA. From this perspective, RNAi offers a strategy to specifically target DENV genomic RNA for degradation. One of the effectors which mediate RNAi is siRNA which can direct the endonuclease activity of the cytoplasmic RISC to mRNA carrying a complementary sequence (*Dykxhoorn & Lieberman, 2006; Castonotto and Rossi, 2009*). It has been demonstrated that vector-expressed shRNA (*Xia et al, 2002; Uprichard et al, 2005; Zhang et al, 2004)* can be transported to the cytoplasm by exportin-5 and processed by dicer to siRNA (*Cullen, 2006*), which can then induce RNAi-mediated gene silencing in mammalian cells. The utility of RNAi as an antiviral strategy against many viruses has been reported (*Dykxhoorn & Lieberman, 2006; Leonard & Schaffer, 2006; Grimm & Kay, 2006*). A recent report showed that an siRNA (designated as ‘sh5b’) complementary to a sequence conserved across the 4 DENV serotypes, delivered into infected cells using a rAd vector, could function as a pan-DENV inhibitor (*Korrapati et al, 2012*). However, low levels of DENV genomic RNA were found in the presence of sh5b RNA. Based on the argument above that the DENV RdRp-mediated error-prone replication can give rise to resistance, it was felt that more than one site on DENV genomic RNA must be targeted, to preclude the emergence of RNAi-resistant mutant populations.

5.2.1 rAd-sh5c

The sh5b target site identified in the previously reported work is located in the 5’ NTR. This study also identified additional putative siRNA target sites in the NTRs: sh5a (located in the 5’ NTRs of DENVs-1, -2 and -3) and sh3c (located in the 3’ NTRs of all four DENV serotypes). Of these only the sh5b target site was evaluated using the rAd vector (*Korrapati et al, 2012*). It was decided to assess the feasibility of targeting these sites as well using rAd-mediated shRNA delivery. Examination of the DENV ORF revealed a putative siRNA target site towards the 5’ end within the ‘C’-encoding sequences (*Figure 4.18, page 51*). This site, designated ‘sh5c’ was completely conserved in two DENV serotypes (DENV-

2, -4), with a single base mismatch (at position 17) in the other two serotypes (DENV-1, -3). This target was also chosen for further investigation based on two considerations: (i) no other 21 nt conserved sequence could be discerned elsewhere within the ORF; and (ii) the sh5b siRNA was demonstrated experimentally to be effective against all 4 DENV serotypes, despite a 3 nt mismatch in DENV-4 at the 3' end (*Korrapati et al, 2012*). As done earlier, a rAd vectors encoding all these three shRNAs (sh5a, sh3c and sh5c) were constructed (*Figure 4.23, page 55*) and characterized thoroughly by restriction and PCR analyses (*Figure 4.24, page 56*) before testing their efficacy on DENV replication.

The effect of sh5a, sh3c and sh5c siRNA on DENV replication was analyzed using multiple assays designed to evaluate viral protein synthesis, genomic RNA replication and infectious virus particle production. In all these experiments, the previously described rAd-sh5b and rAd-shscr, were used as positive and negative controls, respectively, to enable reliable assessment of the effect of the three new rAds created in this study. It is to be noted that DENV NS1 can serve as a marker of viral replication (*Ludert et al, 2008; Schul et al, 2007; Korrapati et al, 2012*). Thus, the initial experiments were based on the measurement of reduction in NS1 secretion in the culture supernatant in DENV-infected cells that had been pre-exposed to rAds expressing different shRNAs.

Initial experiments revealed that in Vero cells pre-infected with rAd-sh5c, NS1 antigen synthesis by all four DENVs was decreased by at least 65%. The level of NS1 decrease seen with rAd-sh5c was not observed with either rAd-sh5a or rAd-sh3c (*Figure 4.26, page 58*), suggesting that the corresponding siRNA target sites are not as accessible to the RNAi machinery as the sh5c target site. For reasons not quite clear, it was also observed that both rAd-sh5a and rAd-sh3c were associated with varying degrees of CPE. Consequently, these were not investigated further.

The data referred to above suggest that RNAi induced by pre-infection with rAd-sh5c was successful in downregulating the replication of all four DENVs, presumably by targeting the viral RNA for degradation. This in turn was reflected by a corresponding decrease in viral protein synthesis. Interestingly, the RNAi antiviral state induced by rAd-sh5c persisted for several days (*Figure 4.27, page 58*). As viremia persists for 4-5 days in DF patients, the durability of the antiviral state associated with RNAi could be of potential therapeutic benefit.

In the investigation above, DENV replication was assessed, in terms of NS1 production in Vero cells, in which RNAi was already induced ('AD' mode). In a natural setting, a therapeutic intervention can be envisaged only after DENV infection has been confirmed based on laboratory diagnosis. The next series of experiments were modified to approximate the natural situation. To this end, DENV infection was first established in Vero cells and then followed with induction of RNAi using rAd infection ('DA' mode). Accordingly, rAdsh-5b reported previously (*Korrapati et al, 2012*) and rAd-sh5c (created in this work) were evaluated singly and in combination, in a 'DA' mode experiment (*Figure 4.28, page 59*). This experiment revealed that rAd-delivered sh5b siRNA could inhibit NS1 synthesis by all four DENVs >90%. It is noteworthy that this represents the first demonstration that induction of rAd-mediated RNAi is effective in curtailing ongoing replication of all four DENVs. A mixture of rAd-sh5b and rAd-sh5c was also equally potent in inhibiting NS1 antigen synthesis by all four DENVs in the 'DA' mode experiment. The pan-DENV inhibitory potential of both rAd-sh5b and rAd-sh5c, in the DA mode, was re-confirmed by analysis of viral genomic RNA, by qRT-PCR (*Figure 4.29, page 60; Table 4.5, page 61)*, as well as infectious DENV production, by plaque assay (*Table 4.6, page 62*). The

data from these experiments essentially mirrored the NS1 data discussed above. Taken together, the overall results of the DA mode experiments appear to suggest that the pan-DENV inhibitory efficacy of rAd-sh5b alone and a mixture of rAd-sh5b plus rAd-sh5c are more or less comparable. If so, what is the contribution of rAd-sh5c? Based on the observation that rAd-sh5c alone, in the absence of rAd-sh5b, manifested significant pan-DENV inhibitory potency, it can be concluded that both rAd-sh5b as well as rAd-sh5c, when present together, can each target each of one of the four DENVs. This is evident in the plaque assay data observed with DENVs-1 and -4. In both these instances, it can be seen that either rAd-sh5b or rAd-sh5c, is not as effective as a mixture of both rAds. This essentially is a reflection of the capacity of both rAds to target DENV genomic RNA independently. In other words, the ability to deploy RNAi targeted to two distinct sites on the DENV viral genomic RNA can raise the genetic barrier against the emergence of resistance.

Ad5 has been documented to interact with host antiviral response pathways. It is known that small RNA molecules, designated VA RNAs, expressed by Ad5 can interact with RISC, and thereby downregulate the RNAi pathway (*Lu & Cullen, 2004; Aparicio et al, 2006*). Given this, how appropriate is it to use it as vector for intracellular delivery of shRNAs? In this context, it is relevant that VA RNAs are severely under-expressed by rAd vectors such as those employed in this work, which are designed to be E1-deleted (*Lu & Cullen, 2004*). Studies using rAd vectors expressing shRNAs have not found any significant impact on either the RNAi or the interferon response pathways of the host cells (*Narvaiza et al, 2006*). In addition, a rAd vector encoding a scrambled shRNA was found to have no discernible effect on the replication of any of the four DENV serotypes (*Korrapati et al, 2012*). It has also been held that rAd vectors may not be suitable for human use. This stems from the fact that the majority of the human population is Ad5 seropositive and the resultant possibility that the anti-Ad5 antibodies could complex to the rAd vector and eliminate it. However, it is pertinent to point out that rAd complexed to anti-Ad5 antibodies can enter macrophages and dendritic cells *via* FcR pathway (*Pilankatta et al, 2010; Leopold et al, 2006*). Given that these cell types represent *in vivo* sites of DENV replication (*Wu et al, 2000; Chen & Wang, 2002*), prior Ad5 immunity in fact has the potential to deliver RNAi into the very cells which support DENV replication. Collectively, available evidence supports the use of rAd vector for intracellular delivery of RNAi to curtail DENV replication.

In conclusion, it may be stated that this work has accomplished the following: (i) it has identified two new and novel small molecule compounds, a benzimidazole derivative (MB21) and a quinoline derivative (BT24). Both these molecules, which are capable of inhibiting cloned DENV-2 NS2b-NS3Pro, are potent inhibitors of the replication of all four DENVs in cultured cells; (ii) the work has demonstrated the feasibility of targeting DENV genomic RNA by deploying the endogenous RNAi pathway. In the context of the absence of a proof-reading mechanism during DENV genomic RNA replication, the identification of two different approaches, one targeting a viral enzyme (using two different small molecule inhibitors), and the other targeting the viral RNA itself (at two distinct sites) by RNAi, opens up the possibility of developing effective combination therapies that set a high genetic barrier to minimize the emergence of resistance.

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CHAPTER 6: CONCLUSIONS

6.1 General Conclusions

Currently, dengue drug development is essentially in its infancy. Efforts at developing dengue drugs have focused so far on investigating potential drugs on a single DENV serotype alone. Thus, the feasibility of developing drug(s) with the potential to be effective against all four DENVs is unknown. Given that DENVs are RNA viruses which rely on error-prone replication machinery, it is likely that a single effective drug could fail in the midst of therapy due to the emergence of resistance. A combination of drugs is one way to raise the genetic barrier to the emergence of resistance. Thus, the feasibility of developing multiple pan-DENV drugs is an area that needs to be explored. This represents a significant gap in the context of dengue drug discovery efforts. The work in this thesis describes efforts undertaken to address this gap. This thesis shows that the DENV protease, essential for successful completion of the viral life cycle, and the viral genomic RNA itself, which functions as a template for both viral translation and replication, can serve as targets for antiviral drug development. Importantly, the work demonstrates the feasibility of inhibiting all four DENVs. Additionally, the work has also shown the feasibility of developing more than one drug and more than one approach to achieving potent inhibition of all four DENVs. The salient accomplishments/outcomes from the work are summarized below.

6.2 Specific Conclusions

- DENV-2 NS2b-NS3pro enzyme was successfully expressed in *E. coli* and purified to >90% homogeneity; it was characterized physically (SDS-PAGE and Western blot analysis) and functionally (protease activity using a fluorogenic peptide substrate).
- A high throughput (96-well plate) protease inhibitor screening assay was set up and validated using the known serine proteinase inhibitor, aprotinin.
- An in-house small molecule library of ~3000 compounds was screened in two successive phases. In each phase, initial screening was done at one drug concentration, followed by selected compounds at several serial dilutions.
- Two rounds of screening in phase I identified the benzimidazole molecule MB21 as a potent inhibitor of DENV-2 NS2b-NS3pro enzyme activity (IC_{50}=5.9µM). *In silico* docking analysis revealed that MB21 binds to a hydrophobic cleft of an allosteric site close to the active site of the protease enzyme.
- MB21 (at 30µM) which was found to be not cytotoxic to Vero cells was shown to inhibit the replication of all four DENVs using two independent assays: (i) synthesis of the viral antigen NS1, considered a marker of DENV replication, was inhibited by MB21 in the case of all four DENV serotypes, based on NS1 ELISA; (ii) the production of infectious virus by all four DENVs was also inhibited, based on determination of residual viral titer using plaque assay. The magnitude of inhibition in both assays was statistically significant.
- Two rounds of screening in phase II identified the quinoline molecule BT24 as a much more potent inhibitor of the purified recombinant NS2b-NS3pro enzyme (IC_{50}=0.5µM).This molecule fits in to the substrate binding site of the protease and presumably acts by interfering with productive NS2b-NS3 interaction, based on *in silico* docking analysis.
- In cell-based assays, BT24 was also found to be pan-DENV inhibitor. This was confirmed based on NS1 ELISA and plaque assay, for monitoring viral antigen synthesis and infectious virus production, respectively; additionally, the pan-DENV inhibitory capacity of BT24 was also corroborated by real-time analysis of viral genomic RNA.

- Despite their pan-DENV inhibitory potential, appreciable levels residual viral replication was discerned. This prompted the exploration of RNAi as an antiviral strategy to bring down the levels of DENV genomic RNA.
- A new siRNA target site, ‘sh5c’ was identified to be fairly conserved in the viral genomic RNAs among the four DENV serotypes. A replication-defective adenoviral (rAd) vector encoding a short hairpin RNA complementary to the ‘sh5c’ sequence on DENV genomic RNA (rAd-sh5c) was designed and created.
- The rAd-sh5c vector was physically characterized by analyzing its genomic DNA by restriction digestion and insert-specific PCR. In parallel, two more rAds (rAd-sh5a and rAd-sh3c), targeting two additional conserved putative siRNA target sites, identified in a previous study, were also created and characterized.
- Of the 3 rAds created, rAd-sh5c was found to be the most efficient at inhibiting the replication of all four DENVs by at least 65%, based on NS1 ELISA data. Further, the ‘antiviral state’ induced by rAd-delivered RNAi was found to be durable, lasting several days.
- The DENV-inhibitory activity of rAd-mediated RNAi was evident even when DENV infection was pre-established in Vero cells before introducing rAds encoding different shRNAs.
- Comparison of rAd-sh5c (created in this study) with rAd-sh5b (reported earlier) revealed the latter to be a more potent pan-DENV inhibitor. This was established on the basis of reduction in viral antigen synthesis, RNA synthesis and infectious virus production.
- The observation that rAd-sh5c *per se* did possess appreciable inhibitory potency suggests that a mixture of rAdsh5b+rAdsh5c would minimize the emergence of escape mutants that can manifest immunity to RNAi.

Finally, this work has demonstrated the feasibility of developing two protease inhibitors, MB21 and BT24, with both possessing the capacity to inhibit the replication of all four DENVs in cultured cells; it has also identified an additional, conserved siRNA target site, ‘sh5c’ on the DENV genome and demonstrated that a rAd vector can deliver a specific shRNA into infected cells to bring about significant inhibition of all four DENV serotypes (Figure 6.1).

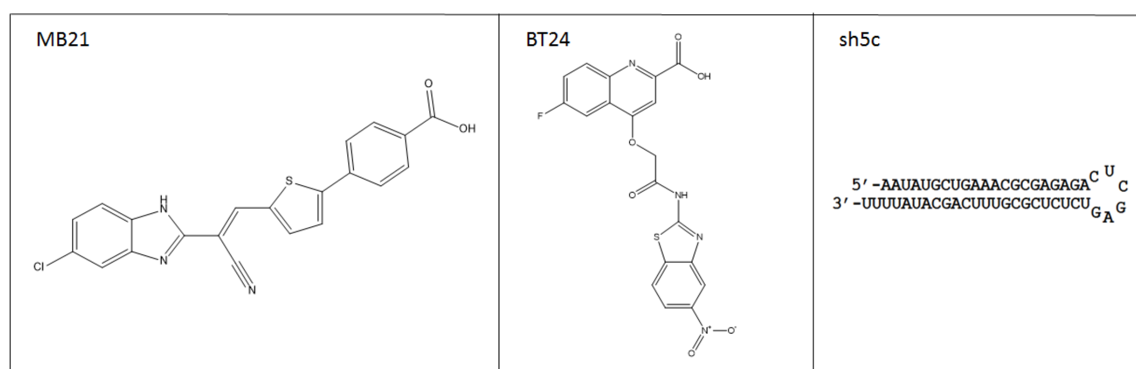

Figure 6.1: Pan-DENV inhibitors identified in this study

6.3 Limitations

This is essentially a preliminary study based on (i) *in vitro* biochemical enzyme assay and (ii) cell-based assay to identify putative pan-DENV inhibitors.

Incomplete inhibition: There is residual viral replication which can potentially give rise to resistance. This can overcome the antiviral effect. The reasons for incomplete inhibition are not clear. The cell-based experiments have examined only one dose of the drugs (MB21 and BT24) and rAds (rAd-sh5b and rAd-sh5c). In case of MB21 and BT21, it is likely that adequate intracellular concentrations were not attained to achieve more potent inhibition. In the case of the rAds, entry into cells would not be an issue. However, there may be subtle serotype differences at play which determine the accessibility of the putative siRNA target sites to rAd-mediated RNAi.

In vivo targets: As both MB21 and BT24 have been selected based on their ability to inhibit DENV-2 NS2b-NS3pro in an enzyme assay, it could be assumed that these drugs exert their antiviral effect by targeting the protease in infected cells. The precise mechanism of action of these drugs and the identity of their true *in vivo* target(s) need to be elucidated.

In vivo efficacy: The data generated in this work is not sufficient to be able to predict the *in vivo* efficacy of both the protease inhibitors as well as the shRNA encoding rAds. This can be ascertained only using an appropriate small animal model system.

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CHAPTER 7: FUTURE PERSPECTIVES

The work presented in this thesis has: (a) identified two small molecule inhibitors, MB21 (a benzimidazole derivative), and BT24 (a quinoline derivative), of the DENV NS3 protease from an in-house library of ~3000 compounds; (b) identified a conserved siRNA target (sh5c) site in the DENV genome to silence DENV viral RNA; (c) developed a replication-defective adenovirus vector (rAd-sh5c) to deliver an siRNA targeting this site into DENV-infected cells. Initial cell-based assays have demonstrated that both the small molecules (MB21 and BT24), as well as the rAd-sh5c vector, are independently effective at inhibiting all the four DENV serotypes. Some of the implications and future perspectives of this work are discussed below.

7.1 Targeting DENV NS3 protease with small molecule inhibitors

One concern in the context of developing antiviral drugs for dengue stems from the imperfect replication machinery utilized by DENVs. The lack of proof-reading function of DENV RdRp enzyme can result in errors in replication. This opens up the possibility that one or more such errors in the DENV genomic RNA sequence may confer drug resistance. A practical way of addressing such a situation would be to develop a cocktail of drugs to minimize the possibility of emergence of such drug resistant mutants. In this context, the availability of the two pan-DENV inhibitors will enable experimental testing of a mixture of both these drugs, on each of the four DENVs, to determine the frequency of emergence of drug resistance.

Apart from studies on drug resistance emergence, it would be important to modify the basic MB21 and BT24 scaffolds using medicinal chemistry to improve inhibitory potency from the micromolar range to sub-nanomolar range.

As a next step, these drugs would need to be tested *in vivo* using a small animal model, such as the DENV-susceptible AG129 mouse. Under certain experimental conditions, these mice can manifest some of the symptoms of dengue disease. The effect of these drugs in mitigating the disease symptoms in this model can indicate if further development of these drugs is warranted. The next step would be to evaluate the absorption, distribution, metabolism and excretion (ADME studies) to assess the feasibility of further development.

7.2 Targeting DENV genomic RNA using rAd-delivered RNAi

RNAi interference is potentially useful as an antiviral strategy. While it has been shown to be capable of inhibiting many viruses, challenges in targeted siRNA delivery have precluded its utility. In this context, the use of a rAd vector has important implications that need to be carefully investigated. Two aspects are relevant to the proposed use of a rAd vector as a potentially useful tool to deliver RNAi-mediated silencing of DENV RNA: (i) as adenovirus is ubiquitous in nature, the prevalence of anti-adenoviral antibodies in the human population is very high. Reports exist which have shown that anti-adenovirus antibodies can bind to adenovirus and target its uptake into Fc receptor bearing monocytes and macrophages. (ii) These very cells are the sites of DENVs replication *in vivo*, in infected individuals. Taken together, this actually makes adenoviral vectors useful in the context of using them to deploy RNAi against DENVs. The demonstration in this work that an adenoviral vector can be utilized to deliver siRNA into infected cells raises the potentially interesting possibility that pre-existing anti-adenovirus antibodies could bind to the rAd vector encoding siRNA and deliver it into the very cells in

which DENV replicates *in vivo*. This exciting possibility needs to be investigated both *in vitro* and *in vivo*, in an animal model. The outcome of such experimentation can shed further light on the utility of rAd-mediated RNAi to silence DENV expression.

Finally, in the context of possible emergence of mutant DENVs that can circumvent inhibition, one may envisage using the NS3 protease inhibitors in conjunction with RNAi-mediated silencing to further raise the genetic barrier against possible drug resistance.

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Note: This thesis cites regular journal papers (section 8.1) as well as patents (section 8.2) and clinical trials (8.3).

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8.2 Patents

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APPENDICES

List of publications/presentations

1. **Beesetti H**, Khanna N and Swaminathan S. (2014). Drugs for dengue-a patent review (2010-2014). *Expert opinion on therapeutic patents*. **24(11)**:1171-1184.

2. Raut R*, **Beesetti H***,Tyagi P, Khanna I, Jain SK, Jeankumar VU, Yogeeswari P, Sriram D and Swaminathan S. (2015). A small molecule inhibitor of dengue virus type 2 protease inhibits the replication of all four dengue virus serotypes in cell culture. *Virology Journal*. **12**:16.
** Equal Contributors*

3. **Beesetti H**, Khanna N and Swaminathan S. (2016). Investigational drugs in early development for treating dengue infection. *Expert Opin Inves Drugs*. **25(9)**:1059-1069.

Presentations:

1. Mishra B, Advait, **Beesetti H**, Swaminathan S, and Raviprasad A. Elucidating the role of essential RNA secondary structural elements in dengue biology and their implication in dengue virulence. 17th International Congress on Infectious Diseases (organized by the International Society for Infectious Diseases, ISID), March 2-5, 2016, Hyderabad.

2. Raut R, **Beesetti H***, Tyagi P, Khanna I, Jain SK, Jeankumar VU, Yogeeswari P*, Sriram D and Swaminathan S. A small molecule inhibitor of dengue virus type 2 protease inhibits the replication of all four dengue virus serotypes in cell culture. Sipra Innovative Pharma Research Awards-2015, Hyderabad.
   **presenters*

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Brief biography of the student

Ms. Hemalatha Beesetti completed her Bachelor's in Biotechnology and Bioinformatics from Acharya Nagarjuna University and was awarded "Akhila Bharathiya Saraswathi Prathiba Puraskar award" by ABVP on standing first in her course. She has done her Master's in Biotechnology and Bioinformatics from SRM University, Chennai and was awarded Gold medal on standing first in the University. She has a book publication with ISBN-No: 978-3-8443-1378-9 titled "p21ras"-The Hidden target for Cancer-An *in silico* analysis published by Lap Lambert Academic publication, Germany in 2011. She worked as DBT-JRF in Assam University for 6 months with GATE fellowship. She later joined BITS in 2013 with DST-INSPIRE fellowship from Department of Science and Technology (DST) for pursuing PhD. She was awarded SRF by DST in 2015 on successful completion of 2 years with 2 international publications.

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Brief biography of the supervisor

Dr. Swaminathan is recognized nationally and internationally for his work on dengue. He joined BITS Pilani-Hyderabad campus as a full professor in July 2013 after serving as a senior scientist for 17 years at the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi. His background is in protein biochemistry, molecular biology and molecular virology. He has over 28 years of post-PhD research experience centred on adenoviruses and dengue viruses. He has supervised/co-supervised fifteen national/international Ph.D. students, mentored three post-docs and published his work in several international peer-reviewed journals such as Proceedings of the National Academy of Science, USA, EMBO Journal, Nucleic Acids Research, Journal of Virology, Gene Therapy, Journal of Biological Chemistry, Vaccine, American Journal of Tropical Medicine & Hygiene, PLoS ONE, PLoS Neglected Tropical Diseases and Lancet Infectious Diseases, besides contributing invited scholarly reviews and book chapters.

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EXPERT OPINION

1. Introduction
2. Dengue viruses
3. Experimental dengue drugs
4. DENV drugs in clinical development phase
5. Conclusion
6. Expert opinion

Drugs for dengue: a patent review (2010 – 2014)

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Introduction: Almost half the global population is estimated to be at risk of contracting dengue infection. Of the 400 million infections estimated to occur annually, 4 million can be potentially life-threatening leading to vascular leakage and shock. The only treatment available to severe dengue patients is fluid replacement therapy and supportive care. A drug for treating dengue is an urgent need.
Areas covered: This article endeavors to provide an overview of the experimental dengue drugs being developed around the world as reflected in the recent patent literature spanning the last few years (2010 – 2014).
Expert opinion: Dengue drug development is essentially in its infancy and currently hobbled by multiple factors including a poor understanding of the molecular mechanism of severe disease and lack of reliable small animal model for preclinical drug evaluation. More intense R&D coupled to setting up product development partnerships to facilitate the efficient movement of a drug molecule from the laboratory to the clinic is needed to make antiviral therapy for dengue a reality in the coming future.

Keywords: dengue antiviral, entry blocker, herbal antiviral, NS3 protease inhibitor

Expert Opin. Ther. Patents [Early Online]

1. Introduction

An estimated 3.6 billion people around the world are at risk of dengue [1]. This is a mosquito-borne viral disease caused by four antigenically closely related serotypes of dengue viruses (DENV-1, -2, -3 and -4), which belong to the genus *Flavivirus*, family *Flaviviridae* [2]. A recent study estimated that ~ 400 million people suffer DENV infections a year with nearly a quarter of these manifesting clinically overt disease [3]. Clinical symptoms of DENV infections range from self-limiting mild dengue fever (DF) to potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [4]. A multitude of factors including among others, the failure to eradicate the mosquito vector, massive and unplanned urbanization, overpopulation, increasing global travel and the increasing co-circulation of all four DENV serotypes, have collectively contributed to dengue disease emerging as a very significant public health challenge of the century [5-7]. Consequently, at the current time, dengue is endemic to > 100 tropical and subtropical countries around the globe [4], whose cities experience sudden and explosive epidemics periodically [8].

Tackling dengue effectively continues to be a challenge. Each DENV serotype can cause the full spectrum of dengue disease. This makes it mandatory for any prophylactic or therapeutic to be effective against all four DENV serotypes. Further, the mechanism underlying the pathogenesis of severe disease is far from clear. Most importantly, a small animal model that can effectively recapitulate all manifestations of dengue disease accurately is not available, making it extremely difficult to understand the pathogenesis as well as to evaluate investigational drugs. However, the increasing realization that dengue does not respect geographical boundaries has spurred considerable efforts to explore dengue vaccines and drugs, with the major

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Article highlights.

- About half the global population is at risk of dengue, a viral disease that can be potentially life threatening.
- Currently, the only treatment available is supportive care in a hospital setting.
- Drugs that can reduce viremia levels in the early phase of infection may halt progression of dengue to severe disease.
- Strategies to develop dengue drugs target multiple viral and host factors.
- Dengue drug discovery is being accomplished through high-throughput computational, target- and infected cell-based screening assays.
- Several 'hits', with inhibitory potency in the micromolar range, have been identified. Data from the few clinical trials completed to date are not encouraging.
- Dengue drug development faces enormous challenges stemming from lack of: i) a clear understanding of disease pathogenesis and ii) a reliable animal model of the disease.
- As dengue drugs are needed most in resource-poor countries, not-for-profit product development partnerships are required to facilitate drug development.

This box summarizes key points contained in the article.

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focus on the former. Live attenuated virus (LAV) vaccine candidates, which held much promise, have run into unexpected hurdles [9,10], presumably stemming from the enigmatic role of dengue antibodies both in protection as well as pathogenesis [11] and a poorly defined phenomenon known as viral interference when all LAVs corresponding to the four DENV serotypes are co-administered [12]. This setback has sharply underscored the urgent need for dengue drugs. This article provides a brief background to dengue and then endeavors to provide an overview of the different experimental dengue drugs described in the patent literature in the recent past (2010 – 2014). For more comprehensive information on experimental dengue drugs described in the peer-reviewed literature, the reader is directed to several recent excellent comprehensive review articles on the subject [13,14].

2. Dengue viruses

2.1 Genomic organization

DENVs share a common genomic organization with the rest of the flaviviruses. The DENV genome is a single-stranded (ss) (+) sense RNA molecule, which is ~ 11 kilobases long. It contains one single open reading frame (ORF), flanked by non-translated regions (NTRs) of ~ 100 nucleotides (nts) at its 5′ end and ~ 450 nts at its 3′ end. This ORF encodes a ~ 3400 amino-acid residue long polyprotein which is subjected to the combined action of viral and host proteases during and after translation, converting it into three structural proteins, the capsid (C), the membrane (M, which is synthesized first as a precursor, prM) and the envelope (E), and seven nonstructural (NS) proteins, NS1, 2a, 2b, 3, 4a, 4b and 5 [2].

In the mature dengue virion, the genomic RNA is associated with the C protein in a nucleocapsid complex, surrounded by a host-derived lipid bilayer studded with the E and prM(M) proteins. The prM protein plays a role in virus maturation, whereas the E protein mediates host cell-surface receptor binding and host membrane fusion. The NS proteins on the other hand are concerned principally with virus replication. Of these, the roles of NS3 and NS5 are best characterized. Both are multifunctional enzymatic proteins present in the cytoplasm of the infected cell and jointly function as the DENV replicase. The NS3 protein has a specific serine protease activity localized to its N terminus and a RNA helicase-cum-triphosphatase activity at its C terminus. The NS5 protein on the other hand manifests a methyltranferase activity at its N terminus and an RNA-dependent-RNA polymerase (RdRp) activity at its C terminus. Not much is known about the remaining NS proteins, all of which are nonenzymatic. NS1 is a secreted protein, whereas NS2a, 2b, 4a and 4b are membrane-associated proteins. All of these are essential for viral replication [2]. Additionally, NS1 is implicated in pathogenesis [15], NS2b in providing cofactor function to the NS3 protease (NS3pro) [16] and NS4b in countering the host antiviral response [17]. The NTRs flanking the ORF mediate RNA–RNA and RNA–protein interactions crucial to DENV replication and viability through conserved, complementary sequences and stem-loop structures, respectively [2,18].

2.2 Life cycle

Current understanding of the DENV life cycle has been reviewed extensively [6,19,20]. A brief overview is presented here in the context of antiviral drug development. The DENV life cycle commences when a virion effects entry into a susceptible host cell. Entry is mediated by the interaction of the E glycoprotein with the host cell-surface receptor. The E protein is organized into three discrete domains, envelope domain I (EDI), EDII and EDIII [21]. Of these, EDIII mediates host receptor recognition [22]. The precise nature of the DENV receptor is not very clear. Several cell-surface molecules such as glycosaminoglycans, dendritic-cell-specific intracellular adhesion molecule-3 grabbing non-integrin, DC-SIGN, mannose receptor [23], syndecan [24] and many more have been identified as potential DENV receptors. Receptor binding is followed by clathrin-mediated endocytosis [25]. Once internalized, the acidic pH of the endosome triggers conformational changes in the E glycoprotein leading to fusion of the viral and host membranes. This results in the release of the viral genome into the cytoplasm where it serves as a template for translation. The resulting polyprotein precursor matures to give rise to the 10 viral proteins mentioned above. The availability of the NS proteins provides an as yet unidentified cue that switches the genomic RNA to function as a template for transcription. Viral RNA synthesis proceeds through a complementary 'minus' strand intermediate.

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Box 1. Attributes of an ideal DENV drug.

Effective against all 4 DENVs
Rapid action
Capacity to reduce viral titers ≥ 2 logs
Low or no significant toxicity
High genetic barrier to emergence of resistance
Stable to high temperature and humidity
Affordable in dengue-endemic countries

DENV: Dengue virus.

Box 2. Potential advantages of DENV drugs.

Prophylactic in endemic regions
Therapeutic in preventing dengue fever to dengue hemorrhagic fever/dengue shock syndrome progression
Treating complications from unequal immune protection
Curbing transmission during outbreaks
Lowering disease burden, healthcare costs

DENV: Dengue virus.

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Replication occurs in discrete host membrane-derived compartments called vesicle packets. Virion assembly which takes place in the lumen of the rough endoplasmic reticulum (ER) involves coating of the newly synthesized RNA with C protein to generate the nucleocapsid core. The prM and E proteins associate into heterodimers and surround the nucleocapsid to generate the immature virion particle which buds out into the Golgi vesicles. During its passage through the *trans*-Golgi network, maturation of the virion particle takes place with host furin-mediated cleavage of the prM protein to M protein, with concomitant structural rearrangements, marking the final maturation event. Finally, the mature virion exits the cell by exocytosis [6,19,20].

2.3 Dengue disease and pathogenesis

Dengue disease manifests itself as mild DF, accompanied by headache, retro-orbital pain, severe general body ache and skin rashes, or more severe and potentially fatal DHF/DSS, characterized by very high viremia, thrombocytopenia, hemorrhage and vascular leakage [4,10]. It has been suggested that these three disease conditions may represent progressively severe stages of a continuous spectrum of dengue disease [10]. Although the mechanism underlying progression of DF to DHF/DSS is not clearly understood, pathogenesis presumably has a multifactorial basis [6]. DENV infections tend to be serious in infants who have antibodies to a different DENV serotype acquired through maternal transfer, and in children and adults having antibodies to a heterotypic DENV serotype through a primary infection [11,26,27]. This underlies the widely accepted antibody-mediated enhancement (ADE) hypothesis that cross-reactive non-neutralizing antibodies can bind heterotypic DENV and promote their uptake into monocytes and macrophages *via* the Fc receptor (FcR) pathway, leading to increased virus replication and virus load [11]. This is consistent with observations of high levels of viremia in severe cases of heterotypic DENV infections [28,29]. It is thought that the body reacts to this high virus load by producing high levels of cytokines, which damage the endothelium and lead to capillary leakage [6].

3. Experimental dengue drugs

Supportive fluid therapy under trained medical supervision is currently the only treatment option available to patients suffering from the severe forms of dengue disease [10]. The > 10-fold higher levels of viremia observed in DHF/DSS patients as compared to DF patients [28,29] suggests that reducing the virus load rapidly during the early phase of dengue disease may be linked to favorable prognosis. This, in the context of the recent availability of rapid diagnostic tests based on NS1 antigen detection [7], provides the rationale that underlies current efforts that seek to develop antiviral therapeutic agents against dengue [14].

Conceptually, an antiviral drug against DENV may be targeted against one or more of the critical steps in the DENV life cycle to achieve effective downregulation of virus replication and thereby achieve a reduction in virus load. During natural DENV infection, viremia declines very rapidly in 24 – 48 h [29]. Thus, early diagnostic confirmation of dengue must be followed by treatment with a DENV antiviral drug that can elicit a quick reduction in virus load. The desirable attributes of an ideal DENV drug are listed in **Box 1**. That a dengue antiviral drug is feasible stems from the availability of modern drug design techniques that have led to successful development of antiviral agents against other viruses such as HIV type 1 (HIV-1), hepatitis B virus (HBV) and influenza virus that cause chronic (HIV-1 and HBV) as well as acute (influenza) disease [14]. Another factor contributing to the feasibility of drugs for dengue that target the virus stems from the availability of structural data for almost all the DENV proteins [30]. This is being used to carry out structure-based *in silico* docking analysis to aid rational drug design. It is anticipated that when a DENV drug becomes available it can have several potential applications in addressing dengue as a public health problem (**Box 2**).

High-throughput screening (HTS) strategies are being pursued to develop useful dengue drugs. Broadly, these approaches seek to target the virus to inhibit either its translation and/or replication or factors co-opted by the virus to effect entry into, maturation in and egress from, the host cell. An alternate approach that does not rely on specific virus or host target knowledge is based on screening for antiviral agents using replication-based high-throughput cell-based assays. This article confines itself to an overview of the recent patent literature that encompasses candidate DENV drugs being developed, using one or more of the approaches targeting viral, host or unknown factors (**Tables 1,2 and 3**).

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Table 1. Entry blockers.

Drug	Target	Activity*	Mouse efficacy‡	Patent no [ref.]
mAb 14c10.8	E	0.328 μg/ml§	+ (D1)	US0259871 (2013) [31]
mAb 9E-H2L2	E	0.021 – 0.167 μg/ml¶	+ (D2)	WO089647 (2013) [33]
mAb 9F12	Envelope domain III	0.02 – 1.95 ng/μl¶	+ (D2)	WO093335 (2010) [36]
RDW031	Bilayer	-	-	US0016244 (2010) [37]
Dendrimer	DC-SIGN	-	-	WO000721 (2011) [38]
-	Syndecan	-	-	US0189275# (2013) [24]

*Entry-blocking activity of mAb was assayed in terms of virus neutralization in a cell-culture-based assay; values shown are mAb concentrations that can reduce virus infectivity by 50%.
‡*In vivo* efficacy tested using AG129 mouse model; the '+' sign indicates that the drug manifested *in vivo* efficacy when tested against DENV-1 (D1) or DENV-2 (D2).
§Tested against DENV-1.
¶mAb active against all four DENVs.
#This patent does not disclose any drug; it claims the possible utility of host syndecan as an antiviral target.
-: Data not reported/available.

Table 2. Enzyme inhibitors.

Drug	Target	Activity (assay)	Patent no [ref.]
Piperine*	NS3Pro‡*(D2)*	-	MY142050 (2010) [47]
Ivermectin	NS3Pro	-	US0035284; WO134135 (2013) [45]
8-OH-quinoline	NS3Pro‡*(W)*	2.35 – 63.12 μM¶ (DENV-2 replicon assay)	US0038962 (2014) [46]
Ivermectin§	NS3 helicase‡*(Ku/Ko)*	0.7 μM¶ (DENV-2 VYRA)	US0208778 (2012) [55]
2,6, di-amino 2′ C-methyl Pu-NMP	RdRp	1.3 μM¶ HCV replicon assay	CA2836579 (2012) [60]
6-substituted 3′-azido 2′3′-dideoxy Pu-NMP	RdRp	-	WO091386 (2010) [61]
I-2,I-9,I-10	RdRp	1.9 – 15 μM# (DENV-4 VYRA)	CA2837242; WO168348 (2012) [59]
CPD-428	RdRp	0.05 μM¶ (HCV1a replicon assay)	US0190297 (2013) [63]
CM-9-78 (DNJ derivative)	α-glucosidase	6.8 μM¶ (DENV-2)	US0189771 (2011) [66]
UV-4** (DNJ derivative)	α-glucosidase	-	US0237567 (2013) [67]

*Obtained from the plant *Piper nigrum*.
‡Tested in biochemical assay using recombinant enzyme from ^{D2}DENV-2, ^{W}West Nile virus, $^{Ku/Ko}$Kunjin and Kokobera viruses.
§Hits identified by *in silico* screening of small molecule libraries.
¶Activity data shown are EC_{50} values.
#Activity data shown are IC_{50} values.
**Showed *in vivo* efficacy against DENV-2 S221 challenge.
-: Data not reported/available; DENV: Dengue virus; DNJ: Deoxynojirimycin; HCV: Hepatitis C virus; Pu-NMP: Purine nucleoside monophosphate; RdRp: RNA-dependent-RNA polymerase; VYRA: Virus-yield reduction assay.

Table 3. Miscellaneous targeted drugs.

Drug	Target	Activity	Mouse efficacy	Patent no [ref.]
C-derived peptide	C-lipid droplet interaction	-	-	WO159187 (2012) [70]
DC-3 (small interfering RNA)	Genomic RNA	> 2 log reduction in virus titers (pan DENV active)	+	WO 012835 (2013) [74]
IL-28, IL-29	PKR, OAS, Mx1	0.0075 – 0.032 μg/ml (DENV CPE assay)	-	US0020919 (2012) [73]
QL-XII-47	Cysteinome	> 3 log reduction in virus titers (pan-DENV active)	-	WO154778 (2013) [71]

DENV: Dengue virus.

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Information in the domain of peer-reviewed publications has been recently reviewed comprehensively elsewhere [13,14].

3.1 Drugs against known targets

3.1.1 Entry blockers

Inhibitors that can potentially block entry of DENVs into susceptible host cells are designed to target viral E glycoprotein–host receptor interaction. These inhibitors may be agents that selectively recognize either the E glycoprotein or the host cell-surface molecule(s) implicated in virus entry. Another alternative to preclude entry described in the patent literature is to cause viral membrane disruption.

Arising from the role of the E glycoprotein in mediating DENV entry into host cells, anti-E mAbs have been developed to abort host cell entry. Investigators from Singapore [31] have revealed the isolation of a mAb from Epstein–Barr virus-immortalized memory B cells of a patient who had recovered from a recent DENV-1 infection. This mAb, designated mAb 14c10.8, is specific to DENV-1, and binds to a quaternary epitope on the E dimer encompassing a site on EDI and the EDI-EDII hinge of one E monomer, and EDIII of the second E monomer, based on cryo-electron-microscopy (of DENV-1 complexed to Fab of mAb 14c10). This mAb was able to specifically neutralize DENV-1 by 50% at a concentration of 0.328 µg/ml in an *in vitro* Plaque Reduction Neutralization Test ($PRNT_{50}$ titer = 0.328 µg/ml). Further, it also manifested homotypic, but not heterotypic ADE in FcR-bearing K562 cells. This could be abrogated using either an FcR mutant (N297Q) or the Fab fragment of mAb 14c10.8. Significantly, mAb 14c10.8, administered either 24 h before (at 1 – 5 µg/mouse) or 24 h after (at 250 µg/mouse) DENV-1 infection conferred protection in the AG129 mouse model, which is a double knock-out strain lacking IFN α/β and γ receptors [32]. Based on this, it has been claimed that passive immunization with mAb 14c10.8 (at neutralizing, but not enhancing concentrations) may have both prophylactic and therapeutic potential in the context of DENV-1 infections [31].

A second group from Singapore has developed a panel of pan-DENV-specific mAbs using plasmablasts from two secondary dengue patients who had high-titer anti-DENV antibodies specific to the primary DENV serotype at the time of fever onset ensuing secondary DENV infection [33]. Using a flow cytometry-based virus neutralization assay [34], the 50% neutralization titers (NT_{50}) were in the range of 0.01 – 1 µg/ml for most of these mAbs. All these antibodies were specific only to the DENV E protein with none targeted to either prM or NS1. Also, none of these anti-E antibodies bound to EDIII, consistent with previously reported observation that natural DENV infections do not elicit a significant fraction of EDIII-specific antibody response [35]. Interestingly, ~ 8% of these mAbs neutralized all four DENV serotypes. The most potent of these, mAb 9E-H2L2, derived from the patient who had experienced a primary DENV-2 infection followed by a secondary DENV-3 infection, manifested NT_{50} titers of 0.04, 0.044, 0.167 and 0.021 µg/ml, respectively, against DENV-1, -2, -3 and -4. With regard to protection *in vivo* using the AG129 model mentioned above [32], several of these antibodies administered 5 – 24 h (100 µg/mouse) prior to DENV challenge at 3×10^6 plaque-forming units (PFU)/mouse, conferred protection, in terms of reduction in viremia, only to that serotype of DENV seen by the patient (from whom these mAbs were derived) during primary infection. This suggests that the capacity of these mAbs to neutralize additional DENV serotypes (other than the primary DENV serotype) is merely a manifestation of cross-reactivity and does not correlate with protective efficacy [33].

Yet another E-specific mAb has been disclosed which binds to an epitope defined by amino-acid residues K-305, K-307, K-310 and G-330 located within EDIII. This EDIII-specific antibody, mAb 9F12, was revealed to be a potent inhibitor of all four DENV serotypes with $PRNT_{50}$ values in the range of 0.2 – 1.95 ng/µl [36]. As EDIII is implicated in host receptor recognition and binding [22], EDIII-specific mAb 9F12 is likely to block binding of DENV to the host cell receptor. Consistent with this, a fusion inhibition assay in C6/36 cells performed in the presence and absence of mAb 9F12 failed to result in any difference in the degree of syncytia formation [36]. *In vivo* efficacy experiments using the AG129 model [32] showed that 0.1 mg mAb 9F12 administered 24 h prior to challenge with 2×10^6 PFU of DENV-2 (TSV01) conferred reasonable degree of protection on the basis of approximately fourfold reduction in viremia and approximately twofold reduction in NS1 antigenemia [36]. Efficacy data for the remaining three DENV serotypes have not been disclosed.

Lipophilic compounds such as butylated hydroxyl toluene (BHT) and its derivative, 3,5-di-*t*-butyl-4-hydroxy benzoic acid (BG4) which can disrupt lipid membranes can act as virucides of a variety of enveloped viruses. Essentially, disrupting the integrity of enveloped viruses by these virucides renders them incapable of successful entry into host cells. In an effort to enhance both the solubility and potency of such virucides, a recent invention has disclosed a glucose ester of butylated hydroxy benzoic acid, designated 'RDW031'. This compound, which can act as a prodrug at significantly lower doses compared to BHT or BG4, has been shown to manifest potent antiviral activity against herpes simplex virus, HSV-1 (an enveloped virus), but not against poliovirus (a nonenveloped virus). As DENVs are also enveloped viruses, it has been suggested that they may also be susceptible to this class of drugs [37].

While the antiviral strategies above target the E glycoprotein or the lipid envelope of the virus to block DENV entry into host cells, alternate approaches seek to target syndecan [24] and DC-SIGN [38], host cell-surface molecules implicated in mediating virus entry. Syndecans are cell-surface heparin sulfate proteoglycans involved in a multitude of functions that include cell-to-cell and cell-to-matrix interactions, migration, proliferation and differentiation [39,40]. Many viruses such as

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HSV-1 [41], HIV-1 [42] and hepatitis C virus (HCV) [43] are known to co-opt cell-surface syndecans as attachment receptors. Consistent with this, it was disclosed recently that DENV-2 (strain 16681) also utilizes syndecans during infection of several endothelial cell lines in culture [24]. Analysis of mutations in the putative heparin binding sites on E and small interfering RNA (siRNA)-mediated knock-down of syndecan expression have demonstrated that DENV-2 infection of dermal microvascular endothelial cells and brain microvascular endothelial cells are apparently mediated by syndecan-2, whereas in primary human umbilical vein endothelial cells, it is syndecan-4. This finding identifies syndecan(s) as potential host target(s) for developing DENV entry blocking antiviral agents.

Another arm of the glycan-mediated pathogen entry involves DC-SIGN. This immune system lectin binds highly mannosylated glycans of many pathogens such as HIV-1 [44]. A molecule, designated compound 12, has been developed by linking four pseudomannotriose moieties to a bis (methoxyol) propanoic dendrimer. It has been disclosed that at 50 µM, compound 12, caused a > 90% reduction in HIV-1 p24 expression [38]. As the role of DC-SIGN in mediating DENV entry is well-documented [23], it is anticipated that this drug may be potentially effective against DENV infection as well.

3.1.2 Enzyme inhibitors

3.1.2.1 NS3pro inhibitors

The patent literature discloses putative protease inhibitors identified either through computational screening [45] or through *in vitro* biochemical assays using cloned flaviviral proteases [46,47].

High-throughput computational screening of a library of commercially readily available small neutral molecules using the EUDOC docking program [48] led to the identification of an anthracene-based lead compound, ARDP0006. This compound predicted to interact with the active site, and P1 pocket residues of NS3pro was shown to inhibit DENV-2 NS3pro activity *in vitro* as well as DENV-2 replication in cultured cells efficiently with EC_{50} of 4.2 ± 1.9 µM [49]. A subsequent structure–activity relationship analysis of commercially procured ARDP0006 analogs, which identified key functional groups essential for NS2b-NS3pro inhibitory efficacy, concluded that the anthracene ring system could be a robust scaffold for developing potent inhibitors [45]. These inventors also screened DENV-2NS2b-NS3pro against a collection of ~ 2000 compounds (MicroSource Spectrum Collection), comprising a range of structurally diverse molecules. This initiative identified chemically distinct compounds, such as ivermectin, selamectin, tyrothricin, alexidine hydrochloride, hematoxylin pentaacetate and methylbenzethonium chloride [45]. Ivermectin and selamectin are analogs of milbemycin, produced by *Streptomyces* species and used to treat various parasitic infections in humans (ivermectin) and animals (ivermectin and selamectin). Tyrothricin, a mixture of cyclic polypeptides (tyrocidins and gramicidins) produced by *Bacillus brevis* is a broad-spectrum antibiotic [50]. Alexidine hydrochloride used as a mouthwash ingredient for its antibiotic activity [51] also possesses anticancer activity [52]. Methylbenzethonium chloride is a drug used in the treatment of skin-related conditions such as diaper rash and cutaneous leishmaniasis [53].

Inventors from Georgetown University experimentally screened a collection of several different small molecule libraries (including NINDS Bioactives, Chemdiv 2, Maybridge 3, ICBG fungal extracts, Enamine 1, I.F. Lab 1 and Bionet 2) against cloned West Nile virus (WNV) protease in conjunction with a specific fluorogenic substrate [46]. This initiative identified several WNV protease inhibitors of which 8-hydroxyquinoline derivatives were the most active. It was further discovered that some of these WNV protease inhibitors could inhibit the replication of WNV replicons in cell-culture-based reporter assay. Interestingly, these WNV inhibitors were also effective in curtailing the replication of DENV-2 replicons, albeit at comparatively lower efficacy. For example, in the DENV-2 replicon assay, compounds 33, 34 and 35 manifested EC_{50} values of 10.63, 34.99, and 2.35 µM, respectively (corresponding WNV replicon assay EC_{50} values were 7.95, 14.29 and 3.38 µM, respectively). In contrast, compound 36, which failed to inhibit the replication of WNV replicon, inhibited the DENV-2 replicon with an EC_{50} value of 63.12 µM, reflecting subtle differences in the susceptibilities of the WNV and DENV-2 proteases. Aside from these synthetic molecules, piperine, an alkaloid from the plant *Piper nigrum* has also been disclosed to be a moderate inhibitor of recombinant DENV-2 NS2b-NS3pro using an *in vitro* assay [47]. It is envisaged that this alkaloid could be a precursor to developing more potent DENV protease inhibitors. Herbal origin inhibitors that target unknown viral and/ or host factors are described below (Section 3.2.2).

3.1.2.2 NS3 helicase inhibitors

DENV helicase activity of NS3, which localizes to the C-terminal 75% of the full length NS3 protein, is presumed to facilitate viral RNA synthesis by resolving secondary structures present in the genomic viral RNA and in unwinding the double-stranded RNA intermediate to facilitate viral RNA replication. As this activity is energy-dependent, the NS3 helicase (NS3hel), like other members of its class, also possesses ATPase activity [2]. In the context of the observation that a DENV-2 helicase mutant fails to replicate [54], it may be envisaged that inhibitors that can block NS3hel activity could have therapeutic potential. But, NS3hel has proven to be a difficult drug target mainly because its mechanism of action has not been elucidated completely [20]. However, a consortium of European inventors adopted a novel *in silico* strategy to identify NS3hel inhibitors. First, they determined the site of ss RNA access on the helicase molecule *in silico*, followed by virtual screening for potential binders that target this site [55]. This screening exercise was performed using

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> 1200 compounds from a commercially available Library of Pharmacologically Active Compounds, Sigma-Aldrich. This identified several potential binders that target the ssRNA-NS3hel interaction site. Interestingly, ivermectin, disclosed to inhibit NS3pro above [45], was also active against cloned flaviviral NS3hel of Kunjin and Kobobera viruses. It did not inhibit helicases encoded by polio-, pox and herpes viruses. Further, ivermectin was disclosed to display inhibition of the reporter activity of a dengue replicon as well as antiviral activity against DENV-2 (NGC) in a virus-yield reduction assay (EC_{50} = 0.7 µM) [55]. These data suggest that the antiviral activity of ivermectin in DENV-infected cells presumably functions through inhibition of both protease and helicase activities of NS3.

3.1.2.3 RdRp inhibitors

The availability of nucleoside analog (NA) inhibitors as drugs against viral polymerases of HIV-1 [56], HBV [57] and HCV [58] justifies the search for DENV polymerase inhibitors. NAs are prodrug substrates of host kinases which convert them into nucleoside triphosphates (NTPs), which in turn interfere with the replication machinery, presumably by inhibiting the action of the polymerases. A few NAs are disclosed in the patent literature with some tested against HCV and others predicted to be of use against flaviviral RdRps [59-62]. However, most of these described below have not been tested against any of the DENVs.

Inventors from Hoffmann La Roche [59] have disclosed a series of NAs which they tested against representative strains of the four DENV serotypes in Huh-7 cells for protection against virus infection and in immature monocyte-derived dendritic cells (iDC) from human volunteers for reduction in viral RNA replication. Using the iDC infection assay to determine the reduction in viral RNA levels at 24 h after infection with DENV-4, these inventors obtained IC_{50} of 1.9, 16 and 15 µM, in the presence of NAs designated as I-2, I-9 and I-10, respectively. A purine monophosphate prodrug (2, 6-diamino 2′-C-methyl purine nucleoside monophosphate prodrug) was found to inhibit recombinant HCV NS5b RNA polymerase in an *in vitro* enzyme assay as well as the replication of HCV replicon in a cell-based assay (EC_{50} = 1.3 µM). This HCV RdRp-tested NA inhibitor has been claimed as a potential inhibitor of its DENV counterpart as well [60]. A series of NAs based on 6-substituted 3′-azido-2′,3′ dideoxy purine nucleoside monophosphates has been proposed as superior broad-spectrum antiviral agents. It has been claimed that in this NA series, the substituent at position 6 of the purine ring is protected to allow *in vivo* generation of a greater variety of 6-substituted purine NTPs as potent antiviral agents [61]. Novartis has revealed that 2-branched nucleosides may be used to inhibit flaviviral infections [62].

Analogs of nitrogenous bases also have potential antiviral activity, presumably because they too can interfere with the proper functioning of the nucleic acid replication machinery. Purine analogs wherein the imidazole ring is replaced either by 1,3-thiazole or by 1,3-oxazole ring, in conjunction with certain combinations of substituents on these scaffolds possess significant antiviral activity. Several such purine analogs have been identified to possess anti-flaviviral activity against HCV. For example, compound 428 when tested against HCV using a HCV-1a replicon displayed an EC_{50} value of 0.05 µM [63]. As for the NAs above, none of the purine analogs has been tested on DENVs.

3.1.2.4 α-glucosidase inhibitors

Infectious virion production in the life cycle of enveloped viruses such as DENVs is dependent on the action of the host ER α-glucosidases, which play a vital role in the maturation of the E glycoprotein. These enzymes trim the high-mannose N-linked glycan moieties on the nascent glycoprotein and thereby facilitate their interaction with the chaperones calnexin and calreticulin, during their maturation [64]. Iminosugars such as deoxynojirimycin (DNJ) which can serve as substrate analogs and competitively inhibit α-glucosidases have emerged as broad-spectrum antivirals against enveloped viruses [65]. But, their utility is limited by efficacy and toxicity issues. A novel iminosugar derived from DNJ, with superior antiviral activity, designated as CM-9-78, has been disclosed by Block *et al* [66]. This compound inhibited DENV-2 replication in baby hamster kidney (BHK) cells with an EC_{50} value of 6.8 µM. Further, these inventors have shown that another DNJ derivative called CM-10-18, together with ribavirin, a NA drug used in treating HCV infections [59], manifests a synergistic antiviral effect against DENV-2 both in cell culture and in AG129 animal model systems [66]. Another series of DNJ derivatives, designated UV-1 through UV-5, have been observed to protect Vero cells from DENV-2 infection in a dose-dependent manner [67]. Further, these compounds could also cause significant reduction in the release of virus from infected cells. Importantly, compound UV-4 when tested in the AG129 model improved the survival of DENV-2 strain S221 [68] challenged mice significantly ($p < 0.05$) at doses as low as 10 mg/kg body weight [67].

3.1.3 Miscellaneous inhibitors

Antiviral approaches aimed at exploiting dependence of DENV on host cell lipid droplets (LDs) or putative cysteine-reactive host factors have been explored in some patent applications. Stimulation of the host IFN-mediated innate antiviral defense system or deployment of the endogenous RNAi pathway is also being explored as possible therapeutic approaches.

It has been shown that successful completion of the DENV life cycle involves the interaction of the viral C protein with ER-derived LDs during the viral life cycle [69]. Based on this, a group of inventors have developed two overlapping peptides to disrupt this interaction and achieve inhibition. These peptides, derived from the N terminus of the C protein, have been shown to inhibit the interaction of recombinant C protein with BHK cell-derived LDs, using atomic

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force microscopy-based force spectroscopy [70]. An alternate approach, disclosed by Gray *et al.* [71] targets the host's reactive 'cysteinome' [72]. Based on the notion that DENVs may rely on host proteins with reactive cysteine residues to successfully complete their life cycle, these inventors developed cysteine-directed agents to inhibit DENV replication. One molecule developed by these inventors, QL-XII-47, a tricyclic quinolone derivative, was found to be a broad-spectrum antiviral capable of inhibiting several different viruses including all four DENV serotypes. In a virus-yield reduction assay, this drug (at 3 μM concentration) caused > 3 log decrease in DENV-2 titers. Additionally, it was found to be without significantly cytotoxicity up to 20 μM concentration. However, the nature of the host target(s) is unknown.

It has been revealed that IFN-pathway could be stimulated to achieve general antiviral effect using IL-28 and IL-29 [73]. These inventors have demonstrated the antiviral potential of these cytokines in terms of their ability to induce the expression of IFN-stimulated genes such as *PKR*, *OAS* and *Mx*1 in mice. DENV-infected Vero cells were protected from cytopathic effects, based on neutral red dye-uptake assay, by IL-28 and IL-29 with EC_{50} values in the range of 0.0075 – 0.032 μg/ml.

In contrast to all other approaches that target either a viral or host protein, Stein and Frueh [74] have targeted the DENV RNA genome by exploiting the endogenous RNAi pathway. These inventors designed a pan-DENV-specific siRNA, designated as DC-3, which targets the highly conserved 5′ CS on the DENV genome [2]. They found that the DC-3 siRNA reduced the titers of representative strains of each of the four DENV serotypes by ~ 2 logs. In addition, using an AG129-based ADE model, DC-3 siRNA-treated mice survived for an average duration of 15 days compared to the 5 days survival time of the control group [74].

3.2 Anti-DENV drugs against unknown targets

3.2.1 *Synthetic small molecules*

A series of phenylethanone compounds with different substituents has been disclosed by a group of German inventors [75] as potential drug leads against dengue. Using q-RT–PCR assay to determine viral RNA levels in infected Vero cells, one molecule, CPD-242, was disclosed to inhibit DENV-1, -2, -3 and -4 replications with EC_{50} values of 0.04, 0.04, 0.57 and 7.87 μM, respectively. Importantly, this compound showed encouraging *in vivo* results. An AG129-based dengue viremia model [32] was used to evaluate the *in vivo* efficacy of CPD-242. Based on q-RT–PCR analysis of viral RNA in of blood samples drawn from DENV-2 (2×10^6 PFU) exposed animals, which were treated with CPD-242, EC_{50} was found to be 0.01 μM. Further, using a dengue mortality model, again based on AG129 mice which are highly susceptible to a mutant DENV-2 strain, D2Y98P, [76], CPD-242 (at 60 mg/kg body weight, twice daily) was found to significantly delay mortality ($p < 0.01$) [75].

Inventors from the Chinese Academy of Sciences have disclosed a series of quinazoline compounds with potential anti-flavivirus drug properties. Based on the reduction in replication of DENV-2 replicon in BHK cells and HCV replicon in Huh 7.5 cells, it was observed that many of these quinazoline compounds inhibited the replication of both flaviviral replicons efficiently. For example, one of the most potent drugs listed by these inventors, 5-*t*-butoxy-2,4-diaminoquinazoline (compound Yhhu-1036) manifested IC_{50} values of 0.009 μM and 1.23 μM in the DENV-2 and HCV replicon assays, respectively [77].

It has been disclosed by Dinneen and Morris [78] that acyl guanidines have potent broad-spectrum antiviral activity. For example, 5-methyl-2-naphthoyl guanidine is capable of inhibiting HIV replication in primary human macrophages efficiently in a p24-based ELISA with an IC_{50} = 1.56 μM. While it is anticipated by the inventors that both lentiviruses (such as HIV) and flaviviruses (such as HCV and DENV) would be susceptible to these acyl guanidines, experimental evidence has been provided in support of inhibitory activity against HIV alone [78].

A HTS campaign of a library of > 200,000 compounds (SIGA chemical library) has discovered that compounds broadly categorized into 2-aryl-benzothiazoles [79], thienopyridines [80] and benzenesulfonamides [81] are capable of protecting Vero cells from DENV-2-induced cytopathicity quite effectively, with EC_{50} values < 5 μM. Several of these are claimed to be effective against all four DENV serotypes. For example, the compound *N*-(4-diethylsulfonyl-phenyl)-2-(4-oxo-4H-quinazoline-3-yl)-acetamide inhibited DENV-1, -2, -3 and -4 with EC_{50} values of 4.3, 0.3, 1 and 1.7 μM, respectively [81].

3.2.2 *Herbal inhibitors*

Apart from piperine, a plant-derived molecule with moderate DENV protease-inhibitory activity referred to above [47], an assortment of plant origin inhibitors, targeting yet to be identified viral and/or host factors, can be found in the recent patent literature. Artemisinin is a sesquiterpene lactone with antimalarial activity, obtained from *Artemisia annua*, a Chinese medicinal plant. Artemisinin is also documented to possess broad-spectrum antiviral action against several different viruses, a property also shared by its semisynthetic derivative artesunate [82]. In the context of malarial treatment, artemisinin is preferably used in combination with another partner drug in order to counter the emergence of artemisinin resistance [83]. A novel artemisinin combination therapy (ACT) based on the use of a mixture of artemisinin and berberine, an alkaloid from *Berberis* plants, useful in the treatment and prevention of malaria has been disclosed to be effective against dengue as well [84]. It has been claimed that this drug mixture has been useful in mitigating the symptoms of DF in patients in the Philippines based on clinical criteria [84]. Alternate ACTs disclosed for the treatment of DF in the patent literature include mixtures of artesunate and an antiviral agent such as carrageenan [85]. Carrageenan is a linear sulfated galactose polysaccharide isolated from red seaweeds and is known

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Table 4. Dengue virus drugs in clinical trials.

Trial identifier	Drug	Developer	Phase	Trial site	Current status (end date)
NCT02045069*	Ivermectin	Mahidol University	II/III	Thailand	Yet to initiate
NCT02061358*	UV-4B	Unither Virology	I	?	Yet to initiate
NCT01973855*	Ribavirin	Guangzhou 8th People's Hospital	II	China	Ongoing (December 2015)
ISRCTN03147572[‡]	Lovastatin	Oxford University Clinical Research Unit & Wellcome Trust	I	Vietnam	Ongoing [90] (January 2015)
NCT00849602*	Chloroquine	University of Sao Paulo	I/II	Brazil	? (June 2009)
ISRCTN38002730[‡]	Chloroquine	Oxford University Clinical Research Unit & Wellcome Trust	I	Vietnam	Completed [91] (July 2008)
ISRCTN3957523[‡]	Prednisolone	Oxford University Clinical Research Unit & Wellcome Trust	I	Vietnam	Completed [92] (January 2011)
NCT02016027*	*Carica folia* extract	Fr. Muller Homeopathic Medical College	I	India	Completed[NR] (December 2013)
NCT01096576*	Balapiravir	Hoffmann-La Roche	I	Vietnam	Completed [93] (April 2011)
NCT01619969*	Celgosivir	Singapore Gen Hospital & Duke-NUS Graduate Med School	I/II	Singapore	Completed [94] (July 2013)

*Data from the International Standard Randomized Controlled Trial Number Register.
[‡]Data from the NIH ClinicalTrials.Gov Register.
?: Unknown; NR: Not reported.

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to be a DENV-2 inhibitor [86]. In contrast to the aforementioned ACTs that utilize a combination of two drugs, a recent disclosure advocates 'Tri-ACT' that entails sequential administration of artemether (methyl ethyl ether derivative of dihydroartemisinin), followed by artesunate and then berberine, for the treatment of infection by several pathogens including DENVs [87].

Another phytochemical, 5, 6, 7-trihydroxy flavone (obtained from the plant *Scutellaria baicalensis*), also known as baicalein, has been demonstrated to inhibit DENV-2 replication in Vero cells in culture by an unknown mechanism [88]. It has been claimed that this drug may not only act directly on DENV to inactivate it but also exert antiviral effect by interfering with virus attachment as well as intracellular replication [88]. In contrast to the well-defined plant-derived antiviral molecules mentioned thus far, inventors from India have revealed that alcohol extracts of the plant *Cissampelos pareira* possess significant antiviral activity against representative strains of all four DENV serotypes based on reduced NS1 synthesis and virus-yield reduction in cell-based assays [89].

4. DENV drugs in clinical development phase

Of the different experimental DENV drugs in development, very few have progressed to clinical trials. Many initially promising molecules have failed to progress further due to unsatisfactory profile as a potential drug, based on several characteristics including physicochemical properties, cellular permeability, cytotoxicity, plasma stability, oral bio-availability, preclinical efficacy and preclinical toxicity. This is illustrated by an adenosine analog, known as NITD-008, which could inhibit DENV replication efficiently and also suppress viremia and protect mice against lethal DENV challenge. But, it showed severe toxicity in rats and dogs after a 2-week oral dosing regimen (50 mg/kg/day) and had to be abandoned [14]. The drugs in clinical trials are in various stages ranging from those that are being planned to those that have been completed [90-94]. Results for some of these have been reported (**Table 4**). This includes chloroquine (CQ) [91], a drug with modest antiviral activity against several viruses [95], prednisolone [92], a corticosteroid expected to provide benefit through its anti-inflammatory action and two drugs, balapiravir [93] and celgosivir [94], initially tested against HCV.

The CQ trial revealed that the drug did not reduce the duration of viremia in dengue patients, while tending to be associated with adverse events [91]. On the other hand, prednisolone which did not precipitate significant adverse events also was without any therapeutic effect [92]. Balapiravir (also known as R1479), a NA, which was found to be unsuitable for HCV treatment (due to adverse events stemming from long term treatment), was recently tested for its efficacy against DENV in a Phase I trial [93]. Surprisingly, despite manifesting anti-DENV activity *in vitro*, balapiravir did not provide any clinical benefit to dengue patients, assessed in terms of fever duration, serum viremia titers and NS1 antigen levels. The reasons for the failure of balapiravir, despite plasma drug levels in treated patients being comparable to *in vitro* EC_{50} values, remain to be investigated. Celgosivir (also known as Bu-Cast), a drug that inhibits the host enzyme, α-glucosidase I, which did not show therapeutic efficacy against HCV, was also tested in dengue patients in a Phase Ib trial recently [94], based on encouraging efficacy data obtained in intracranial challenge [96], viremia [32] and ADE-induced lethal [97] mouse models. Yet, celgosivir did not afford any statistically significant therapeutic benefit to

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dengue patients [94], presumably reflecting the inadequacy of the existing mice models [32,96,97] in reliably predicting outcome in the human context.

5. Conclusion

That a drug for treating DENV infections is an unmet and urgent need is now well recognized. The primary purpose of an anti-DENV drug would be to prevent the progression of mild dengue disease to severe dengue. This is premised on the observation that viral titers in DHF/DSS patients are at least an order of magnitude higher than those in DF patients [28,29]. Thus, a DENV antiviral capable of suppressing viremia by ≥ 10 folds is anticipated to confer clinical benefit.

Putative DENV drugs may target either viral and/or host factors essential for successful completion of the DENV life cycle. The recent patent literature on experimental DENV drugs reveals various approaches being pursued in the quest for antiviral therapy against DENV infections. These include mAbs and drugs that may target DENV entry into susceptible cells [24,31,33,36-38], inhibitors to block the activity of enzymes encoded by DENV as well as the host cells [45-48,55,59-63,65-67], and others seeking to stimulate the host antiviral defense system [73] or tap into the endogenous RNAi pathway [74]. There is also a range of synthetic [75,77-81] and plant-origin [84,85,87-89] inhibitors, with as yet unknown targets, being identified through DENV-infected cell-based assays. Several potential 'hits' have been identified against single DENV serotypes. Many are still to be tested against the remaining three DENV serotypes. Most have been tested against recombinant enzymes in biochemical assays or as replication inhibitors in cell-based assays. Very few have been tested for protective efficacy in the AG129 mouse model. Of these, a small subset has progressed to clinical trials (**Table 4**). Some of these trials are ongoing/yet to be started, while others have been completed. Results from the completed trials are far from encouraging as illustrated by the data from balapiravir [93] and celgosivir [94] studies mentioned above. The reasons for these failures need to be understood and many more challenges overcome in the ongoing efforts to develop dengue drugs. The positive outcomes from the ongoing efforts to develop DENV drugs are the elucidation of the crystal/solution structures of almost the entire DENV proteome and the establishment of robust computational, biochemical and cell-based HTS assays.

6. Expert opinion

No specific antiviral therapy exists for dengue. The only treatment available for severe dengue patients is fluid replacement therapy under medical supervision. The increasing awareness that a preventive dengue vaccine may continue to be elusive coupled to the spread of the dengue vectors beyond the tropical boundaries has directed attention towards dengue drug development. That drugs are available for other chronic and acute viral diseases lends support to the notion that it may be feasible to develop drugs against DENV infections as well.

Based on the > 1 log difference seen in viral titers in DHF/DSS patients in comparison to DF patients, it is believed that a decrease in viremia, equivalent to this magnitude in the early phase of DENV infection, may in fact preclude progression to DHF/DSS. As DENV NS1 antigen-based early detection kits have become available in recent years, a drug can be valuable for timely treatment. Given the enormous public health threat posed by dengue with approximately half the global population 'at risk', an antiviral drug for dengue is an unmet need. This realization has resulted in the initiation of DENV drug development efforts around the world. The major objective of such initiatives is to develop a drug(s) capable of reducing viral titers by ~ 10-fold rapidly at an early stage. But will such a drug confer clinical benefit to the DF patient? This remains to be seen. A related objective would be to identify prognostic biomarkers to help predict which DF patient is likely to develop DHF/DSS.

The recent patent landscape on DENV drugs reveals multiple approaches being pursued. Essentially, at this time, DENV drug development is in the discovery phase. Several 'hits' have been identified using different screening approaches. Typically, these have been based on either a target-based biochemical assay or a cell-based assay confined to a single DENV serotype. These are yet to be tested against the remaining three serotypes. It is important for an ideal DENV inhibitor to be pan-DENV effective as all four serotypes tend to co-circulate in many regions of the world. Further, only some have been tested in the only model available to date based on AG129 mice for *in vivo* efficacy. Some drugs have reached clinical phase of development, but early indications are not very encouraging. It is necessary to pursue aggressive screening campaigns so that adequate new investigational drugs become available for development.

Developing drugs for dengue poses unique challenges on many fronts. The disease is caused by multiple DENV serotypes with all co-circulating in many parts of the world. An effective drug must act on all four DENVs. The molecular basis of severe dengue disease is far from clearly understood. Particularly, the host factors that are involved in pathogenesis of the severe dengue are virtually unknown. Most importantly, a reliable small animal model is not available for credible preclinical evaluation of *in vivo* efficacy of investigational drugs.

Developing a drug entails a time-, effort- and cost-intensive endeavor starting from basic research, screening for hits, lead identification and optimization, preclinical development, clinical trials for safety and efficacy and final drug licensure/registration. This sequence of events is dependent on multiple players to ensure smooth movement of the product from the research laboratory to the clinic. As dengue disease is endemic in the resource-poor nations where potential patients have little purchasing power, it has remained one of the neglected

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diseases. One of the challenges arising from this situation is that an assembly of interacting collaborative players, referred to above for product development, does not exist at the present time for dengue drug development. This could be addressed by evolving not-for-profit product development partnerships to facilitate DENV drug development initiatives along the lines evolved for facilitating dengue vaccine development.

Declaration of interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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A small molecule inhibitor of dengue virus type 2 protease inhibits the replication of all four dengue virus serotypes in cell culture

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Abstract

Background: Dengue has emerged as the most significant of arboviral diseases in the 21st century. It is endemic to >100 tropical and sub-tropical countries around the world placing an estimated 3.6 billion people at risk. It is caused by four genetically similar but antigenically distinct, serotypes of dengue viruses. There is neither a vaccine to prevent nor a drug to treat dengue infections, at the present time. The major objective of this work was to explore the possibility of identifying a small molecule inhibitor of the dengue virus protease and assessing its ability to suppress viral replication in cultured cells.

Methods: We cloned, expressed and purified recombinant dengue virus type 2 protease. Using an optimized and validated fluorogenic peptide substrate cleavage assay to monitor the activity of this cloned dengue protease we randomly screened ~1000 small molecules from an 'in-house' library to identify potential dengue protease inhibitors.

Results: A benzimidazole derivative, named MB21, was found to be the most potent in inhibiting the cloned protease (IC_{50} = 5.95 μM). *In silico* docking analysis indicated that MB21 binds to the protease in the vicinity of the active site. Analysis of kinetic parameters of the enzyme reaction suggested that MB21 presumably functions as a mixed type inhibitor. Significantly, this molecule identified as an inhibitor of dengue type 2 protease was also effective in inhibiting each one of the four serotypes of dengue viruses in infected cells in culture, based on analysis of viral antigen synthesis and infectious virus production. Interestingly, MB21 did not manifest any discernible cytotoxicity.

Conclusions: This work strengthens the notion that a single drug molecule can be effective against all four dengue virus serotypes. The molecule MB21 could be a potential candidate for 'hit-to-lead' optimization, and may pave the way towards developing a pan-dengue virus antiviral drug.

Keywords: Dengue fever, Dengue virus, NS2b-NS3 protease, Dengue protease inhibitor, Antiviral therapy

Background

Dengue is an arboviral disease which is currently a very significant global public health concern [1-3]. The disease is endemic to >100 tropical and sub-tropical countries. Of the ~3.6 billion people estimated to be at risk of dengue, ~400 million people experience dengue infections annually [4]. Four antigenically distinct serotypes of dengue viruses (DENV-1, −2, −3 and −4) of the genus *Flavivirus*, family *Flaviviridae*, cause this disease [5]. Clinically, the disease has been distinguished as either mild dengue fever (DF) or potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [6]. Despite decades of efforts, a preventive dengue vaccine is not available [7,8]. Ongoing efforts have revealed the existence of challenging hurdles in dengue vaccine development [9-11]. This has spurred attention towards exploring the feasibility of developing therapeutic drugs [12,13]. Observations that the virus titers in DHF/DSS patients are an order of magnitude higher in comparison to DF patients [14,15], suggest that a drug

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which can bring about ~1 log reduction in virus replication may be able to prevent the progression of DF to DHF/DSS.

The DENV genome is a single-stranded positive sense, ~11 kilobases (Kb) long RNA molecule [5]. It carries a 5' cap, but no poly A tail and contains a single large open reading frame (ORF) sandwiched between two non-translated regions located at either end. The ORF is translated in the infected host cell cytoplasm into >3000 amino acid (aa) residue long polyprotein precursor. Co- and post-translational processing of this precursor by host and viral proteases generates ten viral proteins, of which three are structural and the rest, non-structural (NS) proteins [5]. One of these latter proteins, NS3, by virtue of its function as the viral protease is crucial in the polyprotein maturation process [16]. The protease activity of NS3, located in the N-terminal one-third of the full-length molecule, contains the classic catalytic triad seen in serine proteases and relies on cofactor function, mapping to a hydrophilic 40 aa residue domain of another viral protein, NS2b [17]. Mutations in either component of the flaviviral protease that compromise its function lead to abrogation of replication [18,19]. This two component protease, NS2b-NS3Pro, has emerged as a potential antiviral drug target in recent years [16].

Ideally, a DENV inhibitor must be effective against all four DENV serotypes. This is because, each of the four DENVs can cause the full spectrum of dengue disease, and all four DENV serotypes tend to co-circulate in hyperendemic regions [2,3]. Functional profiling studies indicate that the NS2b-NS3Pro of the four DENV serotypes share very similar peptide substrate structure activity relationships [20]. Based on this notion, we have explored the feasibility of identifying an inhibitory molecule with pan-DENV-specificity by empirical screening of an 'in-house' library of ~1000 small molecular weight compounds. This paper presents the identification of a DENV-2 NS2b-NS3 protease (NS2b-NS3Pro)-inhibitory molecule which could inhibit the replication of all four DENV serotypes in infected cells in culture.

Results and discussion

Recombinant DENV-2 NS2b-NS3Pro

We cloned and expressed DENV-2 protease in *E. coli* and purified it to >90% homogeneity using modifications of previously reported methods [20-22]. The design of a synthetic *NS2b-NS3Pro* gene, its expression in *E. coli* and its purification by Ni^{2+}-NTA affinity chromatography are summarized in Additional file 1: Figures S1 and S2. Using the synthetic fluorogenic peptide Benzoyl-Nle-Lys-Arg-Arg-4-methylcoumarin-7-amide (Bz-nKRR-AMC), which has been shown to be a better substrate compared to peptides containing endogenous dengue cleavage sites [20], we confirmed that our purified DENV-2 NS2b-NS3Pro is enzymatically active based on the increase in fluorescence that accompanies peptide cleavage (Figure 1). Assay conditions were optimized to identify enzyme and substrate concentration ranges compatible with a linear dose–response (Figures 1A, and B). To validate this assay for inhibitor screening, we tested the effect of the protease inhibitor aprotinin, on the catalytic activity of DENV-2 NS2b-NS3Pro enzyme. Aprotinin is a serine protease inhibitor which can bind NS2b-NS3 strongly [20], and inhibit it effectively at nanomolar concentrations [21]. Our data showed that aprotinin inhibited the recombinant protease activity effectively (IC_{50} = 20nM; Figure 1C).

Compound MB21 inhibits DENV-2 NS2b-NS3Pro

With a functionally validated DENV NS2b-NS3pro assay in hand, we next proceeded to screen an 'in-house' library of ~1000 small molecular weight compounds to identify potential inhibitors. Recent work has shown that this library contains antimicrobial compounds [23,24]. An initial screen wherein these compounds were tested at a single concentration (25 μM), identified 25 compounds which manifested >80% inhibition of the recombinant NS2b-NS3Pro. One of these, a benzimidazole compound, MB21, was the most potent, manifesting an IC_{50} of 5.9 μM against the recombinant DENV-2 NS2b-NS3pro enzyme (Figure 2A). Three additional benzimidazole compounds, RB02, RA14 and RA16, also inhibited the cloned DENV-2 protease, albeit at comparatively lower efficiency (Additional file 1: Figure S3). We used *in silico* molecular docking to understand how MB21 may interact with DENV-2 NS2b-NS3Pro. This analysis showed that MB21 bound to the DENV- protease with its benzimidazole moiety embedded well within the hydrophobic cleft of an allosteric site [25], in the vicinity of the catalytic triad, as depicted in Figure 2 (panels B and C). Features of MB21 binding observed here correlate with earlier reports on allosteric binding [25,26]. To understand better the mechanism of action of MB21 on DENV-2 NS2b-NS3Pro, we determined the efficiency of protease action over a range of substrate concentrations in the absence and presence of varying MB21 concentrations (Figure 3A). These data were analyzed using Lineweaver-Burke double reciprocal plot (Figure 3B). We observed that both the kinetic parameters, K_m and V_{max}, were changed by MB21. These data lead to the conclusion that MB21 may act by as a mixed inhibitor of DENV-2 NS2b-NS3Pro. This is consistent with the *in silico* docking data which show that MB21 binds to an allosteric site.

As a next step, we sought to assess if MB21 which inhibited cloned viral NS2b-NS3Pro efficiently had any inhibitory activity on the parent virus itself. To this end we used a cell-based assay in which we infected Vero

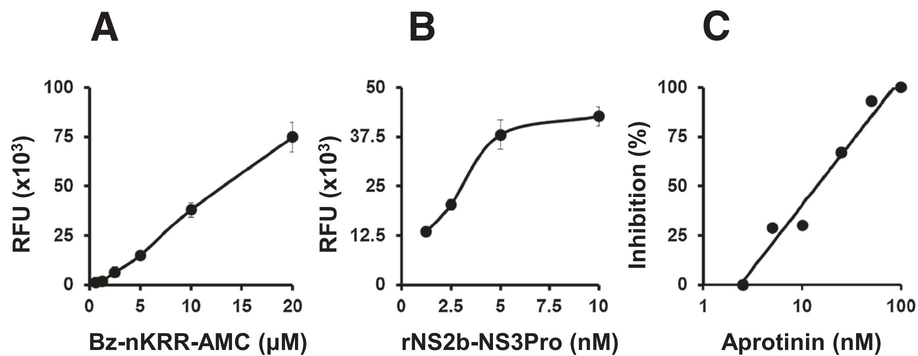

Figure 1 DENV-2 NS2b-NS3Pro enzyme assay, optimization and validation. (A) Kinetics of NS2b-NS3Pro action as a function of substrate concentration (at 5nM enzyme). **(B)** Rate of enzyme catalysis as a function of enzyme concentration (at 10 μM substrate). **(C)** Activity of the cloned NS2b-NS3Pro as function of aprotinin concentration (5nM enzyme, 10 μM substrate, 20 min incubation). Activity in the absence of aprotinin was taken as 100% (RFU = relative fluorescence units).

cells with DENV in the presence of MB21 and monitored the effect of the drug on virus replication. However, before testing MB21 for its DENV inhibitory potential, we sought to ascertain if this compound manifested any cytotoxicity on Vero cells. Interestingly, we found that MB21 at concentrations up to 50 μM (in 0.5% DMSO vehicle) did not manifest any discernible cytotoxicity compared to controls (treated with 0.5% DMSO). This was essentially the case even at 100 μM MB21 (in 1% DMSO), as evidenced by comparable cell viability between 100 μM MB21-treated and 1% DMSO-treated cells, as shown in Figure 4A. It is to be noted, however, that 1% DMSO by itself, caused ~30% loss of cell viability, compared to cells that were not treated with DMSO. Based on these results, we conclude that MB21 up to 100 μM final concentration does not manifest discernible cytotoxicity on Vero cells.

It has been shown previously that DENV replication in cultured cells [27,28] and in animal models [29] can be monitored by determining the levels of the viral antigen NS1 using immunoassays. Recently we showed that the levels of NS1 antigen secreted into culture supernatants of infected cells closely mirror the viral genomic RNA levels, measured using quantitative RT-PCR, for all four DENV serotypes [27].

MB21 is a pan-DENV inhibitor

Given the high degree of functional similarity of the NS2b-NS3Pro enzymes among the four DENV serotypes [20], we anticipated that MB21 may indeed be capable of inhibiting the replication of all four DENV serotypes. To test this, we infected Vero cells with each of the DENV serotypes separately, in the absence or presence of MB21 (at 30 μM final concentration), and monitored

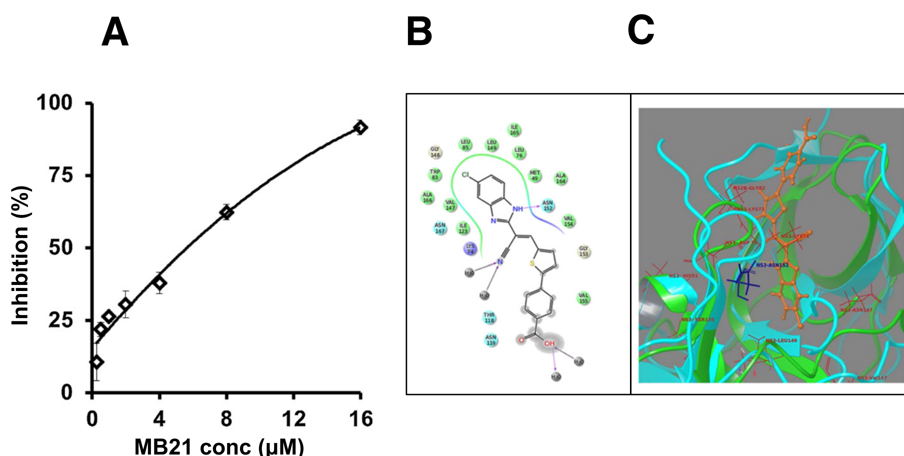

Figure 2 Inhibition of DENV-2 NS2b-NS3Pro by MB21 and *in silico* analysis of the interaction between the two. (A) Inhibition of protease activity of cloned DENV-2 NS2b-NS3Pro as a function of MB21 concentration. **(B)** Computer generated 2D ligand interaction picture depicting the interaction between MB21 and DENV-2 NS2b-NS3Pro. Hydrophobic residues are shown in green. **(C)** Interaction of MB21 at the allosteric pocket in the vicinity of the catalytic triad.

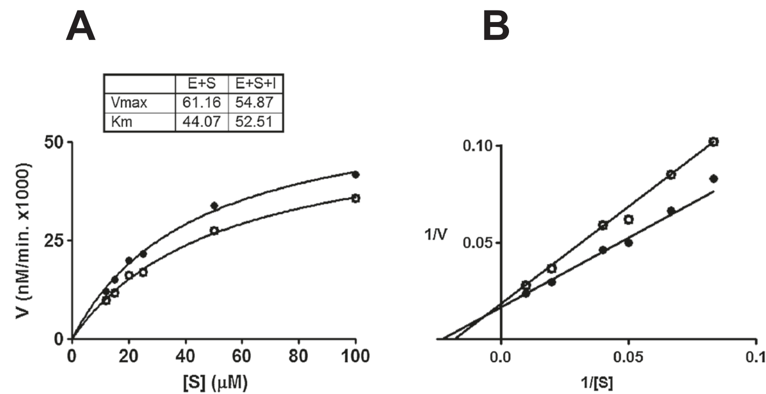

Figure 3 Mode of inhibition of DENV-2 NS2b-NS3Pro by MB21. (A) Enzymatic activity of DENV-2 NS2b-NS3Pro as a function of substrate concentration in the absence (filled circles) and presence of 6 μM MB21 (empty circles). The table shows kinetic parameters in presence (E + S + I) and absence (E + S) of MB21. E, S and I denote the enzyme, DENV-2 NS2b-NS3Pro, the substrate, Bz-nKRR-AMC, and the inhibitor, MB21. **(B)** Lineweaver-Burke plot of DENV-2 NS2b-NS3Pro activity in the absence (filled circles) and presence of 6 μM MB21 (empty circles). Data were analyzed using GraphPad Prism software.

NS1 antigen synthesis as a marker of DENV replication. Culture supernatants were withdrawn at regular intervals over a 1 week period and analyzed for NS1 antigen levels using a commercially available ELISA kit. The data comparing the kinetics of viral NS1 antigen secretion, by each one of the DENV serotypes, into the culture supernatant in the absence and presence of MB21 are presented in Figure 4 (panels B to E). These results show that MB21 did indeed causes a statistically significant reduction of NS1 antigen levels secreted by all four DENV serotypes.

If NS1 secretion goes down, mirroring the down-regulation of viral replication, it follows that final viral titers must also be reduced in the presence of MB21. To ascertain this possibility, we measured DENV titers in a virus yield reduction assay. In this experiment, viral titers in DENV-infected culture supernatants (MB21 treated as well as untreated) harvested at day 3 post-infection were measured using a standard plaque assay (Additional file 1: Figure S4). It was seen that at all dilutions of the culture supernatant tested, the number of plaques in the presence of MB21 was significantly lower compared to that in the absence of the drug. This experiment was performed with all four DENV serotypes. The viral titers calculated from the plaque counts are summarized in Table 1. These data reveal that MB21 could inhibit DENV-1, −2, −3 and −4 titers by 50, 82, 75 and 73%, respectively. This was found to be statistically significant. The observed reduction in DENV titers correlates with the decrease in viral antigen synthesis (Figure 4, panels B-E) and corroborates NS1 as a marker for DENV replication.

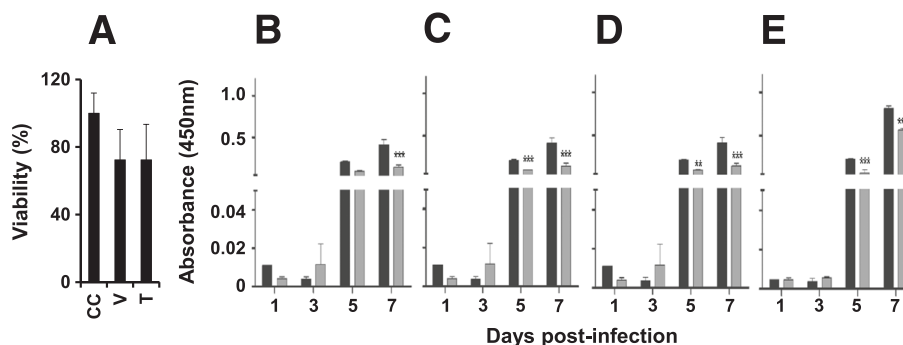

Figure 4 Evaluation of antiviral activity using cell-based assay. (A) Histogram showing the viability of Vero cells that received no drug ('CC', cell control), 1% DMSO vehicle alone ('V') or 10 μM MB21 in 1% DMSO vehicle ('T'). Panels B-E depict the effect of MB21 on NS1 secretion by DENV-infected cells. Vero cells were infected with DENV-1 **(B)**, DENV-2 **(C)**, DENV-3 **(D)** or DENV-4 **(E)** either in the absence (black bars) or presence (grey bars) of MB21. Culture supernatants withdrawn at the indicated time points during the 1 week experiment, were tested for viral antigen levels using the Dengue NS1 ELISA kit (the NS1 ELISA absorbance scale on the Y-axis is the same for panels **B**-**E**). Data shown are mean values (n = 3). The vertical bars represent standard deviation, SD. Two-way ANOVA and Bonferroni post-test analysis was done using GraphPad Prism. *P* values were either significant (**) or very significant (***).

Table 1 DENV titers[a] (x10^6 pfu[b]/ml) in the absence and presence of MB21

DENV serotype[c]	Without MB21	With MB21[d]	*P* value[e]
1	**1.06 ± 0.03**	**0.52 ± 0.02**	**0.0028*** **
2	1.54 ± 0.12	0.27 ± 0.01	0.0045***
3	0.59 ± 0.01	0.14 ± 0.04	0.0065***
4	0.22 ± 0.02	0.05 ± 0.03	0.031**

[a]Titers were determined by plaque assay on Vero cells.
[b]Pfu = plaque forming units.
[c]The viral strains used were: DENV-1: West Pac 74; DENV-2: S-16803; DENV-3: CH54389; and DENV-4: TVP-360.
[d]MB21 used at 30 μM final concentration.
[e]*P* values were calculated using Graphpad software; *P* values were either significant (**) or very significant (***).

Collectively, our data support the conclusion that MB21 is a pan-DENV inhibitor. The precise mechanism of the pan-DENV inhibitory activity of MB21 needs elucidation. Consistent with protease assay data, preliminary *in silico* docking (Figure 2) suggests that MB21 binds to the protease in the vicinity of its active site. The possibility that this binding may perturb the recently identified allosteric site (Ala125) on the protease [30] needs to be addressed. The likelihood that MB21 may compromise the ability of NS2b-NS3Pro to recruit fatty acid synthase during productive DENV infection [31] is another avenue to be explored.

Conclusions

The need for dengue drugs is being increasingly felt as dengue vaccine continues to be elusive. Based on its critical role in the DENV life cycle, NS2b-NS3Pro has emerged as a potential antiviral target. We set up and validated an *in vitro* DENV protease assay and used it to initiate a random screening campaign to search an 'in-house' small molecule compound library, from which we have recently identified molecules with antimicrobial action [23,24], for putative pan-DENV inhibitor(s). One molecule from this library, a benzimidazole compound, MB21, was a potent inhibitor of the cloned DENV-2 protease, NS2b-NS3Pro (IC_{50} = 5.95 μM). It appeared to bind to an allosteric site in the vicinity of the active site. Examination of steady state enzyme kinetics followed by double reciprocal plot analysis indicated that MB21 affects both K_m and V_{max} and presumably functions as a mixed type inhibitor of NS2b-NS3Pro. Further it did not manifest significant cytotoxicity at concentrations as high as 100 μM. Interestingly, MB21 could suppress NS1 antigen secretion by all four DENVs, suggesting that it could function as a pan-DENV inhibitor. This was corroborated by plaque assay data which showed that viral titers were indeed reduced by MB21 in the case of each of the four DENV serotypes. The precise mechanism of action of MB21 on DENV replication needs to be elucidated. This molecule may provide a lead for further optimization.

Methods

Cells, viruses, reagents

The four DENV serotypes used in this study are the World Health Organization reference strains (DENV-1 West Pac 74, DENV-2 S-16803, DENV-3 CH54389 and DENV-4 TVP-360) and were kindly provided by Dr. De Silva, University of North Carolina, USA. The monkey kidney Vero cell line was from American Type Culture Collection, Virginia, USA. It was maintained in Dulbecco's Modified Eagle medium (DMEM), supplemented with 10% (v/v) heat-inactivated (Δ) fetal calf serum (FCS), in a 10% CO_2 humidified incubator, at 37°C.

The synthetic peptide substrate Bz-nKRR-MCA was custom-synthesized (Peptides International, Louisville, Kentucky, USA). NS1 ELISA kit was from J. Mitra & Co. Pvt Ltd, New Delhi, India. MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay kit was from Invitrogen (Life Technologies, USA).

The BITS in-house small molecule library consisted of diverse small molecules that included benzimidazoles, benzothiazoles, quinolones, thiazoles, thiazolidines, azetidines and spiropiperidones among others. Details of synthesis of the benzimidazoles identified as DENV-2 protease inhibitors are provided in Additional file 2.

Protease and protease inhibition assays

Protease assays were carried out in 100 μl volume in microtiter wells of 96-well plates, essentially as described earlier [20]. A typical protease reaction (100 μl) contained 5nM purified DENV-2 rNS2b-NS3Pro enzyme (15 ng protein) in assay buffer (50 mM Tris–HCl, pH 8.5/1 mM CHAPS/20% glycerol). The reaction was initiated by the addition of peptide substrate Bz-nKRR-AMC (10 mM stock) to a final concentration of 10 μM. The reaction was incubated at 37°C for 20 minutes. Protease activity was measured in terms of the increase in fluorescence that accompanied cleavage of the peptide substrate (λ_{ex}: 380 nm; λ_{em}: 450 nm). Control reactions in which the protease was omitted were run in parallel to correct for background fluorescence of the substrate. To measure protease inhibition, the test compound was incorporated into the protease reaction prior to substrate addition. Enzyme control (EC) reactions set up in parallel contained an equivalent amount of the vehicle (DMSO) without any inhibitor. Half maximal inhibitory concentration (IC_{50}) was defined as the inhibitor (test compound) concentration that decreased protease activity by 50%, with reference to the EC reaction (which was taken as 100%), under the experimental conditions. All assays were run in duplicates or triplicates and each experiment was performed at least twice independently.

Vero cell-based DENV inhibition assay

The titers of the stock viruses used were as follows: DENV-1: 1.6×10^6; DENV-2: 1.3×10^6; DENV-3: 1.5×10^7; & DENV-4: 1.5×10^9 PFU/ml. Vero cells were seeded in 48-well plates (4×10^4 cells/well in 0.5 ml DME + 10%ΔFCS) and incubated for 24 hours (37°C, 5% CO_2). Monolayers were aspirated and treated with 0.5 ml DME + 0.5% with ΔFCS containing 30 μM final concentration of the test compound. After 1 hour incubation medium + test compound was removed and saved. The monolayer was infected with DENV (m.o.i = 0.1; 200 μl/well in DME + 0.5% with ΔFCS). After 2 hours, the virus inoculum was removed and replaced with medium containing test compound. The plate was returned to the incubator. Drug concentration was maintained by the addition of 5 μl stock compound (equivalent to 30 μM final concentration) solution into each well on days 3 and 5 post-infection. Aliquots (20 μl) of culture supernatant were withdrawn at indicated time points up to 7 days for estimation of NS1 antigen by ELISA and viral titers by plaque assay. Appropriate virus controls (VC) for each DENV serotype, wherein the drug treatment was omitted, were run in parallel. All infection experiments were done twice independently. Inhibition by a test compound was assessed with reference to VC which was taken to represent 100% infectivity.

DENV NS1 determination

Culture supernatants collected at various time points, which were stored frozen at −20°C, were thawed and diluted appropriately (1:100 to 1:1000 in DME + 0.5%ΔFCS). Suitable aliquots (50 μl) of this were used to detect DENV NS1 antigen using a commercially available Dengue NS1 ELISA kit (J. Mitra & Co., India), as per the manufacturer's protocol. This kit uses N- and C-terminal domain-specific anti-NS1 antibodies to detect the NS1 antigen produced by all four DENV serotypes.

MTT assay

Vero cells were seeded in a 96-well microtiter plate (5,000cells in 200 μl DME + 5%Δ FBS) were exposed to different test compounds at a range of concentrations (2-100 μM) for four days at 37°C in a 10% CO_2 incubator. Control wells received an equivalent amount of DMSO vehicle without the test compound. Cell viability was assessed based on the reduction of MTT using a commercial kit as per the manufacturer's instructions.

Statistical analysis

The statistical significance between MB21-treated and untreated samples was assessed using GraphPad Prism v6 for Windows. Differences were considered statistically significant when the probability levels (P) were <0.05.

In silico studies

Molecular docking of MB21 onto the three-dimensional crystal structure of DENV-2 NS2b-NS3Pro (pdb code: 2FOM) obtained from the protein data bank (www.rcsb.org) was performed using GLIDE extra precision module (Glide v5.7, Schrodinger, LLC, New York, NY) as described [32].

Additional files

Additional file 1: Supplementary results Figures S1-S4.

Additional file 2: Synthesis of benzimidazoles.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

RR carried out DENV infection assays, NS1 ELISAs and plaque assays. HB carried out the library screening work, participated *in silico* docking studies and helped with the initial manuscript draft. PT helped clone and purify the viral protease. IK and SKJ helped with study design. VUJ and DS carried out chemical synthesis. PY performed *in silico* docking studies. DS and PY helped in study coordination. SS conceived and designed the study, coordinated it and wrote the final manuscript. All authors read and approved the final manuscript.

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REVIEW

Investigational drugs in early development for treating dengue infection

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ABSTRACT

Introduction: Dengue has emerged as the most significant arboviral disease of the current century. A drug for dengue is an urgent unmet need. As conventional drug discovery efforts have not produced any promising clinical candidates, there is a shift toward re-positioning pre-existing drugs for dengue to fast-track dengue drug development.

Areas covered: This article provides an update on the current status of recently completed and ongoing dengue drug trials. All dengue drug trials described in this article were identified from a list of >230 trials that were returned upon searching the World Health Organization's International Clinical Trials Registry Platform web portal using the search term 'dengue' on December 31st, 2015.

Expert opinion: None of the handful of drugs tested so far has yielded encouraging results. Early trial experience has served to emphasize the challenge of drug testing in the short therapeutic time window available, the need for tools to predict 'high-risk' patients early on and the limitations of the existing pre-clinical model systems. Significant investment of efforts and resources is a must before the availability of a safe, effective and inexpensive dengue drug becomes a reality. Currently, supportive fluid therapy remains the only option available for dengue treatment.

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1. Introduction

Dengue viruses (DENVs), of which there are four antigenically distinct serotypes (DENV-1, DENV-2, DENV-3, and DENV-4), are transmitted to humans predominantly through the mosquito vector *Aedes aegypti*. These are '+' sense RNA viruses of the *Flaviviridae* family [1]. Recently updated maps of vector prevalence demonstrate the spread of *Aedes* mosquitoes across all continents [2]. Dengue disease, which can be caused by any of these four DENVs, has been declared by the World Health Organization (WHO) to be the fastest spreading arthropod-borne viral disease of this century. About 3.6 billion people around the world, in more than 100 tropical and sub-tropical countries, are at risk of DENV infection [3]. Many of these countries are hyper-endemic with multiple co-circulating DENV serotypes. The incidence of dengue has registered ~30-fold increase in the past five decades [4]. This resurgence is linked to population expansion, unplanned urbanization, increased global travel, climatic changes, lack of robust public health facilities, and failure to eradicate the vector during this time span [5]. While the true disease burden is a matter of conjecture as many countries where dengue is prevalent lack robust surveillance mechanisms, recent estimates indicate that there are ~400 million infections annually, of which ~25% cause clinical illness [6].

Heightened awareness of dengue as a global public health problem in recent years has spurred significant efforts to develop tools to address it. There has been a special emphasis on the development of dengue vaccine. The enigmatic role of anti-DENV antibodies, in protection as well as pathogenesis, has made vaccine development complex and challenging [7,8]. A key requirement of successful vaccine is that it must afford simultaneous robust and durable protection against all four DENV serotypes. Recent phase III trials of a live attenuated vaccine have revealed that the vaccine is not very effective against DENV-2 [9,10]. This unexpected result, which underscores the need to explore other vaccine alternatives, has also renewed interest in fast-tracking drug discovery and development. The reader is referred to recent reviews for a detailed discussion of the various drugs in pre-clinical development [11,12]. The resources invested in dengue drug discovery are modest in comparison to those invested for other antiviral drugs, and dengue drug discovery and development efforts have not produced a promising clinical candidate. A shift in strategy toward re-purposing existing drugs for dengue is discernible and has led to a limited number of clinical trials. This article will focus on such dengue drug trials reported since 2010 and additional trials that are either planned or underway in the various clinical trial registries. Most of these are randomized, double-blinded, placebo-controlled trials listed in the WHO's International Clinical Trials Registry Platform [13].

2. Dengue disease and its pathogenesis

Symptomatic dengue illness manifests itself as continuum of clinical entities [8], dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS), associated with four criteria, namely, fever, hemorrhage, thrombocytopenia,

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Article highlights

- Dengue is a very significant public health concern of the century.
- It is a mosquito-borne viral disease infecting ~400 million people each year; close to a million of these infections can be potentially fatal.
- Supportive care under trained medical supervision is the only treatment available; a dengue drug is an urgent need.
- Conventional drug discovery efforts have not so far produced any promising leads.
- Old drugs for other indications are being re-purposed for dengue to accelerate dengue drug development.
- Early clinical trials data are not very encouraging; clinical trials have underscored the hurdles in dengue drug development.
- Significant resources and efforts are required before a safe, effective and inexpensive dengue drug becomes available.

This box summarizes key points contained in the article.

and plasma leakage [8,14]. The illness, which initially manifests itself as mild DF, coincident with viremia, is self-limiting, and lasts for about 5–7 days. Viremia begins to decline around 72 h of the illness, and the fever begins to subside by days 4–5 of illness. Clinically, this phase known as defervescence [15], is recognized to be critical. While in most cases, full recovery follows, in a small percentage of patients, the illness becomes severe with accompanying hemorrhage, thrombocytopenia, and plasma leakage (DHF). If fluid loss is substantial, there could be circulatory failure and hypovolemic shock (DSS) [16].

Not much is known about the risk factors that predispose a DF patient to DHF/DSS or the mechanism underlying this progression to severe dengue disease. The pathogenesis of severe disease presumably has a multifactorial basis and is not well understood. However, there appears to be a link between secondary DENV infection and severe dengue disease. Studies in endemic settings have revealed that the viremia levels in DHF/DSS patients (undergoing secondary DENV infection) is an order or two higher in magnitude compared to those in DF patients [17,18]. Increased viremia apparently results from pre-existing heterotypic antibodies (from the primary infection) facilitating entry of the secondary DENV into FcR-bearing cells through a phenomenon termed antibody dependent enhancement (ADE) [19]. It is believed that the immune system responds to the high virus load by overproducing inflammatory cytokines [20], such as tumor necrosis factor-α (TNF-α), which damage the capillary endothelium [5,21]. A role for TNF-α in pathogenesis is suggested by the effect of anti-TNF-α antibodies in protecting against vascular leak-associated lethal dengue infection in a mouse model [22]. In this respect, the mechanism of dengue pathogenesis appears to share some degree of similarity with that of other diseases, such as sepsis and atherosclerosis, which are also associated with endothelial dysfunction [23]. One aspect of vascular endothelium damage appears to involve disruption of the integrity of its glycocalyx lining [24].

3. Drugs for dengue

DHF and DSS are potentially fatal and if untreated can be associated with up to 20% mortality. Supportive fluid therapy by trained medical personnel continues to be the only available strategy to treat severe cases of dengue illness. Judiciously administered fluid therapy in a medical facility can bring case fatality rates down to <1% [25]. A safe and effective dengue drug remains an unmet need. The fact that antiviral drugs have been successfully developed against HIV type 1, hepatitis B, and influenza viruses [12], suggests that it is feasible to apply modern drug discovery and development approaches for dengue therapy as well. The observation of high levels of viremia in DHF/DSS patients compared to DF patients [17,18] has prompted the understanding that a drug, which could lower viremia rapidly during the early stages of dengue disease could lead to favorable prognosis. This rationale has spurred several dengue drug discovery and development initiatives for more than a decade now [12,26]. As all four DENVs co-circulate together in hyper-endemic areas, an ideal dengue antiviral drug should be non-toxic and pan-DENV inhibitory, capable of reducing viral titers >2 logs to alter the course of disease favorably. Viremia in DF declines starting at ~72 h into the illness, highlighting the fact that therapeutic antiviral intervention must be not only early, but aggressive, to ensure favorable outcome in case of patients at risk of progressing to DHF/DSS.

4. Drugs in clinical development

As mentioned earlier, rational dengue drug discovery initiatives that have been underway for over a decade, are yet to identify promising clinical candidates. In parallel efforts, to accelerate the development of a drug for dengue, a handful of small molecules, being utilized/tested for other indications, have entered clinical trials for assessment of their utility for dengue therapy [27–34]. The small molecule drugs [35] in clinical trials are shown in Figure 1. Of these, only two classes of compounds, the iminosugars and nucleoside analogs, could be regarded as antivirals, developed initially as candidate drugs against infection by a related flavivirus, hepatitis C virus (HCV). The iminosugars, which are inhibitors of host endoplasmic reticulum (ER)-resident α-glucosidases, can interfere with maturation of a several viruses, while the nucleoside analogs are designed to inhibit viral RNA-dependent RNA polymerases (RdRp). The remaining compounds being tested as dengue drugs include the anti-malarial drug chloroquine (CQ), the anti-helminthic drug ivermectin, corticosteroids, statins, and dietary mineral and vitamin supplements (Tables 1 and 2) [36–38]. Some of the developing countries where dengue is endemic have initiated trials (Table 3) to test traditional herbal medicines for dengue therapy as well [36,38,39]. Figure 2 provides a schematic overview of the DENV life cycle and indicates the targets of candidate drugs in clinical trials. The following sections provide the rationale underlying the testing of these drugs for dengue and their current clinical status.

4.1. *Anti-parasitic drugs*

Two drugs, CQ (anti-malarial drug) and ivermectin (anti-helminthic drug) have drawn attention as possible dengue drugs for different reasons. CQ, by virtue of its lysosomotropic alkalinizing action has the potential to interfere with endosome acidification process essential to the cellular

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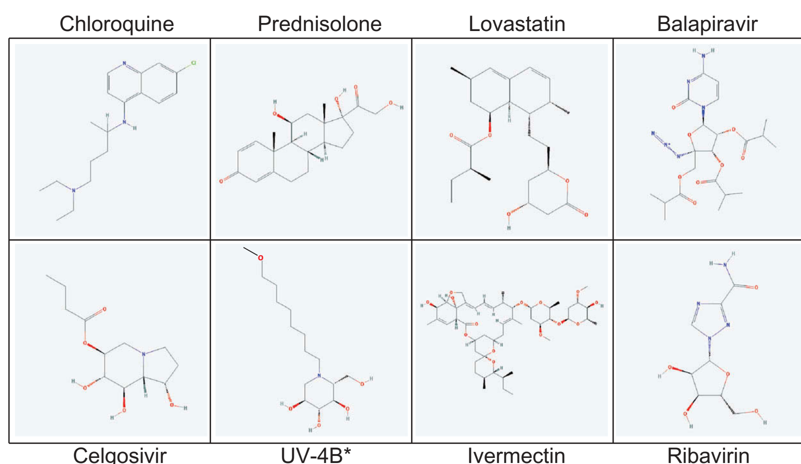

Figure 1. Chemical structures of the small molecules in clinical development for dengue treatment. Compound structures were retrieved from the NCBI PubChem Compound Database [35]. The CIDs for the compounds in the database are as follows. Chloroquine: 2719; Prednisolone: 5755; Lovastatin: 53232; Balapiravir: 11691726; Celgosivir: 60734; Ivermectin: 6321424; and Ribavirin 37542. The asterisk on UV-4B indicates that it is not listed in the PubChem database. N-Nonyl deoxynojirimycin structure (CID: 501640) available in the database was modified by adding a methoxy group to the N-Nonyl side chain at the 9th position to get UV-4B.

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Table 1. Dengue therapeutic clinical trials completed and reported since 2010.

Drug class	Drug (developer)	Trial identifier[a]	Phase[b] (*n*; age)	Trial site	Start and end dates	Ref. [no.]
Anti-malarial	Chloroquine (University of Sao Paulo and Sao Paulo Research Foundation)	NCT00849602	I/II (132; >18 year)	Sao Paulo, Brazil	February–May 2008	Borges et al. [27]
	Chloroquine (Oxford University, UK)	ISRCTN38002730	U (307; >15 year)	Ho Chi Minh City, Viet Nam	July 2006–March 2008	Tricou et al. [32]
Anti-inflammatory	Prednisolone (Oxford University, UK)	ISRCTN39575233	U (225; 5–20 year)	Ho Chi Minh City, Viet Nam	August 2009–January 2011	Tam et al. [31]; Nguyen et al. [29]
	Lovastatin (Oxford University, UK)	ISRCTN03147572	U (330; >18 year)	Ho Chi Minh City, Viet Nam	November 2012–January 2015	Whitehorn et al. [33,34]
Nucleoside analog	Balapiravir	NCT01096576	Phase I (64; 18–65 year)	Ho Chi Minh City, Viet Nam	July 2010–April 2011	Nguyen et al. [30]
Iminosugar	Celgosivir (Singapore General Hospital and Duke NUS Med School, Singapore)	NCT01619969	Phase I/II (50; 21–60 year)	Singapore	July 2012–July 2013	Low et al. [28]

[a]Trial identifiers retrieved from WHO's International Clinical Trials Registry Platform web portal [13]; trial identifiers with 'NCT' prefix are from the US NIH's ClinicalTrials.gov registry [36]; trial identifiers with 'ISRCTN' prefix are from International Standard Registered Clinical/soCial sTudy Number Registry.
[b]In some instances, the trial phase has not been specified; this is indicated as 'U' (unknown).

invasion by many viruses including DENVs. On the other hand, ivermectin, was identified as a putative inhibitor of DENV NS3 protease as well as helicase during *in silico* screening campaigns of commercially available drug molecules [11].

4.1.1. CQ

The anti-malarial drug CQ is an inexpensive, commonly available, and well-tolerated drug. The rationale for testing CQ as an antiviral drug against DENV stems from multiple lines of evidences. CQ can inhibit the replication of members of several viral families including *Flaviviridae* [41]. It is a weak base with a tendency to accumulate in the acidic organelles of cells and interfere with endosome-mediated viral entry. It has been documented that pre-treatment of mammalian cells with CQ can reduce the efficiency of DENV infection [42], while CQ exposure after DENV infection can attenuate the infectivity of the progeny virus [43]. Aside from affecting DENV directly, CQ may also act on host factors. CQ can interfere with endosome acidification necessary for host toll-like receptor-dependent signaling in response to virus infection [44], and also modulate antigen presentation in dendritic cells [45]. Its ability to downregulate pro-inflammatory cytokines [46,47] may explain its potent antipyretic activity. Two different trials, one in Viet Nam and the other in Brazil, have been conducted to test the possible clinical benefit of CQ therapy in dengue patients (Table 1).

The CQ trial (ISRCTN 38002730) in Viet Nam, involved >300 laboratory-confirmed dengue patients (18–28 years) [32]. CQ dosage regimen was similar to that used routinely for *Plasmodium vivax* malaria. This study found no significant differences in viremia clearance times and time to negative NS1 antigenemia between the CQ and placebo arms. Secondary endpoints, namely, fever clearance time, platelet nadir, and hemoconcentration, also did not manifest

Table 2. Ongoing/planned dengue therapeutic trials.

Drug class	Drug (developer)	Trial identifier[a]	Phase (*n*; age)	Trial site	Start and end dates	Comments
Iminosugar	Celgosivir (Singapore Gen Hospital, Duke NUS Med School and 60 Deg Pharma LLC, Singapore)	NCT02569827[b]	Phase Ib/IIa (72; 21–65 year)		July 2016–August 2018	Yet to initiate
	UV-4B (Unither Virology and Quintiles, Inc.)	NCT02061358	Phase I (64; 18–45 year; healthy subjects)	Kansas, USA	July 2014–September 2015	Ongoing/data awaited
Anti-parasitic	Ivermectin (Mahidol University, Thailand)	NCT02045069	Phase II/III (360; >15 years)	Bangkok, Thailand	February 2014–March 2016	Ongoing
Nucleoside analogs	Ribavirin (Guangzhou 8th People's Hospital, China)	NCT01973855[c]	Phase II (300; 18–65 year)	China	January 2012–December 2015	Ongoing/data awaited
Dietary supplement	Zn bis-glycinate (Srinakharinwirot University, Thailand)	TCTR2015111000	Phase II/III (60; 1–15 year)	Thailand	November 2015–June 2016	Ongoing
	Vitamin E (University of Colombo, Sri Lanka)	SLCTR/2015/012	Phase IV (100; 5–12 year)	Sri Lanka	June 2015–September 2015	Ongoing/data awaited

[a]Trial identifiers retrieved from WHO's International Clinical Trials Registry Platform web portal [13]; trial identifiers with 'NCT' prefix are from the US NIH Clinical Trials Registry and Results database [36].; TCTR: Thai Clinical Trials Registry [37]; SLCTR: Sri Lanka Clinical Trials Registry [38].
[b]In addition to celgosivir, this trial will also assess the effect of modipafant (inhibitor of platelet activating factor, an inflammatory mediator).
[c]Ribavirin is being tested in combination with Chinese herbal drug.

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Table 3. Dengue therapeutic trials using herbal extracts from traditional medicine.

Herbal extract (developer)	Trial identifier[a]	Phase[b] (*n*; age)	Trial site	Start and end dates[b]	Comments
DENPAP (Goan Pharma)	CTRI/2014/06/004660	U (15; 18–60 year)	Karnataka, India	May 2014–U	Completed; data not available
Caripill (Micro Labs)	CTRI/2015/05/005806	U (300; 18–60 year)	Karnataka and Andhra	June 2015–U	Ongoing
Papaya leaf extract (Bioextract & JIPMER, India)	CTRI/2014/10/005120	Phase IV (60; 18–60 year)	Puducherry, India	U	Yet to initiate
Papaya leaf mother tincture (Fr Mueller Homeopathic Med College, India)	NCT02016027	Phase I (60; 18–25 year; healthy subjects)	Fr Mueller's College, India	September 2013–December 2013	Completed; data not available
Papaya leaf juice (University of Sri Jayewardenepura, Sri Lanka)	SLCTR/2013/005	U (82; 18–60 year)	Colombo, Sri Lanka	January 2013–June 2014	Completed; data not available
Tanreqing injection (Guangdong Provincial Hospital of Chinese Medicine and Shanghai Kai Bio Pharma)	ChiCTR-IPR-15006778	U (316; 18–70 year)	China	September 2015–U	Ongoing

[a]Trials retrieved from WHO's International Clinical Trials Registry Platform web portal [13]; trial identifiers with 'NCT' prefix are from the US NIH Clinical Trials Registry and Results database [36].; SLCTR: Sri Lanka Clinical Trials Registry [38]; CTRI: Clinical Trials Registry-India [40]; ChiCTR: Chinese Clinical Trials Register [39].
[b]In some instances, the trial phase and/or trial dates have not been specified; this is indicated as 'U' (unknown).

discernible differences between the drug-treated and untreated groups. CQ also did not have any detectable effect on the proportion of activated T cells or plasma levels of cytokines. Despite this lack of an immunomodulatory effect, the CQ group appeared to have lesser number of DHF cases compared to the placebo group. However, this was not statistically significant. In addition, CQ treatment correlated with a higher incidence of mild adverse events [32].

The Brazilian CQ trial (NCT00849602) was a smaller one involving 129 adult dengue patients using a similar dose regimen of CQ as in the Viet Nam CQ trial. Unlike the Vietnam trial, the Brazilian trial subjects were all mild DF patients. CQ did not have any effect on either duration of the disease or its intensity. However, a subset of patients experienced minor benefit. Twelve out of 19 CQ-treated subjects reported substantial decrease in intensity of pain while on the drug. The study authors attribute this to anti-inflammatory action of CQ [27].

The reasons for the failure to observe a CQ-mediated antiviral effect is not clear. The pH-modulating action of CQ observed *in vitro*, which is important for antiviral activity, is presumably not manifested *in vivo*. As pharmacokinetic analysis was not performed, it is not known if CQ achieved adequate inhibitory concentrations *in vivo* at the dosage used.

4.1.2. Ivermectin

Ivermectin, a broad spectrum anti-parasitic drug, licensed for human use for more than two decades [48], has recently been demonstrated to have antiviral action on DENVs. This stems from its ability to inhibit three viral enzymes, NS3 protease, NS3 helicase, and NS5 polymerase of DENVs. The former two enzymes were identified as targets of ivermectin through *in silico* analysis. *In vitro* experimentation corroborated that NS3 helicase is indeed a target, with ivermectin manifesting EC_{50} inhibitory potency in the sub-micromolar range [49]. In

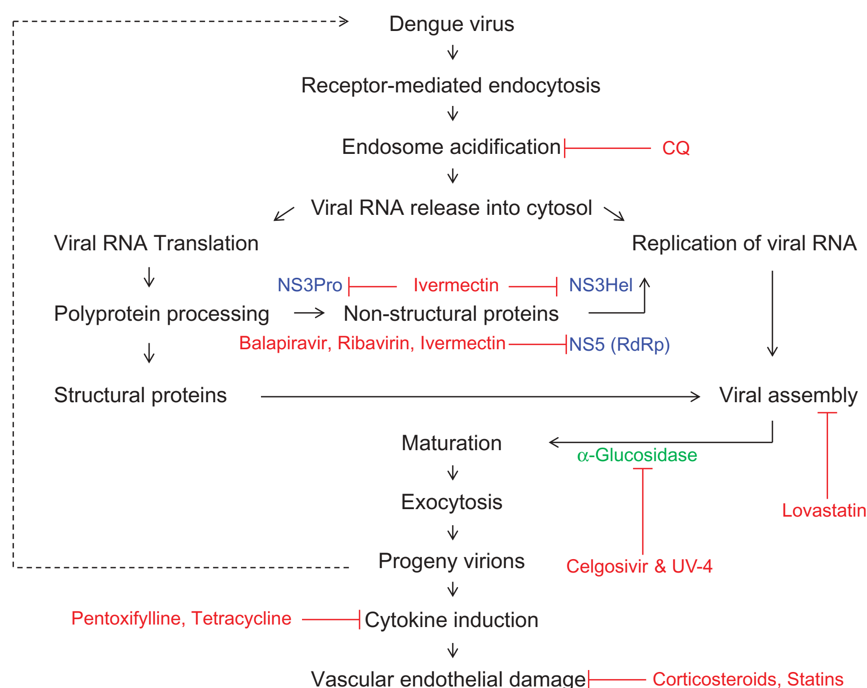

Figure 2. DENV life cycle indicating the sites of action of candidate dengue drugs. Essential steps in the DENV life cycle include endocytosis of the virion following its recognition and binding to an unknown cell surface receptor. Viral and host membrane fusion, triggered by endosome acidification, is followed by uncoating and release of the viral genomic RNA into the cytosol where it serves as the template for translation and replication. Viral RNA translation produces a polyprotein precursor which is cleaved by the combined activity of host protease and the viral NS3 protease (NS3Pro) into processed structural and non-structural (NS) proteins. Viral replication is mediated by the viral NS3 helicase (NS3Hel) and NS5 (the viral RNA-dependent RNA polymerase, RdRp). The structural proteins and the newly replicated viral RNA associate to form immature virions which mature during their transit in the endoplasmic reticulum (ER) and the trans-Golgi network before exiting the cell as mature virions by exocytosis. The host immune response to increased DENV load induces cytokine overproduction which is held to be responsible for the vascular endothelial damage associated with severe dengue disease. The dengue drugs that are described in this review are shown in red. Some of the drug targets are shown either in green (host target) or blue (viral targets). CQ blocks endosome acidification. Statins and corticosteroids are implicated in maintaining/stabilizing the integrity of vascular endothelium. The target of lovastatin in interfering with viral assembly is unknown.

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addition to this, ivermectin is also reported to interfere with DENV infectivity by blocking importin α/β-mediated nuclear localization of DENV RdRp [50]. A later study, which observed ivermectin-mediated inhibition of interaction of NS5 polymerases of both DENV-1 and DENV-2 with the nuclear import machinery, found the drug to be able to inhibit the replication of all four DENVs in infected cells in culture [51]. A phase II/III (NCT02045069) trial to test ivermectin's safety and efficacy in >350 dengue patients is underway in Thailand (Table 2).

4.2. Anti-inflammatory agents

An acute inflammatory response leading to endothelial damage is a common thread that appears to link viral hemorrhage, such as that associated with sever dengue illness [20] and other diseases, such as sepsis and atherosclerosis [23]. This presumably provides the basis for testing corticosteroids and statins, drugs used for the latter non-viral illnesses, for dengue treatment as well. It is likely that these drugs, with their immunomodulatory and anti-inflammatory activities, may be of utility in dengue treatment. In fact, a pilot study showed that decreasing the levels of the pro-inflammatory cytokine TNF-α using a specific inhibitor, pentoxifylline (trental), could be of potential utility in treating DSS [52]. A report from Mexico suggests that inflammatory cytokine production in dengue patients undergo significant reduction during tetracycline therapy [53].

4.2.1. Corticosteroids

Clinically, corticosteroid adjunct immunomodulatory therapy is adopted when the host immune response is suspected to cause disease. Severe dengue, characterized by capillary endothelial damage and plasma leakage, is one such instance. As it tends to manifest itself at a time point when viremia and fever are largely resolved [54], it is held to be precipitated by immunopathological events triggering a strong inflammatory response culminating in damage to capillary endothelium [5,18,21]. Preliminary evidence has implicated transient disruption of the integrity of glycocalyx lining of the vascular endothelium [24]. Further, a role for steroids in protecting the functional integrity of the endothelial glycocalyx lining has emerged in recent years [55,56]. Observational studies suggest that corticosteroids may be of benefit to a patient experiencing DSS [31]. These considerations have prompted efforts to evaluate the effect of corticosteroid therapy in dengue patients.

A recent study in Viet Nam (ISRCTN39575233), examined if steroid therapy administered to >200 laboratory confirmed dengue patients (5–20 years) during the febrile viremic phase, is associated with any potential risk (Table 1). This was a dose escalation study which examined a low (0.5 mg/kg) and a high (2 mg/kg) dose of prednisolone given orally for 3 days within 72 h of fever onset [31]. This study demonstrated that steroid therapy during the early acute phase of dengue did not increase or prolong viremia. In addition, there

were no significant adverse effects except for a trend toward hyperglycemia in the high dose group. The trial investigators did not find any difference in fever clearance times between drug-treated and untreated groups. The trial results also showed no indication of possible efficacy in terms of reduction in the development of severe dengue-related signs and symptoms. However, it is to be noted that the trial was not powered to assess efficacy. As adequate *in vivo* drug levels were likely achieved based on indirect signals, the lack of clinical benefit may be a reflection of having initiated drug therapy too late during the course of illness [31]. Further investigation of whole blood gene expression by microarray analysis revealed that genes expressing T cell and NK cell effector proteins were downregulated in the high-dose prednisolone group. This suggests that prednisolone at the higher dose attenuates the host antiviral response. Cytokine analysis did not reveal any significant differences between corticosteroid-treated and placebo groups. In addition, a comparison of the cytokine profiles during the acute and convalescent phases did not reveal any evidence of immunomodulation [29]. Overall, prednisolone neither affected the host immune response nor influenced disease progression in this trial.

4.2.2. Statins

Aside from their blood cholesterol-lowering activity, statins also manifest anti-inflammatory and immunomodulatory effects. Statins appear to have a role in minimizing the inflammation-induced damage to capillary endothelium [57]. Given that dengue pathogenesis is closely linked to endothelial dysfunction, statins which can have a potentially stabilizing effect on vascular endothelium may be of clinical benefit to dengue patients. In addition, lovastatin is implicated in interfering with virion assembly *in vitro* [58,59]. These considerations, coupled to the well-documented safety profile of statins [60] provide the rationale for testing lovastatin in dengue patients. Recently, sequential phase 1 and 2 trials (Table 1) were completed to evaluate the safety of lovastatin therapy in dengue patients [33,34]. These were randomized, double blinded, placebo-controlled trials, carried out during 2012–2015. Two different doses of 40 and 80 mg lovastatin, given once daily for 5 days in >300 dengue patients (≥18 years), were tested for safety and tolerability. Frequency of adverse events (such as hepatic dysfunction, thrombocytopenia, mucosal bleeding) was comparable in both groups. In addition, no discernible differences were seen in fever clearance and dengue viremia between the treatment groups. Comparison of clinical and virological endpoints did not reveal any clinical benefit to dengue patients in the drug-treated group, except for a hint of benefit to patients infected with DENV-2 [34]. Once again, it is likely that treatment may have commenced too late during the course of illness to observe any clinical benefit.

4.3. RdRp inhibitors

The DENV NS5 polymerase, which is the viral RdRp, is the most conserved flaviviral protein. Inhibitors which target the conserved active site of the polymerase, carry an in-built advantage of high genetic barrier to the emergence of drug resistance. RdRp inhibitors are converted to their triphosphate derivatives *in vivo* and participate in viral RNA synthesis resulting either in chain termination or mutations leading to error catastrophe. Balapiravir and ribavirin are two such drugs, developed originally as inhibitors of HCV RdRp. Given the structural and functional similarities between DENV and HCV RdRps, these inhibitors could be expected to be effective against DENVs as well.

4.3.1. Balapiravir

Balapiravir is an ester prodrug of a nucleoside analog (4′-azidocytidine) initially developed as an antiviral drug for chronic HCV infection [61,62]. It acts as an antiviral drug after its conversion *in vivo* to R1479, which is then phosphorylated to the triphosphate form and gets incorporated into the viral genomic RNA by the viral RdRp enzyme. The drug is well-tolerated (up to 3-g dose) and is effective in decreasing HCV viremia in a dose- and time-dependent manner [63]. However, extended therapy with balapiravir resulted in clinical safety signals, leading to cessation of its further development as an HCV drug. The similarity of balapiravir's target, HCV RdRp to DENV RdRp, in overall structural architecture, and the ability of the drug to inhibit DENV replication *in vitro*, led Hoffmann Hoffmann-La Roche to test balapiravir (Table 1) against DENV in an exploratory, dose-escalating, randomized, placebo-controlled trial in adult male dengue patients, during 2010–2011 [30]. The drug was administered orally within 48 h, twice daily for 5 days. Two dose levels were tested (1.5 or 3.0 g/day, respectively).

Daily assessment of viremia and NS1 antigenemia showed similar kinetics for both virological markers in both drug-treated and untreated groups, despite the drug achieving *in vivo* levels exceeding *in vitro*-determined EC_{50} values. Further, balapiravir did not attenuate the kinetics of plasma cytokine concentrations and whole blood transcriptional profile. Essentially, the data do not support balapiravir as a dengue drug candidate. Several reasons may underlie this failure: one, the patient cohort is too small to detect clinical benefit; two, intervention was not early enough; or three, prodrug to active drug conversion was not efficient and timely. Recent investigations aimed at understanding the cause of balapiravir's lack of efficacy against DENV suggest that prodrug conversion is impaired in DENV-infected cells, and this appears to be mediated by the cytokines induced by DENV infection [64].

4.3.2. Ribavirin

Ribavirin is another prodrug with broad-spectrum antiviral activity. Though it can also inhibit RdRp activity, its anti-flaviviral activity is believed to be mediated through depletion of intracellular GTP pool. Though ribavirin manifests DENV inhibitory activity in infected cells in culture, it is without effect in *in vivo* animal model systems [65,66]. In regard to HCV therapy, while ribavirin administered alone is ineffective [67], it is quite effective as part of a combination therapy with interferon-α [68]. Available information indicates that a phase II trial of ribavirin in combination with traditional Chinese medicine must have recently been concluded in China (Table 2). The trial data are yet to be announced.

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4.4. Iminosugars

Castanospermine (Cast) and deoxynojirimycin (DNJ) are iminosugars, which inhibit host α-glucosidases I and II, ER enzymes that trim glucose residues off *N*-glycans of newly synthesized glycoproteins [69]. This host enzyme-inhibitory action can affect morphogenesis through mis-folding of glycoproteins of many viruses, including DENVs and reduce viral titers [70,71]. Consequently, iminosugars can function as broad-spectrum antiviral agents. Celgosivir, a derivative of castanospermine, and UV-4, derived from deoxynojirimycin (DNJ), are two iminosugar drugs being evaluated as DENV drugs in clinical trials. A celgosivir trial was recently completed (Table 1) in Singapore, and a second trial is also scheduled to commence (Table 2) in Singapore. Another trial in the United States, which is near completion, is designed to assess UV-4B as a dengue drug (Table 2).

4.4.1. Celgosivir

Bu-Cast a 6-*O*-butanoyl prodrug of the plant-derived iminosugar, castanospermine, is also known as celgosivir. This has been tested as a potential drug for HCV infections in phase 1 and 2 trials [72]. The drug was abandoned as it did not offer any improvement in efficacy over existing HCV drugs. *In vitro* experiments have shown that viral replication of all four DENV serotypes may be compromised as a result of NS1 mis-folding [71]. In a mouse model of lethal DENV infection, celgosivir was shown to confer 100% survival in a dosing frequency-dependent manner [73]. Celgosivir may affect other host pathways, such as those involving ER stress proteins [66,70,71]. These in turn could impact DENV replication.

Celgosivir was tested for efficacy in adults with acute dengue fever (Table 1) in a randomized, double-blind, placebo-controlled phase 1b proof-of-concept trial (NCT01619969) recently [28]. Fifty patients, confirmed to be DENV-infected based on clinical and laboratory diagnosis, were inducted into the trial within 48 h of illness onset. Of these, ~64% were identified as secondary DENV infections. Celgosivir was administered to the treatment group for 5 days at 12-h intervals starting with a loading dose of 400 mg with all subsequent doses being 200 mg. Mean virological log reduction and mean area under fever curve for body temperature >37°C were higher in the celgosivir group, compared to placebo group, but were statistically not significant. Time to serum NS1 clearance did not differ significantly between celgosivir and placebo groups, though there was a hint of faster clearance in the subset of secondary DENV infection cases on celgosivir. Of note, diarrhea, an early clinical feature of dengue [74], was more frequent in the celgosivir group. There were 14 cases (11 mild and 3 moderate cases) of diarrhea in the celgosivir group as compared to 4 cases (all mild) in the placebo group. The trial investigators concluded that celgosivir was generally safe and well tolerated, but failed to reduce viral load or fever burden in dengue patients. A phase Ib/IIa trial using a reduced celgosivir dose, but administered at shorter intervals, is scheduled to be initiated shortly (Table 2). This trial will also examine the effect of another drug, modipafant, a potent inhibitor of platelet activating factor on dengue (NCT02569827).

4.4.2. UV-4B

In vivo screening of several DNJ derivatives identified *N*-9-methoxynonyl-DNJ (designated as UV-4; its hydrochloride salt is UV-4B) as a drug capable of protecting AG129 mice from lethal DENV challenge in a dose-dependent manner. This protection correlated with lower viremia, reduced tissue virus load and an attenuation of the cytokine storm [75]. It is of note that the protective action of UV-4B did not affect DENV-specific antibodies, which are key to causing ADE-mediated severe disease in the AG129 model. A more recent study, which utilized next-generation sequencing to analyze DENV genomes passaged in the presence of UV-4B demonstrated that the virus does not acquire fitness-enhancing mutations [76], suggesting that acquisition of UV-4B resistance by DENV may be unlikely. Based on these findings, a clinical trial (NCT02061358) to test the safety and tolerability of UV-4B in healthy volunteers is currently underway (Table 2).

4.5. Traditional medicine

Lack of a robust pipeline of new/re-positioned drug candidates for dengue is beginning to prompt an interest in traditional medicine. Recent years have witnessed an interest in some Asian countries to explore indigenous traditional medicine for treating dengue. Based on their use in traditional settings, extracts from many plants have been reported to manifest antiviral activity against DENV [77–80]. For example, using the knowledge of traditional Indian medicine, it was shown recently that extracts of the plant *Cissampleos pareira* possess pan-DENV inhibitory activity and offer protection against DENV-2 infection in a mouse challenge model [80]. Clinical trials are being set up to test various herbal drugs for possible dengue treatment in India, Sri Lanka, and China (Table 3). Many of these herbal formulations are based on herbal extracts derived from *Carica papaya*. The Sri Lankan trial reported a small, but statistically significant clinical benefit in the treated group in terms of fever duration and progression to DHF. No information is yet available from the other trials. It is to be noted that the efforts to test herbal extracts against dengue is based on their traditional use for treating dengue-like febrile illnesses. The active ingredient in the herbal extract, its target, and its mechanism of action are unknown.

5. Conclusions

A safe and effective drug for dengue is an unmet urgent public health need. Given the magnitude of dengue as a global public health problem [5,6], the resources and efforts invested in this direction are disproportionately small. The conventional drug discovery pipeline has not produced any promising clinical candidates so far. In fact, many of those in the clinical phase are old drugs for which dengue is being evaluated as a new indication. A few of these trials have been completed and few more are ongoing or planned.

Data from the completed drug trials (Table 1) so far have not identified any promising drug candidate. Neither of the two CQ trials found any significant therapeutic benefit in CQ-

treated dengue patients [27,32]. One of these trials also demonstrated that NS1 clearance time may not be a reliable primary endpoint, as NS1 persists even at the time of patient discharge from the hospital in many instances [32]. The prednisolone trial was primarily aimed at evaluating the safety of administering corticosteroid therapy to dengue patients [31]. This study found that prednisolone neither increased nor prolonged viremia in dengue patients. However, the study investigators did note a tendency toward hyperglycemia at the higher drug dose tested. A subsequent investigation of patient samples from this trial delineated a very small 'prednisolone-associated footprint' on the whole blood gene expression profile [29]. Although not powered to assess efficacy, the available data indicated that prednisolone did not attenuate bleeding, thrombocytopenia, and coagulopathy in patients manifesting severe dengue disease [31]. A recent systematic analysis of corticosteroid trials for dengue therapy has concluded that available evidence is inconclusive [81]. Currently, WHO does not advocate corticosteroid therapy for dengue illness [25]. An exploratory dose-escalation study was undertaken to evaluate the safety of administering lovastatin to dengue patients recently [34]. The statin did not influence the frequency of adverse events in the test group compared to the placebo group. Although not designed to evaluate efficacy, a comparison of clinical and virological parameters in the two groups did not reveal any discernible efficacy of lovastatin. The DENV RdRp inhibitor balapiravir, which was effective on DENV replication *in vitro* failed to attenuate the severity of clinical and virological parameters in dengue patients at doses as high as 3 g, despite having achieved plasma drug levels exceeding *in vitro* EC_{50} values [30]. A subsequent investigation showed that prodrug conversion may be inefficient in DENV-infected cells [64]. The iminosugar celgosivir was tested in a proof-of-concept phase 1b trial in Singapore, after obtaining encouraging results in pre-clinical studies [71,73]. Though the drug was considered to be safe and well-tolerated, it failed to manifest any significant therapeutic efficacy [28]. It is significant that the drug tends to accentuate symptoms of diarrhea, which could be a safety signal in the context of fluid loss which is associated with severe dengue disease. This trial also underlines the inadequacy of the AG129 mouse model used in the pre-clinical studies on celgosivir [71,73].

The failure of celgosivir has prompted the drug developers to re-test the drug using a slightly altered dosing regimen. This trial is scheduled to be initiated in mid-2016. Another iminosugar-based drug, UV-4B, is also undergoing clinical trial for safety evaluation in healthy US volunteers. The antiparasitic drug, ivermectin, which has a well-documented record of human usage and safety, is currently being tested in Thailand for efficacy against dengue. Advanced stage clinical trials are also underway to test ribavirin, and dietary supplementation of Zn and vitamin E in Asian countries (Table 2). Finally, some of the Asian countries have initiated testing of indigenous herbal medicines as alternate dengue therapeutic approaches (Table 3). There is a long way to go before an effective dengue drug becomes available. Supportive fluid therapy remains the only available option for the treatment of DHF/DSS.

6. Expert opinion

Dengue drug trials in clinical development phase are in very early stages and largely exploratory. Information from the clinical drug trials so far is meagre and inconclusive. As traditional drug discovery has not produced a promising clinical candidate yet, current trials are mainly focusing on drugs for other indications re-positioned for dengue. This re-positioning is largely guided by limited understanding of dengue pathogenesis. The lack of an animal model that recapitulates all aspects of dengue illness is another constraint. Coupled to the fact that the majority of dengue patients make a full recovery with only a small proportion susceptible to severe dengue disease, efficacy becomes difficult to discern in small trials. Nevertheless, the early clinical trial experience serves to arrive at broad generalizations on some aspects related to time of intervention, drug dose and dosing frequency, disease pathogenesis, and limitations of dengue model systems. Hopefully, this may inform and guide future trials. These aspects, in the context of the trials discussed in this article, are presented in the following.

Most of the patients in these studies were recruited within 72 h of fever onset and included in the trials after laboratory confirmation of dengue infection, most often using the NS1 antigen detection. Thus, at the time of intervention most patients are in the midst of a systemic viral infection manifesting viremia. None of the trials, including one that recruited patients within 48 h of fever onset, found any therapeutic efficacy (Table 1). One possible reason for this failure (assuming of course that drug efficacy *in vivo* is not in question) may be that the time of initiating drug therapy was too late to be of clinical benefit to the patient. Thus, a drug with known DENV-specific antiviral effect needs to be administered very early on to confer therapeutic benefit to the patient. In real life situation, this would pose a challenge, as most patients may not seek medical intervention in the early phase of illness. In addition, currently there is no way to identify patients who are 'at risk' of progressing to severe disease. There are two ways to address this situation [26]. One, it would be necessary to develop appropriate tools of risk prediction so that future dengue drug trials can focus on high-risk patients and increase the possibility of generating conclusive data on the future utility or otherwise of a drug candidate. Two, explore the development of prophylactic dengue drugs. The inherent advantage of the latter approach would be to preclude viremia prior to onset of illness. In an endemic setting, this approach would rely on an early surveillance system to detect dengue outbreaks so that the 'at risk' population can benefit from the prophylactic drug.

There is no information on drug levels and the duration of its persistence *in vivo* to achieve detectable antiviral effect. Safety and tolerability need to be evaluated before this can be addressed. Still a major challenge in this context is that the precise *in vivo* site of DENV replication is not reliably known, though it is believed to occur within cells of the reticuloendothelial system. Thus, it is necessary to identify the actual sites of DENV replication *in vivo*. Once this becomes known, it may be feasible to develop therapies targeted to such cell types.

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Most of the dengue patients in these trials were infected with DENV-1 and DENV-2 [28,30,31] with secondary infections predominating in many instances [28,31,32]. One trial observed a hint of benefit to DENV-2-infected patients [34], while another discerned a slightly better drug efficacy in secondary compared to primary DENV infection cases [28]. This raises the question if the serotype of DENV has a role in determining drug susceptibility? In addition, would therapeutic outcome differ between primary and secondary DENV infections? These questions may be addressed by designing larger trials that stratify trial subjects based on primary versus secondary infections and DENV serotyping. This can be extremely challenging. The time, effort, and cost inherent in such trials could be significantly minimized if tools to predict patients at 'high-risk' of DHF/DSS become available.

Apart from not knowing the primary host cell surface receptor for DENV and the identity of the *in vivo* site of DENV replication, a major hurdle in understanding disease pathogenesis has been the lack of a good animal model of dengue that can reliably mirror all cardinal aspects of the human disease. In recent years, the AG129 mouse has emerged as a valuable model for candidate dengue drug testing [82,83]. Pre-clinical data on celgosivir using the AG129 mouse model were quite encouraging [71,73] and led to it being tested on humans [28]. The limitation of this model system in predicting drug efficacy outcome in humans is underscored by the celgosivir trial data [28]. As non-human primates do not develop dengue disease, humans represent the only available system to study this disease and test the efficacy of therapeutic/prophylactic interventions. While developing and establishing a dengue human infection model (DHIM) will be a formidable and challenging task, it can be a valuable system with the potential to enable better understanding of pathogenesis and accelerate clinical testing of candidate therapeutic/prophylactic drugs. In fact, an initiative by the US army is actively exploring the development of a DHIM [84–86].

Most countries where dengue is endemic are resource-poor countries. As potential patients in these countries have little purchasing power, dengue has remained a neglected disease for long. However, the increased awareness that dengue is not confined by geographical boundaries has resulted in significant fundamental and applied research. Dengue drug development effort is essentially minimal and in its infancy. There is a need to invest more resources and efforts to overcome formidable hurdles before a safe, efficacious, and inexpensive drug becomes available.

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CHAPTER 7: FUTURE PERSPECTIVES

The work presented in this thesis has: (a) identified two small molecule inhibitors, MB21 (a benzimidazole derivative), and BT24 (a quinoline derivative), of the DENV NS3 protease from an in-house library of ~3000 compounds; (b) identified a conserved siRNA target (sh5c) site in the DENV genome to silence DENV viral RNA; (c) developed a replication-defective adenovirus vector (rAd-sh5c) to deliver an siRNA targeting this site into DENV-infected cells. Initial cell-based assays have demonstrated that both the small molecules (MB21 and BT24), as well as the rAd-sh5c vector, are independently effective at inhibiting all the four DENV serotypes. Some of the implications and future perspectives of this work are discussed below.

7.1 Targeting DENV NS3 protease with small molecule inhibitors

One concern in the context of developing antiviral drugs for dengue stems from the imperfect replication machinery utilized by DENVs. The lack of proof-reading function of DENV RdRp enzyme can result in errors in replication. This opens up the possibility that one or more such errors in the DENV genomic RNA sequence may confer drug resistance. A practical way of addressing such a situation would be to develop a cocktail of drugs to minimize the possibility of emergence of such drug resistant mutants. In this context, the availability of the two pan-DENV inhibitors will enable experimental testing of a mixture of both these drugs, on each of the four DENVs, to determine the frequency of emergence of drug resistance.

Apart from studies on drug resistance emergence, it would be important to modify the basic MB21 and BT24 scaffolds using medicinal chemistry to improve inhibitory potency from the micromolar range to sub-nanomolar range.

As a next step, these drugs would need to be tested *in vivo* using a small animal model, such as the DENV-susceptible AG129 mouse. Under certain experimental conditions, these mice can manifest some of the symptoms of dengue disease. The effect of these drugs in mitigating the disease symptoms in this model can indicate if further development of these drugs is warranted. The next step would be to evaluate the absorption, distribution, metabolism and excretion (ADME studies) to assess the feasibility of further development.

7.2 Targeting DENV genomic RNA using rAd-delivered RNAi

RNAi interference is potentially useful as an antiviral strategy. While it has been shown to be capable of inhibiting many viruses, challenges in targeted siRNA delivery have precluded its utility. In this context, the use of a rAd vector has important implications that need to be carefully investigated. Two aspects are relevant to the proposed use of a rAd vector as a potentially useful tool to deliver RNAi-mediated silencing of DENV RNA: (i) as adenovirus is ubiquitous in nature, the prevalence of anti-adenoviral antibodies in the human population is very high. Reports exist which have shown that anti-adenovirus antibodies can bind to adenovirus and target its uptake into Fc receptor bearing monocytes and macrophages. (ii) These very cells are the sites of DENVs replication *in vivo*, in infected individuals. Taken together, this actually makes adenoviral vectors useful in the context of using them to deploy RNAi against DENVs. The demonstration in this work that an adenoviral vector can be utilized to deliver siRNA into infected cells raises the potentially interesting possibility that pre-existing anti-adenovirus antibodies could bind to the rAd vector encoding siRNA and deliver it into the very cells in

which DENV replicates *in vivo*. This exciting possibility needs to be investigated both *in vitro* and *in vivo*, in an animal model. The outcome of such experimentation can shed further light on the utility of rAd-mediated RNAi to silence DENV expression.

Finally, in the context of possible emergence of mutant DENVs that can circumvent inhibition, one may envisage using the NS3 protease inhibitors in conjunction with RNAi-mediated silencing to further raise the genetic barrier against possible drug resistance.

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